Gutachten

für ein 67 ~~35~~mg-Isoflavone und 10mg NADH enthaltendes diätetisches Nahrungsmittel

Gutachten über Isoflavone

1.Wirksamkeit

Phytoöstrogensupplemente und Wechseljahresbeschwerden

Die Wirksamkeit von Phytoöstrogenen in Form von Supplementen wurde in speziellen Kollektiven von Frauen in RCTs nachgewiesen und kann als wissenschaftlich abgesichert angesehen werden. In diesem Sinne wurde in einer rezenten systematischen Übersichtsarbeit über insgesamt 25 RCTs und 6 systematische Übersichtsarbeiten zu diesem Thema folgender Stand der wissenschaftlichen Studien dargelegt (1):

In einer systematischen Übersichtsarbeit von 25 RCTs führten Rotkleeprodukte in einer Meta-Analyse aller Studien, die an unterschiedlichen Patientinnenkollektiven durchgeführt wurden, zu keiner signifikanten Reduktion der Hitzewallungen (weighted mean difference -0.60; 95% Confidence Interval [CI] -1.71-0.51). Von 5 RCTs zur Wirksamkeit von Sojaprodukten zeigten zwei RCTs eine signifikante Reduktion von Hitzewallungen (2). In einer weiteren systematischen Übersichtsarbeit und Meta-Analyse von 12 RCTs zur Wirksamkeit von Sojaprodukten war in 3 von 8 Studien mit einer Behandlungsdauer von >6 Monaten eine signifikante Reduktion von Hitzewallungen nachweisbar (3). Eine systematische Übersichtsarbeit von 10 RCTs fand bei 4 von 10 RCTs eine signifikante Wirksamkeit von Sojaprodukten auf Wechseljahresbeschwerden (4). Kang et al. fanden in einer systematischen Übersichtsarbeit von 7 RCTs in 2 RCTs eine signifikante Reduktion von Hitzewallungen durch Sojaprodukte (5). Diese Vielzahl von Studien und die unterschiedlichen Ergebnisse legen den Schluss nahe, dass offensichtlich die Wirksamkeit von Phytoöstrogensupplementen und anderen Sojaprodukten auf spezielle Kollektive von postmenopausalen Frauen beschränkt ist. Eine Subpopulation von Frauen, für die eine einheitliche Datenlage der durchgeführten

RCTs, also Studien der höchsten Evidenzkategorie, vorliegt, sind Frauen in der frühen Post-Menopause (50-55.Lebensjahr).

In der bereits erwähnten systematischen Übersichtsarbeit (1) wurden in der aktuellen Literatur 5 RCTs mit insgesamt 411 früh natürlich postmenopausalen Frauen identifiziert (6-10). In diesen Studien wurden Isoflavonprotein, Isoflavonkapseln, Rotkleeextrakt und Isoflavontabletten in einer handelsüblichen Dosierung von Isoflavonen zwischen 40mg pro Tag und 100mg pro Tag zwischen 12 und 16 Wochen gegen Plazebo getestet. Fünf von fünf RCTs zeigten eine statistisch signifikante Reduktion von vasomotorischen Symptomen wie z.B. Hitzewallungen. Die Wirksamkeit von Phytoöstrogensupplementen in diesem Kollektiv von Patientinnen lässt den Schluss zu, dass in Studien ohne Selektionskriterien bzgl. natürlicher versus iatrogener Menopause und bzgl. der Dauer seit dem Eintreten der Menopause die Wirksamkeit der Therapie mit Phytoöstrogenen unterschätzt wird. Auf der anderen Seite zeigen die Ergebnisse der vorhandenen RCTs auch, dass in unselektionierten Kollektiven von Frauen die therapeutische Wirksamkeit von Phytoöstrogenen in Bezug auf Wechseljahresbeschwerden nicht gegeben ist.

Diese Ergebnisse der Literatur lassen folgende Einschätzung zur Wirksamkeit von Phytoöstrogensupplementen aus Rotklee und Soja zur Behandlung von Wechseljahresbeschwerden zu. Die letztgenannte aktuellste systematische Übersichtsarbeit (1) sowie die in der Literatur vorhandenen systematischen Übersichtsarbeiten und Meta-Analysen (2,3,4,5,11,12) weisen auf Evidenzniveau I, d.h. basierend auf prospektiv-randomisierten, kontrollierten Studien und/oder Meta-Analysen von prospektiv-randomisierten, kontrollierten Studien, eine Wirksamkeit von Phytoöstrogensupplementen auf Wechseljahresbeschwerden in statistisch signifikantem Ausmass bei früh natürlich postmenopausalen Frauen nach. Eine gegenüber Plazebo nicht nachweisbare Wirksamkeit ist für Frauen mit idiopathischer prämaturer Menopause, prämaturer Menopause nach Chemotherapie oder chirurgischer Ovarektomie, Frauen nach langjähriger Hormonersatztherapie mit Östrogenen und/oder Östrogen-Gestagen-Kombinationstherapie sowie Frauen mit > 3 Jahre zurückliegender Menopause gegeben (1, 13, 14). Aus dem Gesagten ergibt sich daher, dass Phytoöstrogensupplemente bei Frauen unmittelbar nach Eintreten

einer natürlichen Menopause eingesetzt werden können. Die Wirksamkeit von Phytoöstrogensupplementen ist bei dieser Kategorie von Patientinnen als wissenschaftlich einwandfrei nachgewiesen anzusehen.

2. Phytoöstrogensupplemente und Risikobeurteilung

Sicherheit von Phytoöstrogenen

Befürchtungen, Phytoöstrogene könnten aufgrund ihrer estrogenähnlichen Wirkung die Entwicklung von Brustkrebs oder anderen hormonsensitiven Erkrankungen fördern, sind durch die Ergebnisse der vorliegenden klinischen Studien nicht gerechtfertigt. In einer Meta-Analyse von 174 RCTs an über 9000 Frauen konnten wir keine Häufung von Brustkrebs, Endometriumhyperplasie oder Endometriumkarzinom bei Frauen feststellen, die Phytoöstrogensupplemente eingenommen hatten. Auch in diversen Unterkategorien von unerwünschten Nebenwirkungen zeigte sich in den untersuchten Studien keine Häufung von gynäkologischen, neurologischen, muskulo-skeletalen oder unspezifischen Nebenwirkungen. Lediglich in der Kategorie gastrointestinaler Nebenwirkungen fand sich eine überzufällige Häufung von Beschwerden bei Frauen unter Phytoöstrogenen (eigene Daten). Des weiteren ist anzuführen, dass in den vorhandenen epidemiologischen Studien, retrospektiven und prospektiven Fallkontrollstudien und Kohortenstudien kein Hinweis auf eine erhöhte Brustkrebsinzidenz in Zusammenhang mit der Einnahme von Phytoöstrogenen publiziert worden ist. Bislang hat keine einzige der durchgeführten RCTs einen direkten oder indirekten Zusammenhang zwischen der Einnahme von Phytoöstrogenen in handelsüblicher Dosierung und einem gehäuften Auftreten von Brustkrebs gefunden (15).

Weiters ist festzuhalten, dass in vitro-Studien und Tierexperimente grundsätzlich nicht auf Menschen übertragbar sind und dass neben Studien an benignen Brustzellinien, Skelettmuskelzellen und Tiermodellen, die einen proliferationsfördernden Effekt von Sojaisoflavonen und anderen Phytoöstrogenen

nachweisen (16) auch experimentelle Daten zu proliferationshemmenden Effekten von Phytoöstrogenen wie z. B. Prenylflavonen aus *Epimedium brevicornum* (17) publiziert wurden. Ebenso finden sich experimentelle Daten zu einem antioxidativen und damit potenziell antikanzerogenen Effekt von Phytoöstrogenen (18). Wesentlich ist auch, dass die Wirksamkeit von Phytoöstrogenen von der gleichzeitig auf die Zelle einwirkenden Östrogenkonzentration abhängig ist. Kreuter et al. konnten an Brustkrebszellinien (MCF-7-Zellen) nachweisen, dass Soja- und Rotkleeextrakte in Abwesenheit von Östradiol proliferationsfördernd wirken, in Anwesenheit von physiologischen Östradiolkonzentrationen, die der prä-und postmenopausalen Situation entsprechen, jedoch als Östradiol-Antagonisten wirken und die Östradiol-induzierte Zellproliferation hemmen (19).
Der Analogieschluß zwischen Phytoöstrogenen und Östrogenen und einer behaupteten brustkrebsfördernden Wirkung von Östrogenen ist unrichtig und deckt sich nicht mit den Ergebnissen von RCTs am Menschen. Während die Women's Health Initiative (WHI)-Studie ein erhöhtes Risiko für Brustkrebs nach 4.8 Jahren Hormonersatztherapie mit konjugierten Östrogenen und Medroxyprogesteron als Gestagen nachweist (20), führt eine Hormonersatztherapie mit ausschließlich konjugierten Östrogenen nach 7.8 Jahren gegenüber Plazebog z einer statistisch signifikanten Reduktion der Brustkrebshäufigkeit in der per-Protokoll-Analyse (21). Diese Daten zeigen eindeutig, dass 1) in vitro-Daten aus Experimenten an Zellinien und Tiermodellen nicht auf die klinische Situation am Menschen extrapoliert werden können und dass 2) ein grundsätzlicher Unterschied zwischen der Wirkung von Östrogenen auf Brustepithelien in Zellinienexperimenten und Tiermodellen und auf das Brustkrebsrisiko beim Menschen besteht.

In den vorhandenen epidemiologischen Studien, retrospektiven und prospektiven Fallkontrollstudien und Kohortenstudien ist kein Hinweis auf eine erhöhte Brustkrebsinzidenz festgestellt worden.

Weitere Effekte von Phytoöstrogensupplementen

Neben der Linderung von Wechselbeschwerden bei früh natürlich postmenopausalen Frauen durch Phytoöstrogensupplemente und der möglichen brustkrebspräventiven Wirkung von phytoöstrogenreicher Nahrung im Kindheits- und Jugendalter lassen sich weitere positive Effekte von Phytoöstrogenen und Phytoöstrogensupplementen in RCTs nachweisen. So wurde z. B. in einer Meta-Analyse von 38 RCTs eine signifikante Reduktion von Surrogatmarkern der kardiovaskulären Gesundheit wie low-density Lipoproteinen (LDL), high-density Lipoproteinen (HDL) und Triglyzeriden unter der Einnahme von Sojaprotein nachgewiesen (21). Ob eine derartige Reduktion kardiovaskulärer Risikomarker auch geeignet ist kardiovaskuläre klinische Endpunkte wie z.B. den Myokardinfarkt positiv zu beeinflussen, ist unbekannt, da RCTs zu dieser Fragestellung fehlen.

Effekte von Phytoöstrogensupplementen auf Endometrium und Schilddrüse

Klinische Studien der höchsten Evidenzklasse weisen die Sicherheit von Phytoöstrogensupplementen bezüglich des Endometriums nach. In einer RCT an 389 Frauen war nach 12 Monaten Therapie mit Genistein in der Dosierung 54mg/d gegenüber Plazebo keine Veränderung der Endometriumdicke und des Maturationsindex des Endometriums nachweisbar (22). Auch in weiteren RCTs an 62 und 19 Frauen konnte unter der Behandlung von Phytoöstrogensupplementen kein Effekt auf das Endometrium nachgewiesen werden (23, 24). Auch eine RCT an 64 Brustkrebspatientinnen konnte zeigen, dass die Einnahme von 114mg/d an isolierten Isoflavonoiden gegenüber Plazebo keine nachweisbare Wirkung auf das Endometrium aufweist (25). Diese Vielzahl von klinischen Studien der höchsten Evidenzklasse lässt den Schluss zu, das Phytoöstrogensupplemente in handelsüblicher Dosierung bei postmenopausalen Frauen zu keinen klinisch nachweisbaren Veränderungen des Endometriums führen und daher als sicher für das Endometrium anzusehen sind.

Das Schildrüsenenzym Peroxidase, welches die Biosynthese von Schilddrüsenhormonen katalysiert, wird durch Phytoöstrogene teilweise inhibiert.

Dies konnte in experimentellen Studien nachgewiesen werden (26). Auch die hepatische Aktivität des Enzyms Dejodinase, welches für die Konvertierung von Schilddrüsenhormonen notwendig ist, wird durch Phytoöstrogene gehemmt (27). Diese antithyroide Wirkung von Phytoöstrogenen ist allerdings klinisch nicht von Relevanz. Im Tiermodell konnte gezeigt werden, dass die Behandlung mit Genistein in unterschiedlicher Dosierung zwar zu einer Reduktion der Peroxidaseaktivität führt, dies jedoch keine Auswirkungen auf die Funktion der Schilddrüse hat. Die Konzentration der Schilddrüsenhormone, das Schilddrüsengewicht und die Histologie der Schilddrüse zeigten keinerlei Veränderung (28). Dies steht auch im Einklang mit den vorhandenen klinischen Studien. In keiner der 25 RCTs zur klinischen Wirksamkeit von Phytoöstrogensupplementen wurde eine erhöhte Inzidenz von Schilddrüsenfunktionsstörungen beobachtet (1). Aufgrund dieser Datenlage sind Phytoöstrogensupplemente als sicher bzgl. der Schilddrüsenfunktion anzusehen.

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Phytoestrogens in clinical practice: a review of the literature

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Objective: To review clinical studies assessing the effect of phytoestrogen supplementation on the signs and symptoms of the climacteric syndrome and on the incidence of breast cancer, cardiovascular disease, and skeletal fractures.
Design: Literature research using PubMed and the Cochrane controlled trials register.
Setting: None.
Patient(s): None.
Intervention(s): None.
Main Outcome Measure(s): None.
Result(s): Six systematic reviews and meta-analyses of 25 randomized, controlled trials (RCTs) assessing the use of phytoestrogens for the treatment of the climacteric syndrome were identified. Systematic reviews of RCTs show contradictory results, and meta-analyses demonstrate no statistically significant reduction of vasomotor symptoms for phytoestrogens. Individual RCTs report significant reductions in vasomotor symptoms for red clover and soy phytoestrogens. In selected patient populations, such as in women with early natural postmenopause and mild to moderate vasomotor symptoms, a systematic review of five RCTs found a significant reduction of hot flashes in five out of five RCTs. Twenty-two case-control and cohort studies examined the incidence of breast cancer among women with and without a diet high in phytoestrogens. A meta-analysis of 21 studies found a significantly reduced incidence of breast cancer among past phytoestrogen users. RCTs document beneficial effects of phytoestrogens on surrogate parameters such as bone mineral density, vasodilation, platelet aggregation, insulin resistance, and serum concentrations of triglycerides, high-density lipoprotein, and low-density lipoprotein. None of the available RCTs documents a protective effect of phytoestrogens for the clinical end points of breast cancer, bone fracture, or cardiovascular events.
Conclusion(s): Based on the available evidence, phytoestrogens should only be used in selected women, i.e., those presenting with mild to moderate vasomotor symptoms in early natural postmenopause. None of the compounds investigated so far have been proven to protect against breast cancer, bone fracture, or cardiovascular disease. (Fertil Steril® 2007;87:1243–9. ©2007 by American Society for Reproductive Medicine.)

Key Words: Phytoestrogens, climacteric syndrome, hormone replacement, soy, red clover, alternative, review

Phytoestrogens have been widely used in various cultures and time periods. Empirical data and epidemiologic research indicate that the incidence of hormone-dependent diseases is reduced in countries with a high dietary content of phytoestrogens (1, 2). Thus, modern clinical and molecular biological research has increasingly focused on plant-derived phytoestrogens such as isoflavones, lignans, dihydrochalcones, and coumestans. The most widely studied phytoestrogenic compounds are isoflavones found in red clover and soy, such as genistein, formononetin, biochanin A, and daidzein (3).

The widely publicized and discussed results of the Women's Health Initiative (WHI) and the One Million Women Study have considerably increased the interest in the use of phytoestrogens. Because phytoestrogens interact with the estrogen receptor, inducing agonistic as well as antagonistic effects, the primary preventive potential as well as the oncologic safety of a prolonged use of phytoestrogens are of considerable interest. We summarize the clinical and experimental data on the use of phytoestrogens for the alleviation of signs and symptoms of the climacteric syndrome. Numerous randomized, controlled trials (RCTs) have investigated

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the therapeutic efficacy of phytoestrogens, allowing for an evidence-based recommendation for clinical practice. In addition, we summarize clinical evidence investigating the effects of phytoestrogens on the skeletal system and the cardiovascular system, the incidence of breast cancer, and skin health.

PHYTOESTROGENS AND THE CLIMACTERIC SYNDROME

A number of controlled and uncontrolled clinical studies have indicated that a dietary increase in phytoestrogens in addition to a regular diet (4) and phytoestrogen supplementation (5–9) are associated with an increase in quality of life and a significant amelioration of the signs and symptoms of the climacteric syndrome, as summarized by the Kuppermann index (10). On the other hand, several studies could not demonstrate a clinical effect, especially with regard to hot flashes (11). Several reasons may account for these and other discrepancies between studies, among them differences in patient selection, phytoestrogen content, and symptom assessment, and variations in endogenous estrogen production, intestinal resorption, and activation of orally ingested isoflavones.

Study Selection

We searched PubMed and the Cochrane controlled trials register (January 06, 2006, for search terms: climacteric syndrome, hot flashes, phytoestrogens, isoflavones, treatment) to identify RCTs, systematic reviews, and meta-analyses of RCTs assessing the efficacy of phytoestrogens in women with the climacteric syndrome. Studies were included if they were published as complete reports in English. Studies were eligible if they were RCTs comparing placebo or no intervention with phytoestrogens in perimenopausal and/or postmenopausal women with hot flashes. Phytoestrogens were defined as substances with a defined amount of isoflavones, lignans, or coumestans. Studies investigating black cohosh were not included in this review because the mechanism of action of this botanical is unknown. Studies assessing menopausal symptoms other than hot flashes were excluded. Two reviewers assessed eligibility of the studies and extracted data. Missing information and additional trials were not sought from the investigators. To identify subgroups of women selectively benefiting from phytoestrogen treatment, we used criteria proposed by the U.S. Food and Drug Administration (FDA) for the conduct of studies evaluating hormone replacement therapies (12) and by Messina and Hughes (13), who proposed to exclude women with iatrogenic menopause after treatment for oncologic diseases in the assessment of the efficacy of phytoestrogens.

Following criteria used in previous meta-analyses (11), we recorded the adequacy of treatment allocation concealment according to criteria published by Schulz et al. (14). Trials were considered to have adequate concealment if they described satisfactory procedures to conceal treatment allocation such as coded identical containers or centralized randomization. We also recorded blinding participants and outcome assessors to treatment, use of intention-to-treat analysis, and the number of participants who withdrew or were lost to follow-up evaluation. When possible, effect sizes were calculated to provide a measure of the magnitude of a treatment effect for each study. The effect sizes represent the difference in mean hot flash frequency outcomes divided by the standard deviation. Negative effect size values favor placebo, and positive effect sizes favor phytoestrogen. In the scale suggested by Cohen (15), cutoff values of 0.2, 0.5, and 0.8 reflect small, moderate, and large treatment effects, respectively.

If possible, outcome data were pooled and analyzed using the Cochrane Collaboration Review Manager (RevMan 4.2; Update Software, Oxford, United Kingdom). Weighted mean differences, the difference between treatment and control pooled means at end point, along with 95% confidence intervals (CI), were calculated for continuous variables.

Efficacy of Phytoestrogens

We identified 25 RCTs (5–9, 16–35) and six systematic reviews and meta-analyses of RCTs (3, 11, 36–39). In a systematic review and meta-analysis of 25 RCTs, red clover products were not shown to be effective for the treatment of hot flashes (weighted mean difference −0.60; 95% CI, −1.71–0.51). Of the five soy extract trials reporting hot flush frequency, three trials, including the two largest trials, were negative (11). In another systematic review of 29 RCTs of alternative therapies for hot flashes and other menopausal symptoms, the investigators identified 12 RCTs examining soy or soy extracts (3). Only three of eight studies with treatment phases >6 months showed a significant improvement in hot flashes. The effects were modest, and most benefits disappeared after 6 weeks. This review cites two RCTs documenting no statistically significant benefit for red clover products. Similarly, Huntley and Ernst (36) identified 10 RCTs assessing soy preparations for the treatment of perimenopausal symptoms and found four of 10 RCTs to be positive, suggesting soy preparations are beneficial for perimenopausal symptoms. Six RCTs were negative, with one of the six showing a positive trend.

Another review of botanical compounds for the treatment of menopause-related symptoms identified RCTs investigating soy isoflavone extracts with varying compositions and dosages of soy supplements. Results from five RCTs assessing the efficacy of semipurified isoflavone red clover (*Trifolium pratense* L.) leaf extracts were contradictory. The largest study showed no benefit for reducing symptoms associated with menopause for two different red clover isoflavone products compared with placebo. No significant adverse events were reported in the investigated studies (37). A meta-analysis by Nelson et al. (38) investigated alternative treatments for menopausal symptoms. The following compounds were found to significantly reduce the number of daily hot flashes compared with placebo: seven RCTs of selective serotonin reuptake inhibitors (SSRIs) or serotonin

norepinephrine reuptake inhibitors (SNRIs) (mean difference, −1.13; 95% CI, −1.70 to −0.57), four RCTs of clonidine (−0.95; 95% CI, −1.44 to −0.47), and two RCTs of gabapentin (−2.05; 95% CI, −2.80 to −1.30). In contrast, the frequency was not reduced in a meta-analysis of 17 RCTs of red clover isoflavone extracts and soy isoflavone extracts (39). Kang et al. (39) identified seven RCTs, of which five RCTs reported no beneficial effect on hot flashes of soy protein. Two RCTs reported a significant reduction of hot flashes in the group treated with soy product over placebo. Hot flash frequency was reduced by 45% and 28%, respectively, in these two trials, but the placebo effect was also high, with reductions of 30% and 18%.

These data indicate that phytoestrogens have modest if any effects, and that these effects may be limited to selected patient populations. Thus, in an effort to better define possible patient subgroups who may selectively benefit from phytoestrogens, we tried to identify studies examining only early postmenopausal women with natural menopause and mild to moderate vasomotor symptoms using previously published recommendations (12, 13).

We identified 25 RCTs examining phytoestrogens for the treatment of hot flashes (5–9, 16–35). We excluded 20 studies from further evaluation (16–35), leaving five RCTs with 411 early postmenopausal women with natural menopause (5–9). Tables 1 and 2 show the study and participant characteristics of this highly selected set of studies. All of the trials included postmenopausal women and reported double-blinding. Two of the five trials (5, 7) provided enough information to confirm adequate concealment of treatment allocation. Three of the five trials (5–7) reported intention-to-treat analysis. The minimal dose of isoflavones used in these studies was 40 mg per day, the maximal dose was 100 mg per day. The phytoestrogens tested were soy in four trials (5–7, 9) and red clover extract in one trial (8). The number of women included in these studies varied between 30 and 122.

Table 3 shows the outcomes of trials and the symptom score results. All of the five individual studies demonstrated a statistically significant reduction of vasomotor symptoms. Four of five trials reported continuous variables such as mean percentage change of hot flashes per day or Kupperman index (5, 7–9). Only one study (6) reported absolute numbers. Therefore, a pooled analysis for the identification of mean weighted differences was not performed. One study reported sufficient data for the calculation of effect sizes (6). This study favored placebo with a moderate effect size (0.53).

In summary, this meta-analysis of a predefined, selected patient population indicates that the efficacy of isoflavones as a treatment for the signs and symptoms of the climacteric syndrome is reduced in women with idiopathic premature ovarian failure, premature ovarian failure after cancer treatment, after long-term hormone therapy, and when isoflavone treatment is initiated >3 years after menopause (12, 13). Consequently, phytoestrogens may be used most effectively in early postmenopausal women with a natural menopause and mild to moderate climacteric syndrome. Meta-analyses of intervention studies with isoflavones without specific selection criteria may thus underestimate the clinical efficacy of this therapy. This may serve as a possible explanation for contradictory results of RCTs in unselected patient populations (3, 11, 36–39). From a clinical perspective, phytoestrogens may be used most appropriately as first-line treatment for women presenting with mild to moderate vasomotor symptoms in early natural postmenopause. This is also in accordance with the recommendations of the North American Menopause Society (40).

PHYTOESTROGENS AND BREAST CANCER

In Vitro Data

In vitro experiments using benign breast cell lines, skeletal muscle cell cells, and rodent models have found that phytoestrogens exert a proliferation-enhancing effect (41, 42). Although these studies principally document that phytoestrogens can lead to cell proliferation, other in vitro and in vivo studies have demonstrated a concentration-dependent biphasic antiproliferative effect of isoflavones (43). Phytoestrogens can, under certain conditions, function as antioxidants and protect against oxidant-induced DNA damage (44). In vivo studies in rodents also indicate that low-dose dietary isoflavones abrogate tamoxifen-associated mammary tumor prevention (45). Thus, the experimental data available so far are not conclusive. Epidemiologic data and prospective case-control studies, on the other hand, clearly demonstrate

TABLE 1

Study characteristics and isoflavone dosages.

Study (reference)	Cases (n)	Controls (n)	Compound	*P* value[a]	Isoflavone dosage
Albertazzi et al., 1998 (5)	51	53	Isoflavone protein	<.001	76 mg/day
Faure et al., 2002 (6)	39	36	Isoflavone capsules	<.01	70 mg/day
Han et al., 2002 (7)	40	40	Isoflavone capsules	<.01	100 mg/day
Jeri, 2002 (8)	15	15	Red Clover extract	<.001	40 mg/day
Upmalis et al., 2000 (9)	59	63	Isoflavone tablets	.03	50 mg/day

[a] Highest *P* value for improvement of symptoms.

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TABLE 2

Characteristics of studies and participants.

Study (reference)	Participants (dropouts)	Inclusion criteria	Participant characteristics	Study duration
Albertazzi et al., 1998 (5)	104 (25)	Amenorrhea ≥6 months or oophorectomy, or FSH >50 IU/L and E_2 <35 pg/mL; ≥7 HF/day	Italy; MA 53 years; MDM 3.9 years	12 weeks
Faure et al., 2002 (6)	75 (20)	Amenorrhea ≥6 months or FSH >40 IU/L and E_2 <35 pg/mL; ≥7 HF/day	France; MA 53 years;	16 weeks
Han et al., 2002 (7)	82 (2)	Age 45–55 years and amenorrhea ≥12 months or FSH >25 IU/L and E_2 <35 pg/mL; HF present	Brazil; MA 53 years; MDM 2.4yrs	4 months
Jeri, 2002 (8)	30 (0)	Age <60 years and amenorrhea >1 year or FSH >30 IU/L; ≥5 HF/day	Peru; MA 52 years; MDM -	14 weeks
Upmalis et al., 2000 (9)	177 (55)	Age >50 years and amenorrhea ≥6 months or FSH >40 IU/L and E_2 <25 pg/mL; ≥5 HF/day	U.S.; MA 55 years; MDM	12 weeks

Note: E_2, estradiol; FSH, follicle-stimulating hormone; HF, hot flashes; MA, mean age; MDM, median duration after menopause.

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that countries with a high dietary content of isoflavones have lower incidence rates of breast and prostate cancer compared with countries with a low dietary content of phytoestrogens, which argues against a principally cell-proliferative effect of phytoestrogens.

Clinical evidence from uncontrolled trials is consistent with a protective effect of phytoestrogens against the development of breast cancer. For example, a meta-analysis of 14 case-control and 7 cohort studies found a pooled risk reduction of 25% (Relative Risk [RR] 0.75; 95% Confidence Interval [CI] 0.59 to 0.95) for past soyfood, tofu, miso, or isoflavone intake (46). A recent nested case-control study within a Dutch prospective cohort found high plasma levels of genistein to be associated with a 32% reduced odds of developing breast cancer (47). In contrast to this evidence, three prospective trials evaluated this issue (1, 48, 49). All of these prospective trials showed no statistically significant reduction in breast cancer incidence. There has been no prospective, randomized study evaluating phytoestrogens as a primary preventive therapy of breast cancer. It is of note that a protective effect of isoflavones could not be reproduced in European or U.S. studies such as the San Francisco Bay study (50). Some researchers have claimed that the effect of a phytoestrogen diet depends—at least in part—on the individual age of the woman when starting the diet. Asian women are exposed to phytoestrogens starting with early childhood, but the typical Western initiation of a phytoestrogen-rich diet or phytoestrogen supplementation in perimenopause or postmenopause may be too late to exert a protective effect on the breast. Phytoestrogens may induce differentiation of breast epithelium during early childhood and puberty, thus making the breast epithelium less sensitive to noxious agents such as chemical carcinogens. Breast epithelia may no longer be sensitive to phytoestrogens after pregnancy. This model of a lifetime-dependent effect of protective agents has been demonstrated by Russo and Russo (51).

Putting these data into a clinical perspective, women planning to use phytoestrogens can be advised that phytoestrogens can be used safely on a long-term basis. There are no data indicating that prolonged use of a phytoestrogen-rich diet induces malignant growth of hormone-dependent tissue. On the other hand, none of the compounds investigated so far has been proven to protect against breast cancer. There are no data with respect to other malignancies, but there is no indication that phytoestrogens increase the risk of malignant disease.

PHYTOESTROGENS AND THE SKELETAL SYSTEM

Isoflavones have been demonstrated to increase the synthesis of vitamin D in a number of nonrenal cell types (52). Due to this mechanism, isoflavones are thought to contribute to bone mineral density. A prospective, randomized

TABLE 3

Clinical outcomes of trials and symptom score results.

Study (reference) and intervention	HF/day at baseline (mean SD or median range)	HF/day at study end (mean SD)	Decrease HF/day (*P* value)	Symptom score
Albertazzi et al., 1998 (5)				
Soy	11.4 (10.7–12.7)	6.4 (–)	–5.0	None reported
Placebo	10.9 (10.2–11.8)	7.5 (–)	–3.4	
			$P<.01$	
Faure et al., 2002 (6)				
Soy	10.1 (6.4)	3.9 (0.7)	–6.4	None reported
Placebo	9.4 (3.4)	7.0 (1.2)	–2.2	
			$P=.01$	
Han et al., 2002 (7)				
Soy	—	—	—	Kupperman index
Placebo	—	—		–19.7 (soy) vs +1.3 (placebo)
	—			$P<.01$
Jeri, 2002 (8)				
Red clover	7.0 (0.5)	3.6 (0.3)	–3.4	None reported
Placebo	5.7 (0.4)	5.1 (0.3)	–0.6	
			$P=.001$	
Upmalis et al., 2000 (9)				
Soy	8.8 (6.2)	—	Mean % HF/d	None reported
Placebo	9.4 (6.0)	—	–20 (placebo) vs –28 (soy)	
			$P<.05$	

Note: HF, hot flashes; SD, standard deviation.

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study of 66 women found that 88 mg of isoflavones per day have a protective effect on the skeletal system, increasing the bone mineral density in the lumbar spine (53). On the other hand, a large study of 202 postmenopausal women found no benefit after 12 months of daily 99-mg isoflavone supplementation (52 mg of genistein, 41 mg of daidzein, and 6 mg of glycitein) (54). This double-blind, randomized trial does not support the hypothesis that the use of a soy protein supplement containing isoflavones improves cognitive function, bone mineral density, or plasma lipids in healthy postmenopausal women when started at the age of 60 years or later. Based on these data, isoflavones cannot be clinically recommended as a valuable intervention to increase bone health. No data are available to assess the efficacy of isoflavones as a means to treat women with osteoporosis and should thus not be recommended as the sole treatment in this indication. No RCTs are available, documenting the efficacy of phytoestrogens regarding the prevention of osteoporosis or bone fracture.

PHYTOESTROGENS AND THE CARDIOVASCULAR SYSTEM

Endothelium-derived nitric oxide (NO) is a potent vasodilator and mediates the effects of antihypertensive drugs such as nitroglycerin. Isoflavones have been shown to stimulate the activity of the endothelial NO synthase (NOS3), thus inducing vasodilation via NO (55). Isoflavones also have antithrombotic and antiatherogenic effects. For example, genistein and daidzein decrease monocyte chemoattractant protein-1 and collagen-induced platelet aggregation in a dose-dependent manner (56). In addition, 54 mg/day of genistein are sufficient to significantly decrease fasting glucose, fasting insulin, and insulin resistance (57). Clinical studies have assessed the effect of isoflavones on surrogate end points of cardiovascular health. A meta-analysis of 38 controlled human studies of soy consumption provides evidence for its positive effect on lipid profiles, including reduction in levels of low-density lipoproteins (LDL) and triglycerides and increases in high-density lipoprotein (HDL) (58). In a population-based study of 301 postmenopausal women, however, intake of between 0.2 mg and 11.4 mg of isoflavones did not alter systolic or diastolic blood pressure, or the prevalence of hypertension (59). In accordance, a 12-month, double-blind, randomized trial compared the effects of soy protein containing 99 mg of isoflavones/day (aglycone weights) with those of milk protein (placebo) and found no effect on blood pressure and

endothelial function in 202 postmenopausal women aged 60 to 75 years (60).

No RCTs regarding clinical end points of cardiovascular health (i.e., myocardial infarction, angina pectoris) are available to date. Therefore, phytoestrogens cannot be recommended as a primary preventive intervention to reduce the risk of cardiovascular disease. Women using phytoestrogens may be advised, however, that phytoestrogens used for the treatment of the climacteric syndrome have additional benefits on the cardiovascular system by reducing established risk factors of atherosclerosis such as hyperlipidemia and hypertension. Whether this translates into a clinical benefit regarding the reduction of clinical end points such as myocardial infraction is unknown.

PHYTOESTROGENS AND SKIN HEALTH

Isoflavones may have a protective effect on skin health. It has been hypothesized that skin elasticity is improved by increased local blood flow. In addition, the carcinogenic effect of exogenous noxious agents such as ionizing radiation and chemical compounds may be alleviated by the antioxidant and anti-inflammatory properties of phytoestrogens (61). Also, red clover protects from inflammation and immune suppression induced by ultraviolet radiation (62). No data with respect to clinical end points of skin health have been published.

STANDARDIZATION OF PHYTOESTROGENS

Because phytoestrogens are listed as food supplements, quality standards regarding manufacturing and isoflavone content are important. Phytoestrogen compounds used to treat women with climacteric syndrome should contain 40 to 100 mg of isoflavones consisting of variable combinations of different aglycans (i.e., genistein, daidzein, glycitein, formononetin, and biochanin A). A daily dose of 40–100 mg of isoflavones is toxicologically safe and is consistent with the dietary content of isoflavones in Asian countries (63). Interactions between an isoflavone supplementation in this dosage and other pharmaceutic interventions are not known.

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Phytoestrogens for treatment of menopausal symptoms: a systematic review.

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Department of Medicine, University of Minnesota, Minneapolis, MN, USA.

OBJECTIVE: To assess the efficacy and tolerability of phytoestrogens for treatment of menopausal symptoms. DATA SOURCES: We searched the Cochrane Library and MEDLINE from 1966 to March 2004, using a detailed list of terms related to phytoestrogens and menopausal symptoms and also hand-searched abstracts from relevant meetings. METHODS OF STUDY SELECTION: Randomized trials were eligible if they involved symptomatic perimenopausal or postmenopausal women, compared phytoestrogen with placebo or control, reported hot flush frequency or menopausal symptom scores, and were at least 4 weeks in duration. TABULATION, INTEGRATION, AND RESULTS: Data were extracted onto standardized forms using a prospectively developed protocol. Twenty-five trials involving 2,348 participants met criteria. At baseline, the mean age was 53.1 years, mean duration of menopause was 4.3 years, and mean daily hot flush frequency was 7.1. Mean study duration was 17 weeks. Trials were grouped into categories according to type of phytoestrogen: soy foods, beverages, or powders (n = 11); soy extracts (n = 9); and red clover extracts (n = 5). Of the 8 soy food trials reporting hot flush frequency outcomes, 7 were negative. Five trials of soy foods provided information to calculate effect sizes; these were in the small-to-medium range, favoring placebo in 3 trials and soy in 2. Of the 5 soy extract trials reporting hot flush frequency, 3 (including the 2 largest trials) were negative. Effect sizes were calculated for 2 soy extract trials: one favored placebo with small effect size and the other favored soy with moderate effect size. Red clover trials showed no improvement in hot flush frequency (weighted mean difference -0.60, 95% confidence interval -1.71 to 0.51). Adverse effects were primarily gastrointestinal and taste intolerance in the soy food and beverage trials.

CONCLUSION: The available evidence suggests that phytoestrogens available as soy foods, soy extracts, and red clover extracts do not improve hot flushes or other menopausal symptoms.

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Complementary and Alternative Medicine for Menopausal Symptoms: A Review of Randomized, Controlled Trials

Fredi Kronenberg, PhD, and Adriane Fugh-Berman, MD

Background: Women commonly use soy products, herbs, and other complementary and alternative medicine (CAM) therapies for menopausal symptoms. Randomized, controlled trials have evaluated the efficacy and short-term safety of these therapies.

Purpose: To review randomized, controlled trials of CAM therapies for menopausal symptoms in order to better inform practice and guide future research.

Data Sources: Searches of MEDLINE for articles published from January 1966 through March 2002, of the Alternative and Complementary Database (AMED) of the British Library for articles published from January 1985 through December 2000, and of the authors' own extensive files. Search terms were *hot flash/flush, menopause,* and *climacteric,* combined with *phytoestrogens, alternative medicine, herbal medicine, traditional medicine, Traditional Chinese Medicine (TCM), Ayurveda, naturopathy, chiropractic, osteopathy, massage, yoga, relaxation therapy, homeopathy, aromatherapy,* and *therapeutic touch.*

Study Selection: 29 randomized, controlled clinical trials of CAM therapies for hot flashes and other menopausal symptoms were identified; of these, 12 dealt with soy or soy extracts, 10 with herbs, and 7 with other CAM therapies.

Data Extraction: Each author extracted information from half of the studies on the number of patients, study design, outcome measures, and results; the other author then checked these results.

Data Synthesis: Soy seems to have modest benefit for hot flashes, but studies are not conclusive. Isoflavone preparations seem to be less effective than soy foods. Black cohosh may be effective for menopausal symptoms, especially hot flashes, but the lack of adequate long-term safety data (mainly on estrogenic stimulation of the breast or endometrium) precludes recommending long-term use. Single clinical trials have found that dong quai, evening primrose oil, a Chinese herb mixture, vitamin E, and acupuncture do not affect hot flashes; two trials have shown that red clover has no benefit for treating hot flashes.

Conclusions: Black cohosh and foods that contain phytoestrogens show promise for the treatment of menopausal symptoms. Clinical trials do not support the use of other herbs or CAM therapies. Long-term safety data on individual isoflavones or isoflavone concentrates are not available.

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Women are frequent users of complementary and alternative medicine (CAM) therapies in many countries (1–5), including the United States, where women use CAM therapies more often than men (48.9% vs. 37.8%) (6). Many women report using these therapies for menopausal symptoms (7, 8). A national magazine received more than 15 000 responses to a survey of alternative treatments for menopausal women (7). Primary symptoms included hot flashes, joint pain, sleep problems, forgetfulness, and fatigue; hot flashes (the most common menopausal symptom for women in the United States) (9) were most commonly treated. Therapies of choice were herbal remedies, chiropractic, and meditation. Recently, dietary supplements and foods containing phytoestrogens have become increasingly popular, despite the lack of data from clinical trials.

The science of CAM therapies is still inadequate to sufficiently inform clinicians and the public of the benefits or potential risks of CAM therapies. To provide information for clinicians, we reviewed randomized, controlled clinical trials of CAM therapies for menopausal symptoms.

METHODS

Study Selection

We searched MEDLINE from January 1966 to December 2002, the Alternative and Complementary Database (AMED) of the British Library from January 1985 to December 2000, and our own extensive files. Databases were searched under the terms *hot flash/flush, menopause,* and *climacteric,* combined with *phytoestrogens, alternative medicine, herbal medicine, traditional medicine, Traditional Chinese Medicine (TCM), Ayurveda, naturopathy, chiropractic, osteopathy, massage, yoga, relaxation therapy, homeopathy, aromatherapy,* and *therapeutic touch.* We did not limit the search to English-language literature. Studies that examined single symptoms or conditions that are not clearly associated with menopause (for example, anxiety and lipids) were excluded. All other randomized, controlled trials, regardless of their quality, were included. A total of 29 studies were identified. Each author extracted information on the number of patients, study design, outcome measures, and results for half the studies; the other author then checked results.

Role of the Funding Source

The funding source had no role in the design, conduct, analyses, or reporting of the study or the decision to submit the manuscript for publication.

CAM THERAPIES

Herbal Remedies

Herbs used in the United States for menopausal problems include black cohosh (*Cimicifuga racemosa*), chaste tree berry (*Vitex agnus-castus*), dong quai (*Angelica sinensis*),

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Table 1. **Herbs for Menopausal Symptoms***

Study, Year (Reference)	Country	Design	Patient Characteristics and Age
Black cohosh			
Jacobson et al., 2001 (10)	United States	Randomized, double-blind, placebo-controlled trial	85 women (68 completed the study) with breast cancer and daily hot flashes; 59 women were using tamoxifen concurrently Age, >18 y
Warnecke, 1985 (11)	Germany	Randomized, open, treatment-controlled trial	60 women (48 menopausal; 55 completed study) with menopausal symptoms Age, 45–60 y
Stoll, 1987 (12)	Germany	Randomized, double-blind, placebo-controlled trial	80 women (41 menopausal; 75 completed the study) with climacteric symptoms, >3 hot flashes daily, and "psychic complaints" Age, 46–58 y
Lehmann-Willenbrock and Riedel, 1988 (13)	Germany	Randomized, treatment-controlled trial	60 women (41 menopausal) with climacteric symptoms who had had hysterectomy and had at least 1 ovary Age, ≥40 y
Red clover			
Baber et al., 1999 (14)	Australia	Randomized, double-blind, placebo-controlled crossover trial	51 postmenopausal women (43 completed the study) with >3 hot flashes daily Age, 45–65 y
Knight et al., 1999 (15)	Australia	Randomized, double-blind, placebo-controlled trial	37 postmenopausal women (34 completed the study) with >3 hot flashes daily Age, 40–65
Dong quai			
Hirata et al., 1997 (16)	United States	Randomized, double-blind, placebo-controlled trial	71 postmenopausal women (61 completed the study) with >14 hot flashes weekly (any severity) or >5 hot flashes weekly (moderate or severe) Age, 45–69 y
Evening primrose oil			
Chenoy et al., 1994 (17)	United Kingdom	Randomized, double-blind, placebo-controlled trial	56 postmenopausal women (35 completed the study) with >3 hot flashes daily Age, 45–67 y
Ginseng			
Wiklund et al., 1999 (18)	Sweden	Randomized, double-blind, placebo-controlled multicenter trial	384 postmenopausal women (379 completed the study) with ≥6 hot flashes during 3 of 7 days Age, 45–65 y
Chinese herb mixture			
Davis et al., 2001 (19)	Australia	Randomized, double-blind, placebo-controlled trial	78 postmenopausal women (55 completed the study) with ≥14 hot flashes or night sweats weekly Age, 45–70 y

*CGI = Clinical Global Impression Scale; FSH = follicle-stimulating hormone; HAM-A = Hamilton Anxiety Scale; HDL = high-density lipoprotein; LH = luteinizing hormone; SDS = Self-Assessment Depression Scale; SHBG = sex hormone–binding globulin; VAS = visual analogue scale; VMI = vaginal maturation index; WHQ = Women's Health Questionnaire.
† Manufacturers: Ginsana (G115), Pharmaton, Lugano, Switzerland; Ovestin, Organon, West Orange, New Jersey; Presomen, Solvay Pharmaceuticals, Marietta, Georgia; Promensil, Novogen, Sydney, Australia; Remifemin, GlaxoSmithKline, Pittsburgh, Pennsylvania; Trisequens, Novo Nordisk, Princeton, New Jersey.
‡ Comments are italicized.

ginseng (*Panax ginseng* and other *Panax* species), evening primrose oil (*Oenethera biennis*), motherwort (*Leonurus cardiaca*), red clover (*Trifolium pratense*), and licorice (*Glycyrrhiza glabra*). We identified 10 trials of herbs (**Table 1**) (10–19). Most studies found no significant changes in primary outcome measures. However, these studies were small, of short duration, and far from sufficient to yield definitive conclusions.

The most studied and perhaps most popular herb is black cohosh, traditionally used by Native Americans for

Table 1—**Continued**

Treatment, Dose, and Duration†	Outcome Measures	Findings and Comments‡
Remifemin tablets (40 mg/d herbal extract) vs. placebo Duration, 2 mo	Frequency and intensity of hot flashes; menopause symptom index (6 symptoms); global rating of health scale; FSH and LH levels	No significant difference between groups in frequency and intensity of hot flashes (decreased in both groups); significantly greater decrease in sweating in treatment group than in placebo group ($P = 0.04$); no changes in global rating scale or FSH and LH levels *Study duration was short; medication-induced hot flashes are more difficult to treat than naturally occurring hot flashes*
Remifemin (liquid) (40 drops twice daily) vs. conjugated estrogens (0.25 mg/d) vs. diazepam (2 mg/d) Duration, 3 mo	Kupperman index scores (modified 5 symptoms); HAM-A; SDS; CGI; VMI	Kupperman index, HAM-A, SDS, and CGI showed "highly significant reductions" with all three therapies; VMI: "trend towards estrogenic stimulation" for Remifemin and estrogen *No statistical calculations reported*
Remifemin tablets (4 mg twice daily) vs. conjugated estrogens (0.625 mg/d) vs. placebo Duration, 3 mo	Kupperman index scores (9 symptoms); hot flashes; HAM-A; VMI	Significant improvement in Kupperman index score, HAM-A ($P < 0.001$), and VMI ($P < 0.01$); no change in estrogen or placebo groups; hot flashes decreased from 4.9 to 0.7/d in Remifemin group, 5.2 to 3.2 in estrogen group, and 5.1 to 3.1 in placebo group (significance not indicated) *Lack of effect with estrogen calls other findings into question*
Remifemin tablets (4 mg of triterpene glycosides twice daily) vs. estriol (Ovestin, 1 mg/d) vs. conjugated estrogens (Presomen, 1.25 mg/d) vs. estrogen/progesterone combination (Trisequens) Duration, 6 mo	Kupperman index scores (modified 17 symptoms); LH and FSH levels	Kupperman index scores improved in all groups ($P = 0.01$), no differences among treatment groups; no changes in LH and FSH levels
Promensil (40 mg of total isoflavones) vs. placebo Duration, 7.5 mo (12 wk, then 4-wk washout, then 14 wk)	Frequency of hot flashes; symptoms (Greene menopause scale); LH, FSH, estradiol, and SHBG levels; VMI; endometrial thickness (evaluated using ultrasonography)	No difference between groups in frequency of hot flashes (decreased in both groups) or other outcome measures *Study too short to adequately assess estrogenic effects on endometrium; adverse events not mentioned*
Promensil (40 mg of total isoflavones) vs. Promensil (160 mg of isoflavones) vs. placebo Duration, 3 mo	Frequency of hot flashes; symptoms (Greene menopause scale); FSH, SHBG, and HDL cholesterol levels; VMI	No difference among groups in frequency of hot flashes (decreased in all groups), VMI, FSH level, or SHBG level; HDL cholesterol level increased significantly (18%) ($P = 0.038$) in 40-mg Promensil group only *Although total cholesterol and triglyceride tests were done, results were not reported (blood was collected when patients were not fasting)*
Dong quai (3 capsules 3 times/d; equivalent 4.5 g of dong quai root/d; standardized to 0.5 mg/kg ferulic acid) vs. placebo Duration, 6 mo	Kupperman index score; vasomotor flushes; endometrial thickness; VMI; vaginal dryness; estradiol, estrone, and SHBG levels	No difference between groups in Kupperman index scores (decreased significantly in both groups), number of hot flashes, VMI, or endometrial thickness *Dong quai is not considered estrogenic and not usually prescribed alone in Traditional Chinese Medicine*
Evening primrose oil (2000 mg with 20 mg of vitamin E) twice daily vs. placebo Duration, 6 mo	Hot flashes or sweating episodes	Frequency of daytime hot flashes decreased in placebo but not evening primrose oil group; no difference between groups in frequency of nighttime hot flashes (decreased in both groups)
Ginseng (Ginsana containing 100 mg of standardized extract G115) Duration, 4 mo (2-wk run-in, 14-wk treatment)	Hot flashes and other symptoms (VAS); Psychological General Well-Being index; WHQ (measures of quality of life); FSH and estradiol levels; endometrial thickness (evaluated using ultrasonography); VMI	No significant differences between groups in hot flashes (assessed as part of WHQ) or any main outcome measures
Chinese herbal formula (daily dose of *Rehmannia glutinosa*, 15 g; *Cornus officinalis*, 10 g; *Dioscorea opposita*, 12 g; *Alisma orientalis*, 8 g; *Paeonia suffruticosa*, 8 g; *Poria cocos*, 12 g; *Citrus reticulata*, 5 g; *Lycium chinense*, 20 g; *Albizzia julibrissin*, 15 g; *Ziziphus jujuba*, 10 g; *Eclipta prostrata*, 15 g; *Ligustrum lucidum*, 10 g) vs. bitter placebo Duration, 3 mo	Hot flashes and night sweats (by daily diary); Menopause-Specific Quality-of-Life Questionnaire scores; urinary phytoestrogen excretion	No difference between groups in frequency of vasomotor symptoms or Menopause-Specific Quality-of-Life Questionnaire score (both decreased in both groups); urinary phytoestrogen levels did not change in either group

gynecologic and other conditions. Almost all clinical studies of black cohosh have used the standardized product Remifemin (GlaxoSmithKline, Pittsburgh, Pennsylvania); however, the formulation and the dosage have changed over time. One of four randomized, controlled trials of black cohosh for hot flashes was placebo controlled (10), one used both a treatment and placebo control (12), and two were treatment controlled (11, 13). Three of the four trials found black cohosh to be beneficial for treating hot flashes.

Table 2. **Soy and Soy Extracts for Hot Flashes***

Study, Year (Reference)	Country	Study Design	Patient Characteristics and Age
Van Patten et al., 2002 (34)	Canada	Randomized, double-blind, placebo-controlled trial	157 postmenopausal women (123 completed the study) with breast cancer and hot flashes
Han et al., 2002 (35)	Brazil	Randomized, double-blind, placebo-controlled trial	82 postmenopausal women (80 completed the study) with hot flashes Age, 45–55 y
St. Germain et al., 2001 (36)	United States	Randomized, double-blind, placebo-controlled trial	91 perimenopausal women (69 completed the study) with ≥10 hot flashes weekly Age, 42–62 y
Scambia et al., 2000 (37)	Italy	Randomized, double-blind, placebo controlled trial	39 postmenopausal women (22 completed the study); included surgical and early menopause Age, 29–63 y
Quella et al., 2000 (38)	United States	Randomized, double-blind, placebo-controlled, crossover trial	177 women (149 completed the study) with history of breast cancer and <14 hot flashes weekly; most patients were receiving tamoxifen or raloxifene Age, ≥18 y
Upmalis et al., 2000 (39)	United States	Randomized, double-blind, placebo-controlled, multicenter trial	177 postmenopausal women (117 completed the study) with ≥5 hot flashes daily Age, ≥50 y
Washburn et al., 1999 (40)	United States	Randomized, double-blind, placebo-controlled, crossover trial	51 perimenopausal women (42 completed the study) with >1 hot flash daily Age, 45–55 y
Albertazzi et al., 1998 (41)	Italy	Randomized, double-blind, placebo-controlled multicenter trial	104 postmenopausal women (79 completed the study) with ≥7 hot flashes daily Age, 45–62 y
Dalais et al., 1998 (42)	Australia	Randomized, double-blind, placebo-controlled, crossover trial	52 postmenopausal women (44 completed the study) with ≥14 hot flashes weekly Age, 45–65 y
Brzezinski et al., 1997 (43)	Israel	Randomized, placebo-controlled, unblinded trial	145 peri- and postmenopausal women (114 completed the study) with >1 climacteric symptom Age, 43–65 y
Murkies et al., 1995 (44)	Australia	Randomized, placebo-controlled trial	58 postmenopausal women (47 completed the study) with >14 hot flashes weekly Age, 30–70 y
Baird et al., 1995 (45)	United States	Randomized, controlled trial	97 postmenopausal women (91 completed the study) Age, 45–65 y

* FSH = follicle-stimulating hormone; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LH = luteinizing hormone; SHBG = sex hormone–binding globulin; VMI = vaginal maturation index.
† Manufacturer: SOYSELECT, Indena, Settala, Milan, Italy.
‡ Comments are italicized.

Neither the identity of active compounds nor the mechanism of action of black cohosh is known. Although formononetin, an estrogenic isoflavone, was reported to have been isolated from black cohosh extract (20), another study found no formononetin in black cohosh extract (21). In addition, a recent systematic examination of extracts of

Table 2—**Continued**

Treatment, Dose, and Duration†	Outcome Measures	Findings and Comments‡
Soy beverage (90 mg of isoflavones/d) vs. control (rice beverage) Duration, 12 wk	Hot flash frequency and hot flash score (number × severity)	No difference between groups; hot flash scores decreased in both the soy group (30%) and the control group (40%). More gastrointestinal effects were reported in the soy group
Capsules containing daily dose of 150.9 mg of soy protein and 100 mg of isoflavones (69.9 mg of genistein, 18.6 mg of daidzein, 11.4 mg of glycitein) Duration, 4 mo	Kupperman index score; lipids; body mass index; blood pressure; glucose, FSH, LH, and 17β-estradiol levels	Compared with placebo, treatment decreased menopausal symptoms ($P < 0.01$), decreased total cholesterol and LDL levels ($P < 0.01$), and increased 17β-estradiol levels (from 9 ± 1.2 pg/mL to 19 ± 2.2 pg/mL, $P < 0.001$). No effects on other outcome measures *This was the only study that found increased estradiol levels*
40 g/d of isoflavone-rich soy protein (80.4 mg aglycones) vs. 40 g/d of isoflavone-poor soy protein (4.4 mg/d of aglycones) vs. 40 mg/d of control (whey protein) Duration, 24 wk	Frequency and severity of hot flashes and night sweats; insomnia; fatigue; mood swings; vaginal dryness and other menopausal symptoms	No significant difference between groups in frequency or severity of hot flashes or night sweats at 12 or 24 weeks (hot flashes decreased in all groups); no changes in other outcome measures *Longest study to date; 6-wk data not presented; sporadic symptoms throughout the study (menopausal transition)*
Standardized soy extract (SOYSELECT: 400 mg/d [50 mg/d of isoflavones]) vs. placebo. After 6 wk, conjugated estrogens (0.625 mg/d) were added to treatment; after 8 wk, soy was discontinued and medroxyprogesterone acetate (10 mg/d) was added to the conjugated estrogens Duration, 12 wk (6 wk treatment, 4-wk treatment plus conjugated estrogens; 2-wk conjugated estrogens plus medroxyprogesterone acetate)	Frequency and severity of hot flashes; Greene menopause scale; VMI; endometrial thickness; pulsatility index of uterine artery; total cholesterol, HDL, LDL, triglyceride, FSH, and LH levels	Compared with placebo, treatment decreased frequency and severity of hot flashes ($P < 0.01$ and $P < 0.001$) at 6 wk; soy caused no changes in other outcome measures; conjugated estrogens caused predictable estrogenic changes in both groups
Soy tablets containing daily dose of 150 mg of isoflavones vs. placebo Duration, 8 wk (each phase, 4 wk)	Frequency and intensity of hot flashes and hot flash score (frequency × severity)	No difference between groups in hot flash score or frequency (frequency of hot flashes decreased in both groups); patients did not prefer soy over placebo
Standardized soy isoflavone extract containing 50 mg of genistein plus daidzein daily vs. placebo Duration, 12 wk	Frequency and severity of hot flashes and night sweats; SHBG and FSH levels; VMI; endometrial thickness (evaluated using ultrasonography); *N*-telopeptide; quality-of-life measures; total cholesterol, LDL, HDL, and triglyceride levels	Compared with placebo, treatment reduced frequency of hot flashes at 6 wk ($P = 0.03$); at 12 wk, treatment reduced severity of hot flashes and night sweats ($P = 0.01$), but not frequency of hot flashes or night sweats; no changes in other outcome measures *Relative lack of soy protein in supplement*
20 g of soy protein powder daily (34 mg of isoflavones) in single or split doses vs. control (isoflavone-free carbohydrate) Duration, 18 wk (each phase lasted 6 wk)	Frequency and severity of hot flashes; estrogen symptom score (including hot flashes, vaginal dryness, sleep disturbances); total cholesterol, LDL, HDL, and triglyceride levels; blood pressure	Severity of hot flashes ($P < 0.001$) and estrogen symptom score ($P < 0.05$) improved only in split-dose soy group; no differences among groups in frequency of hot flashes; compared with control, treatment (for both soy groups) decreased total cholesterol and LDL levels ($P < 0.05$); no changes in HDL or triglyceride levels; diastolic blood pressure decreased ($P < 0.01$) only in split-dose soy group *No washout between crossover; baseline number of hot flashes not reported*
60 g of isolated soy protein powder daily (40 g of protein, 76 mg of isoflavones [aglycones]) vs. control (isoflavone-free casein powder containing 40 g of protein) Duration, 12 wk	Frequency and severity of hot flashes; Kupperman index scores; blood pressure	Compared with control, treatment significantly reduced frequency of hot flashes (45% vs. 31%) at 12 wk ($P < 0.01$); no change in Kupperman index score
45 g/d (in form of bread) of soy grits (53 mg/d isoflavones) or linseed (flaxseed) vs. control (wheat meal) Duration, 28 wk (each phase lasted 12 wk, 4-wk washout)	Frequency of hot flashes; VMI; bone mineral content; bone mineral density	Compared with baseline, linseed and wheat (but not soy) decreased frequency of hot flashes; only soy improved VMI (increase of 103%) ($P < 0.04$) and bone mineral content (increase of 5.2%) ($P = 0.03$); no changes in bone mineral density *Increased bone mineral content in soy group over short time is of note*
Phytoestrogen-rich diet containing soybean food and flaxseed (1/4 caloric intake) vs. control (normal diet) Duration, 12 wk	Menopause symptom questionnaire; estradiol, LH, FSH, SHBG, and serum phytoestrogen levels	No differences between groups in menopause symptom questionnaire scores, which decreased in both groups; compared with control, treatment significantly increased SHBG levels ($P < 0.003$) and significantly decreased vaginal dryness ($P = 0.005$) and hot flash "severity score" ($P = 0.004$) assessed as part of the menopause symptom questionnaire *Report states that estradiol level did not change significantly; however, table shows estradiol decreased in both groups; no report of 6-wk data*
45 g/d of soy flour vs. 45 g/d of wheat flour Duration, 12 wk	Frequency of hot flashes and hot flash score (frequency × severity); other menopausal symptoms; VMI; lipid and FSH levels	No significant differences between groups in menopausal symptoms, frequency of hot flashes or hot flash score (decreased in both groups), or other outcome measures; no change in VMI or lipids; compared with baseline, FSH level decreased significantly only in the control group
Soy diet (1/3 caloric intake; estimated 165 mg/d of isoflavones) vs. control (usual diet) Duration, 4 wk	VMI; LH, FSH, and SHBG levels	No significant changes in any outcome measures *Hot flashes not measured; trial very short in duration*

black cohosh collected from 13 locations in the eastern United States as well as Remifemin also found no formononetin in any sample (22). Thus, other compounds must be responsible for its biological activity. Small amounts of biochanin, another isoflavone, have been isolated from *C. racemosa* roots (23). Other candidates for the

biological activity of black cohosh include triterpene glycosides, organic acids, and esters.

Black cohosh is not usually used on a long-term basis, and no clinical trials have lasted for more than 6 months. This is of concern because women using this product for "natural" hormone replacement therapy may take it for years. There are no published data from human trials about long-term safety, particularly regarding endometrial or breast stimulation. Effects on vaginal epithelium are inconclusive; two of five randomized, controlled trials that examined estrogenic effect on vaginal epithelium reported a stimulatory effect (11, 12). In vitro and in vivo studies are not consistent or sufficient. Although black cohosh may be useful for menopausal symptoms, long-term use cannot be presumed to be safe until appropriate safety studies are conducted.

Red clover contains the phytoestrogens formononetin, biochanin A, daidzein, and genistein (24). Two small, 3-month clinical trials conducted in Australia reported no significant benefit of red clover extract for hot flashes (14, 15) (**Table 1**). Several larger trials are in progress. Red clover, a Native American herb, has not been traditionally used on a long-term basis for hot flashes, and it is unknown whether long-term use would have an estrogenic effect on the breast or endometrium.

Dong quai, a Chinese herb traditionally prescribed as a tonic for women, is most commonly used as part of a mixture. It is sold in the United States for use alone or as part of newly formulated, nontraditional herbal combinations. One trial of dong quai found no benefit for hot flashes (16) (**Table 1**). It would be valuable to study Traditional Chinese Medicine (TCM) formulas, prescribed in accordance with TCM diagnostic methods. Dong quai does not contain the typically reported phytoestrogens, and the data on stimulation of estrogen receptor–positive breast cancer cells or binding to estrogen receptors (25, 26) are conflicting. Dong quai contains coumarins and can cause bleeding when administered concurrently with warfarin (27); the furocoumarins contained in dong quai can cause photosensitization (24).

Oil of evening primrose, a good source of the prostaglandin E_1 precursor γ-linolenic acid, was evaluated for hot flashes in one trial; no differences were found between a group of patients using evening primrose oil and a placebo group (17). Single-dose studies are difficult to evaluate when optimum dose and duration of treatments are unknown. Evening primrose oil is a benign treatment.

Ginseng has been used as a tonic for centuries in Asia. One trial found no benefit of ginseng over placebo for menopausal symptoms and quality-of-life measures, although there were positive effects on mood (18). Case reports link ingestion of ginseng with postmenopausal bleeding (28, 29); one case of postmenopausal bleeding occurred after topical use of a ginseng-containing face cream (30). Ginseng also reduced the international normalized ratio in one patient receiving warfarin therapy (27).

In summary, of the herbs that have been tested for hot flashes, only black cohosh has shown a beneficial effect. Questions remain about the long-term safety of most herbs.

Dietary Phytoestrogens

Many food plants contain phytoestrogens, primarily phenolic (rather than steroidal) compounds that include isoflavones, lignans, and coumestans. Isoflavone precursors are found in soy and other types of beans, clover, and alfalfa. Lignan precursors are found in whole grains, seeds (especially flaxseed [linseed]), fruits, vegetables, rye, millet, and legumes (31). Intestinal bacteria convert plant lignans to mammalian lignans (enterolactone and enterodiol) and convert conjugated isoflavones to unconjugated active isoflavones (genistein, daidzein, and equol). Phytoestrogens are estrogenic, and, thus, diet may modulate endocrine actions in the body. High dietary intake of soy products in Japan, China, and Korea has been proposed as one reason for the lower prevalence of menopausal symptoms reported in those countries (32, 33). Soy foods have become popular in the United States for treating hot flashes, despite few strong supporting clinical studies.

We identified 11 clinical trials that examined soy or isoflavone supplementation for hot flashes (**Table 2**) (34–44); one additional study of soy for menopausal women examined vaginal epithelium rather than hot flashes (45). Products studied ranged from soy foods to purified isoflavone preparations. Only 3 of 8 studies with treatment phases that lasted for more than 6 weeks showed significant improvement in hot flashes at the end of the study (35, 41, 43). The longest study to date showed no benefit for hot flashes (or other symptoms) at 24 weeks (36). Comparisons are difficult because of variations in product, dosage, scoring systems for symptoms of hot flashes, and the menopausal status of patients. Published data show only modest effects (primarily on the severity of hot flashes), and most benefits disappeared after 6 weeks (for hot flashes, even 3 months is barely adequate to appropriately assess efficacy). It is of interest that in most of these studies, symptoms decreased in all groups—often as much as 50% to 60% in placebo as well as treatment groups. Studies of longer duration must be done to determine whether this placebo effect would have declined over time in any or all groups. Additional studies of menopausal symptoms are warranted to differentiate among whole foods, soy protein, and isoflavone extracts. Soy foods have been a staple in Asian cuisine for thousands of years and are presumed safe. Supplementing the diet with beans or bean products is a benign intervention. No such presumption of safety can be made for the isolated, often high-dose, isoflavones that are currently sold over the counter.

Vitamin E

Vitamin E, recommended in many popular books for hot flashes, was the subject of poorly controlled studies in the 1940s and 1950s. A double-blind, 3-year study in 1953

compared vitamin E (50 to 100 mg/d) with two estrogen preparations, phenobarbital, and a placebo in 658 women (46). On an 11-symptom menopause index (hot flashes were not analyzed separately), more than 90% of women treated with estrogen and 43% of women treated with phenobarbital responded; vitamin E was no more effective (26%) than placebo (33%). A recent randomized, placebo-controlled crossover study tested vitamin E (400 IU vitamin E succinate twice daily) in 125 breast cancer survivors with at least 14 hot flashes weekly (47). After 4 weeks, vitamin E was associated with one less hot flash daily when compared with placebo. The difference was statistically but not clinically significant; patients did not prefer vitamin E to placebo.

Acupuncture

In a study of the effects of acupuncture on hot flashes, 24 menopausal women were randomly assigned to either an electroacupuncture group (electrical stimulation of acupuncture needles at standardized points) or to a control group (shallow acupuncture needle insertion at the same points) (48). Women were treated twice weekly for 2 weeks and then weekly for an additional 6 weeks. Hot flashes and Kupperman index scores decreased significantly ($P < 0.05$) in both groups, and there were no differences between groups. In addition, sleep dysfunction scale scores did not change. Because shallow needle insertion at correct acupuncture points would be expected to have some effect, the chosen control may have been suboptimal. Acupuncture causes tissue trauma occasionally and serious complications (including pneumothorax and cardiac tamponade) rarely. The most common serious complication has been the transmission of hepatitis viruses or other infections agents via inadequately sterilized needles (49). Disposable needles, the standard of care in the United States, obviate this concern.

Behavioral Therapies

Few studies have examined behavioral methods for moderating hot flashes. One study compared paced respiration (slow, deep breathing) with progressive muscle relaxation or nontherapeutic α-wave electroencephalographic biofeedback (control) in 33 postmenopausal women (50). Paced respiration training for 4 months significantly reduced the frequency of hot flashes by 39% ($P < 0.02$); progressive muscle relaxation training and the control intervention had no significant effect. In a more recent trial by the same investigators, 24 postmenopausal women with at least five hot flashes daily were randomly assigned to either paced respiration or biofeedback control (51). Paced respiration decreased hot flashes significantly (44% [$P < 0.001$]); no change occurred in the control group. No adverse effects were noted.

A randomized, controlled, 7-week study of the relaxation response technique involved 45 women (33 completed the study) between 44 and 66 years of age who were experiencing at least five hot flashes daily (52). The women were randomly assigned to a relaxation response group, a reading group, or a symptom-charting (control) group. The relaxation group was instructed in relaxation response techniques and asked to practice 20 minutes daily; the reading group read leisure material for 20 minutes daily. Compared to baseline, the frequency of hot flashes did not change in any group; the intensity of hot flashes decreased significantly only in the relaxation group ($P < 0.05$). State anxiety did not change in any group; trait anxiety (assessed by using the Spielberger State-Trait Anxiety Inventory) decreased significantly only in the relaxation group ($P < 0.04$). Behavioral therapies seem to be safe; an explanation for treatment effects is needed.

Wild Yam and Progesterone Creams

Topical wild yam (*Dioscorea villosa*) and "natural" (micronized) progesterone creams have been promoted for hot flashes as well as many other conditions (53). Wild yam preparations contain diosgenin, which can be converted to progesterone in a laboratory but not in the human body. A double-blind, placebo-controlled crossover trial tested wild yam cream against placebo for 3 months in 23 symptomatic menopausal women (54). The treatment and placebo groups did not differ in hot flashes or night sweats (both improved slightly in both groups). There were no changes from baseline in body weight; blood pressure; or levels of serum estradiol, serum or salivary progesterone, total cholesterol, triglyceride, high-density lipoprotein cholesterol, follicle-stimulating hormone, and glucose.

Pharmacologically active progesterone creams are also sold over the counter. A randomized, double-blind, placebo-controlled trial on the effect of progesterone cream on bone examined hot flashes as a secondary outcome (55). Healthy postmenopausal women ($n = 102$) applied one-quarter teaspoon of progesterone cream (20 mg of progesterone) or placebo daily for 1 year. The women also received daily multivitamins and calcium (1200 mg daily). The treatment did not affect bone, but among patients who initially reported hot flashes, 5 of 26 women in the control group compared with 25 of 30 women in the treatment group reported "improvement"; this difference was significant ($P < 0.001$). Eight women treated with progesterone cream had vaginal spotting. Endometrial biopsy revealed proliferative endometrium in one woman; biopsies for the other seven women retrieved insufficient tissue for diagnosis. The induction of postmenopausal bleeding is worrisome and should be explored further.

Consumers sometimes use transdermal progesterone creams as the progestin component of hormone replacement therapy; however, three studies have shown that serum levels of progesterone after application of transdermal creams are insufficient to prevent estrogenic stimulation of the endometrium (56–58).

DISCUSSION

Hot flashes are the primary symptom for which menopausal women seek "natural" treatments. In well-designed but small trials, vitamin E, red clover, evening primrose oil, and dong quai were ineffective for hot flashes. Several controlled trials supported the use of black cohosh for menopausal symptoms; the two placebo-controlled studies that specifically examined hot flashes found opposite results. As herbs usually take longer than estrogen therapy to work, the typical 3-month pharmaceutical trial model may be too short to accurately assess the value of an herb (in all studies of herbs, duration of treatment is important). Safety data on long-term use of herbs are inadequate. Because of the popularity of black cohosh, it is extremely important to elucidate both its benefits and potential risks (especially on stimulation of endometrial or breast tissue). Behavioral approaches (such as paced respiration) are safe, have shown some initial promise, and deserve further study. There are sufficient data to discourage the use of natural progesterone cream as the progestational component of hormone replacement therapy or for the treatment of osteoporosis.

Not all herbs and dietary supplements are risk-free. Many herbs are being promoted for nontraditional uses. Newly formulated combination herbal products (often containing novel mixtures or subtherapeutic doses) should be avoided until research data are available; currently, no safety or efficacy data on such combinations have been published. The subtherapeutic doses often found in such preparations may render them harmless, but even that cannot be counted on. Although plant foods are presumed to be safe, the isolated, often concentrated components have not been tested for long-term use. Furthermore, because of the lack of adequate product quality control in the United States, it is difficult to identify the reliable supplement brands. The number of herbal products in the U.S. marketplace is growing rapidly, and recommended dosages are increasing without research supporting the need for these larger doses.

Health care providers and consumers should be aware that despite the potential usefulness of many CAM therapies, scientific research is limited. Product advertising hype far exceeds scientific knowledge, and scientific evaluation is essential to enable an informed choice among treatments. The popularity of CAM therapies and their therapeutic potential necessitate definitive safety and efficacy studies. Because herbs, vitamins, and other natural products cannot be patented, industry-sponsored research will remain limited. Federal and alternative funding sources must bridge this gap.

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Nonhormonal Therapies for Menopausal Hot Flashes

Systematic Review and Meta-analysis

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Context Concern regarding the adverse effects of estrogen and other hormones for treating menopausal symptoms has led to demand for other options; however, the efficacy and adverse effects of nonhormonal therapies are unclear.

Objective To assess the efficacy and adverse effects of nonhormonal therapies for menopausal hot flashes by reviewing published randomized controlled trials.

Data Sources MEDLINE (1966-October 2005), PsycINFO (1974-October 2005), and the Cochrane Controlled Clinical Trials Register Database (1966-October 2005) were searched for relevant trials that provided data on treatment of menopausal hot flashes using 1 or more nonhormonal therapies.

Study Selection All English-language, published, randomized, double-blind, placebo-controlled trials of oral nonhormonal therapies for treating hot flashes in menopausal women measuring and reporting hot flash frequency or severity outcomes.

Data Extraction Trials were identified, subjected to inclusion and exclusion criteria, and reviewed. Data on participants, interventions, and outcomes were extracted and trials were rated for quality based on established criteria. A meta-analysis was conducted for therapies with sufficient trials reporting hot flash frequency outcomes.

Data Synthesis From 4249 abstracts, 43 trials met inclusion criteria, including 10 trials of antidepressants, 10 trials of clonidine, 6 trials of other prescribed medications, and 17 trials of isoflavone extracts. The number of daily hot flashes decreased compared with placebo in meta-analyses of 7 comparisons of selective serotonin reuptake inhibitors (SSRIs) or serotonin norepinephrine reuptake inhibitors (SNRIs) (mean difference, −1.13; 95% confidence interval [CI], −1.70 to −0.57), 4 trials of clonidine (−0.95; 95% CI, −1.44 to −0.47), and 2 trials of gabapentin (−2.05; 95% CI, −2.80 to −1.30). Frequency was not reduced in meta-analysis of trials of red clover isoflavone extracts and results were mixed for soy isoflavone extracts. Evidence of the efficacy of other therapies is limited due to the small number of trials and their deficiencies. Trials do not compare different therapies head-to-head and relative efficacy cannot be determined.

Conclusion The SSRIs or SNRIs, clonidine, and gabapentin trials provide evidence for efficacy; however, effects are less than for estrogen, few trials have been published and most have methodological deficiencies, generalizability is limited, and adverse effects and cost may restrict use for many women. These therapies may be most useful for highly symptomatic women who cannot take estrogen but are not optimal choices for most women.

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Hot flashes are the most common symptom related to menopausal transition.[1] They are experienced by more than 50% of menopausal women, can persist for several years after menopause, and for some women can interfere with activities or sleep to such a degree that treatment is requested.[1] A hot flash, or flush, is the spontaneous sensation of warmth, often associated with perspiration, palpitations, and anxiety, resulting from a vasomotor response to decreasing estrogen levels. It is hypothesized that endorphin concentrations in the hypothalamus decrease with declining estrogen production, enhancing release of norepinephrine and serotonin. This lowers the set point in the thermoregulatory nucleus and leads to inappropriate heat-loss mechanisms.[2-4]

Estrogen has been used as a hormone supplement for nearly 60 years to treat menopausal symptoms, and

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randomized controlled trials (RCTs) indicate that estrogen reduces the frequency of hot flashes by 77%[5] or by approximately 2.5 to 3 hot flashes daily[6] compared with placebo. However, recent studies reporting adverse effects of estrogen,[7-9] such as cardiovascular events and breast cancer, raise important concerns about its use. Following the publication of the Women's Health Initiative trial in 2002 comparing estrogen with placebo, annual dispensed prescriptions for estrogen therapy in the United States declined from 91 million users in 2001 to 57 million users in 2003.[10] Treatment of menopausal symptoms remains an indication for estrogen, although the US Food and Drug Administration advises physicians to use the smallest effective dose for the shortest duration possible.[11]

Concerns about the adverse effects of estrogen have led to increased interest in alternative therapies for improving menopausal symptoms. Women with breast cancer who cannot take estrogen are also seeking alternatives. A survey of women aged 45 to 65 years enrolled in a health maintenance organization conducted before the Women's Health Initiative publications reported that 22% of women used alternative therapies to manage menopausal symptoms.[12] These included stress management, nonprescription remedies, chiropractic and naturopathic care, massage therapy, dietary soy, herbs, and acupuncture.[12] The idea that therapies targeting various nonestrogen components of the vasomotor response could influence symptoms is reasonable; however, evidence of their efficacy and adverse effects is generally lacking or unclear.

The purpose of our review is to compare the efficacy and adverse effects of therapies other than agents primarily composed of estrogen, progestin or progesterone, or androgen for menopausal hot flashes based on published RCTs. For simplicity, these are referred to as nonhormonal therapies, although some therapies such as plant isoflavones have weak estrogenic and antiestrogenic activities. This review uses a methodology that determines the strengths and limits of available evidence to provide a comparative assessment of treatment options.

Therapies included in this review and meta-analysis were selected from a broader systematic review of treatments for menopausal symptoms conducted by the investigators.[1] Trials reported from November 2004 through October 2005 were added to the previous review.[1] To compare efficacy across therapies, including estrogen, we included trials using ingested agents and comparable placebo treatments that reported hot flash outcomes. These included trials of antidepressants, other prescribed medications such as clonidine, and isoflavone extracts derived from red clover and soy. Trials of other therapies, such as dietary forms of isoflavones (flour, powder, food items), herbs and other dietary supplements, acupuncture, energy therapies, and behavioral therapies such as relaxation techniques, were not included because of nonstandardization of doses and forms and dissimilar approaches to blinding and using controls. Available trials of these therapies do not support their efficacy in improving hot flashes and are described elsewhere.[1]

METHODS

We identified relevant trials from comprehensive searches of MEDLINE (1966-October 2005), PsycINFO (1974-October 2005), and the Cochrane Controlled Clinical Trials Register Database (1966-October 2005). We also searched MANTIS (1880-July 2004) and Allied and Complementary Medicine Database (AMED) (1985-August 2004) databases, but these yielded no relevant trials. Specific search terms are available from the authors.[1] Additional articles were obtained by manually searching recent systematic reviews,[13-15] reference lists of relevant articles and Web sites, and by consulting experts. We entered citations into an electronic database using EndNote version 6.0 (Thomson ISI ResearchSoft, Carlsbad, Calif).

Only published English-language, randomized, double-blind, placebo-controlled trials providing data on treatment of menopausal hot flashes using 1 or more nonhormonal therapies as described above were included. Head-to-head trials without a placebo group that compared nonhormonal therapies with estrogen or other medications were excluded because of difficulty interpreting results without a placebo.

Participants included women experiencing menopausal hot flashes who were recruited from health care settings or the general population. Trials enrolling women with breast cancer were included and additional data unique to them, such as concomitant use of tamoxifen or other selective estrogen receptor modulators (SERMs), were obtained. Trials of women with other major diseases or estrogen use within 1 month of commencement of the study were excluded.

Trials were included if they measured frequency or severity of hot flashes. Frequency was obtained by self-report using symptom diaries. Severity measures were also self-reported but in a variety of ways, such as using a graded scale or a composite measure (frequency × severity).[16] Hot flash frequency and composite measures have demonstrated validity and reliability, and are highly correlated.[16] Some trials used cumulative symptoms scores, such as those obtained with the Kupperman Index[17] and Greene Climacteric Scale,[18] to describe hot flashes as well as various other menopausal symptoms. However, use of these scores is problematic because some have not been validated, they contain different components and cannot be directly compared, and measures of vasomotor symptoms may not be specifically expressed. Outcomes were determined by the differences in hot flashes measured at baseline compared with the end of the trial. Treatment effects were defined as the differences in outcomes between the treatment and placebo groups at the end of the trial.

From each trial, we abstracted patient characteristics, eligibility crite-

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ria, interventions (treatment, dose, duration), comparisons, numbers enrolled and lost to follow-up, method of outcome ascertainment, results for each outcome, and adverse effects. Intention-to-treat results were recorded if available. Benefits and adverse effects of therapies were considered equally important and both types of outcomes were abstracted and summarized.

We reviewed all eligible trials for quality and applied a best-evidence approach that emphasized trials with the highest quality and most rigorous design.[19] Two reviewers independently rated the quality of trials based on internal and external validity using criteria developed by the US Preventive Services Task Force.[20] These criteria incorporate key elements of the CONSORT checklist.[21] We also considered additional criteria relating to trial size (≥50 patients per group) and duration (≥4 weeks) based on methodological research on studies of hot flashes.[16] Interreviewer agreement was high overall (κ=0.78). When reviewers disagreed, a final rating was reached through consensus with a third reviewer (H.D.N.).

We performed a meta-analysis to determine combined estimates of the efficacy of therapies in reducing hot flash frequency, the most uniformly reported outcome measure across trials. Trials eligible for meta-analysis provided adequate data for hot flash frequency outcomes. In some cases, we obtained additional data by contacting investigators.[22,23] The mean difference (SE) in number of hot flashes per day between treatment and control groups was obtained or estimated from each trial. When the mean difference (SE) was estimated, we assumed correlation between baseline and end points to be zero to produce the most conservative estimates. Most crossover trials in this review did not report adequate crossover analysis and did not include washout periods, limiting interpretation of summary statistics. To combine crossover trials with trials of parallel design in a single meta-analysis, we used data from the first period only.[24]

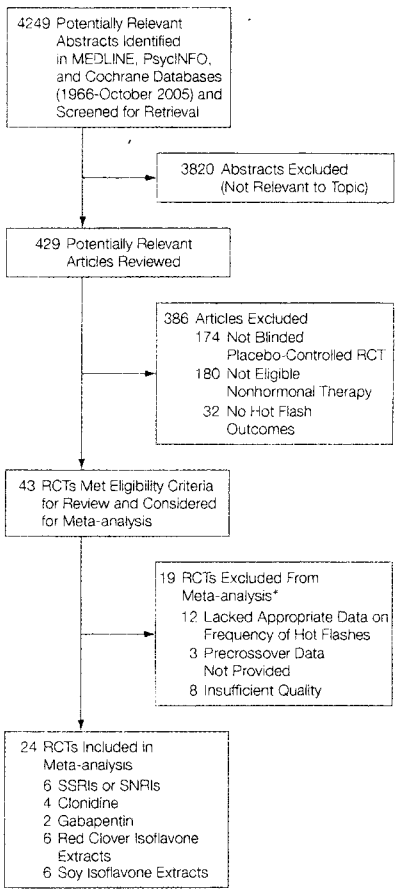
We used a random effects model[25] because there was evidence of heterogeneity between trials based on the χ^2 test for heterogeneity at a significance level of P=.10 and the I^2 statistic, percentage of total variation across studies due to heterogeneity rather than chance, of more than 25%.[26] Trials were stratified by type of therapy and duration of follow-up when data allowed. We performed sensitivity analysis to evaluate the effects of dose, concurrent use of SERMs, quality of trials (eliminating trials rated poor quality), and degrees of correlation between baseline and outcome measures.

We assessed publication bias by using funnel plots and Egger's linear regression method.[27] No publication bias was detected by these methods, however, their interpretation is limited by the small numbers of trials for each therapy.[28] All analyses were performed using Stata version 9.0 (StataCorp LP, College Station, Tex).

RESULTS

Of 4249 abstracts, 43 trials met inclusion criteria, which included 10 trials of antidepressants, 10 trials of clonidine, 6 trials of other prescribed medications, and 17 trials of isoflavone extracts (FIGURE 1).

Figure 1. Search and Selection of Trials



RCTs indicates randomized controlled trials; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin norepinephrine reuptake inhibitors. Articles identified from database searches were subjected to inclusion and exclusion criteria for the systematic review and meta-analysis.
*Some trials had more than 1 reason for exclusion.

Antidepressant Medications

Ten trials of antidepressant medications met inclusion criteria (TABLE 1).[22,23,29-36] These included 6 trials of selective serotonin reuptake inhibitors (SSRIs) or serotonin norepinephrine reuptake inhibitors (SNRIs) (paroxetine,[29,30] venlafaxine,[22,31] fluoxetine,[23,32] and citalopram[23]), 3 trials of the antidopaminergic drug veralipride,[33-35] and 1 trial of a selective monoamine oxidase-A inhibitor moclobemide.[36]

Two trials reported significantly reduced hot flash frequency and severity with paroxetine compared with placebo.[29,30] Women enrolled in a good-quality trial taking paroxetine controlled release (12.5 mg/d or 25 mg/d) experienced fewer daily hot flashes than women taking placebo (3.2-3.3 vs 1.8 fewer episodes, P=.01).[29] In a fair-quality trial that included predominantly women with breast cancer using tamoxifen, those women taking 10- and 20-mg/d doses of paroxetine also had fewer daily hot flash episodes compared with placebo (50%-51% vs 16%, P<.001).[30] Composite scores (frequency × severity) were reduced by 27% to 35% more than placebo in the 2 trials.[29,30] Although treatment effects were comparable between dose levels, more women taking higher doses experienced adverse effects in both trials, including headache, nausea, and insomnia or drowsiness.[29,30]

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Table 1. Placebo-Controlled Trials of Antidepressant Medications*

Source	No. of Participants	Sample	Therapy	Length of Trial	Main Outcomes/Results	Main Adverse Effects	Trial Quality
Stearns et al.[29] 2003	165	≥14 Hot flashes per wk; 7% have breast cancer; 7% use tamoxifen or raloxifene; mean age, 54-55 y (range, 36-76 y); 17 US sites	Paroxetine CR, 12.5 mg/d Paroxetine CR, 25 mg/d	6 wk	Reduced frequency (3.2-3.3 vs 1.8 fewer episodes per d, $P = .01$); reduced composite score (62%-65% vs 38%, $P = .03$), with both doses vs placebo	26 Withdrawals, 14 from adverse effects; 20% using 12.5 mg/d and 31% using 25 mg/d had adverse effects likely related to paroxetine, most commonly headache, nausea, and insomnia	Good
Stearns et al.[30] 2005	151	≥14 Hot flashes per wk; >80% have breast cancer; >60% use tamoxifen; mean age, 50-55 y (range, 27-76 y); 6 US sites	Paroxetine, 10 mg/d Paroxetine, 20 mg/d	4 wk, each phase of crossover	Reduced frequency (50%-51% vs 16%, $P<.001$); reduced composite score (54% for both doses vs 19%, $P<.001$), with paroxetine vs placebo	39 Withdrawals, 17 from adverse effects; 17% using 20 mg/d withdrew due to toxicity vs 5% using 10 mg/d, most commonly for drowsiness and nausea	Fair†
Evans et al.[22] 2005	80	>14 Hot flashes per wk; mean age, 52-53 y; United States	Venlafaxine XR, 75 mg/d	12 wk	No differences in frequency or severity; greater reduction in self-perceived hot flash score with venlafaxine vs placebo (51% vs 15%, $P<.001$)	19 Withdrawals; dry mouth, sleeplessness, and decreased appetite reported	Fair†‡
Loprinzi et al.[31] 2000	221	>14 Hot flashes per wk; all have breast cancer or perceived high risk; 69% use tamoxifen; aged >18 y; United States	Venlafaxine XR, 37.5 mg/d Venlafaxine XR, 75 mg/d Venlafaxine XR, 150 mg/d	4 wk	Reduced frequency (30%-58% vs 19%, $P<.001$); reduced composite score (37%-61% vs 27%, $P<.001$), with all doses vs placebo; greater effect with 75 mg/d and 150 mg/d; similar effects in women using tamoxifen	30 Withdrawals; dry mouth, decreased appetite, nausea, and constipation were frequent with higher doses	Good
Loprinzi et al.[32] 2002	81	>14 Hot flashes per wk; all have breast cancer or perceived high risk; 54% use tamoxifen; most aged ≥50 y; United States	Fluoxetine, 20 mg/d	4 wk, each phase of crossover	Before crossover: no differences in frequency or composite score; summary statistics: reduced frequency (1.5 episodes per d less, $P = .01$), reduced composite score (24% more reduction, $P = .02$), with fluoxetine vs placebo	15 Withdrawals; no differences in symptoms between groups	Fair‡
Suvanto-Luukkonen et al.[23] 2005	150	Natural postmenopause with symptoms, no mental illness; mean age, 54 y (range, 45-66 y); Finland	Fluoxetine, 20 mg/d increased to 30 mg/d at 6 mo Citalopram, 20 mg/d increased to 30 mg/d at 6 mo	9 mo	Reduced frequency (>50% decrease in 58%-64% of women) and Kupperman Index scores in all groups from baseline ($P<.001$); between-group differences not reported	53 Withdrawals; similar rates in each group; 15 reported adverse effects, most commonly nausea and dry mouth; 7 of 8 dose reductions were in treatment groups; 1 case of pulmonary embolism in the citalopram group	Fair†
David et al.[33] 1988	50	≥6 Hot flashes per d; mean age, 55 y (range, 32-81 y), menopause clinic in Israel	Veralipride, 100 mg/d	20 d courses in 4 phases of crossover	Reduced frequency and severity from baseline with veralipride vs placebo (81%-93% vs 30%); no between-group differences	3 Withdrawals; 14 reported bilateral galactorrhea, 6 gastrointestinal complaints, 4 asthenia, and 2 tachypnea and dyspnea	Poor‡§‖
Melis et al.[34] 1988	40	Postmenopause with severe hot flashes; mean age, 48-56 y; Italy	Veralipride, 100 mg/d	30 d	Reduced composite score with veralipride vs placebo (66% vs 26%, $P<.05$); more women noted improvement with veralipride vs placebo (85% vs 50%, $P<.05$)	No withdrawals; 3 reported mastodynia	Poor‡¶
Zichella et al.[35] 1986	30	Postmenopause with severe hot flashes; mean age, 45-55 y; Italy	Veralipride, 100 mg/d	20 d	Reduced composite score with veralipride vs placebo (−2.3 vs −0.6 points, $P<.05$)	Withdrawals not reported; 10 reported mastodynia and 2 galactorrhea	Poor†‡§¶
Tarim et al.[36] 2002	30	≥14 Hot flashes per wk; mean age, 51 y (range, 35-55 y), menopause clinic in Turkey	Moclobemide, 150 mg/d Moclobemide, 300 mg/d	5 wk	Reduced frequency and composite score from baseline in all groups (score reduction: 150 mg/d [70%], 300 mg/d [35%], placebo [24%]); between-group differences not reported	2 Withdrawals due to somnolence	Poor‡‖

Abbreviations: CR, controlled release; XR, extended release.
*Composite score equals frequency × severity. Six trials of 4 antidepressant medications were included in meta-analysis.[22,23,29-32]
†Less than 80% follow-up or not reported.
‡Size of trial groups was less than 50 participants.
§Length of trial was less than 4 weeks.
‖Inadequate analysis or unable to determine.
¶No frequency data.

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A good-quality trial comparing 3 doses of venlafaxine extended release (37.5, 75, or 150 mg/d) with placebo in women with breast cancer or with self-perceived high risk of breast cancer reported decreased hot flash frequency (30%-58% vs 19%, $P<.001$) and decreased composite scores (37%-61% vs 27%, $P<.001$) with all doses of venlafaxine.[31] Effects were greater with the 2 higher doses compared with the lowest dose, although it is not clear from the data that these differences were statistically significant.[31] Women using tamoxifen had similar results as nonusers. A smaller fair-quality trial of women without breast cancer reported no differences in frequency or composite score for women using venlafaxine extended release (75 mg/d) compared with placebo.[22] In this trial, venlafaxine users had a greater reduction in a measure of their perception of how hot flashes interfere with daily living (51% improved with venlafaxine vs 15% with placebo, $P<.001$). Adverse effects of venlafaxine included dry mouth, decreased appetite, nausea, constipation, and sleeplessness, and were more common at higher doses.[22,31]

A fair-quality trial of fluoxetine (20 mg/d) in women with breast cancer or with self-perceived high risk of breast cancer indicated no significant differences in hot flash frequency or composite score between treatment and placebo groups before crossover.[32] Another fair-quality trial in women without breast cancer compared fluoxetine (30 mg/d), citalopram (30 mg/d), and placebo and found no significant differences in frequency of hot flashes and overall Kupperman Index scores.[23] Main adverse effects included nausea and dry mouth.[23]

Veralipride was compared with placebo in 3 poor-quality trials.[33-35] Trials were conducted in the 1980s, enrolled 50 or less patients, and reported limited data. Two trials of veralipride (100 mg/d) reported reduced hot flash composite scores compared with placebo,[34,35] and in 1 trial, more women noted subjective improvement with veralipride compared with placebo (85% vs 50%, $P<.05$).[34] Another trial reported changes from baseline consistent with the other trials but did not report between-group comparisons.[33] Mastodynia, galactorrhea, and gastrointestinal complaints occurred more frequently in the veralipride group in these trials.[33-35]

A poor-quality trial of moclobemide reported reduced hot flash composite scores at 2 doses (70% reduction at 150 mg/d and 35% reduction at 300 mg/d), although comparisons with placebo (24% reduction) were not reported.[36] Two patients withdrew from the trial because of somnolence from moclobemide.

Other Prescribed Medications

Sixteen RCTs of other prescribed medications met inclusion criteria, including 10 trials of the centrally active α-adrenergic agonist antihypertensive clonidine,[37-46] 3 trials of the α-adrenergic agonist antihypertensive methyldopa,[47-49] 2 trials of the γ-aminobutyric acid analog anticonvulsive gabapentin,[50,51] and 1 trial of the antispasmodic/sedative combination ergotamine, phenobarbital, and levorotatory alkaloids.[52]

Of 10 trials comparing clonidine with placebo,[37-46] all except 3 fair-quality trials[38,45,46] met criteria for poor quality due to few patients, lack of clear inclusion and exclusion criteria, high attrition or loss to follow-up, no washout period in crossover trials, lack of data for precrossover comparisons, and short treatment duration (TABLE 2). Four trials[39,42,45,46] reported reduced frequency of hot flashes with clonidine compared with placebo and 5 did not.[37,38,41,43,44] Two trials[45,46] reporting reduced hot flash frequency included women with breast cancer taking tamoxifen; reduced severity[45] and composite score[46] were also reported. Two other trials[41,42] reported reduced hot flash severity with clonidine compared with placebo, although 3 trials found no differences.[37,38,40] Adverse effects were reported in 8 trials; dry mouth[38-40,45] and insomnia or drowsiness[39,40,45,46] occurred more frequently in women taking clonidine than placebo. Blood pressure was not adversely affected by clonidine.[37-39,41,43,44]

Three poor-quality crossover trials compared methyldopa with placebo (TABLE 3).[47-49] All trials provided precrossover statistics and none found significant differences in hot flash frequency.[47-49] One trial[49] reported that methyldopa was more effective than placebo in improving scores on a visual analog scale. Fatigue or drowsiness, dizziness, and dry mouth occurred more often among women using methyldopa than occurred with placebo.[47-49] No significant changes in blood pressure were noted[47,49]; however, 1 study[48] reported orthostatic hypotension in 1 patient.

Two trials of gabapentin,[50,51] a fair-quality trial[50] and a good-quality trial of women with breast cancer using tamoxifen,[51] reported reduced hot flash frequency and severity compared with placebo (Table 3). Women taking a gabapentin dose of 900 mg/d experienced reductions in hot flash frequency in both trials (45% vs 29% for placebo, $P=.02$[50]; 44% vs 15% for placebo, $P<.001$[51]) but not when taking a 300-mg/d dose.[51] Somnolence, fatigue, dizziness, rash, heart palpitations, and peripheral edema were reported with gabapentin use.

A poor-quality trial compared Bellergal Retard, a combination of 0.6-mg ergotamine, 40-mg phenobarbital, and 0.2-mg levorotatory alkaloids, with placebo (Table 3).[52] The number of hot flashes, severity, and sweating decreased in both the Bellergal Retard and placebo groups, but these reductions were not significantly different between groups. Adverse effects were similar between groups and included dry mouth, dizziness, and sleepiness.

Isoflavone Extracts

Seventeen RCTs of isoflavone extracts met inclusion criteria. Trials evaluated red clover isoflavones,[53-58] which contained genistein, daidzein, formononetin, and biochanin, and soy isoflavones,[59-69] which contained

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Table 2. Placebo-Controlled Trials of Clonidine*

Source	No. of Participants	Sample	Therapy	Length of Trial	Main Outcomes/Results	Main Adverse Effects	Trial Quality
Bolli and Simpson,[37] 1975	12	Women with hypertension and menopausal hot flashes; mean age, 51 y; New Zealand	0.0375 mg twice daily 0.075 mg twice daily when unresponsive to lower dose	2 wk, each phase of crossover	Reduced frequency in both the 0.075-mg and placebo groups from baseline; no between-group differences in frequency, severity, or duration	1 Woman reported nausea	Poor†‡§
Clayden et al,[38] 1974	100	Menopausal hot flashes; mean age, 50 y (range, 41-62 y); England	0.025 mg twice daily; increased if needed to maximum 0.075 mg twice daily	4 wk, each phase of crossover	Reduced frequency and improved severity and duration with clonidine from baseline; no between-group differences in mean change in frequency	14 Withdrawals; 4 for adverse effects; 3 with leg pain and ankle swelling; more patients taking clonidine had dry mouth	Fair‖
Edington et al,[39] 1980	93	>30 Hot flashes per wk; mean age, 47 y (range, 27-71 y); Canada	0.05 mg twice daily	4 wk, each phase of crossover	Reduced number of hot flashes (averaged over all 4 trials), with clonidine vs placebo (0.5 fewer episodes per d, $P<.05$)	15 Withdrawals; 6 for adverse effects (2 placebo and 4 clonidine); more patients taking clonidine had dry mouth, insomnia, and headache	Poor†‡‖
Lindsay and Hart,[40] 1978	100	Natural menopause with symptoms; mean age, 46 y (range, 35-60 y); menopause clinic in England	0.050 mg twice daily; increased if needed to maximum of 0.05 mg 3 times daily	6 wk, each phase of crossover	Improved hot flashes in both groups from baseline; no between-group differences (Blatt Menopausal Index, flushing attacks scale)	59 Withdrawals, 2 for nausea, dry mouth, and insomnia; 2 reported increased nocturia	Poor†‖¶
Nagamani et al,[41] 1987	30	Postmenopause with hot flashes; 80% had surgical menopause; mean age, 41 y (range, 25-58 y); United States	0.1 mg/d, transdermal	8 wk	Reduced frequency for both groups from baseline; no between-group differences# More women taking clonidine reported improvement on subjective measures of frequency (80% vs 36%, $P<.04$), severity (73% vs 29%, $P<.04$), and duration (67% vs 21%, $P<.03$)	Minor skin reaction	Poor†‡‖
Nappi et al,[42] 1991	35	Women undergoing oophorectomy; mean age, 44 y (range, 30-50 y); Italy	Clonidine, 0.075 mg twice daily Lisuride, 0.2 mg twice daily Transdermal 17B estradiol, 0.050 mg/d	4 wk	Reduced frequency and intensity with estradiol, clonidine, and lisuride vs placebo ($P<.01$)	Not reported	Poor†‡‖
Salmi and Punnonen,[43] 1979	40	Postmenopause with symptoms; mean age, 51 y (range, 41-57 y); Finland	0.025 mg twice daily; increased if needed to maximum 0.075 mg twice daily	6 wk, each phase of crossover	No between-group differences in intensity	3 Withdrawals; no differences in adverse effects	Poor‡‖¶
Wren and Brown,[44] 1986	19	Postmenopause with symptoms; mean age, 51 y (range, 46-56 y); menopause clinic in Australia	0.05 mg twice daily	4 wk, each phase of crossover	No between-group differences in frequency	5 Withdrawals due to nonresponse, 3 reported giddiness	Poor†‡‖
Goldberg et al,[45] 1994	116	>7 Hot flashes per wk; all have breast cancer and use tamoxifen; mean age, 54 y (range, 30-76 y); United States	0.1 mg/d, transdermal	4 wk, each phase of crossover	Before crossover: reduced frequency (44% vs 27%, $P<.04$), severity (12% vs 2%, $P<.03$), and composite score (56% vs 30%, $P<.04$) for clonidine vs placebo; summary statistics: no between-group differences for frequency, severity, or score, patients preferred clonidine vs placebo ($P=.02$)	No withdrawals for adverse effects; increased dry mouth, constipation, itchiness under the patch, and drowsiness with clonidine	Fair‖
Pandya et al,[46] 2000	198	≥1 Hot flashes per d; all have breast cancer and use tamoxifen, mean age, 53-55 y (range, 35-77 y); United States	0.1 mg/d	8 wk treatment and 4 wk follow-up	Reduced frequency (24% vs 14%, $P=.09$), hot flash score (29% vs 16%, $P=.07$), and duration (22% reduction vs 17% increase, $P=.02$) with clonidine vs placebo; no between-group differences in severity	45 Withdrawals; difficulty sleeping with clonidine vs placebo (41% vs 21%, $P=.02$)	Fair†

*Composite score equals frequency × severity. Precrossover data were not reported for references 37, 39, and 44. Four trials of clonidine were included in meta-analysis.[38,41,45,46]
†Less than 80% follow-up or not reported.
‡Size of trial groups is less than 50 participants.
§Length of trial was less than 4 weeks.
‖Inadequate analysis or unable to determine.
¶No frequency data.
#Calculated P value; not reported in study.

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predominantly daidzein, genistein, and their glucoconjugates.

Red clover isoflavones were compared with placebo in 6 trials; 1 trial rated good quality, 3 trials rated fair quality, and 2 trials rated poor quality (TABLE 4).[53-58] All 6 trials used promensil, which contained a higher proportion of biochanin and genistein, and 1 trial used rimostil,[57] which contained a higher proportion of formononetin and daidzein. Only 1 fair-quality trial[58] indicated improved hot flash frequency with promensil compared with placebo (44% reduction vs 0%, $P = .02$); no trials reported differences in severity scores on the Greene Climacteric Scale[54,56-58] or in a symptom diary.[53] Reduction in hot flashes was significantly faster with promensil than with placebo in 1 trial.[57]

Soy isoflavones were compared with placebo in 11 trials (TABLE 5).[59-69] Three trials, 2 rated fair and 1 rated poor, reported reduced hot flash frequency with soy isoflavones compared with placebo using genistein (54 mg/d),[59] genistein and daidzein (70 mg/d),[61] and genistein and daidzein (50 mg/d).[67] Three other fair-quality trials found no differences in hot flash frequency compared with placebo using similar preparations.[65,66,68] Severity scores compared with placebo improved in 3 trials using genistein, diadzein, and glycitein (33 mg/d)[62] or genistein and daidzein (50 mg/d).[67,68] Five other trials,[60,63,64,66,69] 4 trials of women with breast cancer,[63,64,66,69] found no differences in severity scores with soy isoflavones compared with placebo.

Adverse effects did not differ between isoflavone and placebo groups, although they were not well characterized in several trials. Gastrointestinal symptoms were generally the most common adverse effect with both isoflavone and placebo. Six trials evaluated endometrial thickness and found

Table 3. Placebo-Controlled Trials of Methyldopa, Gabapentin, and Bellergal Retard*

Source	No. of Participants	Sample	Therapy	Length of Trial	Main Outcomes/Results	Main Adverse Effects	Trial Quality
Andersen et al.[47] 1986	40	Menopause with hot flashes; mean age, 51 y (range, 46-60 y); Norway	Methyldopa, 375 mg/d; increased by 1 tablet every 2 wk as needed to maximum dose of 1125 mg/d	Up to 8 wk, each phase of crossover	Reduced frequency from baseline for placebo and methyldopa; no between-group differences; women taking methyldopa were less bothered by hot flashes (visual analog scale) vs placebo ($P < .05$)	14 Withdrawals; 6 from adverse effects, similar in both groups	Poor†‡§
Hammond et al.[48] 1984	10	≥30 Hot flashes per wk; mean age, 46 y (range, 36-54 y); United States	Methyldopa, 250 mg twice daily; increased after 1 wk to 3 times daily	4 wk, each phase of crossover	Reduced frequency with methyldopa and placebo from baseline; no between-group differences	1 Withdrawal; adverse effects in 6 of 10 women using methyldopa, including dizziness, nausea, and fatigue	Poor‡§
Nesheim and Saetre,[49] 1981	40	Women referred for menopausal hot flashes; age not reported; Norway	Methyldopa, 250 mg twice daily; increased if needed to maximum of 500 mg twice daily	30 d, each phase of crossover	Before crossover: reduced frequency with methyldopa from baseline ($P < .05$); no between-group differences Summary statistics: improved visual analog score with methyldopa vs placebo ($P = .002$)	12 Withdrawals; 15 taking methyldopa reported adverse effects, including tiredness, dry mouth, and edema	Poor†‡§
Guttuso et al.[50] 2003	59	≥7 Hot flashes per d; mean age, 53 y; United States	Gabapentin, 300 mg 3 times daily	12 wk	Reduced frequency (45% vs 29%, $P = .02$) and composite score (54% vs 31%, $P = .01$) for gabapentin vs placebo	5 Withdrawals; 4 withdrew from the gabapentin group from dizziness, rash, heart palpitations, and peripheral edema	Fair‡
Pandya et al.[51] 2005	420	≥2 Hot flashes per d; all have breast cancer; 71% use tamoxifen; mean age, 55 y; United States	Gabapentin, 100 mg 3 times daily Gabapentin, 300 mg 3 times daily	8 wk	Reduced frequency (44% vs 15%, $P < .001$) and severity (46% vs 15%, $P < .001$) for gabapentin (900 mg/d) vs placebo; no differences between gabapentin (300 mg/d) and placebo	73 Withdrawals; 22 for adverse effects, including increased somnolence or fatigue in gabapentin groups; more withdrawals in placebo group for lack of effect	Good
Bergmans et al.[52] 1987	71	Natural menopause with hot flashes; age not reported; the Netherlands	Bellergal Retard, 1 tablet twice daily (0.6-mg ergotamine, 40-mg phenobarbital, 0.2-mg levorotatory alkaloids)	8 wk	Reduced number of hot flashes and sweating episodes for both groups from baseline; no between-group differences; patient and investigator opinion favored Bellergal Retard (51% vs 21% placebo)	28 Withdrawals; 19 taking Bellergal Retard had adverse effects and 9 withdrew; 18 taking placebo had adverse effects and 6 withdrew; most common symptoms were dry mouth, dizziness, and sleepiness	Poor†‡§

*Composite score equals frequency × severity. Two trials of gabapentin were included in meta-analysis.[50,51]
†Less than 80% follow-up or not reported.
‡Size of trial groups is less than 50 participants.
§Inadequate analysis or unable to determine.

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no differences between isoflavone and placebo groups during the course of the trials.[54,59,62,63,67,68]

Meta-analysis

Trials of SSRIs or SNRIs, clonidine, gabapentin, red clover isoflavones, and soy isoflavones provided data for the meta-analysis. Trials were excluded from the meta-analysis because they lacked adequate frequency data, did not provide precrossover statistics, or because there were inadequate numbers of fair- or good-quality trials to combine.

Seven comparisons from 6 trials of SSRIs or SNRIs were included in the meta-analysis.[22,23,29-32] These included paroxetine (10 mg/d, 12.5 mg/d [controlled release], 20 mg/d, 25 mg/d [controlled release]), venlafaxine extended release (37.5 mg/d, 75 mg/d [2 trials]), fluoxetine (20 mg/d), and citalopram (20 mg/d). Data for 150-mg/d dose of venlafaxine extended release were not included because this dose was substantially higher than the other dosages. The combined weighted mean difference in the number of daily hot flashes for trials of SSRIs or SNRIs compared with placebo was −1.13 (95% confidence interval [CI], −1.70 to −0.57) (FIGURE 2). Sensitivity analysis indicated that the 4 trials enrolling women with breast cancer and SERM use reported significantly decreased hot flashes (mean difference, −1.40; 95% CI, −1.97 to −0.82),[29-32] although the 2 trials of women without breast cancer and SERM use did not (−0.17; 95% CI, −1.41 to 1.07).[22,23]

Four trials of clonidine provided data for the meta-analysis[38,41,45,46]: 3 trials used 0.1-mg/d dose[41,45,46] and 1 trial used a range of doses (0.05-0.15 mg/d).[38] The combined weighted mean difference in the number of daily hot flashes for clonidine compared with placebo in 4 trials was −0.95 (95% CI, −1.44 to −0.47) after 4 weeks use and in 2 trials was −1.63 (95% CI, −2.76 to −0.50) after 8 weeks use (FIGURE 3). In the 4-week trials, eliminating the poor-quality trial[41] from the analysis did not influence results (weighted mean difference, −0.95; 95% CI, −1.45 to −0.46). The 2 trials enrolling women with breast cancer and SERM use reported significantly decreased hot flashes (weighted mean difference, −1.00; 95% CI, −1.51 to −0.49),[45,46] although the 2 trials of women without breast cancer and SERM use did not (−0.53; 95% CI, −2.09 to 1.04).[38,41]

Two trials of gabapentin (900 mg/d),[50,51] including a trial of women

Table 4. Placebo-Controlled Trials of Red Clover Isoflavone Extracts*

Source	No. of Participants	Sample	Therapy	Length of Trial	Main Outcomes/Results	Main Adverse Effects	Trial Quality
Atkinson et al,[53] 2004	205	99 With hot flashes; mean age, 55 y (range, 49-65 y); breast screening unit, Cambridge, England	Promensil, 40 mg/d	12 mo	Reduced frequency (1 less per d) and menopausal symptom diary score (<1 point) in symptomatic women in both groups; no between-group differences	28 Withdrawals for various reasons, most for nonmedical issues	Fair†
Baber et al,[54] 1999	51	≥3 Hot flashes per d; mean age, 45-65 y; menopause clinic in Sydney, Australia	Promensil, 40 mg/d	12 wk, each phase of crossover	Reduced frequency (17%-20%) and overall Greene Climacteric Scale score (2.6-3.0 points) in both groups; no between-group differences	8 Withdrawals for various reasons not related to study; no between-group differences in endometrial thickness	Fair†
Jeri,[55] 2002	30	≥5 Hot flashes per d; mean age, 51-52 y; Peru	Promensil, 40 mg/d	16 wk	Reduced frequency (48.5%, $P<.001$) and severity index (47%, $P<.001$) with promensil; between-group differences not reported	Not reported	Poor†‡
Knight et al,[56] 1999	37	≥3 Hot flashes per d; mean age, 54.5 y (range, 40-65 y); university gynecology clinic in Sydney, Australia	Promensil, 40 mg/d Promensil, 160 mg/d	12 wk	Reduced frequency (29%-35%) and overall Greene Climacteric Scale score (26%-46%) in all groups; no between-group differences	2 Withdrawals; adverse effects not reported	Poor†
Tice et al,[57] 2003	252	≥35 Hot flashes per wk; mean age, 52 y (range, 45-60 y); 3 US communities	Promensil, 82 mg/d Rimostil, 57 mg/d	12 wk	Reduced frequency (34%-41%) and vasomotor component of the Greene Climacteric Scale score in all groups; no between group differences; reduction in hot flashes was faster for promensil vs placebo ($P = .03$)	6 Withdrawals, 1 with nausea; no significant associations of adverse effects with treatment	Good
van de Weijer and Barentsen,[58] 2002	30	>5 Hot flashes per d; mean age, 53-54 y (range, 49-65 y); the Netherlands	Promensil, 80 mg/d	12 wk	Reduced frequency with promensil vs placebo (44% vs 0%, $P = .02$); no difference in overall Greene Climacteric Scale score	3 Withdrawals from promensil, 3 from placebo for nonresponse; adverse effects not reported	Fair†

*All 6 trials of 2 types of red clover isoflavone extracts were included in meta-analysis.[53-58]
†Size of trial groups is less than 50 participants.
‡Less than 80% follow-up or not reported and inadequate analysis or unable to determine.

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Table 5. Placebo-Controlled Trials of Soy Isoflavone Extracts*

Source	No. of Participants	Sample	Therapy	Length of Trial	Main Outcomes/Results	Main Adverse Effects	Trial Quality
Crisafulli et al.[59] 2004	90	Natural menopause with symptoms; mean age, 51-52 y (range, 47-57 y); participants of prior bone loss trial in Italy	Genistein, 54 mg/d Estradiol, 1 mg/d combined with noresthisterone	1 y	Reduced frequency for genistein vs placebo (24% mean difference, $P<.01$); reduction with estrogen more than genistein (30% mean difference, $P<.05$)	7 Withdrawals from genistein group; no between-group differences in endometrial thickness	Fair†
Duffy et al.[60] 2003	36	Menopause, no specific symptom criteria; mean age, 57-59 y (range, 50-65 y); England	Soy capsule, 60 mg/d of isoflavones	12 wk	No between-group differences in the vasomotor component of the Greene Climacteric Scale score	3 Withdrawals due to concurrent medication use	Poor†‡§
Faure et al.[61] 2002	75	≥7 Hot flashes per d; mean age, 53-54 y; clinics in France	Soy capsule, 70 mg/d of genistein and daidzein	16 wk	Reduced frequency with soy vs placebo (61% vs 21%, $P = .01$)	6 Withdrawals from soy group, 14 from placebo; 2 withdrawals from placebo group for adverse events	Fair†‖
Han et al.[62] 2002	82	Natural menopause with hot flashes; mean age, 48-49 y (range, 45-55 y); gynecology clinics in Brazil	Soy capsule 3 times daily (each with 50 mg of soy protein, 23 mg of genistein, 6 mg of diadzein, and 4 mg of glycitein)	5 mo	Improved Kupperman Index score for vasomotor symptoms with soy vs placebo ($P<.01$)	2 Withdrawals, 1 for nausea, 1 for nonresponse; no between-group differences in endometrial thickness	Fair†‡
MacGregor et al.[63] 2005	72	Menopause with symptoms; all have breast cancer; 78% use tamoxifen; mean age, 33-70 y; Scotland	Soy capsule 2 twice daily (each with 17.5 mg of isoflavones)	12 wk	No between-group differences in severity of hot flashes	22 Withdrawals, most for nonresponse; adverse effects similar between groups	Fair†‡
Nikander et al.[64] 2003	62	Menopause with hot flashes; all have breast cancer; 5% use tamoxifen >5 mo prior; mean age, 54 y (range, 35-69 y); Finland	Soy tablet, 114 mg 3 twice daily (36% diadzein, 6% genistein, 58% glycitein)	3 mo, each phase of crossover	Improved overall Kupperman index in both groups and improved hot flash intensity with placebo; no between-group differences¶	6 Withdrawals; 2 with abdominal pain and 1 with recurrent breast cancer in soy group, 1 with vaginal bleeding in placebo group	Fair†‡§
Penotti et al.[65] 2003	62	≥7 Hot flashes per d; mean age, 53 y (range, 45-60 y); menopause clinic in Italy	Soy tablet, 72 mg/d (36 mg/d of isoflavone and 48 mg/d of soy saponine)	6 mo	40% reduction in frequency in both groups; no between-group differences	13 Withdrawals; 1 taking soy developed diarrhea, others withdrew because of lack of response; no between-group differences in endometrial thickness	Fair†§‖
Quella et al.[66] 2000	177	≥14 Hot flashes per wk; all have breast cancer; 78% use tamoxifen; aged >18 y; United States	Soy tablet, 600 mg 3 times daily (each with 40%-45% genistein, 40%-45% diadzein, and 10%-20% glycitein)	4 wk, each phase of crossover	No differences in frequency or severity score between groups¶; 36% of placebo vs 24% of soy tablet reported reduction in frequency by half ($P = .01$)	33 Withdrawals; no between-group differences in adverse effects	Fair§
Scambia et al.[67] 2000	39	Menopause, no specific symptom criteria; mean age, 53-54 y (range, 29-63 y); Italy	Soy tablet, 400 mg/d (50 mg/d of genistein and daidzein)	6 wk	Reduced frequency (40% vs 25%, $P<.05$) and vasomotor component of the Greene Climacteric Scale score with soy tablet vs placebo	No withdrawals; no adverse effects reported; no between-group differences in endometrial thickness	Poor†§
Upmalis et al.[68] 2000	177	≥5 Hot flashes per d; mean age, 54-55 y; 15 US sites	Soy tablet, 50 mg/d of genistein and daidzein	12 wk	Reduced frequency of hot flashes and night sweats in both groups; no between-group differences; reduced hot flash severity with soy vs placebo ($P = .01$)	55 Withdrawals; 30 taking soy tablet and 39 placebo reported adverse effects; respiratory, gastrointestinal, and genital/reproductive symptoms were most common; no between-group differences in endometrial thickness	Fair‖
Secreto et al.[69] 2004	262	Menopause, no specific symptom criteria; women with breast cancer included (<25%); none use tamoxifen; aged >35 y; 3 centers in Italy	Soy isoflavones, 80 mg/d (50% daidzein, 35% glycitein, 15% genistein) with 3 mg/d of melatonin Soy isoflavones, 80 mg/d Melatonin, 3 mg/d	3 mo	Improved vasomotor component of the Greene Climacteric Scale score in all groups: 69% placebo; 61% melatonin alone; 75% isoflavone alone; 62% isoflavones and melatonin; no between-group differences	20 Discontinued therapy, 12 due to adverse events; higher overall drop out rates in melatonin and placebo groups vs soy groups; gastrointestinal symptoms were most frequently reported	Poor‡

*Six trials of soy isoflavone extracts were included in meta-analysis.[59,61,65-68]
†Size of trial groups is less than 50 participants.
‡No frequency data.
§Inadequate analysis or unable to determine.
‖Less than 80% follow-up or not reported.
¶Precrossover data were not reported.

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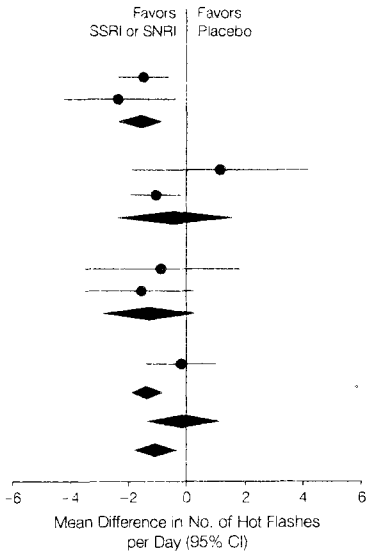
with breast cancer using tamoxifen,[51] provided a combined weighted mean difference in the number of daily hot flashes of −2.05 (95% CI, −2.80 to −1.30) compared with placebo.

Six trials of 2 types of red clover isoflavones provided data for the meta-analysis, including promensil[53-58] (40 mg/d, 80-82 mg/d, 160 mg/d) and rimostil (57 mg/d).[57] The combined weighted mean difference in the number of daily hot flashes for red clover isoflavones compared with placebo was −0.44 (95% CI, −1.47 to 0.58) (FIGURE 4). Quality of trials and type

Figure 2. Trials of Selective Serotonin Reuptake Inhibitors (SSRIs) or Serotonin Norepinephrine Reuptake Inhibitors (SNRIs)

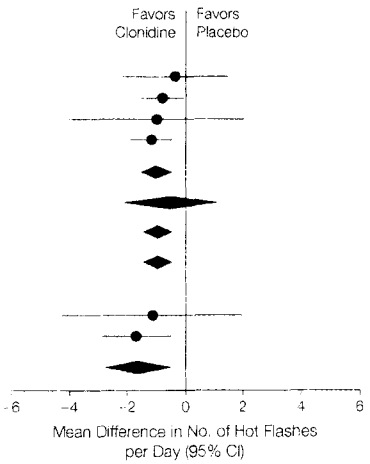
Source	Dose	No. of Participants	Duration of Trial	Quality	Mean Difference (95% CI)
Paroxetine Trials					
Stearns et al,[29] 2003	12.5 or 25 mg/d*	165	6 wk	Good	−1.52 (−2.36 to −0.69)
Stearns et al,[30] 2005	10 or 20 mg/d	151	4 wk	Fair	−2.43 (−4.43 to −0.42)
Combined					−1.66 (−2.43 to −0.89)
Venlafaxine Trials					
Evans et al,[22] 2005	75 mg/d*	80	12 wk	Fair	1.10 (−1.94 to 4.14)
Loprinzi et al,[31] 2000	37.5 or 75 mg/d*†	167	4 wk	Good	−1.09 (−1.99 to −0.18)
Combined					−0.49 (−2.40 to 1.41)
Fluoxetine Trials					
Loprinzi et al,[32] 2002	20 mg/d	81	4 wk	Fair	−0.90 (−3.78 to 1.98)
Suvanto-Luukkonen et al,[23] 2005	20 mg/d‡	100	3 mo	Fair	−1.60 (−3.63 to 0.43)
Combined					−1.37 (−3.03 to 0.29)
Citalopram Trials					
Suvanto-Luukkonen et al,[23] 2005	20 mg/d‡	100	3 mo	Fair	−0.20 (−1.45 to 1.05)
Trials With SERM Use Combined§					−1.40 (−1.97 to −0.82)
Trials Without SERM Use Combined‖					−0.17 (−1.41 to 1.07)
All Trials Combined					−1.13 (−1.70 to −0.57)



CI indicates confidence interval; SERM, selective estrogen receptor modulator. Six trials of 4 SSRIs or SNRIs provided data for meta-analysis.[22,23,29-32]
*Controlled release forms of paroxetine and extended release forms of venlafaxine.
†Data for 150-mg/d dose were not included because this dose was substantially higher than the other dosages.
‡Data for 20-mg/d dose at 3 months of follow-up were included to improve consistency with the other trials.
§Includes references 29-32.
‖Includes references 22 and 23.

Figure 3. Trials of Clonidine

Source	No. of Participants	Quality	Mean Difference (95% CI)
4-wk Trials			
Clayden et al,[38] 1974	100	Fair	−0.36 (−2.18 to 1.47)
Goldberg et al,[45] 1994	116	Fair	−0.79 (−1.55 to −0.04)
Nagamani et al,[41] 1987	30	Poor	−1.00 (−4.03 to 2.03)
Pandya et al,[46] 2000	198	Fair	−1.17 (−1.87 to −0.47)
Trials With SERM Use Combined*			−1.00 (−1.51 to −0.49)
Trials Without SERM Use Combined†			−0.53 (−2.09 to 1.04)
Fair-Quality Trials Combined			−0.95 (−1.45 to −0.46)
All 4-wk Trials Combined			−0.95 (−1.44 to −0.47)
8-wk Trials			
Nagamani et al,[41] 1987	30	Poor	−1.14 (−4.28 to 1.99)
Pandya et al,[46] 2000	198	Fair	−1.70 (−2.91 to −0.49)
All 8-wk Trials Combined			−1.63 (−2.76 to −0.50)



CI indicates confidence interval; SERM, selective estrogen receptor modulator. Four trials of clonidine provided data for meta-analysis: 3 trials used 0.1-mg/d dose[45,46] and 1 trial used a range of doses (0.05-0.15 mg/d).[38]
*Includes references 45 and 46.
†Includes references 38 and 41.

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of red clover isoflavone did not influence results. No trials were conducted in women with breast cancer using SERMs.

Six trials of soy isoflavones (50-70 mg/d and 150 mg/d) provided data for the meta-analysis.[59-61,65-68] The combined weighted mean difference in the number of daily hot flashes for soy isoflavones compared with placebo was −1.15 (95% CI, −2.33 to 0.03) after 4- to 6-weeks use in 5 trials, −0.97 (95% CI, −1.82 to −0.12) after 12- to 16-weeks use in 4 trials, and −1.22 (95% CI, −2.02 to −0.42) after 6 months in 2 trials (FIGURE 5). In the 4- to 6-week trials, results shifted slightly when removing a trial of women with breast cancer and SERM use[66] (weighted mean difference, −1.48; 95% CI, −2.49 to −0.48) but not when removing the single poor-quality trial.[67]

Sensitivity analysis evaluating the effects of dose and degree of correlation between baseline and outcome measures did not significantly influence results for any of the therapies. Interpretation of the sensitivity analysis for any of the variables considered is limited because of the small number of trials available.

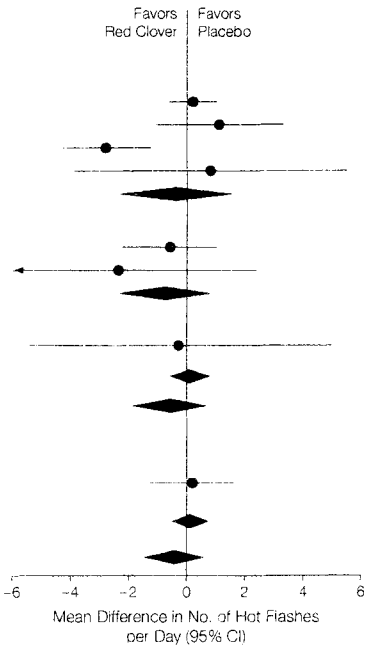
COMMENT

This systematic review and meta-analysis of double-blind, randomized, placebo-controlled trials of nonhormonal therapies provides supportive evidence for the efficacy of SSRIs or SNRIs, clonidine, and gabapentin in reducing the frequency and severity of menopausal hot flashes based on a small number of fair-good trials (SSRIs or SNRIs and gabapentin) or poor-fair trials (clonidine) (TABLE 6). The trials do not support the efficacy of red clover isoflavone extracts and present mixed results for soy isoflavone extracts. Evidence for other therapies is limited due to the small number of trials and their deficiencies. Few trials compare different therapies head-to-head and relative efficacy cannot be determined.

Although only 3 trials of the 7 comparisons of SSRIs or SNRIs with placebo indicated statistically significant reductions in hot flash frequency, the combined estimate indicated a reduction of approximately 1 hot flash per day. A meta-analysis of similarly designed RCTs of estrogen indicated reductions of approximately 2.5 to 3 hot flashes per day.[6] However, direct comparisons with estrogen are not available and the study populations may not be similar. The SSRI or SNRI estimate is influenced by 2 large trials of paroxitine[29,30] and a large trial of venlafaxine[31] that showed significant reductions in hot flash frequency. Whether this effect can be expected from the other SSRIs is not known. Trials evaluating multiple doses of paroxetine[29,30] found no differences in the effects of dose on hot flashes, although higher doses of venlafaxine were more effective than lower doses in 1 trial.[31] Adverse effects were greater with higher doses supporting the use of the lowest effective dose for individual patients. The mechanism of action of SSRIs or SNRIs in relieving hot flashes is not known, but it has

Figure 4. Trials of Red Clover Isoflavone Extracts

Source	No. of Participants	Duration of Trial	Quality	Mean Difference (95% CI)
Promensil Trials				
Promensil 40 mg/d				
Atkinson et al,[53] 2004	99	12 mo	Fair	0.20 (−0.63 to 1.03)
Baber et al,[54] 1999	51	12 wk	Fair	1.10 (−1.10 to 3.30)
Jeri,[55] 2002	30	16 wk	Poor	−2.80 (−4.31 to −1.29)
Knight et al,[56] 1999	24	12 wk	Poor	0.80 (−3.90 to 5.50)
Combined				−0.40 (−2.33 to 1.53)
Promensil 80-82 mg/d				
Tice et al,[57] 2003	169	12 wk	Good	−0.60 (−2.25 to 1.05)
van de Weijer and Barentsen et al,[58] 2002	30	12 wk	Fair	−2.37 (−7.20 to 2.46)
Combined				−0.79 (−2.35 to 0.78)
Promensil 160 mg/d				
Knight et al,[56] 1999	25	12 wk	Poor	−0.30 (−5.54 to 4.94)
Fair-Good Quality Promensil Trials Combined				0.10 (−0.60 to 0.79)
All Promensil Trials Combined				−0.59 (−1.84 to 0.67)
Rimostil Trial				
Rimostil 57 mg/d				
Tice et al,[57] 2003	168	12 wk	Good	0.20 (−1.26 to 1.66)
All Fair-Good Quality Trials Combined				0.11 (−1.51 to 0.74)
All Trials Combined				−0.44 (−1.47 to 0.58)



CI indicates confidence interval. Six trials of 2 types of red clover isoflavones provided data for meta-analysis.[53-58]

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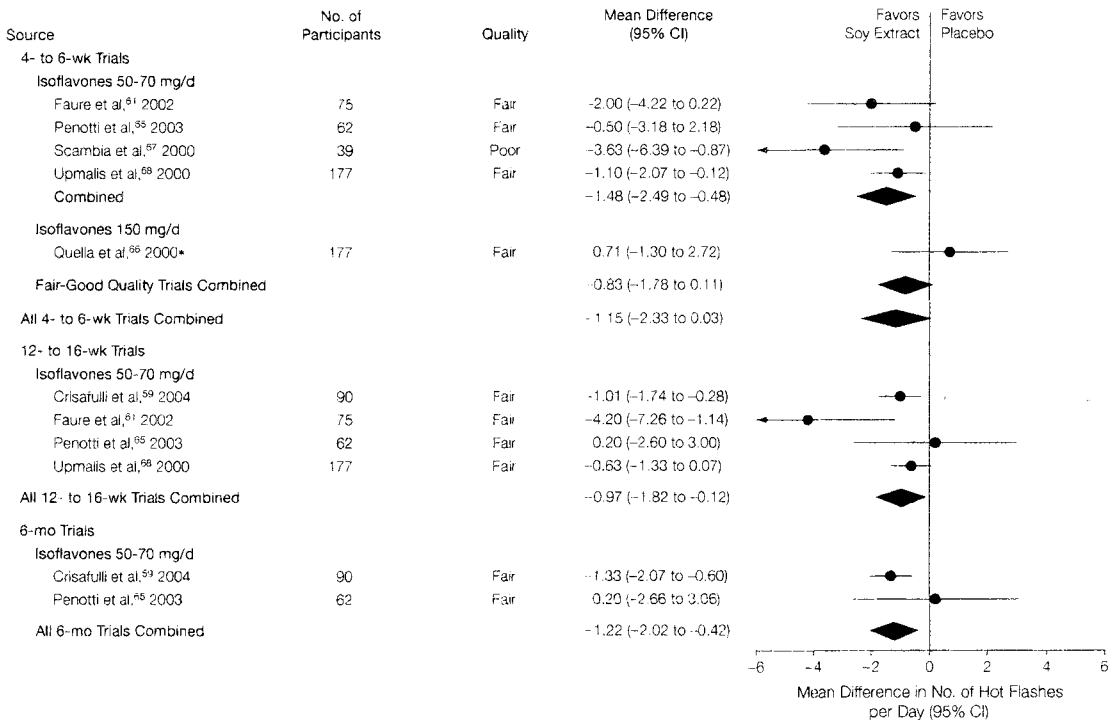
been hypothesized that temperature increases associated with hot flashes could be linked to an overloading of serotonin-receptor sites in the hypothalamus.[39] In these trials, hot flashes improved earlier than expected for psychiatric symptoms and were effective regardless of coexisting depression and anxiety, supporting a separate mechanism.[30]

Ten trials of clonidine reported inconsistent results, with approximately half of trials demonstrating significantly reduced hot flash frequency or severity. Although most trials met

Figure 5. Trials of Soy Isoflavone Extracts



CI indicates confidence interval. Six trials of soy isoflavone extracts provided data for meta-analysis.[59,61,65-68]
*Participants have breast cancer, with 78% taking tamoxifen.

Table 6. Summary of Trials

Therapy	No. of Trials in Review	Trial Quality	No. of Comparisons in Meta-analysis	Effects on Hot Flashes vs Placebo: Mean Difference in No. of Daily Hot Flashes vs Placebo (95% CI)*	Effects on Hot Flashes vs Placebo: Severity or Composite Score (% Difference)†
SSRIs or SNRIs	6	Fair-good	7	−1.13 (−1.70 to −0.57)	Improved in 4 of 6 trials (10-36)
Veralipride	3	Poor	0	‡	Improved in 2 of 2 trials (40)
Moclobemide	1	Poor	0	‡	‡
Clonidine	10	Poor-fair	4 at 4 wk	−0.95 (−1.44 to −0.47)	Improved in 4 of 7 trials (13-26)
			2 at 8 wk	−1.63 (−2.76 to −0.05)	
Methlydopa	3	Poor-fair	0	No difference	Improved in 1 of 2 trials
Gabapentin	2	Fair-good	2	−2.05 (−2.80 to −1.30)	Improved in 2 of 2 trials (23-30)
Beilergal Retard	1	Poor	0	No difference	No difference
Red clover isoflavone extracts	6	Poor-good	8	−0.44 (−1.47 to 0.58)	No difference
Soy isoflavone extracts	11	Poor-fair	5 at 4-6 wk	−1.15 (−2.33 to 0.03)	Improved in 3 of 7 trials
			4 at 12-16 wk	−0.97 (−1.82 to −0.12)	
			2 at 6 mo	−1.22 (−2.02 to −0.42)	

Abbreviations: CI, confidence interval; SNRI, serotonin norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.
*Based on meta-analysis of eligible trials.
†Composite score equals frequency × severity.
‡Between-group differences not reported.

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criteria for poor quality, the 3 fair-quality trials provided a combined estimate of approximately 1 hot flash per day reduction with clonidine.[38,45,46] Clonidine may relieve hot flashes by reducing peripheral vascular reactivity.

The combined estimate from 2 trials of gabapentin (900 mg/d) indicated a reduction of 2 hot flashes per day compared with placebo,[50,51] although the use of 300-mg/d dose did not provide benefit.[51] The mechanism of action of gabapentin, effective for treating seizure disorders and neuropathic pain, is unclear. Increased activity of neurotransmitters in the hypothalamus as a consequence of up-regulation of the gabapentin binding site from estrogen withdrawal has been proposed.[51]

Hot flash frequency was not reduced when all trials of red clover isoflavone extracts were combined, and results for soy isoflavone extracts were contradictory even among the largest and highest quality trials.[59,61-66,68] These results are consistent with other recent systematic reviews.[13,14] We also reviewed trials of other forms of soy isoflavones, such as flour, powder, and food items, in another study.[1] However, these trials are difficult to compare because of variability of components and doses. Overall, evidence does not support benefit in relieving hot flashes. Isoflavones derived from red clover and soy have weak estrogenic and antiestrogenic activities and are able to bind to estrogen receptors, potentially mediating hot flashes triggered by estrogen deficiency.[70,71] This mechanism of action could also cause adverse effects. Although 6 treatment trials included in this review reported no endometrial thickening with short-term therapy, a trial reporting adverse effects of isoflavones indicated an increased rate of endometrial hyperplasia after 5 years of 150 mg/d of soy isoflavone supplement use.[72]

Eleven trials in this review included women with breast cancer, many of whom were using tamoxifen.* Hot flashes in women with breast cancer are particularly problematic because they are common in this population,[73] estrogen therapy is contraindicated, and some agents such as paroxetine may interfere with tamoxifen.[74] Although breast cancer was not active during the course of the trials, it is not known if breast cancer itself influences vasomotor symptoms. Hot flashes are an adverse effect of tamoxifen, and it is not clear if patients enrolled in treatment trials were experiencing hot flashes induced primarily by menopause or tamoxifen. This difference may be irrelevant if mechanisms are similar. Trials considering tamoxifen users separately from nonusers found comparable results.[16]

Our review and meta-analysis is limited by potential publication bias and bias from including only English-language publications. Most treatment trials of hot flashes, regardless of therapy, share methodological challenges. Placebo effects are large and drop out rates are often high in placebo groups due to lack of effect. This must be considered when designing trials and recruiting patients. Other limitations include use of highly selected small samples of women; relatively short and variable lengths of follow-up; inadequate reporting of loss to follow-up, maintenance of comparable groups, contamination, and methods of analysis; use of dissimilar measures and outcomes that are often not standardized or validated; and unclear inclusion and exclusion criteria. Reporting of adverse effects varies by study and usually involves self-reported symptoms experienced during the course of the trial. As a result, data on adverse effects are limited and not sufficiently systematic to draw conclusions. Comprehensive methods including long-term follow-up are needed to more completely determine adverse outcomes. Larger, more rigorous, and more standardized trials, which include head-to-head as well as placebo comparisons, are needed to accurately determine relative benefits and adverse effects of a wider array of therapies.

Despite increasing interest in therapies for menopausal hot flashes that avoid use of estrogen, the efficacy and safety of other options currently are not well supported. The SSRIs or SNRIs, clonidine, and gabapentin trials provide some evidence of efficacy. However, effects are less than for estrogen therapy; few trials have been published and most have methodological deficiencies, and generalizability beyond the small clinical populations studied could be limited. Adverse effects and cost may prohibit use for many women. Although these therapies may be most useful for highly symptomatic women who cannot take estrogen, they are not optimal choices for most women.

*References 29-32, 45, 46, 51, 63, 64, 66, 69.

Author Contributions: Dr Nelson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Nelson, Vesco, Haney, Nedrow, Miller, Humphrey.
Acquisition of data: Nelson, Vesco, Haney, Nedrow, Miller, Walker, Humphrey.
Analysis and interpretation of data: Nelson, Vesco, Haney, Fu, Nedrow, Miller, Nicolaidis, Humphrey.
Drafting of the manuscript: Nelson, Vesco, Haney, Fu, Nedrow, Miller, Walker.
Critical revision of the manuscript for important intellectual content: Nelson, Vesco, Fu, Nedrow, Miller, Nicolaidis, Humphrey.
Statistical analysis: Nelson, Fu.
Obtained funding: Nelson.
Administrative, technical, or material support: Nelson, Walker.
Study supervision: Nelson, Humphrey.
Financial Disclosures: Dr Haney reported being involved in clinical trials for Sanofi-Synthelabo and Pfizer prior to work on this systemic review, none of which involved any drugs mentioned in this article, any treatments for hot flashes, or other menopausal symptoms. No other authors reported financial disclosures.
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>
> —Henri Poincaré (1854-1912)

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The nature and utility of the phytoestrogens: a review of the evidence.

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Non-prescription remedies are becoming increasingly popular particularly amongst postmenopausal who in this market are the largest consumers. Phytoestrogens are a large family of plant derived molecules possessing various degrees oestrogen like activity. Food or food supplements containing phytoestrogen are often been advocated as an alternative to hormonal replacement therapy (HRT) in women with contraindications to the use of conventional oestrogen replacement, or simply wanting a more 'natural' alternatives. There have been several studies performed with phytoestrogen in various aspects of the postmenopausal women health. Results have been sometimes conflicting and difficult to interpret. The lack of knowledge of what precisely is the active ingredient, its minimally effective doses, the lack of standardisation of the preparations used as well as the large individual variability of metabolism of precursors introduced with the diet may all have played a role in confusing the issue about effectiveness of these compounds. Phytoestrogen fall in the gray area between food and drugs hence in spite of the vast public interest, there are no interests in company producing these supplements in investing in research from which they will not exclusively benefit from. It is difficult for the physician to know how to advise patients on this matter. In this paper we critically review the clinical data available to date in an attempt to answer some of the most commonly asked questions about dose and type of phytoestrogens supplementation most likely to be effective in different aspects of climacteric woman health.

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Effects of a standardized soy extract on hot flushes: a multicenter, double-blind, randomized, placebo-controlled study

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Abstract

Objective: To investigate the effect of an oral soy isoflavone extract (Phytosoya) on hot flushes in menopausal women.

Design: The study was conducted on outpatients according to a multicenter, randomized, double-blind, placebo-controlled, parallel-group design. A total of 75 patients in natural or surgical menopause suffering from at least seven hot flushes per day were randomized to receive during 4 months either soy isoflavone extract (total of 70 mg genistin and daidzin per day) or placebo.

Results: There is evidence to suggest that 16 weeks of treatment with soy extract can help reduce the mean number of hot flushes per 24 hours in menopausal women. Withdrawals during this trial made it difficult to obtain an unbiased estimate of the true treatment effect, but numerous sensitivity analyses lend support to the suggestion that taking soy extract can be beneficial in the treatment of hot flushes. In particular, women taking soy extract had a 38% reduction in the mean number of hot flushes by week 4 and a 51% reduction by week 8. By the end of week 16, patients taking soy extract had a 61% reduction in their daily hot flushes versus a 21% reduction obtained with the placebo. "Responders" (defined as patients whose hot flushes were reduced by at least 50% at the end of treatment period) were 65.8% in the soy extract group and 34.2% in the placebo group ($p < 0.005$).

Conclusion: Soy isoflavone extract may help to reduce the frequency of hot flushes in climacteric women and provides an attractive addition to the choices available for relief of hot flushes.

Key words: Hormone replacement therapy – Hot flush – Isoflavone – Menopause – Soy.

Hormone replacement therapy (HRT) is recognized as the most effective treatment for relief of the short-term symptoms of menopause.[1,2] However, in certain clinical settings, the use of estrogen in some women is undesirable or contraindicated. Other women may have a resolution of symptoms with estrogen replacement, but side effects — such as vaginal bleeding, breast tenderness, and bloating — result in discontinuation of therapy.[3,4] Despite clinical benefits attributed to the use of HRT in postmenopausal women, compliance ranges from 10% to 50%.[5] Therefore, alternative therapies are needed for women who refuse, are not compliant, or have contraindications for HRT.

Epidemiological data indicate that fewer than 25% of Japanese and 18% of Chinese climacteric women[6,7] complain of hot flushes compared with 85% of North American women[8] and 70% to 80% of European women.[9] Furthermore, Asian women have a low incidence of estrogen-dependent cancers, cardiovascular disease, and osteoporosis compared with Western women.[10,11] Many theories have been proposed to explain these differences, including cultural perceptions, attitudes toward aging and menopause, diet, physical activity, body mass, and the use of herbs and supplements containing phytoestrogens.

Several classes of phytoestrogens exist. When considering health effects, the major types of phytoestrogens of current interest are the lignans and isoflavones. The isoflavones include the biochemicals genistein, daidzein, glycitein, biochanin A, and formononetin. Genistein and daidzein are found in rich supply in soybeans and soy products as well as in red clover.[12–14]

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The structure of the isoflavone molecule resembles those of many estrogenic compounds, including the physiologic estrogen 17β-estradiol and the synthetic antiestrogen tamoxifen, prompting investigation of its mechanisms of action according to estrogenic and antiestrogenic activities.[15-18] The isoflavones have a common phenolic structure that seems to be a prerequisite for binding to estrogen receptors (ERs). Isoflavones seem to have more binding affinity for ERβ than for ERα.[19] Therefore, given the different tissue distribution of the α and β receptors, there is a clear potential that isoflavones could exhibit tissue-selective effects.

Isoflavones may exert a weak antagonistic effect on the ER,[20] thereby having an antiestrogenic effect on uterine and breast tissue,[21] where excess estrogen may stimulate synthesis. Alternatively, isoflavones may combine with the ER, albeit with lower affinity than 17β-estradiol,[22] and stimulate estrogen activity, thus having an estrogenic effect on bone[20] and blood vessels.[23]

Studies have been reported in which isoflavone-rich foodstuffs, such as soy, produce a moderate decrease in the incidence and severity of hot flushes when added to the diet of menopausal women. Some contradictory results could be due to the large chemical heterogeneity of soy derivatives used. To test the hypothesis that dietary intake of isoflavones may have a therapeutic benefit, a double-blind, placebo-controlled trial was conducted in menopausal women in which the test group received supplementation of dietary isoflavone intake provided as a semipurified isoflavone extract in capsule form.

METHODS

Patients

The patients were all postmenopausal women requesting treatment for hot flushes. In all cases, at least 6 months had passed since the last menstrual period. To be eligible for the study, participants must have had a minimum of seven moderate to severe hot flushes (including night sweats) per 24 hours during the 2 weeks of the prestudy period. Moderate hot flushes were defined as a warm sensation associated with sweating that left the patient able to continue her daily activity. Severe hot flushes were defined as a hot sensation associated with sweating so intense that the patient had to stop her activity.

Patients also were required to have a baseline follicle-stimulating hormone concentration greater than 40 IU/L and a serum estradiol concentration less than 35 pg/mL. HRT or any other drug used for the treatment of climacteric symptoms, such as vitamin E or clonidine, was not allowed throughout the study period. Patients who had received HRT in the past had to have stopped treatment at least 6 weeks before the prestudy period.

All participants signed an informed consent at the beginning of the study, and the study was approved by the Ethical Committee of the Nîmes hospital.

Test substances

Phytosoya is a standardized isoflavone supplement prepared from soy extract in 325-mg capsule form containing 17.5 mg per capsule of total isoflavones and containing the four primary isoflavones: genistein, daidzein, and their methylated precursors biochanin and formononetin. A placebo capsule was formulated with a similar appearance and taste. The placebo capsule contained cellulose microcrystalline and sodium magnesium stearic in the same amount as the soy capsules.

The patients were either given 2 × 2 capsules of soy extract a day or a placebo capsule.

Study design

This was a 16-week, double-blind, randomized, multicenter, parallel trial. The patients were first seen at the screening visit and were then assessed 2 weeks later, before randomization, and then again at treatment weeks 4, 8, and 16. For the entire study period, including the prestudy period, each participant filled out a special card daily to evaluate the number of moderate to severe hot flushes (including night sweats) and brought it to each visit.

Adverse events were collected at each visit. For each event, the nature, duration, severity, outcome, and causal link with the study treatment as evaluated by the investigator were recorded in the case report form. Overall safety assessments by the patient and investigator were recorded.

A sample size of 60 patients (30 per treatment arm) was required to detect, with 90% power, a treatment difference of 3 hot flushes per 24 hours, assuming a standard deviation of 3.8 hot flushes per day. The test was performed two-sided at a significance level of $\alpha = 0.05$. The primary efficacy criterion was the mean change from baseline of the number of daily moderate and severe hot flushes (including night sweats) in each month of treatment. The primary analysis was based on the two-way repeated measures analysis of variance model, including the following factors: treatment effect, time effect, and treatment by time interaction.

TABLE 1. *Demographic data (mean ± standard deviation)*

	Soy group (n = 39)	Placebo group (n = 36)
Age (y)	53.0 ± 5.6	53.9 ± 4.1
Weight (kg)	66.2 ± 10.8	64.9 ± 9.8
Body mass index (kg/m^2)	24.9 ± 3.9	24.9 ± 3.5
Number of hot flushes per 24 h	10.1 ± 6.4	9.4 ± 3.4

Data were analyzed using both an intention to treat (ITT) and a per protocol (PP) approach. For the ITT analysis, the last recorded treatment value was used in accordance with the "last observation carried forward" (LOCF)[24] principle and, therefore, included patients who withdrew prematurely from the trial. For the PP analysis, only data observed at the 16-week endpoint were analyzed and, therefore, this analysis disregarded patients who withdrew prematurely.

Responders were classified as patients whose number of hot flushes had been reduced by at least 50% at the end of the treatment period.

RESULTS

A total of 75 postmenopausal women were randomized in this study (39 in the soy extract group and 36 in the placebo group). These patients were well balanced with respect to baseline characteristics, and there was no statistically significant difference between baseline values in the two groups (Table 1). At baseline, the median number of hot flushes (mean ± standard deviation) was 10.1 ± 6.4 for the group taking soy extract and 9.4 ± 3.4 for the placebo group.

Table 2 shows that 6 (15%) patients withdrew from the soy extract group [4 (10%) due to treatment inefficacy] and 14 (39%) withdrew from the placebo group [11 (28%) due to treatment inefficacy]. In addition, three patients presented outlying data. These problems have been addressed by running sensitivity analyses to obtain a sensible estimate of the treatment effect.

The absolute mean number of hot flushes for soy extract and placebo at all time points are provided in Table 3 for LOCF data and in Table 4 for observed data. From baseline to week 16 (LOCF values), the mean numbers of hot flushes (mean ± standard error of the mean) decreased to −6.4 ± 1.0 and 2.2 ± 1.2 in participants who were taking soy extract and placebo, respectively.

In the ITT analysis, repeated measures analysis of variance testing differences between treatment groups indicated treatment effect (p = 0.01) on the change in frequency of hot flushes, whereas time (p = 0.33) had no significant effect on hot flushes frequency (Table 5).

TABLE 2. *Premature study termination*

Reasons	Soy extract group (%)	Placebo group (%)
Inefficacy	4 (10)	11 (31)
Adverse drug reaction	0 (0)	2 (6)
Lost to follow up	1 (3)	1 (3)
Others	1 (3)	0 (0)

TABLE 3. *Last observation carried forward data for mean number of hot flushes at the given week*

	Week 4	Week 8	Week 16
Raw			
Phytosoya			
n	38	38	38
Mean (SEM)	6.1 (0.9)	4.7 (0.6)	3.9 (0.7)
Placebo			
n	35	35	35
Mean (SEM)	7.1 (0.9)	6.6 (1.0)	7.0 (1.2)
Change from BL			
Phytosoya			
n	38	38	38
Mean (SEM)	−4.2 (0.8)	−5.6 (0.9)	−6.4 (1.0)
Placebo			
n	34	34	34
Mean (SEM)	−2.2 (0.8)	−2.7 (0.7)	−2.2 (1.2)
% change from BL			
Phytosoya			
n	38	38	38
Mean (SEM)	−38.8 (6.1)	−51.1 (5.7)	−61.2 (5.9)
Placebo			
n	34	34	34
Mean (SEM)	−24.8 (6.4)	−32.9 (6.6)	−20.8 (16.9)

BL, baseline; SEM, standard error of the mean.

The p values from the PP analyses that only take into account the observed data at the 16-week endpoint are not significant, but the 95% confidence intervals estimating the treatment effect are supportive of the findings for the ITT/LOCF analysis (Table 5). The fact that there were a substantial number of withdrawals in this trial (Table 2), and that they occurred disproportionately, complicates the unbiased estimation of the true treatment effect. Indeed, it is difficult to say which statistical analysis is the most appropriate (ie, including or excluding outliers, using ITT/LOCF data or PP/observed data). However, one can remark that all results from the various analyses and the corresponding 95% confidence intervals estimating the treatment effect remain consistent in their suggestion that treatment with soy extract has a positive effect in the reduction of hot flushes for menopausal women (Table 5).

Figure 1 shows the variation of hot flushes during the 16 weeks of treatment in the two groups. Using the criteria of ≥50% reduction in number of hot flushes from baseline as evidence of a clinically relevant response, 65.8% of patients treated with soy extract were classi-

TABLE 4. *Observed data for mean number of hot flushes at the given week*

	Baseline	Week 4	Week 8	Week 16
Raw				
Phytosoya				
n	39	38	36	32
Mean (SEM)	10.1 (1.0)	6.1 (0.9)	4.8 (0.6)	3.3 (0.7)
Placebo				
n	35	35	32	22
Mean (SEM)	9.4 (0.6)	7.1 (0.9)	6.5 (1.0)	5.8 (1.6)
Change from BL				
Phytosoya				
n	NA	38	36	32
Mean (SEM)		−4.2 (0.8)	−5.4 (0.9)	−6.5 (1.1)
Placebo				
n		34	31	21
Mean (SEM)		−2.2 (0.8)	−3.0 (0.7)	−3.3 (1.8)
% change from BL				
Phytosoya				
n	NA	38	36	32
Mean (SEM)		−38.8 (6.1)	−50.7 (6.0)	−66.6 (6.2)
Placebo				
n		34	31	21
Mean (SEM)		−24.8 (6.4)	−37.2 (6.6)	−27.9 (26.7)

NA, Nonapplicant; BL, baseline; SEM, standard error of the mean.

fied as responders, compared with 32.4% of patients on placebo.

The standardized soy extract did not modify any other menopausal symptoms (data not shown). No significant changes in systolic and diastolic blood pressure were observed in either treatment group. There was a low incidence of medication-related adverse experiences in both groups. No patient in the soy extract group and two patients in the placebo group withdrew prematurely because of an adverse event (vertigo and weight gain) from the study.

DISCUSSION

The results of this study are consistent with our hypothesis that soy protein rich in isoflavones would reduce hot flush frequency in postmenopausal women. Several published studies report improvements in menopausal symptoms with soy protein, soy foods, or soy extract. Washburn et al.[25] investigated the effect of three dietary supplements: placebo, 34 mg of isoflavones consumed once daily, and 34 mg of isoflavones split into two equal doses consumed twice daily. They concluded that a significant improvement was observed for the severity of vasomotor symptoms and for hypoestrogenic symptoms in the twice-daily group compared with the placebo group. Although similar effects were noted in the single dose and the split-dose soy supplementation diets, the effect on vasomotor symptoms was greater on the split-dose diet. This may suggest that having more consistent circulating levels of phytoestrogens may be more efficacious than a higher single dose.

Brzezinski et al.[26] investigated the effects of short-term dietary intervention with phytoestrogen-rich diet on menopausal symptoms in 145 women. The total score of menopausal symptomatology was reduced significantly in both groups. There seemed to be an overall greater improvement in the phytoestrogen diet group than in the control group, but this difference did not reach statistical significance. However, when analyzed separately, the reductions in hot flushes and vaginal dryness scores were more significant ($p = 0.004$ and 0.005 respectively) in the women assigned a phytoestrogen-rich diet than in the controls.

Albertazzi et al.[27] assessed the effects of 60 g isolated soy protein (containing 76 mg isoflavones in the aglycone form). Soy was significantly superior to placebo ($p < 0.01$) in reducing the mean number of hot flushes per 24 hours after 4, 8, and 12 weeks of treatment. By the end of the 12th week, patients taking soy had a 45% reduction in their daily hot flushes versus a 30% reduction obtained with the placebo ($p < 0.01$).

Scambia et al.[28] examined the effect of soy extract (50 mg isoflavones/day) compared with placebo on climacteric symptoms during the course of 6 weeks, after which estrogen replacement therapy was added to both groups. When compared with pretreatment data on week 6 of the study, a significant ($p < 0.01$) reduction in the mean number of hot flushes per week was observed in participants who received the standardized soy extract, whereas a more marked relief was observed in both soy and placebo groups during estrogen replacement therapy administration. Concurrently, the severity of hot flushes, assessed by means of the Greene climacteric scale, was also reduced in the soy group participants.

The most recently published study by Upmalis et al.[29] examined the efficacy of an oral soy isoflavone extract (50 mg genistin and daidzin per day). Decreases in the incidence and severity of hot flushes occurred in as soon as 2 weeks in the soy group, whereas the placebo group experienced no relief for the first 4 weeks. Differences between women evaluated in both groups were statistically significant over 6 weeks ($p = 0.03$). Over 12 weeks, between-group differences approached significance ($p = 0.08$).

Several other studies investigating the effects of isoflavones showed no statistically significant changes in the frequency of hot flushes between active and control groups.

In a recent double-blind, crossover trial of 149 breast cancer survivors with hot flushes using 50 mg of iso-

TABLE 5. *Results from repeated measures models presenting least square means (adjusted means) and their estimated 95% confidence intervals at the specified endpoint*

Model	Treatment effect	Time effect	Treatment *time interaction	Adjusted mean and 95% confidence interval of estimated treatment effect (Phytosoya-placebo) at 16-week endpoint
Repeated measures intention to treat analysis using 16-week LOCF[a] data	0.0103	0.3324	0.3085	−3.6 (−6.2; −0.9)
Repeated measures intention to treat analysis using 16-week LOCF data (after removing outliers)[b]	0.0141	0.4500	0.5178	−2.7 (−4.7; −0.7)
Repeated measures per protocol analysis using 16-week observed data[c]	0.1251	0.3748	0.3978	−2.8 (−6.0; 0.5)
Repeated measures per protocol analysis using 16-week observed data (after removing outliers)	0.2525	0.4758	0.8389	−1.1 (−2.6; 0.5)

LOCF, last observation carried forward.
[a]LOCF is imputed data using the last known value on treatment.
[b]Outliers were #1502 (placebo), who increased from 5 to 31 hot flushes per 24 hours; #1904 (Phytosoya), who increased from 17 to 21 hot flushes per 24 hours; and #3703 (placebo), who increased from 15 to 23 hot flushes per 24 hours.
[c]Observed data at 16 weeks uses only data actually recorded at the 16-week visit and does not therefore take into account study withdrawals.

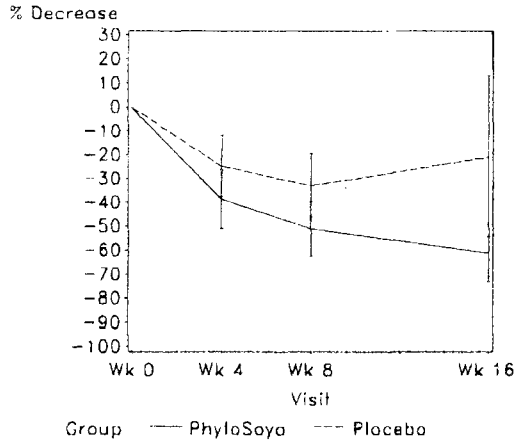

FIG. 1. Percentage decrease from baseline of hot flushes. Results presented as mean ± 2 standard error of mean [LOCF (last observation carried forward) data].

flavones in soy extract tablets, the effects of isoflavones on the frequency and severity of hot flushes were evaluated.[30] The soy tablet in this trial was not more effective than the placebo in reducing hot flushes. Another study examined the change in menopausal symptoms in response to 24 weeks of isoflavone-rich (80.4 mg/day) and isoflavone-poor (4.4 mg/day) soy protein isolate treatment in perimenopausal women.[31] At the completion of the study, no treatment effect was founded on frequency or severity of hot flushes.

Data are often conflicting and difficult to analyze because of variations in the populations studied, duration of dietary soy exposure, study design and variability in responsiveness of some postmenopausal women to phytoestrogen supplementation. But the discrepancies between the negative and positive studies may be related mainly to the specific source of phytoestrogens. Treatments studied have varied from unmodified soy products or phytoestrogen-rich food diets[26] to soy bread (containing soy grits), soy flour, soy protein powder with different doses of isoflavones,[25,27] and tablets containing concentrated subfractions.[28–30] To make direct comparisons between naturally occurring, plant-derived substances and pharmaceutical preparations may be inappropriate. Furthermore, phytoestrogens' metabolite concentration may vary widely between individuals, even with administration of a controlled quantity of isoflavones.

The present study emphasizes many of the problems with pharmaceutical-style intervention studies using naturally occurring dietary compounds. A high withdrawal rate complicated the estimation of the treatment effect, although sensitivity analyses provided support to the suggestion that soy extract taken twice daily can help in the reduction of hot flushes. There is a need for further large studies investigating the areas of clinical effectiveness of isoflavone supplementation in the treatment of menopausal symptoms.

CONCLUSION

In conclusion, on the basis of the results obtained in this pilot short-term trial, this standardized soy extract

(Phytosoya) has a good efficacy on vasomotor symptoms, decreasing hot flushes and night sweats, and it may be safely and effectively used by women who experience vasomotor symptoms and who choose not to take estrogens for personal or medical reasons.

Acknowledgments: We thank A. McNally from Cardinal System for his strong contribution in statistics.

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COMPLEMENTARY AND ALTERNATIVE MEDICINE SERIES

The Use of an Isoflavone Supplement to Relieve Hot Flushes

Arturo R. Jeri, MD

The incidence of menopausal symptoms is low in Asian countries, associated in part with the high levels of dietary isoflavones and lignans present in the soybean products that constitute much of the Asian diet.[1] Numerous studies have demonstrated that dietary supplementation with foods high in isoflavones reduces hot flushes in up to 66% of women.[2] According to 1999 estimates from the Peruvian Menopause Society, 70% to 80% of menopausal Peruvian women report the occurrence of hot flushes, but fewer than 3% of this population uses conventional hormone replacement therapy (HRT).

Most studies of dietary isoflavones have focused on soy legumes (ie, soybeans), which contain genistein and daidzein—only two of the isoflavones known to have significant estrogenic properties. Red clover (Trifolium pratense), also a legume, contains these compounds plus two additional isoflavones, formononetin and biochanin, which have been shown to bind to estrogen receptors to produce estrogen-like effects.[3] This study was undertaken to evaluate the effectiveness of Promensil[3], an isoflavone supplement derived from red clover, in relieving the frequency and severity of hot flushes in postmenopausal women. Promensil was selected for this study because it contains the key isoflavones commonly found in soy foods and is widely available as a consumer product. Evaluation of potential heart disease prevention, favorable lipid influences, and preservation of bone strength was beyond the scope and duration of this study.

MATERIALS AND METHODS

This 16-week, randomized, double-blind prospective study selected 30 healthy, nonvegetarian women who had been postmenopausal for more than 1 year, using nonprobabilistic sampling and randomly dividing them into two groups of 15. Eligibility criteria required that subjects be younger than age 60, have follicle-stimulating hormone (FSH) levels of more than 30 mIU/mL, experience at least five hot flushes daily (averaged for more than 1 week), and not use HRT, antidepressants or other medications, or soy or other estrogen-active plant products for the previous 16 weeks. The median age was 52 + 0.7 years for the treatment group, and 51 + 0.8 years for the control group. All participants were Hispanic, with a middle-class income and good education. There were no statistically significant differences between the two groups with respect to demographics or eligibility criteria. Before starting the study, both groups were asked to rate the severity of their symptoms on a scale of 0 (no symptoms) to 3 (severe, interfering with normal activities).

The 15 treatment-group subjects were given a single daily tablet of Promensil containing 40 mg of standardized isoflavones (genistein, daidzein, formononetin, and biochanin). The 15 control-group subjects received 1 placebo tablet per day, identical in appearance to the active tablets. All subjects were asked to record incidence and severity of hot flushes at the beginning and end of the study. Data were statistically analyzed using the Student t test and c-square test to evaluate the significance of mean differences between the treatment and placebo groups.

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Table 1. Frequency of Hot Flushes

	Treatment Group	Control Group
Baseline (no/day)	7.0 + 0.5	5.7 + 0.4
16 weeks	3.6 + 0.3	5.1 + 0.3
Mean % reduction	48.5 + 7.2	10.5 + 9.6

Table 2. Severity of Hot Flushes

Severity Score	No. of subjects reporting each severity score			
	Treatment Group		Control Group	
	Baseline	16 weeks	Baseline	16 weeks
0 (none)	0	0	0	0
1(light)	0	10	3	3
2 (moderate)	7	5	9	9
3 (severe)	8	0	3	3
Severity Index*	2.53	1.33	2.00	2.00

*Severity Index = No. of subjects reporting each symptom score X symptom score

Total no. of subjects per group

RESULT

At the end of the 16-week study, a reduction in both frequency and severity of hot flushes was reported by women in the treatment group (Tables 1 and 2). The treatment group reported a statistically significant reduction of 48.5% (7.0 + 0.5 to 3.6 + 0.3) in the frequency of hot flushes per day compared with 10.5% (5.7 + 0.4 to 5.1 + 0.3) in the control group. c-Square analysis for proportional differences of frequency of hot flushes revealed that the reduction in the treatment group was statistically significant ($x2 = 25.25$, $P < 0.001$). The severity index for the Promensil group showed a statistically significant reduction from 2.53 to 1.33 (47% reduction), whereas there was no change in the placebo group ($t = 3.67$, $P < 0.001$). There was a shift for the participants in the active group from severe hot flushes ($n = 8$) at baseline to light ($n = 10$) at the study's end.

Mean FSH levels decreased in 14 Promensil-group women from 59.27 + 4.22 IU/mL to 48.60 + 3.93 IU/mL, but increased in nine control-group women from 51.47 + 3.96 IU/mL at baseline to 54.60 + 3.62 IU/mL at 16 weeks. Although the decrease in FSH values in the treatment-group women was statistically significant ($t = 4.39$, $P < 0.001$), the absolute levels remained above the 30-IU/mL threshold—ie, the level at which ovarian estrogen production is considered to have ceased. There was no observed correlation between FSH levels and frequency of hot flushes or between age and frequency of hot flushes at baseline.

Table 3. Content of Four Isoflavones in Promensil[6]

Daidzein (µg/g)	1,532 + 163

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Genistein (µg/g)	2,900 + 65
Formononetin (pg/g)	26,726 + 540
Biochanin A (µg/g)	44,330 + 1,072
Ratio of (diadzein + glycitein)-derived/ genistein	1.36 + 0.07
Aglycones (%)	99.9
Total isoflavones (mg/g)	78.15 + 1.61
Label content per capsule	40.0
Actual content per capsule	41.7

DISCUSSION

Most Peruvian women avoid HRT because they fear that it may lead to breast or uterine cancer, vaginal bleeding, and weight gain,[4] and often they are reluctant to interact with the medical system to obtain medications.

This study demonstrates that dietary supplementation with red clover-derived isoflavones is an effective alternative for relief of vasomotor symptoms in postmenopausal women, reducing both the average daily frequency and severity of hot flushes. A previous study using the same red clover isoflavone supplement failed to demonstrate a statistically significant effect on hot flushes, primarily due to the failure to control for isoflavone intake in the placebo group.[5] However, that study showed a clear correlation between isoflavone excretion and hot flush reduction, and an 18% reduction in high-density lipoprotein levels—even in the absence of an effect on hot flushes. That study also demonstrated the safety of red clover isoflavones in that uterine thickness remained unchanged in the women using supplementation over a 12-week period.[5]

A recent study evaluating the bioavailability of commercial isoflavone supplements confirmed the manufacturer's statements regarding the composition of Promensil tablets. Under high-performance light chromatography, the product was shown to contain predominantly formononetin and biochanin A in the aglycone form, small amounts of their glycosides, and daidzein and genistein (Table 3). In a single subject who was given 1 tablet of Promensil, the plasma concentrations of daidzein and genistein rapidly rose to levels typically observed after the ingestion of soy-containing foods.[6]

Based on the efficacy in hot-flush reduction reported here and findings of improved cardiovascular function, bone density, and safety reported elsewhere,[3,5,7] red clover isoflavone supplementation offers a useful alternative for women seeking relief from acute symptoms of menopause.

Arturo R. Jeri, MD, is medical researcher of the climacteric unit at the Institute of Gynaecology and Reproduction in Lima, Peru.

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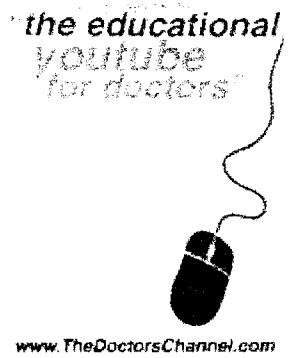

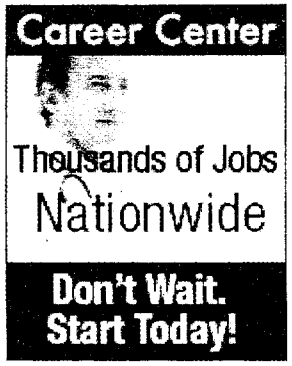

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Benefits of soy isoflavone therapeutic regimen on menopausal symptoms.

Han KK, Soares JM Jr, Haidar MA, de Lima GR, Baracat EC.

Department of Gynecology, Escola Paulista de Medicina/Federal University of Sao Paulo, Sao Paulo, Brazil.

OBJECTIVE:To examine the change in menopausal symptoms and cardiovascular risk factors in response to 4 months of daily 100-mg soy isoflavone in postmenopausal women. METHODS: In this double-blind, placebo-controlled study, 80 women were randomly assigned to isoflavone (n = 40) and placebo (n = 40) treatment. The menopausal Kupperman index was used to assess change in menopausal symptoms at baseline and after 4 months of treatment. Cardiovascular risk factors were assessed by evaluating plasma lipid levels, body mass index, blood pressure, and glucose levels in the participants. To examine the effects of this regime on endogenous hormone levels, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and 17 beta-estradiol were measured. Transvaginal sonography was performed to quantify endometrial thickness. RESULTS: The data showed a decrease in menopausal symptoms (P <.01, paired t test, two-tailed, between baseline and isoflavone groups, and P <.01, unpaired t test, between placebo and isoflavone groups). Total cholesterol and low-density lipoprotein decreased significantly in the isoflavone group compared with the baseline or placebo group (P <.001, paired t test, two-tailed, between baseline and isoflavone groups, and P <.01, unpaired t test, between placebo and isoflavone groups). The isoflavone treatment appeared to have no effect on blood pressure, plasma glucose, and high-density lipoprotein and triglyceride levels. CONCLUSION: This study suggests that isoflavone 100-mg regime treatment may be a safe and effective alternative therapy for menopausal symptoms and may offer a benefit to the cardiovascular system.

PMID: 11864664 [PubMed - indexed for MEDLINE]

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Menopause. 2000 Jul-Aug;7(4):213-4.

Vasomotor symptom relief by soy isoflavone extract tablets in postmenopausal women: a multicenter, double-blind, randomized, placebo-controlled study.

Upmalis DH, Lobo R, Bradley L, Warren M, Cone FL, Lamia CA.

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OBJECTIVE: To determine the safety and efficacy of an oral soy isoflavone extract for relief of menopausal hot flushes. DESIGN: This was a double-blind, randomized, parallel group, outpatient, multicenter (15 sites) study. A total of 177 postmenopausal women (mean age = 55 years) who were experiencing five or more hot flushes per day were randomized to receive either soy isoflavone extract (total of 50 mg genistin and daidzin per day) or placebo. Physical examinations and endometrial and biochemical evaluations were performed upon admission and completion. Body weight, symptoms, and safety were evaluated at all visits. RESULTS: Relief of vasomotor symptoms was observed in both groups. Decreases in the incidence and severity of hot flushes occurred as soon as 2 weeks in the soy group, whereas the placebo group experienced no relief for the first 4 weeks. Differences between evaluable subjects in both groups were statistically significant over 6 weeks ($p = 0.03$). Over 12 weeks, between-group differences approached significance ($p = 0.08$). Endometrial thickness evaluated by ultrasound, lipoproteins, bone markers, sex hormone-binding globulin and follicle-stimulating hormone, and vaginal cytology did not change in either group. CONCLUSIONS: Soy isoflavone extract was effective in reducing frequency and severity of flushes and did not stimulate the endometrium. Soy isoflavone extracts provide an attractive addition to the choices available for relief of hot flushes.

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Longitudinal analysis of the association between vasomotor symptoms and race/ethnicity across the menopausal transition: study of women's health across the nation. [Menopause. 2006]

Review Phytoestrogens for vasomotor menopausal symptoms. [Cochrane Syst Rev. 2007]

Review Botanical and dietary supplements for menopausal symptoms: what works, what does not. [Obstet Gynecol. 2006]

Review Phytoestrogens for treatment of menopausal symptoms: a systematic review. [BMC Obstet Gynecol. 2005]

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1: Maturitas. 2004 Jan 20;47(1):1-9.

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Soy for the treatment of perimenopausal symptoms--a systematic review.

Huntley AL, Ernst E.

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OBJECTIVES: Many women have turned to complementary and alternative medicines for relief from their perimenopausal symptoms. The prevalence of plant-based medicine use among perimenopausal women highlights the need for investigation into these interventions. The aim of this study was to evaluate the benefit of soy preparations for the treatment of perimenopausal symptoms by performing a systematic review of randomised clinical trials (RCTs). METHODS: Literature searches were performed using four computerised databases to identify RCTs of soy preparations for the treatment of perimenopausal symptoms. Manufacturers of soy products were contacted and our own files were also searched. There were no restrictions on the language of publication. Trials were considered if they used mono-preparations of soy or soy isoflavones, and if the outcome measures related to the physical and/or psychological impact of menopause in healthy women and scored at least three on the Jadad scale. RESULTS: Thirteen RCTs were identified that investigated the use of soy preparations for perimenopausal symptoms. Ten of these trials fitted our inclusion criteria. The results of these studies are not conclusive. Four of these randomised controlled trials were positive, suggesting soy preparations are beneficial for perimenopausal symptoms. Six were negative; with one of the six showing a positive trend. CONCLUSIONS: There is some evidence for the efficacy of soy preparations for perimenopausal symptoms. However, the heterogeneity of the studies performed to date means it is difficult to make a definitive statement. Adverse event data from the trials suggest that there are no serious safety concerns with soy products in short-term use.

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Menopause: a review of botanical dietary supplements

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KEYWORDS:
Black cohosh extract;
Botanical extracts;
Menopausal disorders;
Soy isoflavone extract

Since the release of the Women's Health Initiative (WHI) findings, an increasing number of dietary supplement products specifically targeting women in menopause have appeared in the American marketplace. This growth highlights the need for a critical evaluation of the tolerability and effectiveness of these products. The purpose of this article is to assess the evidence for safety and benefit of botanical monopreparations used for relief of menopause-related symptoms. The Cochrane Library and Medline databases were searched from January 1966 to October 2004, using a detailed list of terms related to botanicals and menopausal symptoms. Studies were considered eligible (1) if they were controlled trials of a botanical monopreparation administered orally for a minimum of 6 weeks to perimenopausal or postmenopausal women with hot flashes and (2) if they included a placebo or comparative treatment arm. Topical preparations, botanical combinations, and dietary interventions, such as soy food or protein, were not included. No language restrictions were imposed on the search. A total of 19 studies met the inclusion criteria. The majority of studies indicate that extract of black cohosh (*Actaea racemosa* L.) improves menopause-related symptoms; however, methodologic shortcomings in the trials were identified. To date, 4 case reports of possible hepatotoxicity have been published, although previous safety reviews suggest that black cohosh is well tolerated and that adverse events are rare when it is used appropriately. The results of 6 clinical studies on soy (*Glycine max* L.) isoflavone extracts are mixed. Moreover, the composition and dose of soy supplements varies widely across studies, making comparisons and definitive conclusions difficult. One study challenged the long-term safety of high-dose soy isoflavone extract (150 mg/day for 5 years) on the uterine endometrium. Clinical data from 5 controlled trials assessing the efficacy of semipurified isoflavone red clover (*Trifolium pratense* L.) leaf extracts to reduce hot flash frequency and severity or to relieve symptoms associated with the domains of the Greene Menopausal Symptom Scale are contradictory. The largest study showed no benefit for reducing symptoms associated with menopause for 2 different red clover isoflavone products compared with placebo. No significant adverse events have been reported in the literature. Single clinical trials do not support the use of dong quai (*Angelica sinensis* L.), ginseng (*Panax ginseng* C.A. Mey), or evening primrose seed oil (*Oenothera biennis* L.) for improving menopausal symptoms. We conclude that black cohosh extracts appear to ease menopausal symptoms; ongoing studies funded by the National Institutes of Health (NIH) will provide more definitive safety and efficacy data. Soy isoflavone extracts appear to have minimal to no effect, although definitive conclusions are difficult given the wide variation in product composition and dose. Long-term safety of higher dosage (150 mg/day) soy isoflavone extracts is uncertain. Semipurified isoflavone red clover leaf extracts have minimal to no effect in reducing menopausal symptoms. Dong quai, ginseng extract, and evening primrose seed oil appear to be ineffective in ameliorating menopausal symptoms at the dosages and in the preparations used in these studies.
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The opinions offered at the National Institutes of Health (NIH) State-of-the Science Conference on Management of Menopause-Related Symptoms and published herein are not necessarily those of the National Institute on Aging (NIA) and the Office of Medical Applications of Research (OMAR) or any of the cosponsoring institutes, offices, or centers of the NIH. Although the NIA and OMAR organized this meeting, this article is not intended as a statement of Federal guidelines or policy.

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When the Women's Health Initiative (WHI) was discontinued owing to unanticipated increases in risk for breast cancer, stroke, heart attack, and blood clots among women taking estrogen plus progestin, the search for alternative treatments that were perceived to offer beneficial effects with less risk intensified. Some women turned to botanical dietary supplements with the presumption that these "natural substances" are relatively safe and effective. Whether these presumptions are correct remains to be seen.

Although botanical remedies have been used for centuries, many products in the American marketplace bear little resemblance to the simple preparations of the past. Potent, concentrated products extracted via a range of solvents may be consumed for prolonged periods, often in combination with over-the-counter and prescription drugs—unique circumstances when compared with use of the apothecary of yesteryear. Thus, a "long history" of use cannot be presupposed, and questions of safety and efficacy must continue to be entertained.

The purpose of this article is to systematically assess the evidence for safety and benefit of single constituent botanical products (often referred to as *monopreparations*) for the treatment of menopause-related symptoms. This review is based on the most rigorous scientific studies published in peer-reviewed literature.

Methods

The Cochrane Library and Medline databases were searched from January 1966 to October 2004, using a detailed list of terms related to botanicals and menopausal symptoms. Terms used in the search included the following: menopause, hot flashes, climacteric, herb, botanical, phyto-, phytoestrogen, isoflavone, soy, black cohosh, *Cimicifuga*, *Actaea*, red clover, *Trifolium*, dong quai, *Angelica*, licorice, wild yam, *Dioscorea*, ginseng, *Panax*, evening primrose oil, γ-linolenic acid, hops, kava, *Piper methysticum*, St. John's wort, *Hypericum*, chastetree, *Vitex*, valerian, and motherwort. References were then reviewed to identify additional studies.

Study selection

Studies were considered eligible (1) if they were controlled trials of a botanical monopreparation administered orally for a minimum of 6 weeks to perimenopausal or postmenopausal women with hot flashes and (2) if they included a placebo or comparative treatment arm. Topical preparations, botanical combinations, and dietary interventions, such as soy food or protein, were not included. No language restrictions were imposed. A total of 19 studies met the inclusion criteria.

Results

Black cohosh (*Actaea racemosa* L.; *Cimicifuga racemosa* [L.] Nutt.)

The root and rhizome of black cohosh, an indigenous North American herb, have been researched for >30 years for the relief of menopause-related symptoms. German health authorities endorse the use of black cohosh extract for premenstrual discomfort, dysmenorrhea, and menopause.[1] Similarly, the World Health Organization (WHO)[2] recognizes its use for "treatment of climacteric symptoms such as hot flushes, profuse sweating, sleeping disorders and nervous irritability." The North American Menopause Society[3] recommends black cohosh, in conjunction with lifestyle approaches, as a treatment option for women with mild menopause-related symptoms.

Of 13 published trials identified for black cohosh, 5 met criteria for inclusion in this review (**Table 1**).[4–8]

Efficacy

Most studies indicate that black cohosh extract reduces some symptoms associated with menopause; however, methodologic shortcomings and variations in product and dosage limit definitive conclusions. The randomized, double-blind study by Stoll[5] reported a statistically significant ($P < 0.001$) reduction in Kupperman Index (KI) and Hamilton Anxiety Rating Scale (HAM-A) scores among women assigned to black cohosh compared with those given conjugated estrogens or placebo. Daily hot flash incidence decreased from 4.9 to 0.7 in the black cohosh group, from 5.2 to 3.2 in the estrogen group, and from 5.1 to 3.1 in the placebo group. Attrition bias may have occurred; 12 of 30 women dropped out of the estrogen group between weeks 5 and 8 due to "perceived lack of efficacy," a finding that, in itself, raises questions about the study. Lehmann-Willenbrock and Riedel[6] found black cohosh reduced KI scores as effectively as hormone therapy (HT). However, their study suffered from small sample size, lack of a placebo arm, lack of blinding, and no description of the randomization process. The trial by Warnecke[4] reported "highly significant reduction" in all outcome measures, but it did not report calculations or provide analytical details. The study lacked a placebo arm and included diazepam—a drug not typically used for relief of menopausal symptoms—in 1 treatment arm. Wuttke and coworkers[8] found a statistically significant reduction in the Menopause Rating Scale (MRS) score among women taking black cohosh compared with those assigned to placebo; however, reduction in hot flashes (item 1 on the MRS) did not differ significantly between the black cohosh and placebo groups. Jacobson and colleagues[7] failed to detect any reduction in hot flash frequency or severity among breast cancer survivors taking black cohosh. This study included a large number of participants (69%) taking tamoxifen, a drug

Table 1 Controlled trials of black cohosh

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Warnecke[4]	60	Peri- and postmenopausal women	CEs (0.6 mg/day) or diazepam (2 mg/day) for 12 wk	40 drops Remifemin* liquid extract bid (4 mg/day 27-deoxyactein) for 12 wk	KI, HAM-A score, Global Impressions, and Self-Assessment Depression Scale	All 3 groups showed significant decrease in neurovegetative and psychological symptoms. Black cohosh and estrogen groups experienced proliferation of vaginal epithelium. No significant adverse effects noted.
Stoll[5]	80	Postmenopausal women	CEs (0.625 mg/day) or placebo for 12 wk	Remifemin 4 mg (27-deoxyactein) bid for 12 wk (equivalent to 80 mg bid extract)	KI, HAM-A score	Remifemin group had most pronounced reduction in KI and HAM-A scores compared with estrogen and placebo groups ($P < 0.001$). Remifemin group had most significant change in proliferation of vaginal epithelium ($P < 0.01$).
Lehmann-Willenbrock and Riedel[6]	60	Surgically menopausal women	Estriol (1 mg/day) or CEs (1.25 mg/day) or estrogen + progestin therapy (2 mg/day estradiol + 1 mg/day norethisterone acetate) for 6 mo	Remifemin 4 mg bid (27-deoxyactein) for 6 mo (equivalent to 80 mg bid extract)	KI	Authors concluded that all 3 groups had decreased KI; no significant difference. Remifemin group had no change in LH or FSH level.
Jacobson et al[7]	85	Women with vasomotor symptoms and history of breast cancer	Placebo for 60 days	20 mg bid of an unspecified black cohosh product for 60 days	4-day hot flash diary; menopausal symptom questionnaire	Black cohosh was not significantly more efficacious than placebo for hot flash number or intensity; sweating was the only symptom with significantly greater improvement over placebo. No change in FSH or LH level in either group.
Wuttke et al[8]	62	Postmenopausal women	CE (0.6 mg/day); or placebo for 12 wk	CR BNO 1055/ Menofem† (40 mg/day) for 12 wk	MRS	Statistically significant reduction in MRS score; however, reduction in hot flashes (item 1 on MRS) did not differ significantly between groups. Beneficial effect on bone metabolism and vaginal cytology reported in both CE and CR BNO groups. CR BNO had no effect on endometrial thickness, which was increased by CE.

CE = conjugated estrogen; FSH = follicle-stimulating hormone; HAM-A = Hamilton Anxiety Rating Scale; KI = Kupperman Index; LH = luteinizing hormone; MRS = Menopause Rating Scale.

*Remifemin (black cohosh; Schaper & Brummer GmbH & Co. KG, Salzgitter, Germany). Remifemin preparations are standardized to contain 1 mg triterpene glycosides (expressed as 27-deoxyactein (23-epi-26-deoxyactein) in each dose, equivalent to 20 mg root/rhizome.

†Menofem (CR BNO aqueous ethanolic extract of black cohosh; Bionorica AG, Neumarket, Germany).

Table 2 Placebo-controlled trial of dong quai

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Hirata et al[25]	71	Postmenopausal women	Placebo for 24 wk	Dong quai, 3 capsules tid (4.5 g/day) for 24 wk	KI, hot flash frequency	No difference between groups in serum hormone levels, vaginal cytology, hot flashes, KI scores

KI = Kupperman Index.

known to induce hot flashes, and thus limited the generalization of the results to women going through natural menopause and not taking tamoxifen.

Safety

Our understanding of the mechanism of action of black cohosh is a work in progress, but recent research suggests a nonhormonal effect.[9] An abstract reporting increased metastases from breast to lung in mice given black cohosh has raised questions concerning safety for women with breast cancer[10]; however, other studies in animals have not found an effect on mammary tumors.[11,12] Four case reports[13–16] purportedly link black cohosh use with acute liver disease in 5 patients. Evaluation of these reports is difficult, however, because 2 of the 5 cases involved combination herbal products, 3 cases failed to analyze suspected products for purity and identification, and 1 case did not report the brand or dose of black cohosh consumed. No serious adverse events have been reported in published clinical trials, and 2 safety reviews have found black cohosh extract to be well tolerated and adverse events to be rare when it is taken for up to 6 months.[17,18]

Dose and preparation

There is a wide variety of black cohosh products and formulations available in the American marketplace, including combination preparations. Clinical trials have been conducted on 2 proprietary preparations. The majority of studies used Remifemin (Schaper & Brummer GmbH & Co. KG, Salzgitter, Germany), although the method of extraction in this preparation has changed over time from hydroethanolic (60% ethanol by volume) to isopropyl alcohol (40% by volume) and the dosage form has changed from liquid to tablets, thus complicating any comparison of research trials. In 2 recent studies, investigators used CR BNO 1055, an aqueous ethanolic extract (58% vol/vol), sold as Klimadynon and Menofem (Bionorica AG, Neumarket, Germany). It is unclear whether pharmacologic equivalence can be assumed between products. The dose of extract used in clinical trials is 40 to 160 mg/day.

Conclusion

Most published reviews[19–24] lend support for the contention that black cohosh extract is beneficial for the relief of menopause-related symptoms; however, clinical trials published to date suffer from methodologic shortcomings that necessarily temper endorsement of this botanical. It is hoped that ongoing National Institutes of Health (NIH)–funded clinical studies will provide more definitive information regarding safety and efficacy of black cohosh for the alleviation of menopausal symptoms.

Dong quai (*Angelica sinensis* [Oliv.] Diels)

Dong quai root has been used to treat a variety of disorders in traditional Chinese medicine (TCM) for ≥20 centuries and is now quite popular in the US marketplace. Our search revealed 1 published clinical trial on dong quai that met the inclusion criteria (**Table 2**).[25]

Efficacy

A double-blind study by Hirata and coworkers[25] failed to find any benefit for women receiving 4.5 g/day dong quai (aqueous extract standardized to 0.5 mg/kg of ferulic acid) when compared with placebo with respect to relief of hot flashes or other menopausal symptoms captured by the KI over a 24-week period.

Dong quai is not typically used in TCM to treat menopausal symptoms, nor is it typically administered as a single agent. However, since the majority of dong quai products sold in the United States are not prepared in accordance with TCM principles, the findings of Hirata and coworkers[25] may be relevant to over-the-counter use of the botanical.

Safety

There is little information available on the hormonal effects of dong quai. Hirata and coworkers[25] did not detect any change in serum hormone levels or vaginal cytology. One case report of male gynecomastia was purportedly caused by dong quai ingestion.[26] No significant adverse effects are reported in the literature; however, a case report[27] and results of animal research[28] suggest a possible herb-drug interaction between donq quai and warfarin.

Table 3 Placebo-controlled trial of evening primrose seed oil

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Chenoy et al[30]	56	Postmenopausal women	Placebo for 24 wk	4 capsules evening primrose oil capsules bid (4,000 mg/day) for 24 wk	KI, hot flash and sweating episode frequency	No significant difference in any outcome measure between active and placebo groups

KI = Kupperman index.

Dose and preparation

There are numerous dong quai products in the American marketplace, the majority of which are combination preparations. No particular product dominates the marketplace, and most are not standardized to any constituent. The recommended "serving size" varies across manufacturers, but most recommend 3 to 6 g/day, usually taken in 3 divided doses.[29]

Conclusion

Based on the data from Hirata and coworkers,[25] dong quai as a single therapeutic intervention does not appear to be an effective treatment for relieving the vasomotor symptoms of menopause. However, the paucity of data limits definitive conclusions.

Evening primrose seed oil (*Oenothera biennis* L.)

Evening primrose oil is extracted from the seeds of the evening primrose plant, a wildflower native to North America. The seed oil is a rich source of linoleic acid and γ-linolenic acid. Our search identified 1 clinical trial evaluating the efficacy of evening primrose oil for the relief of menopausal symptoms (**Table 3**).[30]

Efficacy

The study by Chenoy and colleagues[30] randomized 56 women reporting >3 hot flashes per day to 2,000 mg evening primrose oil plus 20 mg vitamin E or placebo twice daily for 24 weeks. The investigators did not find any significant benefit for evening primrose oil over placebo in relieving the vasomotor symptoms of menopause. However, the trial was small and suffered from attrition; only 35 participants completed the study.

Safety

Evening primrose oil is generally well tolerated. Mild nausea, diarrhea, flatulence, and bloating have been reported in some individuals.[31] Limited data suggest a possible aggravation of temporal lobe epilepsy.[32]

Dose and preparation

Evening primrose oil products generally are standardized to contain 320 to 360 mg linoleic acid and 40 mg γ-linolenic acid per capsule, although levels vary between manufacturers. Vitamin E may be added to prevent rancidity. The recommended dosage ranges from 3 to 6 g/day.

Conclusion

There was no demonstrated benefit for evening primrose oil over placebo in relieving menopause-related symptoms. However, the paucity of data limits any definitive conclusions.

Ginseng (*Panax ginseng* C.A. Mey)

Ginseng root has held a place of importance in Asian medicine for millennia, and it is among the most popular herbal supplements sold in the American market. The common name, *ginseng*, is used to describe a number of chemically different species of *Panax*. Thus, caution must be exercised when interpreting data between species. The German Commission E[1] and the WHO[33] endorse the use of *Panax ginseng* as a tonic or restorative agent for invigoration and fortification in times of fatigue, debility, or physical or mental exhaustion. We found 1 clinical trial that met our criteria (**Table 4**).[34]

Efficacy

Wiklund and colleagues[34] conducted a randomized, double-blind, placebo-controlled study of ginseng in 384 women reporting ≥6 hot flashes on ≥3 days per week. Subjects received either 200 mg/day ginseng extract or placebo for 16 weeks. With regard to the primary end point, the Psychological General Well-Being (PGWB) index, ginseng showed a tendency only for better overall symptomatic relief ($P < 0.1$). Exploratory analysis of PGWB subsets, however, reported favorable results for ginseng compared with placebo ($P < 0.05$) on the depression, well-being, and health subscales.

Table 4 Placebo-controlled trial of ginseng

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Wiklund et al[34]	384	Symptomatic postmenopausal women	Placebo for 16 wk	4 capsules evening primrose oil bid (4,000 mg/day) for 24 wk	Hot flash and sweating episode frequency, PGWB, WHQ, VAS	No significant difference in any outcome measure between active and placebo groups

PGWB = Psychological General Well-Being index; WHQ = Women's Health Questionnaire; VAS = Visual Analogue scale.

Safety

A systematic review[35] found that *Panax ginseng* monopreparations are rarely associated with adverse events or drug interactions, and the few effects reported were mild and transient. Data from clinical trials suggest that the incidence of adverse events is similar for ginseng and placebo, with subjects reporting headache as well as sleep and gastrointestinal disturbances.

Dose and preparation

There are numerous products and formulations of ginseng available in the United States. Doses of standardized extract range from 100 to 600 mg per day. Ginsana (standardized to 4% ginsenosides; Pharmaton S.A., Lugano, Switzerland, distributed by Pharmaton Natural Health Products) was the product used in the study by Wiklund and colleagues.[34]

Conclusion

Panax ginseng may be helpful with respect to quality-of-life outcomes, such as well-being, mood, and sleep. However, based on the results of 1 large clinical trial, it does not appear to be beneficial for vasomotor symptoms.

Red clover (*Trifolium pratense* L.)

Although red clover blossoms have been used in traditional herbal medicine for centuries, it is the semipurified isoflavone leaf extracts for relief of menopause-related symptoms that interest researchers. Red clover contains isoflavones, which are sometimes referred to as phytoestrogens because of their estrogen-like effects. Isoflavones may act as an estrogen agonist or antagonist depending on the individual compound and level present in the body. We found 5 clinical trials that met our inclusion criteria (**Table 5**).[36–40]

Efficacy

The largest study of semipurified isoflavone red clover leaf extracts by Tice and colleagues[36] randomized 252 postmenopausal women reporting ≥35 hot flashes per week to Promensil (red clover isoflavones; Novogen Ltd., North Ryde, New South Wales, Australia) 82 mg/day, Rimostil (red clover isoflavones; Novogen Ltd.) 57 mg/day, or placebo for 12 weeks. There was no significant benefit for either red clover product over placebo in improving hot flash frequency or Greene Menopause Symptom score. Women receiving Promensil initially had a more rapid decline in hot flash frequency compared with women in the other 2 groups. However, at 12 weeks, the reduction in mean number of daily hot flashes was similar for women receiving Promensil (41%), Rimostil (34%), and placebo (36%).

The crossover study by Baber and colleagues[40] administered Promensil 40 mg/day isoflavones or placebo to 51 menopausal women for 12 weeks, followed by all participants receiving 1 month of placebo, and then 12 weeks of the alternate arm. There were no significant differences in hot flashes, flushing, or other physiologic parameters between the Promensil and placebo groups. Limitations of the study include small sample size, single-blind design, and the finding that some women in the placebo groups had higher urinary isoflavone levels at the end of the trial compared with baseline, raising questions about possible dietary confounders. Knight and coworkers[39] reported no statistically significant difference in alleviation of symptoms between groups receiving isoflavones 40 mg/day, isoflavones 160 mg/day, or placebo (reduction in hot flashes of 29%, 34%, and 29%, respectively). The study was small; only 37 postmenopausal women were enrolled.

In contrast to these negative findings, 2 smaller studies[37,38] found a statistically significant reduction in hot flash frequency among women taking Promensil compared with placebo. van de Weijer and Barentsen[37] randomized 30 women reporting ≥5 hot flashes per day to either Promensil (80 mg/day isoflavones) or placebo for 12 weeks. Hot flash frequency was significantly ($P < 0.01$) lower in women receiving red clover, although no significant difference was seen in the Greene score across groups. The study was limited by its small sample size, lack of detailed description of methodology or calculations, and lack of intention-to-treat analysis. Jeri[38] compared Promensil (40 mg/day isoflavones) with placebo for 16 weeks in a small sample (N = 30) of women in Peru. A significant ($P < 0.001$) reduction in hot flash frequency (48.5%) and severity was reported in the active group compared with the

Table 5 Placebo-controlled trials of red clover

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Tice et al[36]	252	Postmenopausal women	Placebo for 12 wk	Promensil* (82 mg/day isoflavones) or Rimostil† (57 mg/day isoflavones) for 12 wk	Hot flash frequency, changes in QOL	No significant difference between groups in hot flash frequency. Greene scale or adverse events
van de Weijer and Barentsen[37]	30	Postmenopausal women	Placebo for 12 wk	Promensil (80 mg/day isoflavones) for 12 wk	Hot flash frequency, Greene Menopause Symptom score	Reduction in hot flash frequency per day in active treatment (5.4 to 3.4) compared with placebo (5.8 to 6.0; P <0.01). No significant difference in Greene score
Jeri[38]	30	Postmenopausal women	Placebo for 16 wk	Promensil (40 mg/day isoflavones) for 16 wk	Hot flash frequency and severity	Reduction in hot flash frequency (48.5%) and severity (47%) significantly greater with active treatment than placebo (10.5% reduced frequency and no change in severity; P <0.001)
Knight et al[39]	37	Postmenopausal women	Placebo for 12 wk	Promensil (160 mg/day isoflavones) or Promensil (40 mg/day isoflavones) for 12 wk	Hot flash frequency and Greene Menopause Symptom score	No statistically significant difference between 2 red clover arms and placebo
Baber et al[40]	51	Postmenopausal women	Placebo or active for 3 mo followed by 1 mo placebo, then 3 mo alternate arm	Promensil (40 mg/day isoflavones)	Hot flash frequency and Greene Menopause Symptom score	No statistically significant difference between active treatment and placebo groups

QOL = quality of life.
*Promensil (red clover isoflavones; Novogen Ltd., North Ryde, New South Wales, Australia).
†Rimostil (red clover isoflavones; Novogen Ltd.).

placebo group, who experienced a small reduction in frequency (10.5%) and no change in hot flash severity.

Safety

No serious adverse effect has been reported in clinical trials or in the medical literature, but because red clover leaf extracts contain semipurified isoflavones, the question of safety in hormone-sensitive tissue is important, especially when considering high doses over prolonged periods. Atkinson and coworkers[41] found no significant difference in mammographic breast density after 12 months' administration of Promensil (43.5 mg/day isoflavones) compared with placebo in a study in 177 women (aged 49 to 65 years) with increased breast density, nor was there any statistically significant effect on estradiol, follicle-stimulating hormone (FSH), or luteinizing hormone levels. Clifton-Bligh and colleagues [42] found that Rimostil dosages up to 85.5 mg/day taken for 6 months did not increase endometrial thickness. Baber and coworkers[40] found no significant differences in serum estradiol, FSH, sex hormone-binding globulin, vaginal cytology, or endometrial thickness after 3 months' administration of Promensil 40 mg/day. At this time, the safety of red clover leaf extracts in women with a history of breast cancer is unknown. Although not an adverse effect per se, red clover isoflavones can inhibit the drug-metabolizing cytochrome P-450 system enzymes CYP1A1, CYP1B1, and CYP2C9[43]; however, no herb-drug interaction has been reported.

Dose and preparation

Clinical trials for menopause-related symptoms have been conducted primarily on the proprietary semipurified isoflavone leaf extracts marketed as Promensil and as Rimostil. The ratio of genistein plus biochanin A to daidzein plus formononetin is 1.9:1.0, with 40.0 to 43.5 mg total isoflavones per Promensil tablet. Rimostil purportedly contains a ratio of genistein plus biochanin A to daidzein plus formononetin of 0.15:1.0, with <29.5 mg total isoflavones per tablet. It is important to avoid simply comparing the difference in total isoflavones between these products because the isoflavone profiles differ considerably.

Conclusion

The majority of clinical data do not support the efficacy of semipurified isoflavone red clover leaf extracts in reducing hot flash frequency and severity or in relieving symptoms associated with the domains of the Greene Menopausal Symptom Scale.

Soy (*Glycine max* L.)

It has been postulated that dietary intake of soy may explain, in part, the lower reporting of hot flashes by Asian women,[44] compared with women in the United States.[45] This has led to a surge in interest in soy in the research community and among consumers. The present review considers soy extracts only in dietary supplement form. We identified 6 controlled studies of soy extract dietary supplements that met our inclusion criteria (**Table 6**).[46–51]

Efficacy

In aggregate, the 6 studies presented in Table 6 produced equivocal results. The largest study, by Upmalis and coworkers,[51] found a statistically significant reduction in hot flash severity, but no reduction in incidence, in the active treatment group (50 mg/day soy isoflavones; genistein and daidzein) compared with placebo ($P = 0.01$). However, the use of the glycoside form of isoflavones, as opposed to the biologically active aglycones, at the dose administered may have influenced the findings.

The crossover study by Nikander and coworkers[49] failed to find any significant difference between the active treatment (114 mg/day soy isoflavone tablets containing glycitein 58%, daidzein 6%, and genistein 6%) and placebo groups with respect to KI or other outcome measures. Penotti and colleagues[46] did not detect any significant difference in hot flash frequency or severity between postmenopausal women receiving soy isoflavones 72 mg/day (genistein 11 mg, daidzein 36 mg, and glycitein 25 mg) and those receiving placebo. Both groups had a 40% reduction in the number of hot flashes.

In contrast to the negative results from other trials, Scambia and colleagues[50] reported that after 6 weeks, women receiving soy extract 400 mg/day (corresponding to isoflavones 50 mg/day) had a significant reduction in mean number of hot flashes ($P < 0.01$) and mean point values 19 (hot flashes) and 20 (night sweats) on the Greene scale ($P < 0.001$) when compared with those taking placebo. The study was of short duration and was small; only 39 women were enrolled.

Faure and coworkers[47] randomized 75 women reporting ≥7 hot flashes per day to placebo or soy extract (total isoflavones 70 mg/day [genistein, daidzein, biochanin, and formononetin]). Women experiencing a ≥50% reduction in hot flashes were significantly ($P < 0.005$) more likely to be in the soy treatment group (65.8%) than in the placebo group (34.2%). However, the trial suffered from attrition; 39% of the placebo group failed to complete the study.

Han and associates[48] found a significant ($P < 0.01$) reduction in KI score among women taking isoflavones 100 mg/day (genistein 70 mg, daidzein 18 mg, and glycitein 12 mg) compared with placebo. The mean KI score decreased from 44.6 to 24.9 in the active treatment group, whereas there was a small increase (from 40.3 to 41.6) in the placebo group. Lack of placebo response is highly unusual in a menopause study.

Safety

Soy extracts appear to be well tolerated, with few if any serious adverse effects noted in clinical studies. A review by Munro and colleagues[52] concluded that the literature, when viewed in its entirety, supports the notion that soy isoflavones consumed in foods or dietary supplements are safe. The report by Unfer and associates,[53] however, has raised questions of safety with regard to long-term use of higher doses of soy isoflavones. After 5 years, 6 cases (3.8%) of endometrial hyperplasia were detected by endometrial biopsy in women receiving soy extract 1,800 mg/day (soy isoflavones 150 mg/day); no case was found in the placebo group. Investigators reported that the difference in rates of endometrial hyperplasia between the 2 groups was statistically significant ($P < 0.05$), although 41 biopsy specimens from the placebo group were considered unassessable at the final 5-year point.

Dose and preparations

There are numerous soy extracts in the marketplace that provide widely varying levels of isoflavones in a range of mixtures and ratios. The isoflavone dosages used in the majority of clinical trials are in the 50- to 100-mg/day range.

Conclusion

The results from clinical studies are contradictory and difficult to evaluate owing to variation in soy preparations, dosages, and durations of treatment. Many products fail to adequately declare the composition of isoflavones (e.g.,

Table 6 Placebo-controlled trials of soy extracts

Study	N	Sample	Control	Treatment	Primary Outcome Measures	Results
Penotti et al[46]	62	Postmenopausal women	Placebo for 6 mo	1-g tablet bid (72 mg/day soy isoflavones) for 6 mo	Self-reported hot flash frequency (diary)	No significant difference between groups: both had 40% reduction in hot flash incidence
Faure et al[47]	75	Menopausal women	Placebo for 16 wk	2 Phytosoya* capsules bid (70 mg/day soy isoflavones) for 16 wk	Self-reported hot flashes and night sweats (symptom card)	The percentage of "responders" (hot flashes reduced by ≥50% at end of treatment) was 65.8% in the soy extract group and 34.2% in the placebo group (P <0.005)
Han et al[48]	80	Postmenopausal women with breast cancer	Soy protein without isoflavones (50.3 mg) for 16 wk	1 soy extract capsule tid (100 mg/day soy isoflavone) for 16 wk	KI	Greater decrease in KI score with soy extract (44.6 to 24.9) than with placebo (40.3 to 41.6) (P <0.01)
Nikander et al[49]	62	Women with vasomotor symptoms and history of breast ancer	Placebo for 3 mo, followed by 2-mo washout, then 3 mo alternate arm	3 phytoestrogen tablets bid (114 mg/day isoflavones)	KI, VAS, mood	No significant difference between groups in KI, VAS, or other outcome measures
Scambia et al[50]	39	Menopausal women	Placebo for 12 wk	SoySelect† (50 mg/day isoflavones) qd for 12 wk	Hot flash frequency and severity, Greene Menopause Scale	At 6 wk, compared with placebo, soy decreased frequency (P <0.01) and severity (P <0.001) of hot flashes; no change in other outcome measures
Upmalis et al[51]	177	Postmenopausal women	Placebo for 12 wk	Soy extract (50 mg/day isoflavones) qd for 12 wk	Hot flash and night sweat frequency and severity	At 12 wk significant reduction in severity of hot flashes and night sweats (P <0.01) but not frequency

KI = Kupperman Index; VAS = Visual Analogue Scale.
*Phytosoya; Arkopharma, Carros, France.
†Soy Select; Indera SpA, Milan, Italy.

percentage of individual isoflavone, glycoside or aglycone form), further complicating comparison between trials. Substantial interindividual differences in isoflavone metabolism exist in the general population,[54] which may also affect study outcomes. At this time, the scientific evidence cannot support the recommendation of soy isoflavone extracts for the relief of menopausal symptoms.

Summary and discussion

Whereas studies indicate that black cohosh extracts ease menopausal symptoms, most investigations are limited by methodologic shortcomings; additionally, comparison between trials is complicated by variations in dosing and in use of products with different extraction systems. A total of 4 case reports of acute hepatitis involving 5 women using black cohosh have been reported in the literature, although previously published safety reviews suggest that the herb generally is well tolerated. Soy isoflavone extracts appear to exert minimal effects, but dose differences and marked variation in product composition limits definitive conclusions. Long-term safety of higher-dose (150 mg/day) soy isoflavones is uncertain. Most clinical data do not support the efficacy of semipurified isoflavone red clover leaf extracts to reduce hot flash frequency and severity or to relieve symptoms associated within the domains of the Greene Menopausal Symptom Scale. Limited data suggest that dong quai, ginseng extract, and evening primrose seed oil are ineffective in ameliorating menopausal symptoms at the doses and in the preparations used in these studies; no significant adverse effect has been associated with their use.

Reports in the literature on botanicals used to treat menopausal symptoms reveal equivocal findings, with some studies reporting efficacy for specific products and others reaching opposite conclusions. Differences in findings across studies of the same botanical may be a function of less than optimal trial design, variation in products used, duration of treatment, inadequate dosing, and/or use of small or noncomparable population samples. When comparing products that differ in extraction technique, delivery system, ratio of ingredients, and dose, the question of equivalency must be considered. It also makes the "pooling" of trial data problematic. For example, reaching a definitive conclusion on the efficacy of soy isoflavone extracts is difficult when the products vary so markedly in composition. Many of the trials failed to provide adequate definitions for "postmenopausal" or "menopausal," which also may contribute to varied findings. Some treatments may be more effective in the perimenopausal woman than in the postmenopausal woman, or vice versa.

Future research should include long-term safety assessments of botanical preparations used for menopause-related symptoms because women may use these products for prolonged periods. Dose-escalation studies should be conducted before conducting expensive clinical studies on botanical extracts. The development of analytical methods for identifying and verifying the quantity of active ingredients, or marker compounds, in botanical products must be a priority, as is improving quality-control measures from seed to shelf. In addition to implementing rigorous clinical trial designs, researchers must clearly describe the botanical product being studied (i.e., plant part, method of botanical identification, solvent extraction method, etc.) to enable replication and critical analyses of the research.

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Menopause:
Editorial: PDF Only

Guidance for Clinical Evaluation of Combination Estrogen/Progestin-Containing Drug Products Used for Hormone Replacement Therapy of Postmenopausal Women

Abstract

The following guidance was prepared by the staff of the FDA's Division of Metabolism and Endocrine Drug Products to advise drug manufacturers of current standards for the development of combined estrogen/progestin drugs for the treatment of postmenopausal women. As stated in the footnote, "(t)hese recommendations are based on current data and may require modification as additional data become available."

The Division is pleased that the editors of Menopause are willing to publish this guidance in a journal devoted to this subject and hope that the publication will generate a productive discussion of the various issues involved.

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Efficacy of Soyfoods and Soybean Isoflavone Supplements for Alleviating Menopausal Symptoms Is Positively Related to Initial Hot Flush Frequency

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ABSTRACT

Soy has received attention as an alternative to conventional hormone replacement therapy (HRT) largely because it is a unique dietary source of isoflavones. Isoflavones are diphenolic compounds that have both hormonal and nonhormonal properties and are considered to be selective estrogen receptor modulators. The estrogen-like effects of isoflavones in combination with the low reported frequency of hot flushes in Japan has prompted investigation of the effect of soy on menopausal symptoms. The purpose of this review is to evaluate the efficacy of soyfoods and isoflavone supplements for the alleviation of hot flushes. Nineteen trials (13 using a parallel design) involving more than 1,700 women were identified. Six trials were excluded from analysis: two that involved breast cancer patients, two that reported data on severity but not hot flush frequency, one that was not blinded, and one that did not include a control group. Based on a simple regression analysis of the remaining data set (13 trials), there was a statistically significant relationship ($P = .01$) between initial hot flush frequency and treatment efficacy. Initial hot flush frequency explained about 46% of the treatment effects, and hot flush frequency decreased by about 5% (above placebo or control effects) for every additional initial hot flush per day in women whose initial hot flush frequency was five or more per day. Although conclusions based on this analysis should be considered tentative, the available data justify the recommendation that patients with frequent hot flushes consider trying soyfoods or isoflavone supplements for the alleviation of their symptoms.

INTRODUCTION

SOYFOODS HAVE PLAYED an important role in the diets of many Asian countries for centuries. But within the past 10 years, soyfoods and soybean constituents have attracted widespread research attention in the West for their purported health benefits. Many of the newly developed "Westernized" soy products specifically target women because of reports that soy may be helpful for conditions and diseases associated with the menopausal years. Soy is much discussed as a possible alternative to conventional hormone replacement therapy (HRT),[1–6] in large part because it is a unique natural dietary source of isoflavones.[7,8] This perspective may gain even further attention as a result of the recently published disappoint-

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ing findings from the Heart and Estrogen/Progestin Replacement Study (HERS) I/II[9,10] and the Women's Health Initiative.[11] Isoflavones have estrogen-like properties[12–14] and are considered by many experts to be natural dietary selective estrogen receptor modulators (SERMs).[15–17]

Traditionally, one reason, if not the primary reason, that women elected to use HRT was for relief of menopausal symptoms.[18] Because many women may now opt not to use HRT for such purposes, health professionals, especially clinicians, need accurate information about the efficacy of alternative agents and approaches to alleviating hot flushes. Soyfoods and soybean isoflavones are popular alternatives,[19] but thus far no comprehensive review on their efficacy in the alleviation of hot flushes has been published. The intent of this paper is to fill this void and to present the hypothesis that soyfoods and soybean isoflavones effectively alleviate menopausal symptoms in women who have frequent hot flushes. Trials examining the effects of isoflavones derived from red clover were not included in the statistical analysis to avoid any potential differences in efficacy stemming from the difference in isoflavone profile between soybean- and red clover-derived isoflavones. Results of several recent studies on the effects of red clover isoflavones are available elsewhere.[20–24]

BACKGROUND

Isoflavones have traditionally been considered to be weak estrogens, although it is not possible to arrive at a single estimate of potency, which varies according to tissue. In addition, it is well established that serum isoflavone levels in response to modest soyfood consumption can reach the low micromolar range, about 100 to 1,000 times that of estrogen.[25] This suggests, even assuming a relatively weak potency, that isoflavones have the potential to exert biological effects *in vivo*, and in fact they have been reported to do so in several trials that included a variety of different endpoints.[26–30]

However, it is probably more accurate to refer to isoflavones as having estrogen-like (rather than estrogenic) properties, because they behave differently from estrogen. For example, isoflavones bind with much greater affinity to estrogen receptor-β (ER-β) than to ER-α,[12,31] and they are much more potent at triggering transcriptional activity when bound to ER-β rather than ER-α.[32] Also, isoflavones have potentially important nonhormonal effects.[33]

In 1992, Adlercreutz et al.[34] first suggested that the estrogen-like properties of isoflavones might account for the low incidence of hot flushes reportedly experienced by women in Japan, a notion later popularized by Lock.[35,36] In general, the incidence of hot flushes among Asian women tends to be lower[37,38] than among Western women, although many other menopausal symptoms (e.g., shoulder aches, psychological changes) are experienced to a similar extent.[39] Consistent with these observations are recent epidemiological data indicating that American women of Chinese and Japanese ancestry are about one third less likely to report experiencing hot flushes, compared with Caucasian-American women.[40]

Obviously, there are many reasons why Asian women may experience fewer hot flushes than Western women do. For example, it could be that the lower premenopausal estrogen levels among Asian women mitigate the drop in estrogen concentration that occurs as a woman enters the menopause, and thus the trigger for the onset of hot flushes is minimized.[41] However, there exists epidemiological support, albeit limited, for the notion that soy may be a contributing factor. For example, in a small cross-sectional study involving 284 Japanese women 40–59 years of age, fermented but not total soy product intake was inversely related to hot flush severity ($P < .05$).[42] Stronger support came from a Japanese prospective epidemiological study: during 6 years of follow-up among the 101 women who experienced hot flushes, the hazard ratios were 0.42 (P for trend = .005) and 0.47 (P for trend = .002) for intake of total soy product (g/d) and isoflavones (mg/d), respectively, when comparing those in the highest and lowest tertiles of intake.[43]

Finally, in a case-control study by Somekawa et al.[44] involving 478 postmenopausal Japanese

women, the incidence of hot flushes tended to decrease as isoflavone intake increased, although this relationship was not statistically significant. Lack of statistical significance may have been partially related to the isoflavone intake cutoff values used in this study. The lowest intake quartile included women who consumed as much as 35 mg of isoflavones per day. This represents considerable soy intake and is an amount that may have exceeded the threshold isoflavone exposure necessary to experience benefit. In the prospective study by Nagata et al.[43] cited previously, the hazard ratio for hot flushes decreased from 1.00 to 0.78 between the first and second tertile of intake; the average isoflavone intakes in those two groups were 20.5 and 32.6 mg/day, respectively.[43]

One of the difficulties of conducting trials to evaluate the efficacy of agents for hot flush relief is the large placebo effect observed in most studies. According to Loprinzi et al.,[45] placebo decreases hot flushes by an average of 25% over a 3- to 4-week period, about 10% of women experience a 75% or greater reduction in hot flushes with placebo, and another 10% experience a reduction of 50% to 75%. To circumvent this problem, Pan et al.[46] examined the effects of soy on rat tail skin temperature. They found that the rise in tail skin temperature induced by ovariectomy was significantly reduced in rats fed a diet containing isoflavone-rich soy protein (soy+), compared with rats fed the control diet lacking soy ($P = .04$) or a diet containing isoflavone-poor soy protein ($P = .10$). However, the effect of soy+ was not as pronounced as the effect of estrogen. Obviously, ovariectomized rats are not little menopausal women, but at least in this model the placebo effect is eliminated.

CLINICAL TRIALS

Overall description

The first trial assessing the efficacy of soy (soyflour) for the alleviation of menopausal symptoms was published in 1995.[47] Since that time, 11 additional studies[48–58] have examined the effects of soyfoods (7 using isolated soy protein, 1 soyflour, 1 soymilk, 1 textured vegetable [soy] protein, and 1 a combination of traditional soyfoods[58]), and 7 others[59–65] have investigated the effects of isoflavone supplements, on hot flush frequency, or severity, or both (Table 1). These 19 trials were conducted in eight countries, ranged in duration from 4 weeks to 24 months (although 11 of the trials were 12 weeks in length), and exposed women to intakes of 34 to 100 mg isoflavones per day (in most cases, ≥70 mg/day). For comparison, average isoflavone intake for a Japanese adult is about 35 to 40 mg/day.[66]

Twelve of the 19 studies were randomized, double-blinded, parallel trials; 5 were randomized, double-blinded, crossover trials; 1 was a parallel trial that was not blinded; and 1 had no control group. Altogether, in the 12 soyfood trials, 467 women were given the control diet and 482 the soy diet, and in the 7 supplement trials, 323 women were given the placebo and 474 the isoflavones, for a total of 1,746 participants. (The actual number of women in these trials is slightly different, because some women participated in both the control and active arms, and, in three trials[51,52,67] there was more than one soy group but only the group fed the soy protein with the highest isoflavone content was considered for review and analysis.) Because the trial by Brzezinski et al.,[48] which found a statistically significant decrease in hot flush frequency in women fed soyfoods (and some flax) compared with the control group, was not blinded, it was excluded from the analysis, as was the trial by Albert et al.,[63] which found a statistically significant favorable response among women given an isoflavone supplement but did not include a placebo group. Elimination of these two trials brings the totals down to 431 control and 404 experimental subjects in the soyfood trials and 323 placebo and 323 experimental subjects in the isoflavone supplement trials.

Analysis

Of the 11 remaining soyfood trials, only 1 found that women in the active arm experienced a statistically significant decrease in hot flush frequency.[50] This trial is intriguing because the investigators noted that improve-

TABLE 1. FINDINGS FROM TRIALS EXAMINING THE EFFICACY OF SOYFOODS AND ISOFLAVONE SUPPLEMENTS FOR ALLEVIATING HOT FLUSHES

Author (ref. no.)	*Design*	*Duration (wk)*	*Isoflavone exposure (mg/day)*	*Age (yr)*	*Control/ Placebo (N)*	*Soyfood/ Isoflavone (N)*	*Initial hot flushes/day*[a]	*Absolute % change versus inactive arm*[b]
Trials involving soyfoods								
Albertazzi (50)	Parallel	12	76	53	39	40	11.2	↓ 12.5%
Knight (54)	Parallel	12	77	53	11	9	7.6	↓ 22.2%
Woods (58)	Crossover	12	46	45–58	85	85	6.6[c]	↑ 4.6%
Murkies (47)	Parallel	12	53	55	24	23	5.7	↓ 17%
Dalais (49)	Crossover	12	52	54	11	11	3.9[c]	↑ 29%
Washburn (51)	Crossover	6	34	51	51	51	3.7	↓ 4.7%
Burke (57,66)	Parallel	12	58	51	70	65	3.4	↓ 1.4%
St. Germain (52)	Parallel	24	80	50	24	21	2.2[c,d]	↑ 33.2%
Totals					315	305		
Trials involving isoflavone supplements								
Han (62)	Parallel	16	100	49	40	40	10.7	↓ 26.4%
Faure (64)	Parallel	16	70	54	35	38	9.8	↓ 35.8%
Upmallis (60)	Parallel	12	50	55	63	59	8.6[e]	↓ 9%
Hochanadel (65)	Crossover	12	100	45–55	11	11	4.5[c]	↑ 27.0%
Scambia (61)	Parallel	6	50	54	19	20	4.3	↓ 19.5%
Totals					168	168		
Trials excluded from regression analysis and reason for exclusion								
Quella (59)—cancer patients	Crossover	4	—	>18	155	155	7.3	↓ 1%
van Patten (55)—Cancer patients	Parallel	12	—	55	64	59	7.3	↑ 8.4%
Kotsopoulos (53)—no data on frequency	Parallel	12	—	59	40	33	NA	Decrease in severity, 3.7%
Balk (56)—no data on frequency	Parallel	24	—	57	12	7	NA	Increase in severity, 25.8%
Brzezinski (48)—not blinded	Parallel	12	—	52	36	78	NA	Improvement in hot flush score, 19%
Albert (63)—no control	Single arm	16	—	>45	0	151	7.12[c]	↓ 57.9%
Totals					307	483		

NA, not available.
[a]Represents average of control/placebo and soyfood/isoflavone supplement groups.
[b]Unless otherwise indicated, refers to changes in hot flush frequency.
[c]Data on hot flush frequency provided by authors via personal communication.
[d]Only the isoflavone-rich soy protein and the whey group were considered for analysis; results from week 12 of study.
[e]Values estimated from figure in published article.

ment in the soy group lessened as compliance worsened. One additional trial found that isolated soy protein, when consumed two times per day, significantly reduced hot flush severity (although not incidence), compared with the control diet or with consumption of the same amount of isolated soy protein but in one sitting per day.[51]

In contrast to the soyfood trials, four of the six remaining isoflavone supplement trials found that women in the active arm experienced a statistically significant decrease in hot

flush frequency; however, in one of these four studies, the results were significant at 6 weeks ($P = .0275$) but only marginally significant at 12 weeks ($P = .078$).[60] Of the two trials that did not find isoflavone supplements to be effective at any point, one involved only 11 subjects[65] and the other was a large, 4-week trial that involved breast cancer patients, two thirds of whom were taking tamoxifen.[59] This latter trial was the only study conducted for such a short duration. The U. S. Food and Drug Administration (FDA) recommends that trials evaluating the efficacy of drugs such as HRT for alleviation of menopausal symptoms be continued for at least 3 months.[68] In any case, in those trials that found soy or isoflavones to be efficacious, differences between the inactive and active arms were apparent early on, and the gap between the two typically continued to increase beyond 4 weeks.[50,60]

The supplement trial by Han et al.[62] which reported a 26% decrease in hot flush frequency in the isoflavone group (relative to placebo), is particularly interesting because the subjects were specifically instructed to consume the isoflavones three times each day. This pattern is likely to result in higher serum isoflavone levels than would have been seen with consumption of the same total amount in one or two sittings per day, given the known half-life of isoflavones (6–9 hours).[25] The dose of isoflavones used in this trial (100 mg) was also at the high end among all trials, which would further increase serum isoflavone levels. Findings from the study by Han et al.[62] are consistent with the data from Washburn et al.[51] discussed previously.

The lack of statistical significance among the soyfood trials, in and of itself, should not be taken as an indication of a lack of efficacy, because the large placebo effect makes most of these trials underpowered to detect modest effects. Therefore, it is worthwhile to consider the absolute differences between the groups. Of the nine soyfood trials in which no statistically significant effects were found, three reported percentage decreases (absolute differences relative to control) in hot flush frequency or severity (3.7%,[53] 17.0%,[47] and 22.2%[54]), and six reported percentage increases (1.4%,[67] 4.6%,[58] 8.4%,[55] 19.0%,[52] 25.8%,[56] and 29.0%[49]). Although many of the soy trials were of small size, it is apparent that there is essentially an equal chance that women in the soy group will experience an increase rather than a decrease in symptomology. On this basis, it is hard not to conclude that soyfoods lack efficacy.

If that is the case, is one to conclude that isoflavone supplements are more efficacious than soyfoods? First, it is important to acknowledge in addressing this question that the isoflavone supplement trials involved a total of only 323 women in each arm, and that the largest trial[59] ($N = 155$) did not report a statistically significant decrease in hot flush frequency (although, as noted previously, that trial involved breast cancer patients). With this small number of subjects, the favorable findings may be due to chance. Also, hot flush frequency decreased only slightly in the placebo group in two of the four supplement trials, in one[60] by less than 20%, and in another[62] by only 1%. Certainly, decreases of even 25% in the placebo group would have prevented statistically significant effects from being observed between the placebo and isoflavone groups in both trials. But these trials did involve 63[60] and 40[62] women in the placebo groups and therefore should not be dismissed.

The explanation that the results observed were due to "chance" is appealing because there is no obvious biological basis for thinking that isoflavones and soyfoods would differentially affect menopausal symptoms. Isoflavones are the only components of soy thought to be responsible for the hypothesized effects on hot flushes. Furthermore, the bioavailability of isoflavones from soyfoods is similar to that of supplements,[25] and the amount of isoflavones to which women were exposed was similar in the soyfood and the isoflavone supplement trials. Still, differences in "isoflavone delivery vehicle" cannot be completely dismissed as an explanation for the apparent discrepancy in results between the soyfood and the isoflavone supplement trials. However, there is at least one other, even more attractive explanation: that the supplement trials produced favorable findings compared with the soyfood trials because, on average, the baseline level of hot flush frequency was higher among the participants in the supplement trials.

In the four supplement trials in which statistically significant beneficial effects were observed, the number of hot flushes per day initially was 10.7,[62] 9.8,[64] 8.6,[60] and 4.3.[61] Three of these values are on the very high end among all trials conducted. Interestingly, in the three trials in which women in groups fed soyfoods reported markedly fewer hot flushes (only one of which was statistically significant), the number of initial hot flushes per day was 11.2,[50] 7.6,[54] and 5.7.[47] As with the supplement trials, these values are also on the high end.

In contrast, the number of initial hot flushes per day for women in the soyfood trials that did not report decreases was 7.3,[55] 6.6,[58] 3.9,[49] 3.7,[51] 3.4,[67] and 2.2.[52] Furthermore, of these six studies, the one with the highest initial hot flush frequency (7.3/day) involved breast cancer patients, one third of whom were taking tamoxifen.[55] Consistent with the hypothesis proposed here, that soy/isoflavones are effective in women who have more frequent hot flushes, are the results from two trials that examined only severity, neither of which reported beneficial effects of soyfoods. Women in these two trials reported initial values of 0.84[53] and 1.3[56] on a three-point severity scale (0, none; 1, mild; 2, moderate; 3, severe). Although it is not possible to directly translate degree of severity into frequency, it would appear that women in these two groups were not terribly bothered by their symptoms.

Figure 1 presents the correlation between initial frequency of hot flushes and treatment efficacy (i.e., the absolute percentage change in the soy/isoflavone group minus that in the control/placebo group). Based on a simple regression analysis of the whole (see Table 1 and text for reasons four additional trials were excluded from analysis) data set (13 trials), there was a statistically significant relationship ($P = .01$) between initial hot flush frequency and treatment efficacy. More specifically, initial hot flush frequency explained about 46% of the treatment effects, and hot flush frequency decreased about 5% (above placebo/control effects) for every additional initial hot flush per day in women whose initial hot flush frequency was ≥5. Assuming a 20% placebo effect, these findings indicate, for example, that women having approximately 10 hot flushes per day who begin to consume soy or isoflavones can

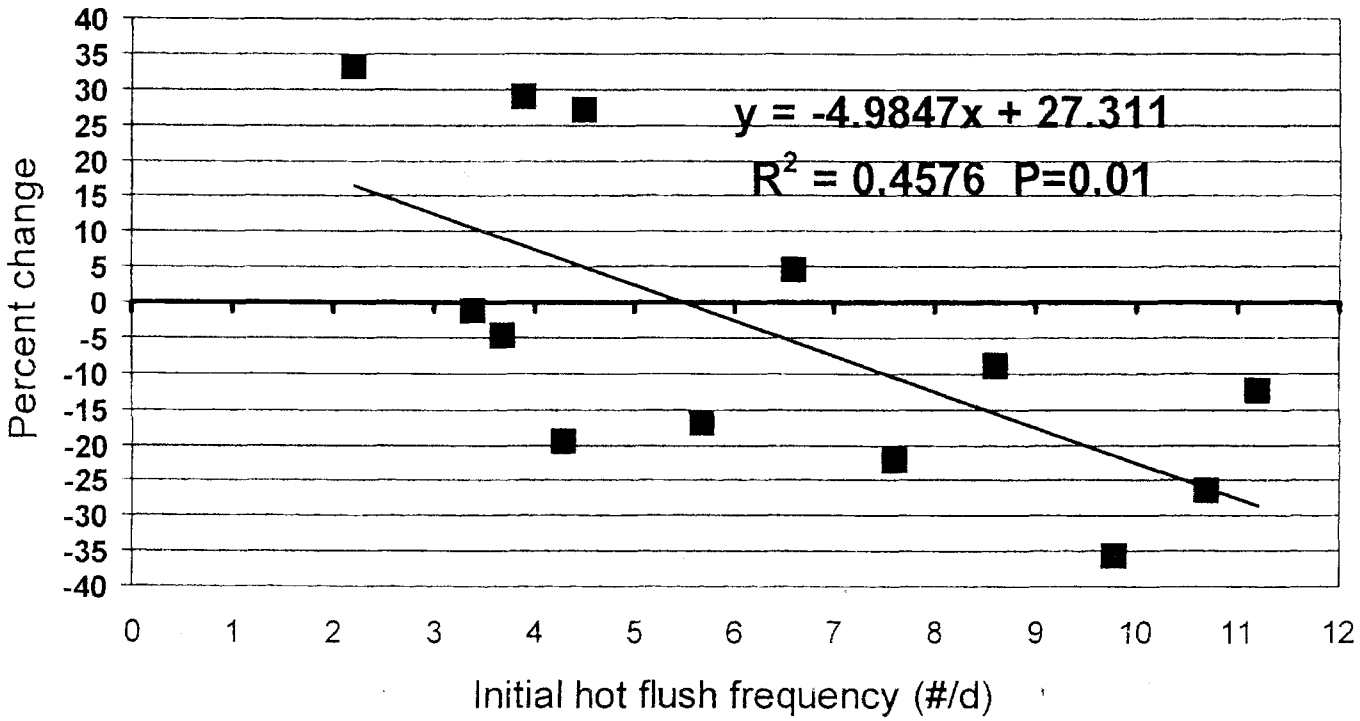

FIG. 1. Relationship between initial hot flush frequency and treatment efficacy of soyfoods and isoflavone supplements.

expect to experience a reduction of about 4.5 hot flushes per day, whereas women with an initial 7 hot flushes per day will experience a reduction of about 3 hot flushes per day. Further analysis of covariance (data not shown) suggested that treatment efficacy was greater in those trials utilizing a parallel, rather than a crossover, design. There is no obvious reason why efficacy would be greater in the parallel trials, but the observation that efficacy is greater in women with more frequent hot flushes is certainly biologically plausible if greater frequency reflects a lower estrogen status. In this type of hormonal environment, the estrogen-like properties of isoflavones may be more apparent.

Given the significant correlation between frequency and efficacy, the recommendation by the FDA that trials examining the efficacy of drugs for menopausal symptom relief include women with more than 60 moderate to severe hot flushes per week is particularly germane.[67] Trials adhering to such guidelines would obviously place them at the very highest frequency level among women in the soy/isoflavone trials included in this review. In fact, all four of the trials meeting this recommendation[50,60,62,64] found statistically significant beneficial effects of soyfoods/isoflavones. Nevertheless, at least three caveats need to be cited when evaluating the hypothesis that the efficacy of soy/isoflavones is related to initial hot flush frequency.

First and foremost, the final analysis included only 13 data points. Second, four trials that did not show efficacy were among those excluded from the correlation. Two trials involving breast cancer patients[55,59] were excluded on the premise that breast cancer patients (regardless of tamoxifen use) may respond differently to soy and isoflavones than healthy women do. Whether this is the case is a matter of debate.[69] Two trials that examined only severity were also excluded because of the lack of data.[53,56] Third, in a small subgroup analysis, Burke et al.[67] failed to find that women with four or more hot flushes per day responded differently to soy than did women with fewer hot flushes per day (the average number of hot flushes per day was 7.4 in the placebo group and 9.0 in the soy group). Among women with more frequent hot flushes, the frequency decreased by 59.5% and 62.2% in the control and soy groups, respectively.[67] However, it is clear that with a placebo effect this large, a modest effect of soy may have been obscured.

It should be noted that the correlation as calculated leaves open the possibility that soy/isoflavones could actually increase hot flushes in women with infrequent hot flushes. But this appears doubtful, and the findings from the three trials that actually reported relatively large increases relative to the control/placebo groups[49,52,65] were likely a result of chance. Two of these three trials involved only 11 subjects in each study arm.[49,65] The other trial[52] involved perimenopausal women, a difficult group to study because of fluctuating hormone levels and because perimenopausal women are reported to experience a higher placebo response than postmenopausal women.[70] Also, the primary endpoint of this particular trial was bone mineral density; the data on hot flushes were collected retrospectively.

Furthermore, it is important that all three trials that reported increases in hot flush frequency relative to placebo also noted decreases in comparison to the baseline values in the soy/isoflavone groups. In the perimenopausal trial[52] and in the trials by Dalais et al.[49] and Hochanadel et al.,[65] hot flush frequency decreased by approximately 17%, 22%, and 12%, respectively, relative to baseline values. Therefore, in contrast to other SERMs such as tamoxifen[71,72] and raloxifene,[73] there is no evidence to suggest that soyfoods or isoflavones exacerbate hot flushes.

Finally, trials examining the impact of isoflavones derived from red clover provide some support for the hypothesis that soyfoods and soybean isoflavones are effective primarily in women with frequent hot flushes. Van de Weijer et al.[20] found that Promensil® reduced hot flush frequency to a statistically significantly greater extent than did placebo. To be enrolled in this trial, women had to have at least 5 hot flushes per day. The authors suggested that the failure of previous trials to show that red clover isoflavones were efficacious may have occurred

because of the inclusion of women who were only mildly symptomatic.[22,23]

CLINICAL RELEVANCE

One might question the clinical relevance of the soy/isoflavone effect because those trials in which soy/isoflavones were effective mostly reported decreases of less than 20% compared with the inactive arm. Clearly, the effects are less than that of estrogen.[74] However, for at least three reasons this degree of improvement should not be dismissed. First, in women with severe symptoms, any improvement is likely to be welcomed. Furthermore, it is not just the soy/isoflavone effect that needs to be considered, but also a possible placebo effect accompanying the act of using soyfoods or isoflavone supplements, such that the overall improvement is likely to be as high as 50%. For women who are having ten hot flushes per day, five per day may look very appealing. Second, some women are likely to experience above-average responses; for these women, soy/isoflavones may be very effective. And third, an intriguing but still speculative body of research suggests that isoflavones and isoflavone-rich soy protein may have skeletal benefits[75–78] and may also reduce coronary heart disease risk by improving arterial compliance[26] and flow-mediated dilation.[27] Of course, soy protein also has a modest cholesterol-lowering effect.[79]

Thus, there appear to be several reasons for menopausal women to ingest isoflavones through foods or pills. Importantly, there is no reason to suspect that isoflavones pose any of the disease risks observed in the HERS I/II and WHI trials.[80] Consequently, although health professionals should take a guarded approach and emphasize that the data are inconsistent and the effects are likely to be modest, they can in good conscience recommend that their clients with frequent hot flushes try soy/isoflavones for relief of their symptoms. In respect to dose, based on the results from the hot flush trials and from studies examining other possible benefits, an initial dose of 50 mg isoflavones per day can be recommended, with an upper limit of approximately 100 mg/day.

FUTURE RESEARCH

In view of the hypothesis suggested in this paper, future trials involving soyfoods and isoflavone supplements are warranted but should focus on women who have frequent hot flushes. In addition, within a given trial, investigators should examine whether there exists a correlation between initial hot flush frequency and the extent of reduction of symptoms among trial subjects. Parenthetically, it may be possible to do this type of analysis retrospectively, using the raw data from existing trials. Although it is unlikely that any single future trial will be sufficiently large to prove or disprove the proposed hypothesis, the results from several smaller trials, even if the findings are not statistically significant, can help to establish whether initial hot flush frequency is related to efficacy.

Ideally, trials should be large enough to also consider the impact of isoflavone metabolism on efficacy. A recent Japanese cross-sectional study of 180 women given a standardized questionnaire to evaluate the severity of menopausal symptoms found that those who produced equol (from the action of intestinal bacteria on the soybean isoflavone daidzein, occurring in approximately 30% to 50% of those who consume soyfoods) recorded the least severe symptomology.[81] Therefore, investigators should establish whether efficacy differs according to equol production. Some research suggests that equol is more estrogen-like than its parent isoflavone daidzein, which could account for this observation.[82–85] There are also large variations in serum isoflavones among subjects in response to the intake of similar amounts of isoflavones.[25,86] Therefore, serum isoflavone levels or perhaps isoflavone excretion should also be considered as a possible variable affecting efficacy.

Finally, it would be interesting to determine whether isoflavones are more effective at preventing the onset of hot flushes when consumed before the menopause (rather than alleviating them once they have already begun). This might account for the very low incidence of hot flushes in Japan. Although this hypothesis would obviously be quite difficult to test clinically, additional epidemiological research could provide some insight.

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In Vitro Effects of Soy Phytoestrogens on Rat L6 Skeletal Muscle Cells

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ABSTRACT Soy isoflavones display estrogenic activity in humans and animals, and thus are referred to as phytoestrogens. This study was performed to observe the effects of the soy isoflavones genistein, daidzein, and glycitein on cell cultures of rat skeletal muscles. [^{3}H]Thymidine incorporation was used to determine cell proliferation, while protein synthesis and degradation were determined by tracking radiolabeled leucine. For the proliferation studies, insulin, estradiol, genistein, daidzein, or glycitein was supplemented at 0, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, or 20 μM, respectively, or in combinations with final concentrations of 0, 0.1, 1, or 10 μM. Genistein reacted most similarly to estradiol, inhibiting proliferation at ≥ 1 μM ($P < .001$). A combination of phytoestrogens resulted in significant inhibition of cell proliferation, but not to the extent observed with genistein alone. For the protein synthesis and degradation experiments, treatments of 0.1 μM dexamethasone or 1 μM concentrations of insulin, genistein, daidzein, or glycitein were used. Phytoestrogens did not inhibit or stimulate protein degradation or synthesis ($P > .05$). A one-tailed univariate analysis of variance revealed a trend ($P \leq .1$) in protein stimulation with genistein and glycitein treatments. These results suggest that the tyrosine kinase inhibiting activity of genistein may be affecting phosphorylation of the mitosis-promoting factor, preventing the advancement of the mitotic cell cycle. In addition, at higher total combined concentrations, daidzein and glycitein may be able to outcompete genistein for receptor sites. These results suggest that soy isoflavones in the diet may potentially modulate normal growth and development in humans and animals that ingest soy-based products.

KEY WORDS: • ***growth*** • ***isoflavone*** • ***myoblast*** • ***phytoestrogen*** • ***proliferation***

INTRODUCTION

CONSUMPTION OF SOY PHYTOESTROGENS is associated with beneficial health properties similar to endogenous estrogens.[1] For example, estrogens improved skeletal muscle cell membrane integrity, and decreased energy loss.[2] In addition, the estrogenic compound estrone stimulated cell proliferation, while 17β-estradiol (E_2) had no effect on proliferation.[3]

Estrogen receptors (ERs) have been quantified in both smooth[4,5] and skeletal[2,5] muscle cells. ERβ is expressed in smooth muscle cells and is likely the receptor type responsible for improved cardiovascular health in the presence of E_2. ERs have also been found in skeletal muscle cells, but have yet to be identified as ERα or as ERβ.[3] Recently mRNA for ERβ has been quantified in human skeletal cells, but no difference in transcript level could be attributed to gender.[6]

Soy isoflavones contain a phenolic ring[7] and show distinct agonistic and antagonistic properties on ERα and ERβ.[8] Genistein has been reported to inhibit L8 myoblast proliferation, fusion, and myotube protein synthesis,[9] whereas genistein, daidzein, and glycitein have successfully inhibited proliferation of smooth muscle cells.[10] In this study, rat L6 skeletal muscle cells were used to assess soy phytoestrogens' effects on cell proliferation, protein degradation, and protein synthesis.

MATERIALS AND METHODS

Rat L6 myogenic cell lines were acquired from American Type Culture Collection (Manassas, VA). Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Cell lines were grown to 80% confluency in 75-cm^2 flasks before passaging and plating. All cultures were maintained at 95% air/5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). Cell culture procedures and analyses were followed as previously described.[11,12]

Experiments 1–3 examined cell proliferation, Experiment 4 examined protein degradation, and Experiment 5 examined protein synthesis stimulation or inhibition. The media for Experiment 1 consisted of DMEM with FBS supplemented with insulin, estradiol (E_2), genistein, daidzein, and glycitein, at 0, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 5, 10, or 20 μM concentrations, respectively. The medium for Experiment 2 was DMEM with FBS with individual phytoestrogens or in combination. Final concentrations included

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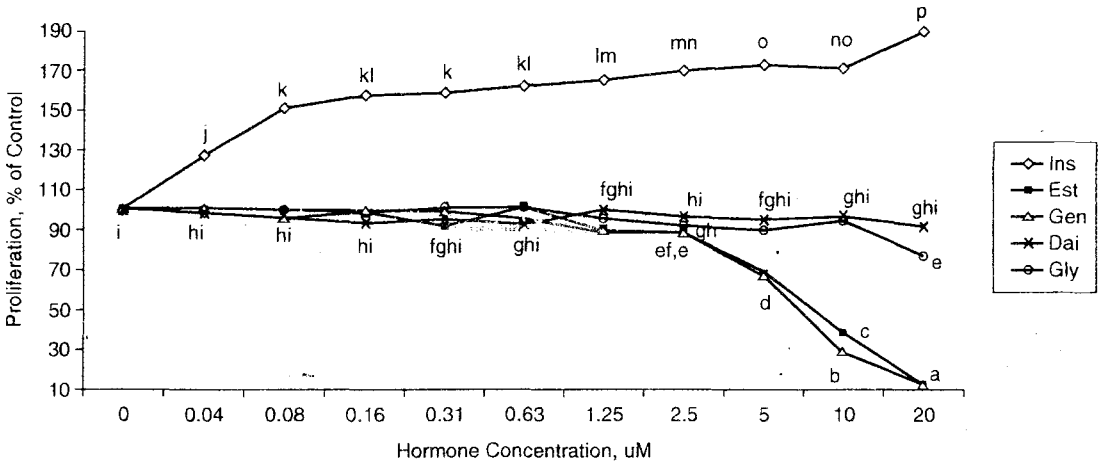

FIG. 1. Graphical representation of the proliferative response of L6 rat myoblasts to increasing dosages of insulin (Ins), E_2 (Est), genistein (Gen), daidzein (Dai), and glycitein (Gly). Points with different letters were significantly different ($P < .0001$). For 0 and 25 μM, $n = 12$, SE = 2.23; for 0.04 μM, 0.08 μM, 0.16 μM, 0.31 μM, 0.63 μM, 5 μM, 10 μM, and 20 μM, $n = 6$, SE = 3.19; for 2.5 μM, $n = 14$, SE = 2.07.

0.1, 1.0, or 10 μM concentrations of genistein, daidzein, or glycitein, respectively, or combined in the naturally occurring proportion of 45% genistein + 45% daidzein + 10% glycitein. To determine the effects for each phytoestrogen within combination levels, a third proliferation study was performed. Experiment 3 consisted of DMEM with FBS plus the combination treatment of 45% genistein + 45% daidzein + 10% glycitein at 0, 0.1, 1.0, and 10 μM final concentrations. In addition, the phytoestrogens were observed as their respective proportion of the combination. Genistein and daidzein were used at 0.045, 0.45, and 4.5 μM; corresponding concentrations for glycitein were 0.01, 0.1, and 1.0 μM, respectively. Media for Experiment 4 consisted of 2 mM Leu-DMEM with 2% FBS alone or supplemented with 1 μM E_2, 1 μM genistein, 1 μM daidzein, 1 μM glycitein, 1 μM daidzein, 1 μM glycitein, or 0.1 μM dexamethasone. Treatments tested in Experiment 5 were DMEM plus 2% FBS alone or supplemented with 1 μM insulin, 0.1 μM dexamethasone, and 1 μM E_2, with 1 μM genistein, 1 μM daidzein, or 1 μM glycitein, respectively.

Data were analyzed using analysis of variance (ANOVA) using the General Linear Model (GLM) of the Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC, 1999). Treatment and trial were set as independent variables, while percentage of control was set as the dependent variable. A 6 × 11 factorial design was used for calculation of F values for protein degradation trials, for treatment, trial, and treatment × trial effects. For protein synthesis trials, a 6 × 6 factorial design was used for calculation of F values; treatment, trial, and treatment × trial effects were tested. *Post hoc* analyses were determined using the PDiff function of SAS.

When significance was detected for degradation, results were analyzed by one-way ANOVA using SPSS version 10.0 for Windows (SPSS, Inc., Chicago, IL), with *post hoc* analysis with Tukey's Honestly Significant Difference test and Scheffé's test. For synthesis trials, results were analyzed using a one-tailed univariate ANOVA (with percent protein formation stimulation/inhibition as the dependent variable, and treatment as the independent variable) for a significance level of $P = .1$.

RESULTS

Cell proliferation trials were conducted with concentrations of experimental supplements ranging from 0 to 20 μM. Results of all trials were analyzed as percentages of nonsupplemented control. At all concentrations analyzed, insulin significantly stimulated proliferative activity of L6 cells (Fig. 1). Concentrations of E_2 and genistein over 2.5 μM had inhibitory ($P < .0001$) effects on proliferation (0, 1.25 μM, $n = 12$, SE = 2.23; 0.04 μM, 0.08 μM, 0.16 μM,

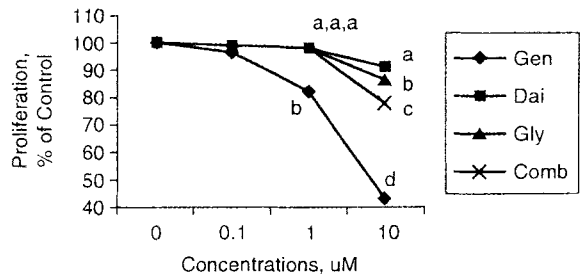

FIG. 2. Effect of single and combined [Comb; 45% genistein (Gen), 45% daidzein (Dai), and 10% glycitein (Gly)] phytoestrogens at various concentrations on proliferation of rat L6 myoblasts. Points with different letters indicate significant differences ($P < .0001$) within a concentration ($n = 3$, SE = 0.422–5.58).

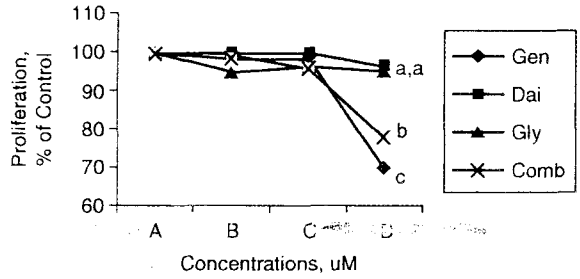

FIG. 3. Effect of single and combined [Comb; 45% genistein (Gen), 45% daidzein (Dai), and 10% glycitein (Gly)] phytoestrogens at various concentrations on proliferation of rat L6 myoblasts ($P < .001$). Point A (0 μM) was the control. Point B treatment levels were 0.045 μM Gen, 0.045 μM Dai, 0.01 μM Gly, and 0.1 μM for the combination. Point C treatment levels were 0.45 μM Gen, 0.45 μM Dai, 0.1 μM Gly, and 1.0 μM for the combination. Point D treatment levels were 4.5 μM Gen, 4.5 μM Dai, 1.0 μM Gly, and 10 μM for the combination ($n = 4$, SE = 0.35–1.62).

0.31 μM, 0.63 μM, 5 μM, 10 μM, 20 μM, $n = 6$, SE = 3.19; 2.5 μM, $n = 14$, SE = 2.07). In cell proliferation trials of comparison plates with 0, 0.1, 1.0, and 10 μM concentrations of genistein, daidzein, and glycitein or with a combination treatment (45% genistein + 45% daidzein + 10% glycitein, yielding final concentrations of 0.1, 1.0, and 10 μM total supplementation; Fig. 2), genistein inhibited ($P < .0001$) proliferation at 1.0 and 10 μM concentrations ($n = 3$, SE = 0.422–5.58) when compared with equimolar isoflavones. At a 10 μM/mL concentration, the combination treatment inhibited ($P < .001$) proliferation compared with daidzein and glycitein alone, but not as profoundly as genistein at this highest concentration ($n = 3$, SE = 0.422–5.58).

In Experiment 2, the isoflavones alone and in combination were compared at equimolar final concentrations. To better understand an individual isoflavone's contribution in the combined inhibition of cell proliferation, another proliferation experiment was conducted. In this experiment, the combination treatments were repeated keeping the 45% genistein, 45% daidzein, and 10% glycitein proportions at 0, 0.1, 1.0, and 10 μM. However, the individual isoflavones were evaluated at concentrations reflecting their respective proportion in the combined treatment (Fig. 3). Once again, the 10 μM combined treatment reduced cell proliferation ($P < .001$), and genistein alone reduced cellular proliferation more than the combined treatment. No inhibition of cell proliferation was observed at 0.1 or 1 μM combined treatment or the individual isoflavones for these concentrations.

In Experiment 4, protein degradation was evaluated (Fig. 4). Averaged values of inhibition/stimulation of protein degradation for individual treatments were standardized to percentages of control (2% FBS + 2 mM Leu-DMEM). An initial analysis by sum of squares, with a 6 × 11 factorial design, indicated that there were differences ($P < .05$) due to replicate and treatment. Results were then analyzed using one-way ANOVA, with *post hoc* Tukey's Honestly Significant Difference and Scheffé's tests ($P < .05$). *Post hoc* analysis revealed that insulin inhibited ($P < .05$) protein degradation while the isoflavones did not affect protein degradation.

Analysis of the protein synthesis data revealed that there were no differences ($P > .05$) due to treatment or replicate (Fig. 5). However, a one-tailed univariate ANOVA revealed ($P = .1$) trends due to treatment. Dexamethasone tended to inhibit ($P = .057$) protein synthesis compared with E_2 and the isoflavones, whereas genistein and glycitein treatments tended to stimulate ($P = .087$) compared with controls.

DISCUSSION

The soy phytoestrogens genistein, daidzein, and glycitein have diverse effects on cellular activity. Genistein inhibits cellular proliferation in non–estrogen-induced cancer cells at concentrations of 1 μM and greater. This might be due to the inhibiting potential of genistein, a known receptor tyrosine kinase inhibitor.[13] At the beginning of the cell cycle, a

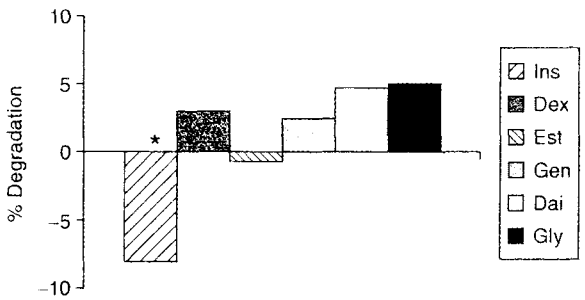

FIG. 4. Results of the protein degradation trials, with 1 μM concentrations of insulin (Ins), E_2 (Est), genistein (Gen), daidzein (Dai), and glycitein (Gly) and 0.1 μM concentration of dexamethasone (Dex). Significant differences are indicated by an asterisk ($P < .05$).

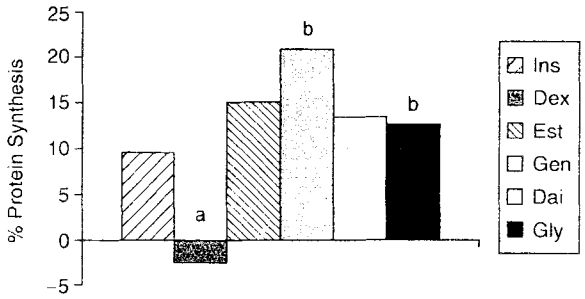

FIG. 5. Graphical representation of percent protein synthesis stimulation or inhibition by 1 μM concentrations of insulin (Ins), E_2 (Est), genistein (Gen), daidzein (Dai), and glycitein (Gly) and 0.1 μM concentration of dexamethasone (Dex). Dex treatment results were different from those of Est, Gen, Dai, and Gly ($P \leq .057$, SE = 9.92). Gen and Gly showed trends in stimulation ($P \leq .087$, SE = 9.92). Letters indicate trends observed when compared with control.

two-subunit complex is composed of cyclin B and Cdc2, the mitosis-promoting factor. The mitosis-promoting factor complex must be properly phosphorylated and dephosphorylated on tyrosine and threonine sites for the cell cycle to advance. At 0.7 μg/mL genistein had a half-maximal effect on receptor tyrosine kinase inhibition.[13] If genistein can act to inhibit tyrosine kinases, it is possible that genistein acts via the tyrosine 45 site on the mitosis-promoting factor complex to inhibit the cell cycle, thus inhibiting proliferation.

Cell proliferation observed in the current study does not coincide with another study[3] where E_2 had no effect on proliferation in L6 skeletal muscle cells *in vitro*. Here, E_2 had an inhibiting effect on these skeletal muscle cells. There were differences in the protocols that might account for these dissimilar findings. In the previous study,[3] serum-free, phenol red-free medium was used for proliferation trials. In the present study cells were incubated at a minimal presence of 2% FBS, in phenol red-containing DMEM. It is possible that the use of phenol red-containing DMEM might have accentuated the effect of E_2. Cells were also completely serum deprived in the study by Kahlert *et al.*,[3] while in the present study cells were incubated in 2% FBS, to insure that cells would maintain viability. Preliminary trials here with complete serum deprivation resulted in little cell proliferation across all treatments, even with insulin treatment (data not shown). The length of serum deprivation differed as well. Kahlert *et al.*[3] conducted a 24-hour serum deprivation, while cell cultures were serum deprived for only 16 hours here. These slight changes may account for the differences observed.

The actions of daidzein and glycitein can be speculated upon. Individually, these two phytoestrogens may not have strong affinity for binding sites when compared with genistein. Kuiper *et al.*[14] found that genistein competes with E_2 for binding to the ER, with preference to ERβ over ERα. However, the observation that glycitein had little effect on proliferation is unexpected. Song *et al.*[15] suggested that glycitein may be more physiologically active than previously thought, having a relatively strong binding affinity to ERs. Thus, the little to no response seen here was unexpected. Perhaps daidzein and glycitein have preferential binding for ERβ over ERα, or vice versa. As the ERs have not been clearly identified in skeletal muscle cells, this could account for the response seen here if one ER is more prevalent than the other receptor.

Insulin's action on protein turnover is documented.[16,17] In protein synthesis, insulin stimulates, while in degradation insulin inhibits. In the present study insulin had an inhibitory effect on protein degradation, but had no effect on protein synthesis. Further analysis of passage number, an indicator of cell age, was analyzed as a possible correlation in the protein degradation trials. No evidence suggested that cell age influenced protein degradation results. As no prior research has been conducted concerning the effects of estrogen or the respective phytoestrogens on protein turnover, it is difficult to determine the norm. In the present study, even though there was a trend observed with genistein and glycitein treatment in protein synthesis, there were no significant effects due to the treatment with E_2 or the phytoestrogens on protein turnover. Possible implications of this study include the potential for affecting fetal development and human health. If phytoestrogens cross into the bloodstream to gestating subjects, skeletal muscle cell, as well as smooth muscle cell, development could be modified. If cellular proliferation is inhibited by the presence of genistein *in vitro*, it could be possible that embryonic cellular proliferation during development could be altered as well. Phytoestrogens could also mimic the membrane protecting activities of estrogen; then skeletal muscle cells would be more resistant to damage during exercise because of improved muscle cell membrane integrity. Indeed, these *in vitro* results suggest that soy isoflavones may potentially modulate growth and development in humans and animals that ingest soy-based products. Future research should focus on the potential *in vivo* beneficial and/or detrimental effects of the soy isoflavones on growth and development.

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Combinatory effects of phytoestrogens and 17ß-estradiol on proliferation and apoptosis in MCF-7 breast cancer cells

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Abstract

Phytoestrogens have been described to be weak estrogens, SERMs or exhibit antiestrogenic properties. However, information about their activity in presence of estrogens is limited. Therefore, we have analysed the dose dependent combinatory activity of the phytoestrogens genistein (Gen), daidzein (Dai) and coumestrol (Cou), and 17ß-estradiol (E2) on cell proliferation and apoptosis induction in human MCF-7 breast cancer cells. Neither additive nor antagonistic effects on proliferation could be observed, but in contrast all phytoestrogens possessed the ability to inhibit apoptosis in the presence of 17ß-estradiol.

In summary, our in vitro results demonstrate that Gen does not exhibit any antiestrogenic properties. The additive growth stimulatory effects of Gen, Dai and Cou in the presence of E2 are not the result of a stimulation of proliferation; these phytoestrogens, at least in MCF-7 cells, could be characterised as inhibitors of apoptosis.

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Keywords: Phytoestrogen; Genistein; Daidzein; Coumestrol; Combinatory effect

1. Introduction

Effects of phytoestrogens on human health are very controversy debated. On one hand, it has been described that the intake of these compounds may protect against hormone-dependent cancers [1] and their use is propagated as a natural alternative to hormonal replacement therapy [2]. On the other hand, the potential risks, like action as endocrine disruptors [3], are still a matter of discussion. Although it is known that the estrogenic potency of these substances is rather low compared to endogenous or synthetic estrogens, information about their activity in presence of those estrogens is limited. To classify distinct phytoestrogens as weak agonists, selective estrogen receptor modulators (SERMs) or antiestrogens, it is essential to study their action also in the presence of endogenous estrogens. Therefore, we have examined the dose-dependent effects of the phytoestrogens genistein (Gen), daidzein (Dai) and coumestrol (Cou) in comparison to 17ß-estradiol (E2), fulvestrant (Ful) and raloxifene (Ral) by analysis of the cell cycle distribution and apoptosis induction in human MCF-7 breast cancer cells. In addition, we have analysed whether the phytoestrogens interfere with the activity of estradiol by co-treating the cells with E2 and the test compounds. Cell cycle distribution and apoptosis induction was analysed by using flow cytometry.

2. Materials and methods

17ß-Estradiol, genistein and coumestrol were provided by Sigma–Aldrich (Steinheim, Germany). Daidzein was obtained from Biomol (Hamburg, Germany). Fulvestrant was provided by Zeneca (Zeneca Pharmaceuticals, Macclesfield, UK) and raloxifene was provided by the Schering AG (Berlin, Germany). The minimum purity of all used phytoestrogens was 98%.

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MCF-7 human breast cancer cells were cultured as described previously [4]. E2, Ful and Ral were added from a concentrated stock solution [10^{-3} M] in ethanol; Gen, Dai and Cou from a concentrated stock solution [10^{-3} M] in DMSO. The cells were cultured for five days with E2 [10^{-13}–10^{-10} M] or Ful, Ral, Gen, Dai and Cou [10^{-8}–10^{-5} M]. Part 2 of the study was performed by co-treating MCF-7 cells with E2 [10^{-12}–10^{-10} M] and Gen, Dai and Cou [10^{-6} M]. The antiestrogen Ful was added in a concentration of [10^{-7} M] and [10^{-6} M] to E2 [10^{-10} M]. Cells were cultured in CCS medium (charcoal-treated calf serum) two days before the administration of the test compounds to exclude estrogenic effects via medium. Experiments were repeated at least five times. Flow cytometry was performed as described before [4].

3. Results

Effects of genistein, daidzein and coumestrol on cell cycle distribution and apoptosis induction were analysed in human MCF-7 cells by flow cytometry. 17ß-Estradiol, the pure antiestrogen fulvestrant and the SERM raloxifene served as reference compounds.

In Table 1 the applied concentrations of the compounds and mean ± standard deviation of S, G_1, and G_2 phases, which result in 100%, are presented. Sub G_0 values represent the percentage of apoptotic cells from the entirety of cells.

Our results demonstrate that administration of the investigated phytoestrogens in doses lower than [10^{-6} M] (Fig. 1) induces only a weak stimulation of proliferation. In contrast, at higher concentrations [10^{-5} M], all phytoestrogens are potent stimulators of proliferation. Administration of Ful has – due to the pure antiestrogenic properties of this substance – no effects on proliferation, but results in a dose-dependent induction of apoptosis. Interestingly the phytoestrogens effects on apoptosis result in a u-shaped doses response curve. All phytoestrogens posses the ability to inhibit the rate of apoptosis at doses of [10^{-6} M] and even at doses where they are unable to stimulate proliferation [10^{-7} M]. In contrast, administration of the phytoestrogens in very high doses [10^{-5} M] results in an induction of apoptosis. Cou displays a proliferation/apoptosis profile similar to E2. Taken together, the maximum effects of the phytoestrogens on the rate of apoptosis occur at doses of [10^{-6} M], whereas the strongest effects on proliferation appear at [10^{-5} M].

To investigate combinatory effects of the phytoestrogens and E2 on apoptosis and proliferation, MCF-7 cells were co-treated with E2 and Gen, Dai and Cou. As a control, cells were also co-treated with combinations of E2 and Ful. The combined application of Ful with E2 reveals a strong and significant rise in apoptosis and a reduction in proliferation

Table 1
Cell cycle analysis of MCF-7 cells after dose-dependent administration of E2 ([10^{-13}–10^{-10} M]) and Gen, Dai, Cou, Ful and Ral (all [10^{-8}–10^{-5} M]); $x \pm s$, $n \geq 3$.

	Apoptosis		Cell cycle		
	[M]	Sub G_0 [%]	S [%]	G_1 [%]	G_2 [%]
CCS		33.1 ± 3.6	12.7 ± 2.2	70.3 ± 1.6	17.1 ± 1.6
E2	10^{-13}	38.4 ± 4.3	10.8 ± 4.4	71.4 ± 3.9	17.8 ± 2.6
	10^{-12}	37.4 ± 11.0	14.5 ± 4.5	70.2 ± 3.5	15.5 ± 2.9
	10^{-10}	17.8 ± 1.6	18.6 ± 3.6	62.4 ± 1.2	19.0 ± 2.7
Ful	10^{-8}	38.0 ± 0.7	10.6 ± 0.4	76.6 ± 2.3	12.8 ± 2.1
	10^{-7}	45.1 ± 1.8	11.0 ± 1.8	74.3 ± 2.2	14.6 ± 0.7
	10^{-6}	49.3 ± 0.8	9.3 ± 0.9	81.2 ± 0.2	9.5 ± 1.1
	10^{-5}	53.8 ± 0.6	11.5 ± 1.1	79.4 ± 0.5	9.0 ± 0.8
Ral	10^{-8}	19.3 ± 0.8	9.1 ± 1.1	82.7 ± 1.8	8.2 ± 0.8
	10^{-7}	18.2 ± 1.5	7.3 ± 0.6	83.0 ± 1.4	9.7 ± 1.1
	10^{-6}	26.2 ± 0.9	9.9 ± 2.1	79.0 ± 2.2	11.1 ± 0.3
	10^{-5}	21.6 ± 1.1	12.1 ± 1.5	78.4 ± 2.8	9.6 ± 1.3
Gen	10^{-8}	34.2 ± 9.2	13.8 ± 2.4	69.9 ± 2.8	16.3 ± 2.2
	10^{-7}	26.2 ± 6.0	13.7 ± 1.9	70.4 ± 3.4	16.0 ± 3.9
	10^{-6}	22.4 ± 3.1	12.7 ± 3.5	69.6 ± 3.5	17.8 ± 4.1
	10^{-5}	26.2 ± 2.9	23.1 ± 8.2	61.4 ± 2.1	15.6 ± 6.6
Dai	10^{-8}	42.2 ± 9.2	14.0 ± 3.5	72.5 ± 2.2	13.6 ± 2.5
	10^{-7}	23.0 ± 4.0	16.2 ± 5.3	66.3 ± 4.2	17.6 ± 3.3
	10^{-6}	19.2 ± 10.0	15.3 ± 1.6	67.3 ± 3.9	17.5 ± 3.3
	10^{-5}	21.4 ± 8.3	20.2 ± 2.8	60.9 ± 2.4	18.9 ± 2.6
Cou	10^{-8}	29.8 ± 7.3	12.9 ± 2.8	70.0 ± 1.2	17.2 ± 1.8
	10^{-7}	18.6 ± 7.6	16.0 ± 1.7	67.0 ± 3.3	17.1 ± 3.5
	10^{-6}	14.4 ± 6.2	17.4 ± 2.9	65.8 ± 2.6	16.9 ± 4.4
	10^{-5}	29.3 ± 3.9	19.3 ± 2.1	62.1 ± 2.7	18.7 ± 3.1

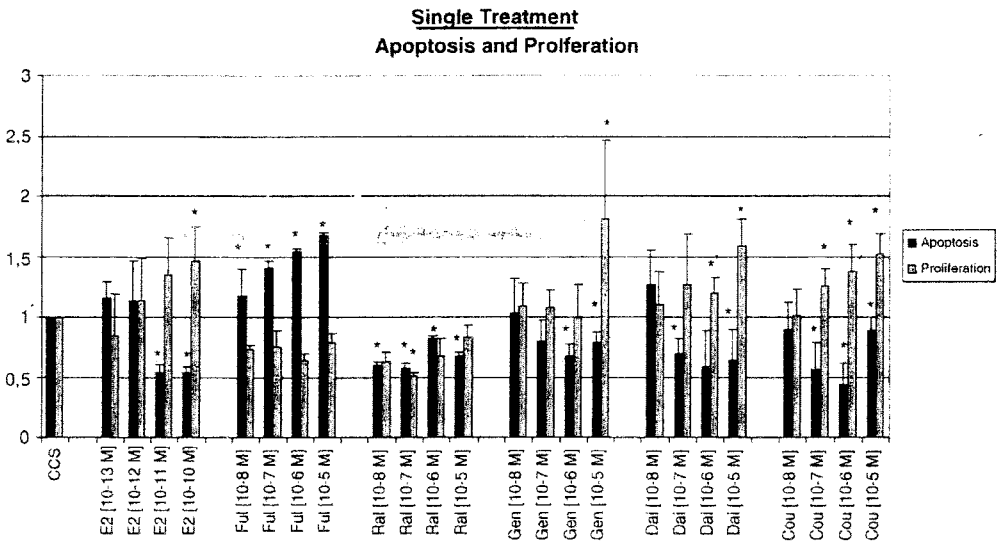

Fig. 1. Apoptosis and proliferation after single treatment. Rate of apoptosis and cell proliferation of MCF-7 cells after dose-dependent administration of E2 ([10^{-13}–10^{-10} M]) and Ful, Ral, Gen, Dai and Cou (all [10^{-8}–10^{-5} M]); the rate of apoptosis is indicated by the percentage of Sub G_0 nuclei of all nuclei measured. The rate of apoptosis in the CCS control group was defined as 1. Cell proliferation is indicated by the percentage of cells in the S phase of the cell cycle. The rate of proliferation in the CCS group was defined as 1. Asterisk (*) means significant against CCS control group ($P \leq 0.05$), Mann–Whitney U-Test.

in comparison to CCS control. In all groups co-treated with E2 and the investigated phytoestrogens, a reduction of the rate of apoptosis could be observed (Fig. 2). This effect is significant against CCS control group at least for the combination of E2 [10^{-10} M] with the phytoestrogens in a dose of [10^{-6} M]. In contrast to apoptosis, co-treatment with E2 and the phytoestrogens does not effect proliferation significantly. There is only a tendency for an increase of proliferation; however this effect is not significant against CCS control.

4. Discussion

Because data about the activity of phytoestrogens in presence of estrogens is limited, the major aim of this study was to examine the combinatory activity of phytoestrogens and 17ß-estradiol (E2) in vitro. As mentioned above, Cou shows a proliferation/apoptosis profile similar to E2. In agreement to previous investigations [5], these data indicate pure agonistic properties of this substance. Interpretation of the proliferation/apoptosis profiles of Gen and Dai is more complicated. At doses up to [10^{-6} M], they mainly act as inhibitors of apoptosis, comparable to the effects of the SERM Ral. These data are also in agreement to own previous data [5] and results of other groups [6], demonstrating SERM like properties of these compounds. In comparison to the pure antiestrogen fulvestrant none of the tested phytoestrogens demonstrate any profile indicating antagonistic properties. This is verified in our combination experiments.

Co-treatment of MCF-7 cells with different doses of E2 and the respective phytoestrogens [10^{-6} M] resulted in none of the used combinations in any antagonisation of the E2 activity (Fig. 2). The rate of proliferation was not affected by any of the phytoestrogen/E2 combinations. However, like shown for single administration, effects could be observed for the combinations with regard to the rate of apoptosis (Fig. 2A). Co-treatment of MCF-7 cells with E2 and Gen, Dai or Cou resulted at least in the combination with E2 [10^{-10} M] in a significantly enhanced inhibition of apoptosis (Fig. 2A).

Summarising the data of our experiments, there is clear evidence that the analysed phytoestrogens are not able to antagonise the effects of E2 in MCF-7 breast cancer cells. All phytoestrogens are acting additive with respect to inhibition of apoptosis, but only Cou also with respect to proliferation. These results are in agreement to previous data indicating a very low potency at least of Gen to induce proliferation in the uterus [7]. In addition, they provide a mechanistically basis to understand controversy data with respect to the effects of phytoestrogens on proliferation of MCF-7 breast cancer cells. Recently, two articles [8,9] describe a strong induction of MCF-7 cell growth after treatment with Gen. However, in none of these presented studies reference compounds were analysed simultaneously, and the investigators did not distinguish between the effects on apoptosis and cell proliferation. Our results demonstrate that Gen and Dai posses only a limited ability to induce proliferation at doses higher than [10^{-6} M], but they inhibit the rate of apoptosis, which can serve as an explanation for an increase of cell number—without a significant stimulation of proliferation. Whereas, Gen and Dai only show the ability to inhibit apoptosis – like the SERM Ral – Cou also stimulates simultaneously proliferation. This is again an argument to classify Gen and Dai as PhytoSERMS and Cou as a phytoagonist. Inhibitory effects on apoptosis were also detected in the MCF-7 combination experiments, demonstrating that this property of the investigated phytoestrogens is also relevant

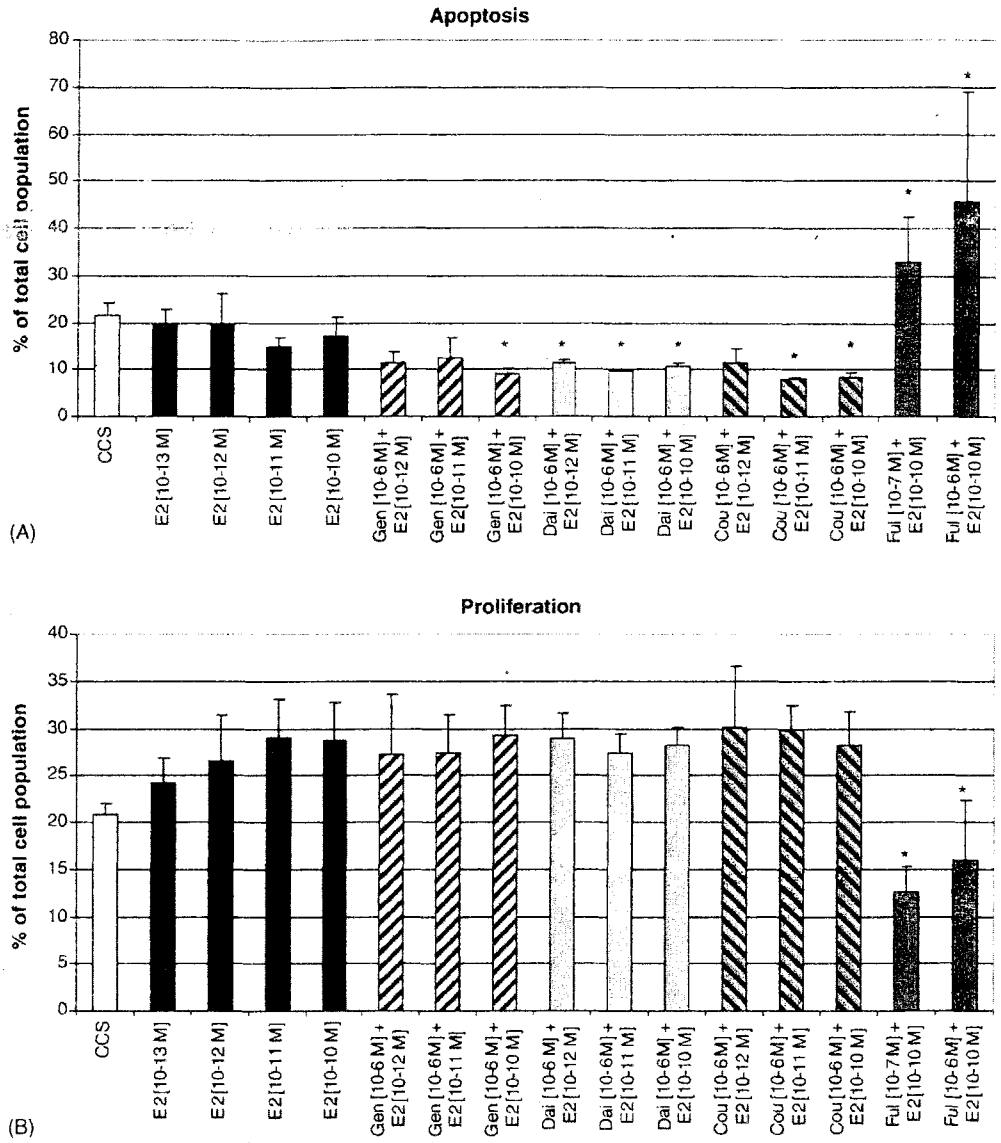

Fig. 2. Apoptosis and proliferation after co-treatment. Rate of apoptosis (A) and cell proliferation (B) of MCF-7 cells after co-treatment with E2 ([10^{-12}–10^{-10} M]) and Gen, Dai, Cou (each [10^{-6} M]), respectively, E2 ([10^{-10} M]) with Ful ([10^{-8}–10^{-7} M]); the rate of apoptosis is indicated by the percentage of Sub G_0 nuclei of all nuclei measured. Cell proliferation is indicated by the percentage of cells in the S phase of the cell cycle. Asterisk (*) means significant against CCS control group ($P \leq 0.05$), Mann–Whitney *U*-Test.

in the presence of E2. With regard to this, the results of our investigations are in the line to data of tumor models, showing an enhanced promotion of tumor growth in the presence of phytoestrogens and low levels of estrogens [10]. Summarising the results of our combination studies in MCF-7 cells, we can conclude that the investigated phytoestrogens do not inhibit the growth of MCF-7 cells by antagonising the growth stimulating effects of E2. In contrast, in MCF-7 cells phytoestrogens seem to stimulate the increase of cell number. Interestingly, this increase is not the result of a stimulation of proliferation; it is the consequence of the inhibition of the rate of apoptosis. In any case, these results support the hypothesis that phytoestrogens may stimulate the progression of an existing estrogen sensitive tumor.

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New Estrogenic Prenylflavone from *Epimedium brevicornum* Inhibits the Growth of Breast Cancer Cells

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Original Paper

Abstract

Estrogens maintain female sexual health. The hormone also drives the growth of estrogen receptor (ER) positive breast tumors, and ER modulators, like tamoxifen, are used to reduce tumor recurrence. To identify phytoestrogens with possible health benefits, we screened several Traditional Chinese Medicines and encountered an extract from the leaves of *Epimedium brevicornum* (EB), with strong (EC_{50}: 1.3 μg/mL) and specific ER-stimulatory activity. It increased estrogen-responsive human breast cancer cell proliferation at low doses, but paradoxically caused profound inhibition of growth at higher doses. Using bioassay-guided fractionation, we isolated and characterized a new prenylflavone, breviflavone B, which exerted biphasic stimulatory and inhibitory effects on breast cancer cell proliferation, mimicking the effects of EB. In contrast to estradiol and genistein, high doses (> 2 μM) of breviflavone B almost eliminated ERα protein; a process that may be mediated through increased proteasome degradation. Pre-clinical studies are needed to explore whether these prenylflavones are of value in estrogen-deficiency states and for prophylaxis of breast cancer.

Key words
Epimedium brevicornum · Berberidaceae · prenylflavone · estrogen · receptor · anti-proliferation · breast · cancer

Introduction

Phytoestrogens are plant-derived non-steroidal compounds that can behave as estrogen mimics. Flavonoids make up the most common form of phytoestrogens and are sub-classified on the basis of structural features into flavones, isoflavones, stilbenes and lignans [1]. Isoflavones, like soy-derived genistein, are the best-known flavonoids and they typically exhibit weak estrogenic activity but because of their consumption as basic foods, may be stoichiometrically sufficient to compete with endogenous estrogen and be pharmacologically active. Estrogens acting via estrogen receptors (ERα and ERβ) are critical in the pathogenesis of breast cancer and selective estrogen receptor modulators (SERMs), like tamoxifen, have important roles for the chemoprevention and treatment of this common malignancy [2]. Epidemiological evidence suggests that plant derived-estrogens ingested with food may have beneficial effects in the prevention of breast cancer and for the treatment of post-menopausal problems [3].

The dried aerial parts of *Epimedium brevicornum* Maxim. (Yin Yang Huo; Berberidaceae) are a widely consumed TCM herb that is believed to benefit conditions described as "impotence, seminal emission, weakness of the limbs and climacteric hypertension" [4]. More than 130 compounds have been isolated from *Epimedium* subspecies, including flavones like icaritin, quercetin, luteolin, apigenin and their glycosides; prenylflavones like yinyanghuo A – E; and phytosterols like β-sitosterol [5]. Compounds and extracts of *Epimedium* have been reported to have anti-platelet [6], anti-hepatotoxic [7], osteoclast-enhancing [8], neuro-

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nal-stimulatory [9] and cytotoxic [10] properties using *in vitro* cell lines, and to enhance lymphocyte proliferation in an *in vivo* mouse model [11]. To our knowledge, no study on the estrogenic properties of EB has been reported. Our study showed that the new prenylflavone, breviflavone B, isolated from the leaves of EB paradoxically regulated breast cancer cells proliferation, and the molecular mechanisms underlying this inhibitory action were explored in this study.

Materials and Methods

Authentication and genetic profiling of botanical raw materials

Dried leaves of *Epimedium brevicornum* Maxim. were obtained from a local TCM retailer (Eu Yan Sang International, Singapore) and its genetic identity compared to authenticated reference materials provided by Prof Liu Ruoyung from the Herbarium of Henan TCM College, Henan, P.R. China. Authenticity of herbal raw materials was checked by amplifying DNA sequences in the ribosomal ITS1 – 5.8S-ITS2 region using conserved plant sequences as primers [12]. Voucher specimens (SBG-EB02 – 040720) have been deposited in the Singapore Herbarium.

Measurement of steroidogenic activity

Sample preparation: Dried leaves of EB (300 g) were extracted twice with EtOH (2 L each) at 37 °C for 1 week. The dried EB EtOH extract was re-dissolved in MeOH:H_2O (9 : 1) and partitioned three times with hexane. The MeOH extract was dried under vacuum and re-dissolved in EtOH to the desired concentration for subsequent experiments.

Hormone-driven reporter-gene bioassays: Briefly, HeLa cells were transiently co-transfected with 50 ng plasmids encoding either human ERα or ERβ plus 100 ng of an estrogen reporter plasmid (MMTV-ERE-LUC) comprising a luciferase reporter gene driven by 4 copies of the vitelogenin estrogen response element cloned into the MMTV promoter [13]. Transient transfections for androgen receptor (AR), progesterone receptor (PR) and glucocorticoid receptor (GR) were performed as described previously [13]. For peroxisome proliferator-activated receptor (PPARα and PPARγ) activity assays, 25 ng of the respective $Gal4_{DBD}$-$PPAR_{LBD}$ expression plasmids and 250 ng of pG5LUC were co-transfected into HeLa cells. Luciferase induction responses for each treatment group were expressed as fold-increase compared to vehicle treated cells. All data points represented the mean ± SE of at least three replicates.

Breast cancer cell proliferation assay

Breast cancer MCF-7 (ER+) cells, pre-conditioned in estrogen-depleted MEM, were allowed to attach with overnight incubation, followed by the addition of 200 μL of fresh culture media containing one or more of the test samples at the desired concentration. After 6 days of incubation, cell proliferation was determined based on the standard protocol for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Cell proliferation of the test compound-treated cells was expressed as fold-increase in optical absorbance compared to that of ethanol-treated cells. ER-negative breast cancer cells (MDA-MB-231) were treated under identical conditions to serve as negative controls. All the experiments were done in 6 replicates.

Purification and isolation of compounds 1, 2, and 3

The dried ethanolic EB extract (20.5 g) was reconstituted in MeOH and eluted through a polyamide (Macherey-Nagel, Germany) column (40.0 cm × 7.5 cm) with MeOH, the eluant (2.5 L) was dried to yield a dark green solid (16.1 g) after rotary evaporation. The solid was dissolved in MeOH, absorbed onto C18 (52 g, Waters, USA) by rotary evaporation, the resulting solid added to the top of a C18 column (40.0 cm × 7.5 cm) that was eluted with a stepwise gradient from 0% (resulting mass 1.9 g), 30% (1.8 g), 50% (2.8 g), 70% (1.5 g), 90% (1.1 g) to 100% MeOH in H_2O (1.7 g) (1 L each). The most active fraction was the 90% MeOH in H_2O fraction, which was separated by preparative HPLC using a Hypersil BDS C18 column (150 mm × 21.2 mm, 5 μm) at 16 mL/min with a gradient system of MeOH-H_2O (3 : 2) to MeOH-H_2O (9 : 1) in 15 min, followed by 8 min with 100% MeOH. Eleven fractions were collected and tested for ERβ activity. Of these, Fr. 6 and Fr. 7, with elution times from 11.16 – 11.91 min and 11.92 – 13.17 min, respectively, exhibited highest ERβ activity. In total, 20 separate HPLC runs were performed to obtain 17.2 mg and 109.9 mg for Fr. 6 and Fr. 7, respectively. These were further fractionated by semi-preparative HPLC. Fr. 6 was chromatographed with a Supelco RP Amide C16 column (150 mm × 10 mm, 5 μm) at 4 mL/min using a gradient from ACN-H_2O (1 : 1) for 10 min followed by ACN-H_2O (3 : 2) for 10 min to yield compound **2** (1.3 mg, Rt = 11.39 min). Fr. 7 was separated with a Phenomenex Luna C18 column (150 mm × 10 mm, 5 μm) at 4 mL/min using ACN-H_2O (9 : 11) isocratic solvent system to yield compound **3** (1.1 mg, Rt = 8.53 min) and compound **1** (0.6 mg, Rt = 12.60 min).

Western blot analysis

The primary antibody used for immunoblotting was anti-ERα mouse monoclonal antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), followed by anti-mouse secondary antibody conjugated to horseradish peroxidase. Proteins were detected using an enhanced chemiluminescence Western blotting analysis system [14]. The intensities of the bands were quantified by Scion imaging software.

Statistical analysis

SPSS 9.0 (SPSS, Chicago IL) was used to perform the data analyses. Each data point in the steroid receptor reporter-gene and cell-proliferation bioassays was the mean (± SE) of at least three determinations. When the interaction was statistically significant, the Student's *t* test was used to determine the statistical significance of differences. All statistical tests were two sided. $P < 0.05$ was taken to be significant.

Results

DNA profiling and authentication of raw materials

DNA was prepared from EB samples obtained from the Singapore retailer and from reference specimens supplied by the Herbarium of Henan TCM College. Amplification by the polymerase chain reaction of the 898 bp ribosomal ITS1 – 5.8S-ITS2 regions was performed. There was 100% match in 9 polymorphic sites (positions 112, 279, 299, 306, 493, 597, 607, 662, 680) between the two samples, confirming the genetic identity of the subspecies. Ethanolic extracts from the Singapore retailer produced consistently high and stable ER activity over a period of 18

months and subsequent experiments were performed using this batch.

Estrogenic properties of EB extracts

Ethanolic extracts of EB displayed significant ER activity that was about 50% of that observed for a saturating dose of estradiol, in estrogen-driven reporter gene bioassays (Fig. **1A**). The effect of ethanolic EB extracts on ERα was dose-dependent with an EC_{50} of 1.3 μg/mL. The dose-response curve of EB was shifted to the right with respect to estradiol and genistein, the EC_{50} of estradiol and genistein being 0.20 ng/mL and 0.10 μg/mL, respectively. EB extract did not have any agonistic effect on AR, PR, GR, PPARα and PPARγ reporter gene systems (Fig. **1B**). At doses of EB < 10 μg/mL, the ERα effects of EB extract predominates, whereas at higher doses its ERβ effects were more pronounced (Fig. **1B**). The effects of ERα and ERβ of estradiol and EB can be blocked completely by tamoxifen.

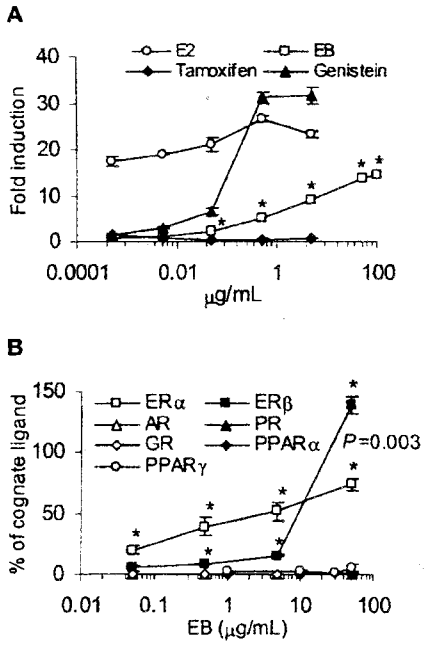

Fig. **1** Estrogenic properties of EB extract. **A** Estrogenic activities of estradiol (E2), EB extract, tamoxifen and genistein were assessed by estrogenic reporter gene systems and expressed as fold-increase in luciferase activity compared to cells exposed to vehicle alone. Statistically significant increases in cell proliferation for EB compared to vehicle are indicated with asterisks (P < 0.0001 to 0.002). **B** Comparative effects of EB extract on ERα, ERβ, AR, PR, GR, PPARα and PPARγ reporter gene systems. Bioactivities are expressed as percentages of saturating doses of reference ligands: estradiol 1 nM, DHT 1 nM, progesterone 100 nM, hydrocortisone 1 nM, PPARα ligand WY14643 100 μM and PPARγ ligand pioglitazone 30 μM. Statistically significant activation of ERα or ERβ compared to vehicle are indicated with asterisks (P = 0.0001 to 0.015; a vs. b, P = 0.003).

As expected, estradiol stimulated the growth of ERα-positive MCF-7, but not ERα-negative MDA cells (Fig. **2A**). Strikingly, EB extract had a dose-dependent biphasic effect on MCF-7 cell proliferation. Low doses of EB extract from ≤ 30 μg/mL significantly increased cell growth. In contrast, doses of EB from 50–200 μg/mL had a significant growth inhibitory effect (Fig. **2B**). Profound suppression of cell growth to below basal levels was observed at doses > 100 μg/mL in three independent experiments. In comparison, estradiol at 4 orders of concentrations, from 10 pM to 100 nM, continued to have stimulatory effects on MCF-7 cells (Fig. **2A**). The inhibitory effect of EB was not due to non-specific toxicity, as high doses of EB did not have any effect on the growth of MDA-MB-231 cells (Fig. **2B**). Genistein maintained proliferative effects from 30 ng/mL to 5 μg/mL (Fig. **2C**). Thus, high doses of EB extract can, unlike estradiol and genistein, paradoxically inhibit the growth of estrogen-responsive breast cancer cells.

Structure-function characterization of compounds 1, 2 and 3

Compound **1** was identified as 3′-prenylapigenin (yinyanghuo D) by comparison of the spectral data with literature values [6].

Compound **2**, yellow powder, $[\alpha]_D^{27}$: −2.16 (*c* 0.025, EtOH) gave a molecular ion in the HR-ESI-MS [$(M-H)^-$ *m/z* = 437.1631, Δmmu 3.1] consistent with the molecular formula $C_{25}H_{26}O_7$, corresponding to thirteen double bond equivalents. Analysis of the NMR data (Table **1**) readily identified the structure as a prenylflavone similar in structure to **1**, but differing in substitution of the pendant aryl system. One symmetrical aryl group, two 1,1-disubstituted double bonds, two hydroxymethines, two olefinic methyls and two benzylic methylenes were observed in the NMR spectra (Table **1**). Important HMBC correlations from δ = 6.52 to 121.0, δ = 7.59 to 39.0, and from δ = 2.94 to 160.5, 129.5 and 128.0 allowed the gross structure to be elucidated as 3′,5′-di-(2-hydroxy-3-methyl-3-butenyl)-apigenin and was named as breviflavone A (Fig. **3**). The stereochemistry of **2** has not yet been elucidated because of the small amount of sample left after biological experiments. Breviflavone A exhibited minimal activity in the ERα and ERβ bioassays.

Compound **3**, a yellow powder, $[\alpha]_D^{27}$: −43.6 (*c* 0.003, EtOH), gave a molecular ion in the HR-ESI-MS [$(M-H)^-$ *m/z* = 437.1579, Δmmu 2.1] consistent with the molecular formula $C_{25}H_{26}O_7$. Comparison of NMR data of **1** [6], **2** and **3** (Table **1**) indicated that **3** was also a prenylflavone. Calculation of the molecular unsaturation showed that in addition to the known three aromatic

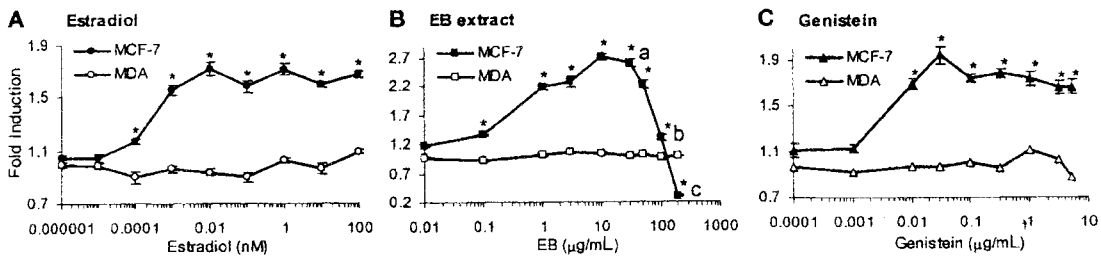

Fig. **2** Effects of estradiol, EB extract and genistein on the growth of breast cancer cells. MCF-7 and MDA-MB-231 cells were exposed to test compounds and their effects on cell proliferation measured with MTT assay. Results are expressed as fold increase (mean ± SE of 6 replicates) in MTT activity over vehicle. Cells were exposed to **A** estradiol, **B** EB extract, or **C** genistein and their effects measured after 6 days (* P < 0.0001, MCF-7 vs. MDA).

Table 1 ^{1}H-NMR and ^{13}C-NMR data of compounds **2** and **3** (CD_3OD, δ in ppm, J in Hz)

C/H No	Compound 2		Compound 3	
	δ_C	δ_H	δ_C	δ_H
2	166.0		166.2	
3	104.0	6.52 (1H, s)	104.0	6.57(1H, s)
4	[illegible]		183.5	
5	164.0		163.1	
6	100.5	6.15 (1H, d, J = 2.0)	100.3	6.20 (1H, d, J = 2.1)
7	168.0		167.0	
8	95.5	6.40 (1H, d, J = 2.0)	95.2	6.43 (1H, d, J = 2.1)
9	159.5		159.3	
10	104.0		105.9	
11	39.0	2.94 (4H, m)	29.0	3.39 (2H, d, J = 7.3)
12	77.5	4.37 (2H, ddd, J = 4.0, 4.0, 3.4)	122.4	5.36 (1H, m)
13	148.0		134.5	
14	112.0	4.92, 4.80 (2H each, J = 2.5)	25.7	1.76 (3H, s)
15	18.0	1.80 (6H, s)	18.1	1.76 (3H, s)
16			71.8	
$2CH_3$ – 16			25.3, 25.8	1.23 (3H, s), 1.30 (3H, s)
1′	121.0		124.9	
2′	129.5	7.59 (2H, s)	122.8	7.84 (1H, brs)
3′	128.0		131.5	
4′	160.5		163.0	
5′			125.9	
6′			129.5	7.67 (1H, *brs*)
4″			73.4	5.39 (1H, d, J = 4.2)
5″			122.4	4.36 (1H, d, J = 4.2)

rings, there was another cyclic system in the molecule. The ^{13}C-NMR data of B ring showed a typical [1′,3′,4′,5′-tetrasubstituted aromatic system (Table **1**). The presence of a (1-hydroxyl-1-methyl)ethyl-dihydrofuran moiety in the molecule was evidenced by the correlations in ^{1}H-^{1}H COSY [δ = 5.39 (1H, d, J = 4.2 Hz) and δ = 4.36 (1H, d, J = 4.2 Hz),] and HMBC spectrum (δ = 4.36 with δ = 25.3, 25.8, 73.4 and 163.0, δ = 5.39 with δ = 71.8, 122.8, 131.5 and 163.0). Comparison of the NMR data with a known prenylflavone, epimedokoreanin A [15], also confirmed that the dihydrofuran ring was fused at C-3′ and C-4′ positions. The two groups on the dihydrofuran ring were *trans*-oriented according to the $J_{4'',5''}$ (4.2 Hz) [16]. Thus, combined analyses of MS, 1D and 2D NMR allowed for the gross structure of compound **3** to be 4″-hydroxy-5″-(1-hydroxy-1-methyl)ethyl-dihydrofuran-(2″,3″:3′,4′)-5′-prenyl-5,7-dihydroxyflavone and named as breviflavone B (Fig. **3**). Breviflavone B exhibited strong ERα stimulatory activity at higher doses, which was > 5-fold higher than that observed for a saturating dose of estradiol and > 2 fold higher than genistein (P = 0.002) (Fig. **4A**) The ERβ dose-response curve of breviflavone B was shifted to the right with respect to genistein with a maximal potency that was higher (P = 0.0071, Fig. **4B**).

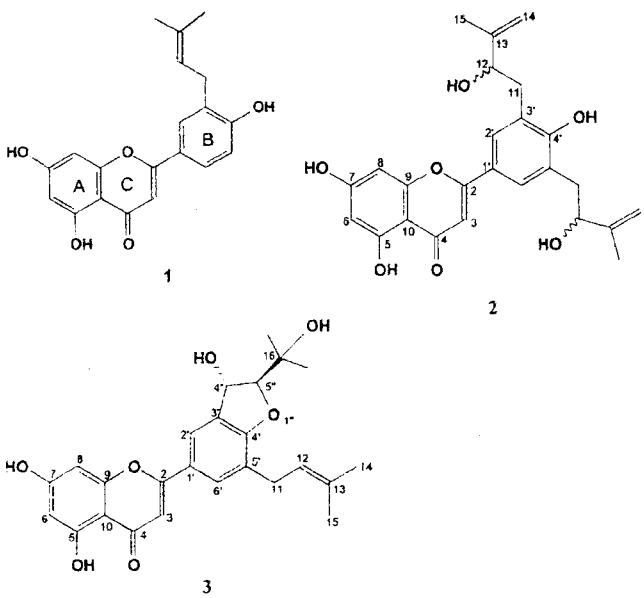

Fig. 3 Structures of compounds **1** (yinyanghuo D), **2** (breviflavone A), and **3** (breviflavone B)

Effects of breviflavone B on growth and ERα protein expression in breast cancer cells

The effects of breviflavone B on growth dynamics of breast cancer cells were studied. Genistein and breviflavone B were able to dose-dependently increase MCF-7 cell proliferation with an EC_{50} of 20 nM and 200 nM, respectively (Fig. **5A**). Peak stimulatory activity of breviflavone B was observed at a dose of 450 nM. However, higher doses (2.2 – 6.6 μM) inhibited the growth of MCF-7 cells. This inhibitory effect was not observed with genistein as its growth-stimulatory properties were maintained from 0.03 – 18μM (Fig. **5A**). To determine the mechanisms of this biphasic growth inhibitory effect, MCF-7 cells were exposed to breviflavone B, genistein, estradiol following which ER proteins were de-

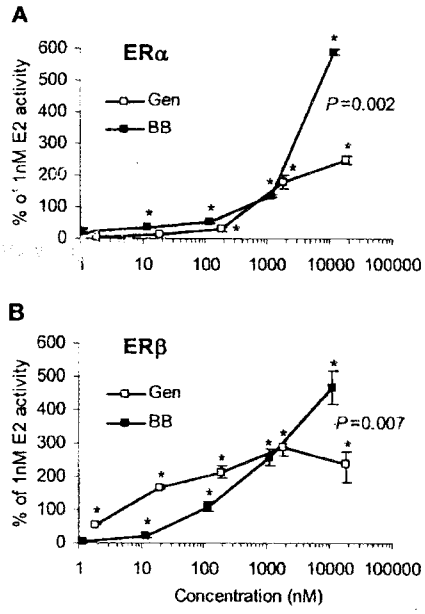

Fig. 4 Estrogenic effects of genistein (Gen) and breviflavone B (BB). HeLa cells were with transiently transfected with plasmids encoding either **A** ERα or **B** ERβ and the reporter gene MMTV-ERE-LUC. Each treatment was performed in triplicate and luciferase reporter gene activity was expressed as percent of 1 nM estradiol. Statistically significant differences between breviflavone B and genistein at the highest doses are indicated. Asterisks indicate significant increases in ER activities compared to vehicle alone ($P < 0.0001$ to 0.028).

tected using ERα specific antibodies. Exposure to estradiol alone did not induce any change in ERα protein levels. Genistein slightly reduced ERα levels. Interestingly breviflavone B inhibited ERα protein expression strongly, reducing it to about 15% of that observed with vehicle only (Fig. **5B** comparing lanes 8 and 1). The inhibitory actions of breviflavone B on ERα protein levels were reversed in the presence of the proteasome inhibitor, lactacystin, suggesting that proteasome degradation of the breviflavone B-ERα complex was increased compared to estradiol-ERα (Fig. **5B**, comparing lanes 8 and 3).

Discussion

Our screening of TCM herbs led to the discovery of the unexpected hormonal properties of EB extracts. Unlike estradiol and genistein, EB extracts have biphasic inhibitory effects on the growth of breast cancer cells. Although the lack of effect on ER-negative MDA cells suggests that this property is estrogen-driven, the underlying molecular mechanisms were puzzling, as the normal response of MCF-7 cells to estrogenic stimulation is increased cell division. Under our experimental conditions, doses of estradiol that were 1000-fold higher, respectively, than its saturating dose were still stimulatory to MCF-7 cells. Similarly genistein maintained its growth-inducing effects at doses that were up to 100-fold higher than its saturating dose.

Purification steps using bioassay-guided column chromatography and repeated preparative HPLC separations were performed to define the chemical entities responsible for estrogenic effects of EB. The most potent bioactive compound isolated, breviflavone B, was a prenylflavone. The effect of breviflavone B was reminiscent of EB extract (comparing Figs. **2B** and **5A**) but at a higher potency, suggesting that this effect of EB was mediated, at least partially, through breviflavone B. In contrast to isoflavones, flavones with estrogenic activity are uncommon. One recently described class of bioactive flavones is the prenylflavones [17]. An example is 8-prenylnaringenin, a strong phytoestrogen from hops that shows binding activity to both estrogen receptor isoforms [18], [19]. Naringenin is a prenylflavone that exhibits partial anti-estrogenic activity in the female rat uterus and MCF-7 human breast cancer cells [20]. Breviflavone B displayed high estrogenic activity and was able to both stimulate and inhibit the growth of ER-responsive breast cancer cells. At high doses, its peak potency is 3–5-fold higher than a saturating dose of estradiol. However unlike estradiol and tamoxifen, breviflavone B can markedly reduce ERα content in breast cancer cells, a process reversible by the proteasome inhibitor, lactacystin, indicating protein degradation through ubiquitin proteasome pathways. In this respect, the action of breviflavone B resembles the action of the pure anti-estrogen, ICI182,780 which binds ERα with high affinity and induces a rapid proteasome-dependent degradation of the receptor [21].

The finding that breviflavone B can markedly reduce ERα protein levels suggests one possible mechanism for biphasic proliferative/anti-proliferative actions on breast cancer cell growth dynamics. At low doses, estrogenic stimulation predominates as sufficient ERα protein is present for estrogenic drive to be exerted, thereby increasing cell proliferation. At higher doses, estrogenic drive and MCF-7 proliferation ceases as ERα protein is reduced to almost undetectable levels. Our data provided new mechanistic insights as to why phytoestrogens such as prenylated flavonoids may be effective in prophylaxis against breast cancer. It is possible that concurrent usage of such compounds with tamoxifen or raloxifene may enhance their effectiveness for breast cancer prophylaxis since these SERMs increased, but breviflavone B and its parent extract decreased, ERα levels in breast cancer cells. Preclinical studies are needed to explore whether the paradoxical estrogenic/anti-estrogenic properties of these phy-

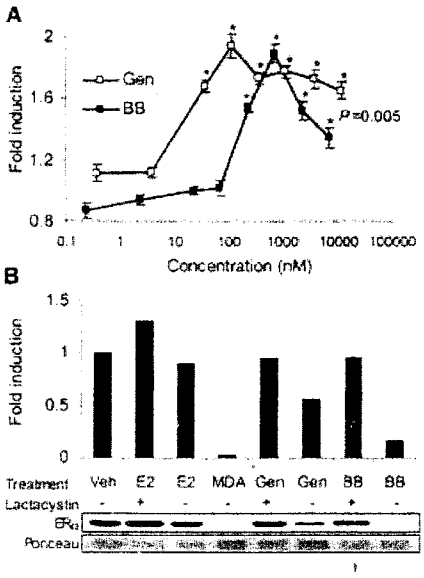

Fig. 5 Effects of breviflavone B and genistein on growth and ERα protein expression in breast cancer cells. **A** Proliferative effects of breviflavone B (BB) and genistein (Gen) on MCF-7 cells. Cell proliferation was expressed as fold-increase over vehicle alone. Asterisks indicate significant differences ($P < 0.0001$ to 0.004) compared with vehicle alone. P value indicates significant differences between genistein and breviflavone B at the two highest doses. **B** MCF-7 cells were treated with 1 μM estradiol (E2), 10 μM genistein (Gen) or 10 μM breviflavone B (BB) in the presence or absence of proteasome inhibitor, lactacystin. MCF-7 cells and MDA-MB-231 cells treated with vehicle were used as positive and negative controls. Specific ERα protein expression was determined by immunoblot analyses.

toestrogens may be harnessed for management of estrogen deficiency states and for prophylaxis of breast cancer.

Acknowledgements

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Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay

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Abstract

Antioxidant species may act in vivo to decrease oxidative damage to DNA, protein and lipids thus reducing the risk of coronary heart disease and cancer. Phytoestrogens are plant compounds which are a major component of traditional Asian diets and which may be protective against certain hormone-dependent cancers (breast and prostate) and against coronary heart disease. They may also be able to function as antioxidants, scavenging potentially harmful free radicals. In this study, the effects of the isoflavonoids (a class of phytoestrogen) genistein and equol on hydrogen peroxide-mediated DNA damage in human lymphocytes were determined using alkaline single-cell gel electrophoresis (the comet assay). Treatment with hydrogen peroxide significantly increased the levels of DNA strand breaks. Pre-treatment of the cells with both genistein and equol offered protection against this damage at concentrations within the physiological range. This protection was greater than that offered by addition of the known antioxidant vitamins ascorbic acid and α-tocopherol, or the compounds 17β-oestradiol and Tamoxifen which have similar structures to isoflavonoids and are known to have weak antioxidant properties. These findings are consistent with the hypothesis that phytoestrogens can, under certain conditions, function as antioxidants and protect against oxidatively-induced DNA damage. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: DNA damage; Phytoestrogens; Antioxidants; Diet

1. Introduction

Evidence suggests that oxidative stress is linked to the aetiology of several chronic diseases, including coronary heart disease [1] and cancer [2]. Epidemiological studies have shown that a diet that includes many fruits and vegetables is associated with a reduced risk of these pathologies [3]. Observational associations have suggested a causal relationship mediated by dietary antioxidants limiting lipid peroxidation and DNA damage.

Phytoestrogens are organic plant compounds found in soy products, legumes, fruits and vegetables, that have been proposed to have many health benefits. In countries following the traditional Asian diet, which is rich in soy foods and therefore, high in phytoestrogen intake, the relative risk of breast and prostate cancer is five- to eight-fold less than that of West-

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ern populations [4]. However, the incidence of breast and prostate cancers in Asian migrants and their offspring approaches that of indigenous Westerners, indicating that diet has an important role in the aetiology of hormone-dependent cancers [5].

The structure of phytoestrogens resembles the steroid hormone 17β-oestradiol and phytoestrogens can bind to the oestrogen receptor. The physiological effects of these compounds are not confined, however, to oestrogen modulation. Phytoestrogens may also be able to function as antioxidant species. Genistein has been shown to enhance antioxidant enzyme activity in mice [6]. Wei et al. showed that genistein significantly inhibits phorbol ester-type tumour promoter-induced H_2O_2 formation both in vitro and in vivo by inhibiting the formation of reactive oxygen species [7]. Genistein also exhibits antioxidant activity by preventing hemolysis of red blood cells by dialuric acid or hydrogen peroxide [8,9] and by protecting microsomal lipid peroxidation induced by an Fe^{2+}–ADP complex [10].

It is accepted that in appropriate conditions virtually all cell components, including the lipids, nucleic acids and proteins, are all sensitive to damage by reactive oxygen species. At normal oxygen tension these mechanisms are enough to maintain homeostasis, and even though oxygen free radicals are produced, they are efficiently removed. However, under circumstances of increased oxidative stress, free radical concentration can increase uncontrollably.

The single-cell gel electrophoresis (comet) assay is a very simple, rapid and sensitive technique to analyse DNA damage at the individual cell level and specifically for detecting DNA strand breaks. The comet assay has been used in various studies to investigate the effect of reactive oxygen species on DNA, and the protective effects of certain dietary antioxidants [11,12]. It has been postulated that antioxidants, mainly of dietary origin, may scavenge free radicals and thus, limit the damage incurred by the DNA and therefore, protect against mutagenesis and cancer [13].

The aim of this study was to determine whether hydrogen peroxide-induced oxidative damage in human lymphocytes could be reduced by the phytoestrogens genistein and equol, compared to the known antioxidant vitamins ascorbic acid and α-tocopherol. Two compounds known to be weak antioxidants, and of similar structure to phytoestrogens, 17β-oestradiol and Tamoxifen [14,15] were also included for comparison.

2. Materials and methods

All chemicals were obtained from Sigma (Poole, Dorset) unless otherwise stated. Equol was obtained from Apin Chemicals Ltd. (Oxon).

2.1. Lymphocyte separation

Human lymphocytes were isolated from a healthy female donor (non-smoker aged 26). Fasting blood was collected into vacutainer cell preparation tubes containing sodium citrate as anticoagulant and Ficoll-Hypaque (Becton Dickinson). The samples were then centrifuged at 3000 rpm at 25°C for 20 min. The lymphocytes form a layer directly above the Ficoll-Hypaque in the vacutainer. They were removed, washed with fresh cold tissue culture medium and then centrifuged for 5 min at 2000 rpm. The cell pellet was re-suspended in foetal calf serum (containing 10% DMSO), aliquoted and stored at −80°C until required. The cells were diluted down to a concentration of 2.5×10^4 cells/ml prior to use.

2.2. Antioxidant pre-treatment

The compounds of interest were kept at −20°C as 10 mg/ml stock solutions in ethanol. They were then diluted to the required concentrations using foetal calf serum. Cells were incubated with different concentrations of the compounds of interest for 30 min at 37°C in a dark incubator together with untreated control samples. Samples were then centrifuged at 3000 rpm for 10 min. In an experiment to test the effects of compound removal after this incubation step, cells incubated with genistein were either treated as per normal protocol at this stage, or had the genistein supernatant removed and were washed using phosphate-buffered saline (PBS) prior to H_2O_2 damage.

2.3. H_2O_2 treatment

Samples were suspended in PBS with 500 μmol/l H_2O_2 for 10 min at 4°C in the dark. Control samples were treated with PBS alone without hydrogen peroxide. Samples were then centrifuged at 3000 rpm for 10 min and the cells washed with PBS. Cells were then

re-suspended in FCS and processed using the established experimental protocol [16].

2.4. Principle of the comet assay

Cells are embedded in agarose on a microscope slide and lysed to remove cellular proteins. The DNA is denatured under alkaline conditions and electrophoresed. During electrophoresis any relaxed or broken DNA fragments migrate further than supercoiled, undamaged DNA. After appropriate staining the DNA resembles a comet with a brightly fluorescent head and a tail whose length and intensity is determined by the level of DNA strand breakage produced within the cells. The tail moment is used as a measure of DNA damage and is defined as the product of the fraction of DNA in the comet tail, and the distance between the means of the head and tail distributions, based on the definition by Olive and Banath [17].

2.5. Statistical analysis

All statistical analyses were carried out using SPSS for Windows 7.5.1.

In view of the small numbers ($n = 4$–6) of repeated experiments, and the non-normal distribution of the data, the non-parametric Mann–Whitney-U test was used to determine the effects of antioxidant supplementation on lymphocyte DNA integrity. Results were considered to be statistically significant if a P-value of <0.05 was recorded.

3. Results

The genotoxic effects of hydrogen peroxide and the protective ability of genistein and equol was compared with ascorbic acid, α-tocopherol, and 17β-oestradiol and Tamoxifen in normal human lymphocytes using

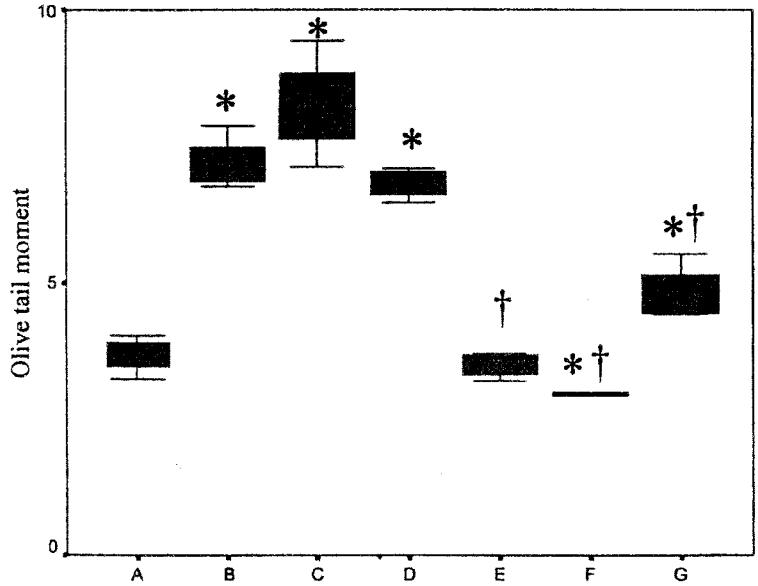

Fig. 1. The effect of supplementation in vitro with different concentrations of genistein on H_2O_2-induced human lymphocyte DNA damage. (A) Control; (B) Lymphocytes treated with 500 μmol/l H_2O_2; (C) Lymphocytes treated with 100 μmol/l genistein + 500 μmol/l H_2O_2; (D) Lymphocytes treated with 10 μmol/l genistein + 500 μmol/l H_2O_2; (E) Lymphocytes treated with 1 μmol/l genistein + 500 μmol/l H_2O_2; (F) Lymphocytes treated with 0.1 μmol/l genistein + 500 μmol/l H_2O_2; (G) Lymphocytes treated with 0.01 μmol/l genistein + 500 μmol/l H_2O_2. Values are medians (black line) with interquartile ranges (boxed area) and minimum–maximum values (*I*). (*) Significantly different to control values ($P < 0.05$, Mann–Whitney-U test). (†) Significantly different to values for samples treated with H_2O_2 only ($P < 0.05$, Mann–Whitney-U test). For each condition $n = 4$.

the comet assay. The comet assay was sensitive and reproducible, with an intraassay CV of 7.63% ($n = 20$).

Genistein produced a significant reduction in DNA damage after cells had been damaged with hydrogen peroxide at concentrations from 0.01–1 μmol/l. Higher concentrations, of 10 and 100 μmol/l had no significant effect on DNA damage, although 100 μmol/l actually seemed to increase the damage caused by incubation with H_2O_2 (Fig. 1). Genistein at 1 μmol/l was also protective regardless of whether genistein was washed from the cells or was present with the hydrogen peroxide throughout the incubation period (Mean (S.E.) olive tail moment with wash 4.95 (0.64); without wash 3.93 (0.22); P = NS, Mann–Whitney-U test).

Equol, similarly to genistein, significantly reduced Olive tail moment, this time at doses from 0.01 to 100 μmol/l. Equol at 0.01 μmol/l reduced DNA damage, but by a lesser extent than at 0.1 μmol/l (Fig. 2). For both genistein and equol, a concentration of 0.1 μmol/l seemed to offer most protection against H_2O_2-induced damage.

When the antioxidant ability of genistein and equol was compared with the other compounds tested it was found that genistein was most protective, followed by equol, ascorbic acid, α-tocopherol and 17β-oestradiol, with Tamoxifen being the least protective (Fig. 2).

When a combination of antioxidants was tested it was found that genistein in combination with ascorbic acid was the most effective combination, with genistein in combination with equol also being more protective than genistein alone. Equol in combination with ascorbic acid offered more protection than either equol or ascorbic acid alone (Fig. 4).

4. Discussion

It can be concluded from these results, that the isoflavonoids genistein and equol, as well as ascorbic

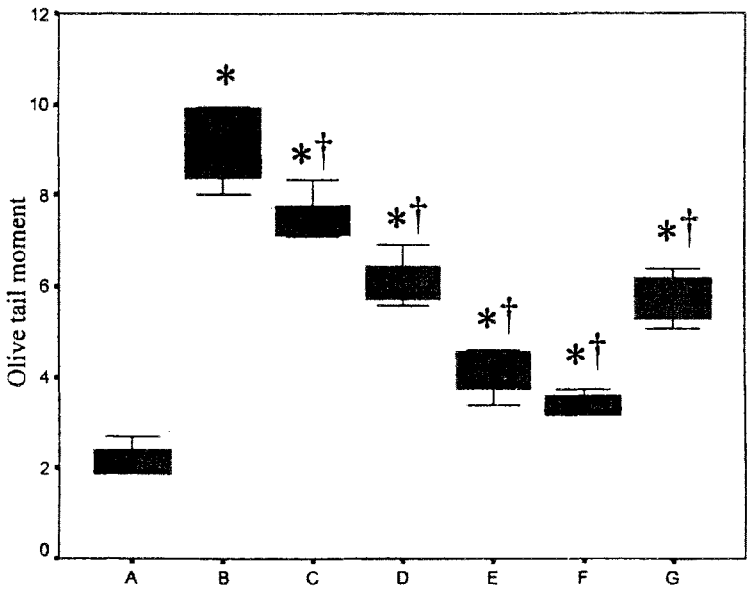

Fig. 2. The effect of supplementation in vitro with different concentrations of equol on H_2O_2-induced human lymphocyte DNA damage. (A) Control; (B) Lymphocytes treated with 500 μmol/l H_2O_2; (C) Lymphocytes treated with 100 μmol/l equol + 500 μmol/l H_2O_2; (D) Lymphocytes treated with 10 μmol/l equol + 500 μmol/l H_2O_2; (E) Lymphocytes treated with 1 μmol/l equol + 500 μmol/l H_2O_2; (F) Lymphocytes treated with 0.1 μmol/l equol + 500 μmol/l H_2O_2; (G) Lymphocytes treated with 0.01 μmol/l equol + 500 μmol/l H_2O_2. Values are medians (black line) with interquartile ranges (boxed area) and minimum–maximum values (*I*). (*) Significantly different to control values ($P < 0.05$, Mann–Whitney-U test). (†) Significantly different to values for samples treated with H_2O_2 only ($P < 0.05$, Mann–Whitney-U test). For each condition $n = 4$.

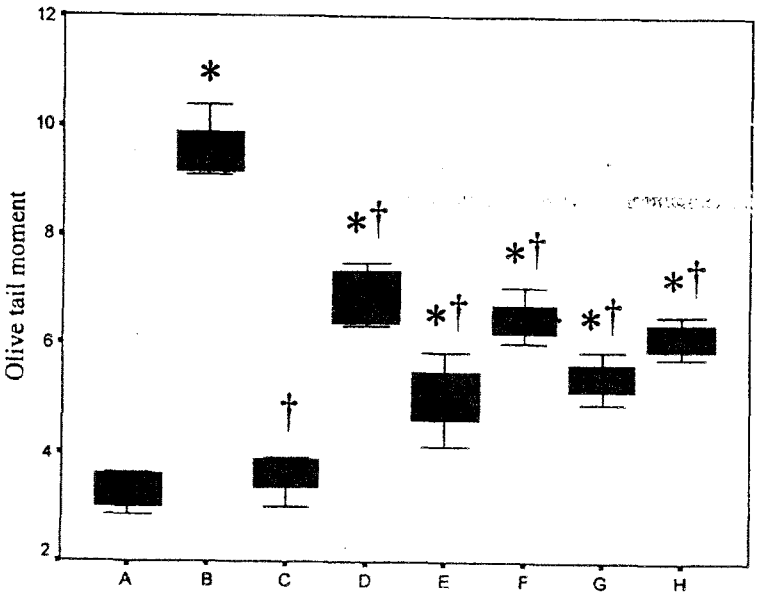

Fig. 3. The effect of supplementation in vitro with different antioxidants on H_2O_2-induced human lymphocyte DNA damage. (A) Control, (B) Lymphocytes treated with 500 μmol/l H_2O_2; (C) Lymphocytes treated with 1 μmol/l genistein + 500 μmol/l H_2O_2; (D) Lymphocytes treated with 1 μmol/l Tamoxifen + 500 μmol/l H_2O_2; (E) Lymphocytes treated with 1 μmol/l equol + 500 μmol/l H_2O_2; (F) Lymphocytes treated with 1 μmol/l oestradiol + 500 μmol/l H_2O_2; (G) Lymphocytes treated with 1 μmol/l ascorbic acid + 500 μmol/l H_2O_2; (H) Lymphocytes treated with 1.24 μmol/l α-tocopherol + 500 μmol/l H_2O_2. Values are medians (black line) with interquartile ranges (boxed area) and minimum–maximum values (*I*). (*) Significantly different to control values ($P < 0.05$, Mann–Whitney-U test). (†) Significantly different to values for samples treated with H_2O_2 only ($P < 0.05$, Mann–Whitney-U test). For each condition $n = 4$.

acid and α-tocopherol, and, to a lesser extent, 17β-oestradiol and Tamoxifen, have antioxidant properties as shown by the comet assay performed on the H_2O_2-damaged lymphocyte cells. The effect of combinations of these compounds requires further study. It is possible that an optimum level or correct balance of these compounds is required before a synergistic or additive response may be observed.

A number of studies have been carried out examining the antioxidant potential of phyotestrogens. These studies are difficult to compare directly, as they use a variety of designs and test systems.

Mitchell et al. [18] tested the antioxidant activity of a variety of phytoestrogens including genistein and equol in a number of in vitro test systems and found that the isoflavones in general had weak activity in comparison to flavonoids. An exception was in the Trolox equivalent antioxidant capacity (TEAC) assay, which did not correlate well with the results in the other systems and where genistein and equol had 1.5–2 times the activity of Trolox. This highlights the importance of test system used, and the marked differences between them. The concentrations of compound used in these assays were greater than those found in serum and the authors concluded that this poor in vitro antioxidant activity may reflect little significance as antioxidants in vivo.

Mitchell and Collins [19] have also carried out a 4-week feeding study in healthy men to assess the effects of a phytoestrogen supplement in the form of soy milk on plasma lipids and levels of DNA damage. There was no significant effect on H_2O_2-induced DNA damage in lymphocytes in those receiving the soy milk supplement as assessed by the comet assay. However, the levels of oxidised pyrimidine base damage over the 4-week period progressively decreased. These studies highlight the differences between (a) difference in vitro systems to determine antioxidant efficiency and (b) in vitro and in vivo systems.

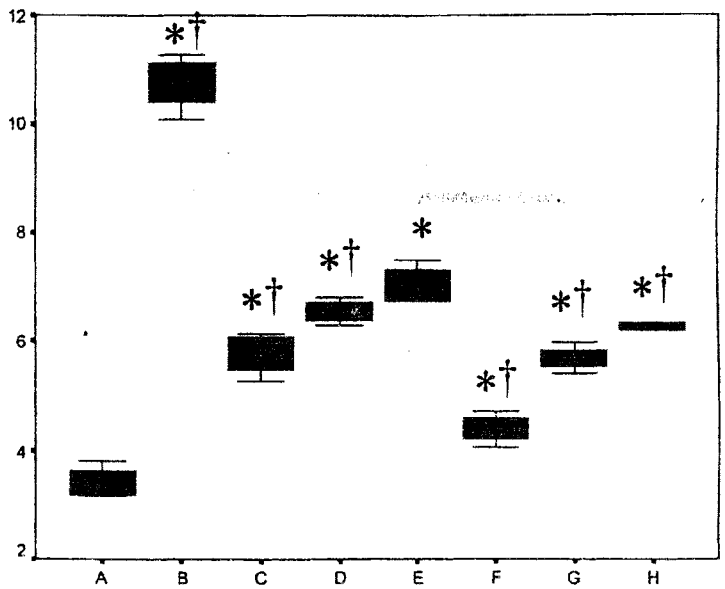

Fig. 4. The effect of supplementation in vitro with different antioxidants both singly and in combination on H_2O_2-induced human lymphocyte DNA damage. (A) Control; (B) Lymphocytes treated with 500 μmol/l H_2O_2; (C) Lymphocytes treated with 1 μmol/l genistein + 500 μmol/l H_2O_2; (D) Lymphocytes treated with 1 μmol/l equol + 500 μmol/l H_2O_2; (E) Lymphocytes treated with 1 μmol/l ascorbic acid + 500 μmol/l H_2O_2; (F) Lymphocytes treated with 1 μmol/l genistein + 1 μmol/l ascorbic acid + 500 μmol/l H_2O_2; (G) Lymphocytes treated with 1 μmol/l genistein + 1 μmol/l equol + 500 μmol/l H_2O_2; (H) Lymphocytes treated with 1 μmol/l equol + 1 μmol/l ascorbic acid + 500 μmol/l H_2O_2. Values are medians (black line) with interquartile ranges (boxed area) and minimum–maximum values (*I*). (*) Significantly different to control values ($P < 0.05$, Mann–Whitney-*U* test). (†) Significantly different to values for samples treated with H_2O_2 only ($P < 0.05$, Mann–Whitney-*U* test). For each condition $n = 4$.

In a similar study to [19] in healthy females over a 1-week intervention period (80 mg phytoestrogens/day) where the supplement was a soy and linseed bar, there were no significant changes in lipid or antioxidant status. However, background DNA damage assessed by the comet assay in isolated lymphocytes was reduced post-supplementation [20].

Similarly, a protective effect has been shown by phytoestrogen supplementation on the susceptibility of LDL to oxidation [21]. Six healthy volunteers received 19 mg isoflavones/day for 2 weeks. Lag phases of LDL oxidation curves were increased post-supplement compared with baseline values. Another study, however, failed to show an inhibition of lipid peroxidation after 8 weeks of isoflavonoid supplementation in subjects with high-normal blood pressure [22], measuring urinary F-2-isoprostanes. This study was randomised, double-blind and placebo-controlled, and was carried out in large numbers ($n = 59$).

Arora et al. [23] suggest that the free radical scavenging properties of isoflavones only partly account for their antioxidant capabilities. Because of their polyphenolic structures, these compounds can donate hydrogen atoms to deleterious oxy-radicals and form the less reactive phenoxyl radicals in the process. The same structure also confers isoflavonoids with an ability to chelate metal-ions. The greater antioxidant potential of the isoflavonoids found against metal-ion-induced peroxidation is likely to be due to a consequence of their combined metal chelating and free radical scavenging abilities.

The chemical structure of genistein and equol determines their antioxidant properties, the number and the position of hydroxyl groups are of critical importance in the antioxidant activity of the compound. Further work must be carried out comparing the various phytoestrogens, taking into account the conflicting results obtained in different antioxidant test systems. Further

work relating to how quickly the compounds are taken up into cells and their mechanism of action, must also be undertaken before the antioxidant potential of these compounds in vivo is fully elucidated.

Phytoestrogens, by acting as antioxidants may have a role to play in the protection against DNA damage and, therefore, a variety of diseases including cancer and coronary heart disease. Since biological effects are dependent on dose, potency, duration, timing and pattern of exposure, further research is required to be able to determine the importance to human health. The present lack of clinical data hampers further understanding of the potential risks and benefits that may result from the consumption of phytoestrogen-rich diets.

5. Conclusion

The isoflavonoids genistein and equol, when assessed for their ability to protect against lymphocyte DNA damage using the comet assay, offered protection against oxidatively-induced DNA damage at normal human physiological concentrations [24]. The known antioxidant vitamins; ascorbic acid and α-tocopherol, also showed protective effects towards H_2O_2-induced damage. The conclusions from these in vitro experiments must remain tentative until more is known about the absorption, distribution, metabolism and biological effects of these compounds within the body. However, the present study supports the possibility that the phytoestrogens genistein, and equol, with their antioxidant properties, may be important in health and disease in protecting against DNA damage through oxidative stress.

Acknowledgements

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Writing Group for the Women's Health Initiative Investigators

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Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women

Principal Results From the Women's Health Initiative Randomized Controlled Trial

Writing Group for the Women's Health Initiative Investigators

Context Despite decades of accumulated observational evidence, the balance of risks and benefits for hormone use in healthy postmenopausal women remains uncertain.

Objective To assess the major health benefits and risks of the most commonly used combined hormone preparation in the United States.

Design Estrogen plus progestin component of the Women's Health Initiative, a randomized controlled primary prevention trial (planned duration, 8.5 years) in which 16608 postmenopausal women aged 50-79 years with an intact uterus at baseline were recruited by 40 US clinical centers in 1993-1998.

Interventions Participants received conjugated equine estrogens, 0.625 mg/d, plus medroxyprogesterone acetate, 2.5 mg/d, in 1 tablet (n=8506) or placebo (n=8102).

Main Outcomes Measures The primary outcome was coronary heart disease (CHD) (nonfatal myocardial infarction and CHD death), with invasive breast cancer as the primary adverse outcome. A global index summarizing the balance of risks and benefits included the 2 primary outcomes plus stroke, pulmonary embolism (PE), endometrial cancer, colorectal cancer, hip fracture, and death due to other causes.

Results On May 31, 2002, after a mean of 5.2 years of follow-up, the data and safety monitoring board recommended stopping the trial of estrogen plus progestin vs placebo because the test statistic for invasive breast cancer exceeded the stopping boundary for this adverse effect and the global index statistic supported risks exceeding benefits. This report includes data on the major clinical outcomes through April 30, 2002. Estimated hazard ratios (HRs) (nominal 95% confidence intervals [CIs]) were as follows: CHD, 1.29 (1.02-1.63) with 286 cases; breast cancer, 1.26 (1.00-1.59) with 290 cases; stroke, 1.41 (1.07-1.85) with 212 cases; PE, 2.13 (1.39-3.25) with 101 cases; colorectal cancer, 0.63 (0.43-0.92) with 112 cases; endometrial cancer, 0.83 (0.47-1.47) with 47 cases; hip fracture, 0.66 (0.45-0.98) with 106 cases; and death due to other causes, 0.92 (0.74-1.14) with 331 cases. Corresponding HRs (nominal 95% CIs) for composite outcomes were 1.22 (1.09-1.36) for total cardiovascular disease (arterial and venous disease), 1.03 (0.90-1.17) for total cancer, 0.76 (0.69-0.85) for combined fractures, 0.98 (0.82-1.18) for total mortality, and 1.15 (1.03-1.28) for the global index. Absolute excess risks per 10000 person-years attributable to estrogen plus progestin were 7 more CHD events, 8 more strokes, 8 more PEs, and 8 more invasive breast cancers, while absolute risk reductions per 10000 person-years were 6 fewer colorectal cancers and 5 fewer hip fractures. The absolute excess risk of events included in the global index was 19 per 10000 person-years.

Conclusions Overall health risks exceeded benefits from use of combined estrogen plus progestin for an average 5.2-year follow-up among healthy postmenopausal US women. All-cause mortality was not affected during the trial. The risk-benefit profile found in this trial is not consistent with the requirements for a viable intervention for primary prevention of chronic diseases, and the results indicate that this regimen should not be initiated or continued for primary prevention of CHD.

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For editorial comment see p 366.

Author Information and Financial Disclosures appear at the end of this article.

THE WOMEN'S HEALTH INITIATIVE (WHI) focuses on defining the risks and benefits of strategies that could potentially reduce the incidence of heart disease, breast and colorectal cancer, and fractures in postmenopausal women. Between 1993 and 1998, the WHI enrolled 161809 postmenopausal women in the age range of 50 to 79 years into a set of clinical trials (trials of low-fat dietary pattern, calcium and vitamin D supplementation, and 2 trials of postmenopausal hormone use) and an observational study at 40 clinical centers in the United States.[1] This article reports principal results for the trial of combined estrogen and progestin in women with a uterus. The trial was stopped early based on health risks that exceeded health benefits over an average follow-up of 5.2 years. A parallel trial of estrogen alone in women who have had a hysterectomy is being continued, and the planned end of this trial is March 2005, by which time the average follow-up will be about 8.5 years.

The WHI clinical trials were designed in 1991-1992 using the accumulated evidence at that time. The primary outcome for the trial of estrogen plus progestin was designated as coronary heart disease (CHD). Potential cardioprotection was based on generally

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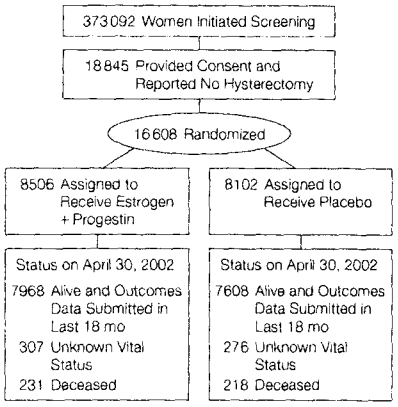
Figure 1. Profile of the Estrogen Plus Progestin Component of the Women's Health Initiative



supportive data on lipid levels in intermediate outcome clinical trials, trials in nonhuman primates, and a large body of observational studies suggesting a 40% to 50% reduction in risk among users of either estrogen alone or, less frequently, combined estrogen and progestin.[2-5] Hip fracture was designated as a secondary outcome, supported by observational data as well as clinical trials showing benefit for bone mineral density.[6,7] Invasive breast cancer was designated as a primary adverse outcome based on observational data.[3,8] Additional clinical outcomes chosen as secondary outcomes that may plausibly be affected by hormone therapy include other cardiovascular diseases; endometrial, colorectal, and other cancers; and other fractures.[3,6,9]

The effect of hormones on overall health was an important consideration in the design and conduct of the WHI clinical trial. In an attempt to summarize important aspects of health benefits vs risks, a global index was defined as the earliest occurrence of CHD, invasive breast cancer, stroke, pulmonary embolism (PE), endometrial cancer, colorectal cancer, hip fracture, or death due to other causes. Compared with total mortality, which may be too insensitive, this index assigns additional weight to the 7 listed diseases. Procedures for monitoring the trial involved semiannual comparisons of the estrogen plus progestin and placebo groups with respect to each of the elements of the global index and to the overall global index.

This report pertains primarily to estrogen plus progestin use among healthy postmenopausal women, since only 7.7% of participating women reported having had prior cardiovascular disease. During the course of the WHI trial, the Heart and Estrogen/progestin Replacement Study (HERS) reported its principal results.[10] HERS was another blinded, randomized controlled trial comparing the same regimen of estrogen plus progestin with placebo among women with a uterus; however, in HERS, all 2763 participating women had documented CHD prior to randomization. The HERS findings of no overall effect on CHD but an apparent increased risk in the first year after randomization seemed surprising given preceding observational studies of hormone use in women with CHD.[3] Subsequent to HERS, some investigators reanalyzed their observational study data and were able to detect an early elevation in CHD risk among women with prior CHD[11-13] but not in ostensibly healthy women,[14] prompting speculation that any early adverse effect of hormones on CHD incidence was confined to women who have experienced prior CHD events.

The WHI is the first randomized trial to directly address whether estrogen plus progestin has a favorable or unfavorable effect on CHD incidence and on overall risks and benefits in predominantly healthy women.

METHODS

Study Population

Detailed eligibility criteria and recruitment methods have been published.[1] Briefly, most women were recruited by population-based direct mailing campaigns to age-eligible women, in conjunction with media awareness programs. Eligibility was defined as age 50 to 79 years at initial screening, postmenopausal, likelihood of residence in the area for 3 years, and provision of written informed consent. A woman was considered postmenopausal if she had experienced no vaginal bleeding for 6 months (12 months for 50- to 54-year-olds), had had a hysterectomy, or had ever used postmenopausal hormones. Major exclusions were related to competing risks (any medical condition likely to be associated with a predicted survival of <3 years), safety (eg, prior breast cancer, other prior cancer within the last 10 years except nonmelanoma skin cancer, low hematocrit or platelet counts), and adherence and retention concerns (eg, alcoholism, dementia).

A 3-month washout period was required before baseline evaluation of women using postmenopausal hormones at initial screening. Women with an intact uterus at initial screening were eligible for the trial of combined postmenopausal hormones, while women with a prior hysterectomy were eligible for the trial of unopposed estrogen. This report is limited to the 16 608 women with an intact uterus at baseline who were enrolled in the trial component of estrogen plus progestin vs placebo. The protocol and consent forms were approved by the institutional review boards for all participating institutions (see Acknowledgment).

Study Regimens, Randomization, and Blinding

Combined estrogen and progestin was provided in 1 daily tablet containing conjugated equine estrogen (CEE), 0.625 mg, and medroxyprogesterone acetate (MPA), 2.5 mg (Prempro, Wyeth Ayerst, Philadelphia, Pa). A matching placebo was provided to the control group. Eligible women were randomly assigned to receive estrogen plus progestin or placebo after eligibility was established and baseline assessments made (FIGURE 1). The randomization procedure was developed at the WHI Clinical Coordinating Center and implemented locally through a distributed study database, using a randomized permuted block algorithm, stratified by clinical center site and age group. All study medication bottles had a unique bottle number and bar code to allow for blinded dispensing.

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Initially, the design allowed women with a uterus to be randomized to receive unopposed estrogen, estrogen plus progestin, or placebo. After the release of the Postmenopausal Estrogen/Progestin Intervention (PEPI) trial results[15] indicating that long-term adherence to unopposed estrogen was not feasible in women with a uterus, the WHI protocol was changed to randomize women with a uterus to only estrogen plus progestin or placebo in equal proportions. The 331 women previously randomized to unopposed estrogen were unblinded and reassigned to estrogen plus progestin. These women are included in the estrogen plus progestin group in this report, resulting in 8506 participants in the estrogen plus progestin group vs 8102 in the placebo group. Analysis of the data excluding the women randomized before this protocol change did not affect the results. Considerable effort was made to maintain blinding of other participants and clinic staff. When required for safety or symptom management, an unblinding officer provided the clinic gynecologist, who was not involved with study outcomes activities, with the treatment assignment.

Follow-up

Study participants were contacted by telephone 6 weeks after randomization to assess symptoms and reinforce adherence. Follow-up for clinical events occurred every 6 months, with annual in-clinic visits required. At each semiannual contact, a standardized interview collected information on designated symptoms and safety concerns, and initial reports of outcome events were obtained using a self-administered questionnaire. Adherence to study interventions was assessed by weighing of returned bottles. The study protocol required annual mammograms and clinical breast examinations; study medications were withheld if safety procedures were not performed, but these participants continued to be followed up. Electrocardiograms were collected at baseline and at follow-up years 3 and 6.

Data Collection, Management, and Quality Assurance

All data were collected on standardized study forms by certified staff according to documented study procedures. Study data were entered into a local clinical center database developed and maintained by the Clinical Coordinating Center and provided to each site in the form of a local area network connected to the Clinical Coordinating Center through a wide area network. Data quality was ensured through standard data entry mechanisms, routine reporting and database checks, random chart audits, and routine site visits.

Maintenance/Discontinuation of Study Medications

During the trial, some flexibility of the dosages of both estrogen and progestin was allowed to manage symptoms such as breast tenderness and vaginal bleeding. Vaginal bleeding was managed according to an algorithm that accounted for the time since randomization, severity of the bleeding, treatment assignment, and endometrial histology. Women who had a hysterectomy after randomization for indications other than cancer were switched to unopposed estrogen or the corresponding placebo without unblinding. These women are included in the original randomization group for analyses.

Permanent discontinuation of study medication was required by protocol for women who developed breast cancer, endometrial pathologic state (hyperplasia not responsive to treatment, atypia, or cancer), deep vein thrombosis (DVT) or PE, malignant melanoma, meningioma, triglyceride level greater than 1000 mg/dL (11.3 mmol/L), or prescription of estrogen, testosterone, or selective estrogen-receptor modulators by their personal physician. Medications were temporarily discontinued in participants who had acute myocardial infarction (MI), stroke, fracture, or major injury involving hospitalization, surgery involving use of anesthesia, any illness resulting in immobilization for more than 1 week, or any other severe illness in which hormone use is temporarily inappropriate.

Outcome Ascertainment

Cardiovascular Disease. Coronary heart disease was defined as acute MI requiring overnight hospitalization, silent MI determined from serial electrocardiograms (ECGs), or CHD death. The diagnosis of acute MI was established according to an algorithm adapted from standardized criteria[16] that included cardiac pain, cardiac enzyme and troponin levels, and ECG readings. The primary analyses included both definite and probable MIs as defined by the algorithm. Myocardial infarction occurring during surgery and aborted MIs were included. An aborted MI was defined as chest pain and ECG evidence of acute MI at presentation, an intervention (eg, thrombolysis) followed by resolution of ECG changes, and all cardiac enzyme levels within normal ranges. Silent MI was diagnosed by comparing baseline and follow-up ECGs at 3 and 6 years after randomization. Coronary death was defined as death consistent with CHD as underlying cause plus 1 or more of the following: preterminal hospitalization with MI within 28 days of death, previous angina or MI and no potentially lethal noncoronary disease, death resulting from a procedure related to coronary artery disease, or death certificate consistent with CHD as the underlying cause. Stroke diagnosis was based on rapid onset of a neurologic deficit lasting more than 24 hours, supported by imaging studies when available. Pulmonary embolism and DVT required clinical symptoms supported by relevant diagnostic studies.

Cancer. Breast, colorectal, endometrial, and other cancers were confirmed by pathological reports when available. Current data indicate that at least 98% of breast, colorectal, and endometrial cancers and 92% of other cancers were documented with pathological reports.

Fractures. Reports of hip, vertebral, and other osteoporotic fractures (including all fractures except those of

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Table 1. Baseline Characteristics of the Women's Health Initiative Estrogen Plus Progestin Trial Participants (N = 16 608) by Randomization Assignment*

Characteristics	Estrogen + Progestin (n = 8506)	Placebo (n = 8102)	P Value†
Age at screening, mean (SD), y	63.2 (7.1)	63.3 (7.1)	.39
Age group at screening, y			
50-59	2839 (33.4)	2683 (33.1)	.80
60-69	3853 (45.3)	3657 (45.1)	
70-79	1814 (21.3)	1762 (21.7)	
Race/ethnicity			
White	7140 (83.9)	6805 (84.0)	.33
Black	549 (6.5)	575 (7.1)	
Hispanic	472 (5.5)	416 (5.1)	
American Indian	26 (0.3)	30 (0.4)	
Asian/Pacific Islander	194 (2.3)	169 (2.1)	
Unknown	125 (1.5)	107 (1.3)	
Hormone use			
Never	6280 (73.9)	6024 (74.4)	.49
Past	1674 (19.7)	1588 (19.6)	
Current‡	548 (6.4)	487 (6.0)	
Duration of prior hormone use, y			
<5	1538 (69.1)	1467 (70.6)	.25
5-10	426 (19.1)	357 (17.2)	
≥10	262 (11.8)	253 (12.2)	
Body mass index, mean (SD), kg/m²§	28.5 (5.8)	28.5 (5.9)	.66
Body mass index, kg/m²			
<25	2579 (30.4)	2479 (30.8)	.89
25-29	2992 (35.3)	2834 (35.2)	
≥30	2899 (34.2)	2737 (34.0)	
Systolic BP, mean (SD), mm Hg	127.6 (17.6)	127.8 (17.5)	.51
Diastolic BP, mean (SD), mm Hg	75.6 (9.1)	75.8 (9.1)	.31
Smoking			
Never	4178 (49.6)	3999 (50.0)	.85
Past	3362 (39.9)	3157 (39.5)	
Current	880 (10.5)	838 (10.5)	
Parity			
Never pregnant/no term pregnancy	856 (10.1)	832 (10.3)	.67
≥1 term pregnancy	7609 (89.9)	7233 (89.7)	
Age at first birth, y‖			
<20	1122 (16.4)	1114 (17.4)	.11
20-29	4985 (73.0)	4685 (73.0)	
≥30	723 (10.6)	621 (9.7)	
Treated for diabetes	374 (4.4)	360 (4.4)	.88
Treated for hypertension or BP ≥140/90 mm Hg	3039 (35.7)	2949 (36.4)	.37
Elevated cholesterol levels requiring medication	944 (12.5)	962 (12.9)	.50
Statin use at baseline¶	590 (6.9)	548 (6.8)	.66
Aspirin use (≥80 mg/d) at baseline	1623 (19.1)	1631 (20.1)	.09
History of myocardial infarction	139 (1.6)	157 (1.9)	.14
History of angina	238 (2.8)	234 (2.9)	.73
History of CABG/PTCA	95 (1.1)	120 (1.5)	.04
History of stroke	61 (0.7)	77 (1.0)	.10
History of DVT or PE	79 (0.9)	62 (0.8)	.25
Female relative had breast cancer	1286 (16.0)	1175 (15.3)	.28
Fracture at age ≥55 y	1031 (13.5)	1029 (13.6)	.87

continued

the ribs, chest/sternum, skull/face, fingers, toes, and cervical vertebrae) were routinely ascertained. All fracture outcomes were verified by radiology reports. Study radiographs were not obtained to ascertain subclinical vertebral fractures.

This report is based on outcomes adjudicated by clinical center physician adjudicators, as used for trial-monitoring purposes. Clinical center physician adjudicators were centrally trained and blinded to treatment assignment and participants' symptoms. Future communications will report results based on centrally adjudicated outcomes and will include a broader range of outcomes with more extensive explanatory analyses. Since this report is presented before the planned study closeout, outcome information is still being collected and adjudicated. Local adjudication is complete for approximately 96% of the designated self-reported events. To date, agreement rates between local and central adjudication are: MI, 84%; revascularization procedures, 97%; PE, 89%; DVT, 84%; stroke, 94%; invasive breast cancer, 98%; endometrial cancer, 96%; colorectal cancer, 98%; hip fracture, 95%; and specific cause of death, 82%. When related cardiovascular conditions are combined (eg, when unstable angina or congestive heart failure is grouped with MI), agreement rates exceed 94% for cardiovascular disease and 90% for specific cause of death.

Statistical Analyses

All primary analyses use time-to-event methods and are based on the intention-to-treat principle. For a given outcome, the time of event was defined as the number of days from randomization to the first postrandomization diagnosis, as determined by the local adjudicator. For silent MIs, the date of the follow-up ECG applied. Participants without a diagnosis were censored for that event at the time of last follow-up contact. Primary outcome comparisons are presented as hazard ratios (HRs) and 95% confidence intervals (CIs) from Cox proportional haz-

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ards analyses,[17] stratified by clinical center, age, prior disease, and randomization status in the low-fat diet trial.

Two forms of CIs are presented, nominal and adjusted. Nominal 95% CIs describe the variability in the estimates that would arise from a simple trial for a single outcome. Although traditional, these CIs do not account for the multiple statistical testing issues (across time and across outcome categories) that occurred in this trial, so the probability is greater than .05 that at least 1 of these CIs will exclude unity under an overall null hypothesis. The adjusted 95% CIs presented herein use group sequential methods to correct for multiple analyses over time. A Bonferroni correction for 7 outcomes as specified in the monitoring plan (described herein) was applied to all clinical outcomes other than CHD and breast cancer, the designated primary and primary adverse effect outcomes, and the global index. The adjusted CIs are closely related to the monitoring procedures and, as such, represent a conservative assessment of the evidence. This report focuses primarily on results using the unadjusted statistics and also relies on consistency across diagnostic categories, supportive data from other studies, and biologic plausibility for interpretation of the findings.

Table 1. Baseline Characteristics of the Women's Health Initiative Estrogen Plus Progestin Trial Participants (N = 16 608) by Randomization Assignment* (cont)

Characteristics	Estrogen + Progestin (n = 8506)	Placebo (n = 8102)	P Value
Gail model 5-year risk of breast cancer, %			
<1	1290 (15.2)	1271 (15.7)	.64
1-<2	5384 (63.3)	5139 (63.4)	
2-<5	1751 (20.6)	1621 (20.0)	
≥5	81 (1.0)	71 (0.9)	
No. of falls in last 12 mo			
0	5168 (66.2)	5172 (67.5)	.18
1	1643 (21.0)	1545 (20.2)	
2	651 (8.3)	645 (8.4)	
≥3	349 (4.5)	303 (4.0)	

*Data are presented as number (percentage) of patients unless otherwise noted. BP indicates blood pressure; CABG/PTCA, coronary artery bypass graft/percutaneous transluminal coronary angioplasty; DVT, deep vein thrombosis; and PE, pulmonary embolism.
†Based on χ^2 tests (categorical variables) or *t* tests (continuous variables).
‡Required a 3-month washout prior to randomization.
§Total number of participants with data available was 8470 for estrogen plus progestin and 8050 for placebo.
‖Among women who reported having a term pregnancy.
¶Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors.

Data and Safety Monitoring

Trial monitoring guidelines for early stopping considerations were based on O'Brien-Fleming boundaries[18] using asymmetric upper and lower boundaries: a 1-sided, .025-level upper boundary for benefit and 1-sided, .05-level lower boundaries for adverse effects. The adverse-effect boundaries were further adjusted with a Bonferroni correction for the 7 major outcomes other than breast cancer that were specifically monitored (CHD, stroke, PE, colorectal cancer, endometrial cancer, hip fracture, and death due to other causes). The global index of monitored outcomes played a supportive role as a summary measure of the overall balance of risks and benefits. Trial monitoring for early stopping considerations was conducted semiannually by an independent data and safety monitoring board (DSMB). Aspects of the monitoring plan have been published.[19]

RESULTS

Trial Monitoring and Early Stopping

Formal monitoring began in the fall of 1997 with the expectation of final analysis in 2005 after an average of approximately 8.5 years of follow-up. Late in 1999, with 5 interim analyses completed, the DSMB observed small but consistent early adverse effects in cardiovascular outcomes and in the global index. None of the disease-specific boundaries had been crossed. In the spring of 2000 and again in the spring of 2001, at the direction of the DSMB, hormone trial participants were given information indicating that increases in MI, stroke, and PE/DVT had been observed and that the trial continued because the balance of risks and benefits remained uncertain.

In reviewing the data for the 10th interim analyses on May 31, 2002, the DSMB found that the adverse effects in cardiovascular diseases persisted, although these results were still within the monitoring boundaries. However, the design-specified weighted log-rank test statistic for breast cancer ($z=-3.19$) crossed the designated boundary ($z=-2.32$) and the global index was supportive of a finding of overall harm ($z=-1.62$). Updated analyses including 2 months of additional data, available by the time of the meeting, did not appreciably change the overall results. On the basis of these data, the DSMB concluded that the evidence for breast cancer harm, along with evidence for some increase in CHD, stroke, and PE, outweighed the evidence of benefit for fractures and possible benefit for colon cancer over the average 5.2-year follow-up period. Therefore, the DSMB recommended early stopping of the estrogen plus progestin component of the trial. Because the balance of risks and benefits in the unopposed-estrogen component remains uncertain, the DSMB recommended continuation of that component of the WHI. Individual trial participants have been informed.

Baseline Characteristics

There were no substantive differences between study groups at baseline; 8506 women were randomized into the estrogen plus progestin group and 8102 into the placebo group (TABLE 1). The mean (SD) age was 63.3 (7.1) years. Two thirds of the women who reported prior or current hormone use had taken combined hormones and one third had used unopposed estrogen.

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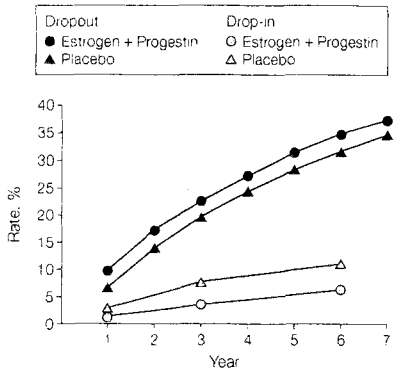
Figure 2. Cumulative Dropout and Drop-in Rates by Randomization Assignment and Follow-up Duration



Dropout refers to women who discontinued study medication; drop-in, women who discontinued study medication and received postmenopausal hormones through their own clinician.

Prevalence of prior cardiovascular disease was low and levels of cardiovascular risk factors were consistent with a generally healthy population of postmenopausal women. An assessment of commonly studied breast cancer risk factors, both individually and combined using the Gail model,[20] indicate that the cohort in general was not at increased risk of breast cancer.

Follow-up, Adherence, and Unblinding

Vital status is known for 16025 randomized participants (96.5%), including 449 (2.7%) known to be deceased. A total of 583 (3.5%) participants were lost to follow-up or stopped providing outcomes information for more than 18 months. The remaining 15576 (93.8%) provided recent outcome information (Figure 1).

At the time of this report, all women had been enrolled for at least 3.5 years, with an average follow-up of 5.2 years and a maximum of 8.5 years. A substantial number of women had stopped taking study drugs at some time (42% of estrogen plus progestin and 38% of placebo). Dropout rates over time (FIGURE 2) exceeded design projections, particularly early on, but compare favorably with community-based adherence to postmenopausal hormones.[21] Some women in both groups initiated hormone use through their own clinician (6.2% in the estrogen plus progestin group and 10.7% in the placebo group cumulatively by the sixth

Table 2. Clinical Outcomes by Randomization Assignment*

Outcomes	No. of Patients (Annualized %) Estrogen + Progestin (n = 8506)	Placebo (n = 8102)	Hazard Ratio	Nominal 95% CI	Adjusted 95% CI
Follow-up time, mean (SD), mo	62.2 (16.1)	61.2 (15.0)	NA	NA	NA
Cardiovascular disease†					
CHD	164 (0.37)	122 (0.30)	1.29	1.02-1.63	0.85-1.97
CHD death	33 (0.07)	26 (0.06)	1.18	0.70-1.97	0.47-2.98
Nonfatal MI	133 (0.30)	96 (0.23)	1.32	1.02-1.72	0.82-2.13
CABG/PTCA	183 (0.42)	171 (0.41)	1.04	0.84-1.28	0.71-1.51
Stroke	127 (0.29)	85 (0.21)	1.41	1.07-1.85	0.86-2.31
Fatal	16 (0.04)	13 (0.03)	1.20	0.58-2.50	0.32-4.49
Nonfatal	94 (0.21)	59 (0.14)	1.50	1.08-2.08	0.83-2.70
Venous thromboembolic disease	151 (0.34)	67 (0.16)	2.11	1.58-2.82	1.26-3.55
Deep vein thrombosis	115 (0.26)	52 (0.13)	2.07	1.49-2.87	1.14-3.74
Pulmonary embolism	70 (0.16)	31 (0.08)	2.13	1.39-3.25	0.99-4.56
Total cardiovascular disease	694 (1.57)	546 (1.32)	1.22	1.09-1.36	1.00-1.49
Cancer					
Invasive breast	166 (0.38)	124 (0.30)	1.26	1.00-1.59	0.83-1.92
Endometrial	22 (0.05)	25 (0.06)	0.83	0.47-1.47	0.29-2.32
Colorectal	45 (0.10)	67 (0.16)	0.63	0.43-0.92	0.32-1.24
Total	502 (1.14)	458 (1.11)	1.03	0.90-1.17	0.86-1.22
Fractures					
Hip	44 (0.10)	62 (0.15)	0.66	0.45-0.98	0.33-1.33
Vertebral	41 (0.09)	60 (0.15)	0.66	0.44-0.98	0.32-1.34
Other osteoporotic‡	579 (1.31)	701 (1.70)	0.77	0.69-0.86	0.63-0.94
Total	650 (1.47)	788 (1.91)	0.76	0.69-0.85	0.63-0.92
Death					
Due to other causes	165 (0.37)	166 (0.40)	0.92	0.74-1.14	0.62-1.35
Total	231 (0.52)	218 (0.53)	0.98	0.82-1.18	0.70-1.37
Global index§	751 (1.70)	623 (1.51)	1.15	1.03-1.28	0.95-1.39

*CI indicates confidence interval; NA, not applicable; CHD, coronary heart disease; MI, myocardial infarction; CABG, coronary artery bypass grafting; and PTCA, percutaneous transluminal coronary angioplasty.

†CHD includes acute MI requiring hospitalization, silent MI determined from serial electrocardiograms, and coronary death. There were 8 silent MIs. Total cardiovascular disease is limited to events during hospitalization except venous thromboembolic disease reported after January 1, 2000.

‡Other osteoporotic fractures include all fractures other than chest/sternum, skull/face, fingers, toes, and cervical vertebrae, as well as hip and vertebral fractures reported separately.

§The global index represents the first event for each participant from among the following types: CHD, stroke, pulmonary embolism, breast cancer, endometrial cancer, colorectal cancer, hip fracture, and death due to other causes.

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year). These "drop-in" rates were also greater than expected.

At the time of this report, clinic gynecologists had been unblinded to treatment assignment for 3444 women in the estrogen plus progestin group and 548 women in the placebo group, primarily to manage persistent vaginal bleeding. During the trial, 248 women in the estrogen plus progestin group and 183 in the placebo group had a hysterectomy.

Table 3. Cause of Death by Randomization Assignment

	No. (Annualized %)	
	Estrogen + Progestin (n = 8506)	Placebo (n = 8102)
Total deaths	231 (0.52)	218 (0.53)
Adjudicated deaths	215 (0.49)	201 (0.49)
Cardiovascular	65 (0.15)	55 (0.13)
Breast cancer	3 (0.01)	2 (<0.01)
Other cancer	104 (0.24)	86 (0.21)
Other known cause	34 (0.08)	41 (0.10)
Unknown cause	9 (0.02)	17 (0.04)

Intermediate Cardiovascular Disease End Points

Blood lipid levels, assessed in an 8.6% subsample of fasting blood specimens collected from women at baseline and year 1, showed greater reductions in low-density lipoprotein cholesterol (-12.7%) and increases in high-density lipoprotein cholesterol (7.3%) and triglycerides (6.9%) with estrogen plus progestin relative to placebo (data not shown), consistent with HERS and PEPI.[10,22] Systolic blood pressure was, on average, 1.0 mm Hg higher in women taking estrogen plus progestin at 1 year, rising to 1.5 mm Hg at 2 years and beyond (data not shown). Diastolic blood pressures did not differ.

Clinical Outcomes

Cardiovascular Disease. Overall CHD rates were low (TABLE 2). The rate of women experiencing CHD events was increased by 29% for women taking estrogen plus progestin relative to placebo (37 vs 30 per 10000 person-years), reaching nominal statistical significance (at the .05 level). Most of the excess was in nonfatal MI. No significant differences were observed in CHD deaths or revascularization procedures (coronary artery bypass grafting or percutaneous transluminal coronary angioplasty). Stroke rates were also higher in women receiving estrogen plus progestin (41% increase; 29 vs 21 per 10000 person-years), with most of the elevation occurring in nonfatal events. Women in the estrogen plus progestin group had 2-fold greater rates of venous thromboembolism (VTE), as well as DVT and PE individually, with almost all associated CIs excluding 1. Rates of VTE were 34 and 16 per 10000 person-years in the estrogen plus progestin and placebo groups, respectively. Total cardiovascular disease, including other events requiring hospitalization, was increased by 22% in the estrogen plus progestin group.

Cancer. The invasive breast cancer rates in the placebo group were consistent with design expectations. The 26% increase (38 vs 30 per 10000 person-years) observed in the estrogen plus progestin group almost reached nominal statistical significance and, as noted herein, the weighted test statistic used for monitoring was highly significant. No significant difference was observed for in situ breast cancers. Follow-up rates for mammography were comparable in the estrogen plus progestin and placebo groups. Colorectal cancer rates were reduced by 37% (10 vs 16 per 10000 person-years), also reaching nominal statistical significance. Endometrial cancer incidence was not affected, nor was lung cancer incidence (54 vs 50; HR, 1.04; 95% CI, 0.71-1.53) or total cancer incidence.

Fractures. This cohort experienced low hip fracture rates (10 per 10000 person-years in the estrogen plus progestin group vs 15 per 10000 person-years in the placebo group). Estrogen plus progestin reduced the observed hip and clinical vertebral fracture rates by one third compared with placebo, both nominally significantly. The reductions in other osteoporotic fractures (23%) and total fractures (24%) were statistically significant (all associated CIs exclude 1).

The global index showed a nominally significant 15% increase in the estrogen plus progestin group (170 vs 151 per 10000 person-years). There were no differences in mortality or cause of death between groups (TABLE 3).

Time Trends

The Kaplan Meier estimates of cumulative hazards (FIGURE 3) for CHD indicate that the difference between treatment groups began to develop soon after randomization. These curves provide little evidence of convergence through 6 years of follow-up. The cumulative hazards for stroke begin to diverge between 1 and 2 years after randomization, and this difference persists beyond the fifth year. For PE, the curves separate soon after randomization and show continuing adverse effects throughout the observation period. For breast cancer, the cumulative hazard functions are comparable through the first 4 years, at which point the curve for estrogen plus progestin begins to rise more rapidly than that for placebo. Curves for colorectal cancer show benefit beginning at 3 years, and curves for hip fracture show increasing cumulative benefit over time. The difference in hazard rates for the global index (FIGURE 4) suggests a gradual increase in adverse effects compared with benefits for estrogen plus progestin through year 5, with a possible narrowing of the difference by year 6; however, HR estimates tend to be unstable beyond 6 years after randomization. Total mortality rates are indistinguishable between estrogen plus progestin and placebo.

Tests for linear trends with time since randomization, based on a Cox proportional hazards model with a time-

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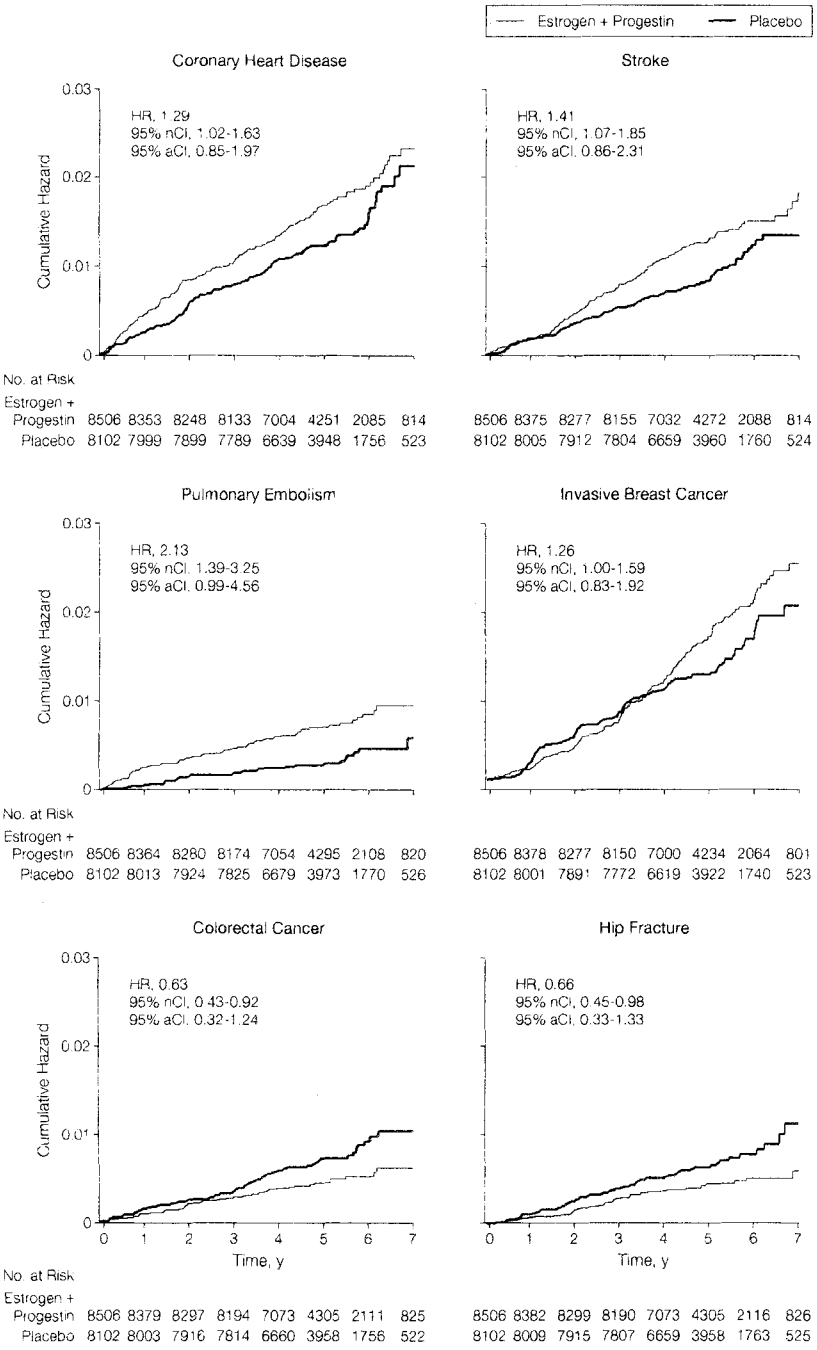
dependent covariate, detected no trend with time for CHD, stroke, colorectal cancer, hip fracture, total mortality, or the global index (TABLE 4). There was some evidence for an increasing risk of breast cancer over time with estrogen plus progestin ($z=2.56$ compared with a nominal z score for statistical significance of 1.96) and a decreasing risk of VTE with time ($z=-2.45$). These results must be viewed cautiously because the number of events in each interval is modest, the data in later years are still incomplete, and later year comparisons are limited to women still at risk of their first event for that outcome.

Figure 3. Kaplan-Meier Estimates of Cumulative Hazards for Selected Clinical Outcomes



HR indicates hazard ratio; nCI, nominal confidence interval; and aCI, adjusted confidence interval.

Subgroup Analyses

Cardiovascular Disease. A small subset of women (n=400; average follow-up, 57.4 months) in WHI reported conditions at baseline that would have made them eligible for HERS, ie, prior MI or revascularization procedures. Among these women with established coronary disease, the HR for subsequent CHD for estrogen plus progestin relative to placebo was 1.28 (95% CI, 0.64-2.56) with 19 vs 16 events. The remaining women, those without prior CHD, had an identical HR for CHD (145 vs 106; HR, 1.28; 95% CI, 1.00-1.65). Few women with a history of VTE were enrolled, but these data suggest a possibility that these women may be at greater risk of future VTE events when taking estrogen plus progestin (7 vs 1; HR, 4.90; 95% CI, 0.58-41.06) than those without a history of VTE (144 vs 66; HR, 2.06; 95% CI, 1.54-2.76). For stroke, prior history did not confer additional risk (1 vs 5 in women with prior stroke; HR, 0.46; 95% CI, 0.05-4.51; 126 vs 80 with no prior stroke; HR, 1.47; 95% CI, 1.11-1.95). No noteworthy interactions with age, race/ethnicity, body mass index, prior hormone use, smoking status, blood pressure, diabetes, aspirin use, or statin use were found for the effect of estrogen plus progestin on CHD, stroke, or VTE.

Breast Cancer. Women reporting prior postmenopausal hormone use had higher HRs for breast cancer associated with estrogen plus progestin use than those who never used postmenopausal hormones (among never users, 114 vs 102; HR, 1.06; 95% CI, 0.81-1.38; for women with <5 years of prior use, 32 vs 15; HR, 2.13; 95% CI, 1.15-3.94; for women with 5-10

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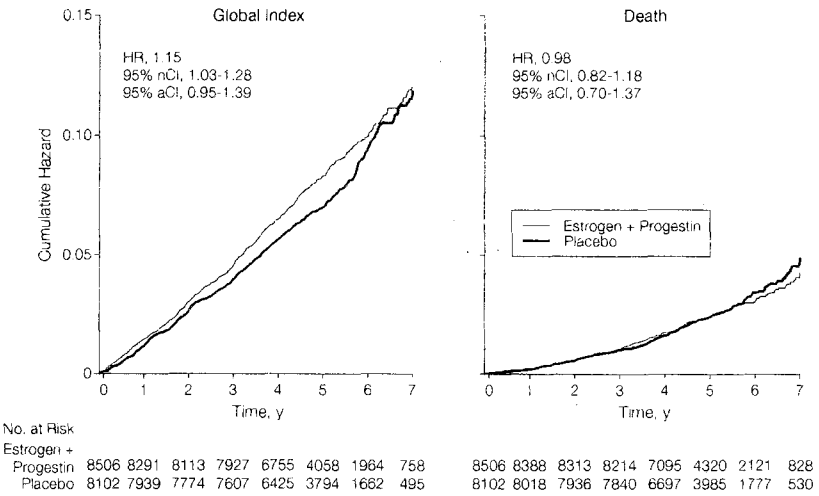
years of prior use, 11 vs 2; HR, 4.61; 95% CI, 1.01-21.02; and for women with ≥10 years of prior use, 9 vs 5; HR, 1.81; 95% CI, 0.60-5.43; test for trend, $z=2.17$). No interactions between estrogen plus progestin and age, race/ethnicity, family history, parity, age at first birth, body mass index, or Gail-model risk score were observed for invasive breast cancer.

Further Analyses

Because a number of women stopped study medications during follow-up, several analyses were performed to examine the sensitivity of the principal HR estimates to actual use of study medications. Analyses that censored a woman's event history 6 months after becoming nonadherent (using <80% of or stopping study drugs) produced the largest changes to estimated effect sizes. This approach increased HRs to 1.51 for CHD, to 1.49 for breast cancer, to 1.67 for stroke, and to 3.29 for VTE. Analyses attributing events to actual hormone use ("as treated," allowing for a 6-month lag) produced more modest changes to these estimates. Analyses excluding women randomized during the period when the unopposed-estrogen component was open to women with a uterus and analyses stratifying by enrollment period did not substantially

Figure 4. Kaplan-Meier Estimates of Cumulative Hazards for Global Index and Death



HR indicates hazard ratio; nCI, nominal confidence interval; and aCI, adjusted confidence interval.

Table 4. Selected Clinical Outcomes by Follow-up Year and Randomization Assignment*

	Year 1			Year 2			Year 3		
Outcomes	E + P	Placebo	Ratio	E + P	Placebo	Ratio	E + P	Placebo	Ratio
No. of participant-years	8435	8050		8353	7980		8268	7888	
Coronary heart disease	43 (0.51)	23 (0.29)	1.78	36 (0.43)	30 (0.38)	1.15	20 (0.24)	18 (0.23)	1.06
Stroke	17 (0.20)	17 (0.21)	0.95	27 (0.32)	15 (0.19)	1.72	30 (0.36)	16 (0.20)	1.79
Venous thromboembolism	49 (0.58)	13 (0.16)	3.60	26 (0.31)	11 (0.14)	2.26	21 (0.25)	12 (0.15)	1.67
Invasive breast cancer	11 (0.13)	17 (0.21)	0.62	26 (0.31)	30 (0.38)	0.83	28 (0.34)	23 (0.29)	1.16
Endometrial cancer	2 (0.02)	2 (0.02)	0.95	4 (0.05)	4 (0.05)	0.96	4 (0.05)	5 (0.06)	0.76
Colorectal cancer	10 (0.12)	15 (0.19)	0.64	11 (0.13)	9 (0.11)	1.17	6 (0.07)	8 (0.10)	0.72
Hip fracture	6 (0.07)	9 (0.11)	0.64	8 (0.10)	13 (0.16)	0.59	11 (0.13)	12 (0.15)	0.87
Total death	22 (0.26)	17 (0.21)	1.24	30 (0.36)	30 (0.38)	0.96	39 (0.47)	35 (0.44)	1.06
Global index	123 (1.46)	96 (1.19)	1.22	134 (1.60)	117 (1.47)	1.09	127 (1.54)	107 (1.36)	1.13

	Year 4			Year 5			Year 6 and Later			
Outcomes	E + P	Placebo	Ratio	E + P	Placebo	Ratio	E + P	Placebo	Ratio	z Score for Trend†
No. of participant-years	7926	7562		5964	5566		5129	4243		
Coronary heart disease	25 (0.32)	24 (0.32)	0.99	23 (0.39)	9 (0.16)	2.38	17 (0.33)	18 (0.42)	0.78	−1.19
Stroke	25 (0.32)	14 (0.19)	1.70	16 (0.27)	8 (0.14)	1.87	12 (0.23)	15 (0.35)	0.66	−0.51
Venous thromboembolism	27 (0.34)	14 (0.19)	1.84	16 (0.27)	6 (0.11)	2.49	12 (0.23)	11 (0.26)	0.90	−2.45
Invasive breast cancer	40 (0.50)	22 (0.29)	1.73	34 (0.57)	12 (0.22)	2.64	27 (0.53)	20 (0.47)	1.12	2.56
Endometrial cancer	10 (0.13)	5 (0.07)	1.91	1 (0.02)	4 (0.07)	0.23	1 (0.02)	5 (0.12)	0.17	−1.58
Colorectal cancer	9 (0.11)	20 (0.26)	0.43	4 (0.07)	8 (0.14)	0.47	5 (0.10)	7 (0.16)	0.59	−0.81
Hip fracture	8 (0.10)	11 (0.15)	0.69	5 (0.08)	8 (0.14)	0.58	6 (0.12)	9 (0.21)	0.55	0.25
Total death	55 (0.69)	48 (0.63)	1.09	41 (0.69)	44 (0.79)	0.87	44 (0.86)	44 (1.04)	0.83	−0.79
Global index	155 (1.96)	127 (1.68)	1.16	112 (1.88)	77 (1.38)	1.36	100 (1.95)	99 (2.33)	0.84	−0.87

*E + P indicates estrogen plus progestin. All outcome data are number of patients (annualized percentage).
†Tests for trends are based on Cox proportional hazards models with time-dependent treatment effects. The z scores shown indicate trends across all years.

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affect the results. These analyses suggest that the intention-to-treat estimates of HRs may somewhat underestimate the effect sizes relative to what would be observed with full adherence to study medications.

COMMENT

The WHI provides evidence from a large randomized trial that addresses the important issue of whether most women with an intact uterus in the decades of life following menopause should consider hormone therapy to prevent chronic disease. The WHI enrolled a cohort of mostly healthy, ethnically diverse women, spanning a large age range (50-79 years at baseline). It is noteworthy that the increased risks for cardiovascular disease and invasive breast cancer were present across racial/ethnic and age strata and were not influenced by the antecedent risk status or prior disease. Hence, the results are likely to be generally applicable to healthy women in this age range. At the time the trial was stopped, the increases in numbers of invasive breast cancers, CHD, stroke, and PE made approximately equal contributions to harm in the estrogen plus progestin group compared with placebo, which were not counterbalanced by the smaller reductions in numbers of hip fractures and colorectal cancers.

Cardiovascular Disease

Even though the trial was stopped early for harm from breast cancer, a sufficient number of CHD events had occurred by 5.2 years of average follow-up to suggest that continuation to the planned end would have been unlikely to yield a favorable result for the primary outcome of CHD. Even if there were a reversal of direction toward benefit of a magnitude seen in the observational studies (ie, a risk reduction of 55%) during the remaining years, conditional power analyses indicate that less than 10% power remained for showing potential benefit if the trial continued.

The WHI finding that estrogen plus progestin does not confer benefit for preventing CHD among women with a uterus concurs with HERS findings among women with clinically apparent CHD,[10] with the Estrogen Replacement for Atherosclerosis trial, in which estrogen plus progestin did not inhibit progression,[23] and with a trial in women with unstable angina that did not observe a reduction in ischemic events.[24] The finding of an increased risk after initiation of treatment in WHI is similar to HERS. In HERS, after 4.1 and 6.8 years of follow-up, hormone therapy did not increase or decrease risk of cardiovascular events in women with CHD.[25] The WHI extends these findings to include a wider range of women, including younger women and those without clinically apparent CHD, and indicates that the risk may persist for some years.

Unlike CHD, the excess risk of stroke in the estrogen plus progestin group was not present in the first year but appeared during the second year and persisted through the fifth year. Preliminary analyses indicate that the modest difference in blood pressure between groups does not contribute much to an explanation of the increase in strokes (data not shown). The findings in WHI for stroke are consistent with but somewhat more extreme than those of HERS, which reported a nonsignificant 23% increase in the treatment group.[26] The results were also more extreme than those of the Women's Estrogen and Stroke Trial of estradiol (without progestin) in women with prior stroke, which found no effect of estrogen on recurrent strokes overall but some increase in the first 6 months.[27] Trials of the effect of estradiol on carotid intima-media thickness have yielded conflicting results.[28,29] At least 1 observational study has suggested that that use of estrogen plus progestin is associated with higher risk of stroke than estrogen alone.[14] In WHI, there was no indication that excess strokes due to estrogen plus progestin were more likely to occur in older women, in women with prior stroke history, by race/ethnicity, or in women with high blood pressure at baseline. Therefore, it appears that estrogen plus progestin increases the risk of strokes in apparently healthy women.

Venous thromboembolism is an expected complication of postmenopausal hormones, and the pattern over time in WHI is consistent with the findings from HERS and several observational studies.[30,31]

Cancer

The WHI is the first randomized controlled trial to confirm that combined estrogen plus progestin does increase the risk of incident breast cancer and to quantify the degree of risk. The WHI could not address the risk of death due to breast cancer because with the relatively short follow-up time, few women in the WHI have thus far died as a result of breast cancer (3 in the active treatment group and 2 in the placebo group). The risk of breast cancer emerged several years after randomization. After an average follow-up of about 5 years, the adverse effect on breast cancer had crossed the monitoring boundary. The 26% excess of breast cancer is consistent with estimates from pooled epidemiological data, which reported a 15% increase for estrogen plus progestin use for less than 5 years and a 53% increase for use for more than 5 years.[32] It is also consistent with the (nonsignificant) 27% increase found after 6.8 years of follow-up in HERS.[33]

With more common use of estrogen plus progestin, several epidemiological studies have reported that estrogen plus progestin appears to be associated with greater risk of breast cancer than estrogen alone.[34-37] In the PEPI trial, women in the 3 estrogen plus progestin groups had much greater increases in mammographic density (a predictor of breast cancer) than women in the estrogen or placebo groups.[38] In WHI, the HR for estrogen plus progestin was not higher in women with a family history or other risk factors for breast cancer, except for reported prior use of postmenopausal hormones. This may suggest a cumulative effect of years of exposure to postmenopausal hormones.

Endometrial cancer rates were low and were not increased by 5 years of es-

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trogen plus progestin exposure. Close monitoring for bleeding and treatment of hyperplasia may contribute to the absence of increased risk of endometrial cancer.

The reduction in colorectal cancer in the hormone group is consistent with observational studies, which have suggested fairly consistently that users of postmenopausal hormones may be at lower risk of colorectal cancer.[39] The mechanisms by which hormone use might reduce risk are unclear. Results from other trials of postmenopausal hormones will help resolve the effects of hormones on colorectal cancer.[40]

Fractures

The reductions in clinical vertebral fractures, other osteoporotic fractures, and combined fractures supported the benefit for hip fractures found in this trial. These findings are consistent with the observational data and limited data from clinical trials[41] and are also consistent with the known ability of estrogen (with or without progestin) to maintain bone mineral density.[42] The WHI is the first trial with definitive data supporting the ability of postmenopausal hormones to prevent fractures at the hip, vertebrae, and other sites.

Overall Risks and Benefits

At the end of the trial, the global index indicated that there were more harmful than beneficial outcomes in the estrogen plus progestin group vs the placebo group. The monitored outcomes included in the global index were selected to represent diseases of serious import that estrogen plus progestin treatment might affect, and do not include a variety of other conditions and measures that may be affected in unfavorable or favorable ways (eg, gallbladder disease, diabetes, quality of life, and cognitive function). The data on these and other outcomes will be the subject of future publications. All-cause mortality was balanced between the groups; however, longer follow-up may be needed to assess the impact of the incident diseases on total mortality.

The absolute excess risk (or risk reduction) attributable to estrogen plus progestin was low. Over 1 year, 10000 women taking estrogen plus progestin compared with placebo might experience 7 more CHD events, 8 more strokes, 8 more PEs, 8 more invasive breast cancers, 6 fewer colorectal cancers, and 5 fewer hip fractures. Combining all the monitored outcomes, women taking estrogen plus progestin might expect 19 more events per year per 10000 women than women taking placebo. Over a longer period, more typical of the duration of treatment that would be needed to prevent chronic disease, the absolute numbers of excess outcomes would increase proportionately.

During the 5.2 years of this trial, the number of women experiencing a global index event was about 100 more per 10000 women taking estrogen plus progestin than taking placebo. If the current findings can be extrapolated to an even longer treatment duration, the absolute risks and benefits associated with estrogen plus progestin for each of these conditions could be substantial and on a population basis could account for tens of thousands of conditions caused, or prevented, by hormone use.

Limitations

This trial tested only 1 drug regimen, CEE, 0.625 mg/d, plus MPA, 2.5 mg/d, in postmenopausal women with an intact uterus. The results do not necessarily apply to lower dosages of these drugs, to other formulations of oral estrogens and progestins, or to estrogens and progestins administered through the transdermal route. It remains possible that transdermal estradiol with progesterone, which more closely mimics the normal physiology and metabolism of endogenous sex hormones, may provide a different risk-benefit profile. The WHI findings for CHD and VTE are supported by findings from HERS, but there is no other evidence from clinical trials for breast cancer and colorectal cancer, and only limited data from trials concerning fractures.

Importantly, this trial could not distinguish the effects of estrogen from those of progestin. The effects of progestin may be important for breast cancer and atherosclerotic diseases, including CHD and stroke. Per protocol, in a separate and adequately powered trial, WHI is testing the hypothesis of whether oral estrogen will prevent CHD in 10739 women who have had a hysterectomy. The monitoring of this trial is similar to that for the trial of estrogen plus progestin. At an average follow-up of 5.2 years, the DSMB has recommended that this trial continue because the balance of overall risks and benefits remains uncertain. These results are expected to be available in 2005, at the planned termination.

The relatively high rates of discontinuation in the active treatment arm (42%) and crossover to active treatment in the placebo arm (10.7%) are a limitation of the study; however, the lack of adherence would tend to decrease the observed treatment effects. Thus, the results presented here may underestimate the magnitude of both adverse effects on cardiovascular disease and breast cancer and the beneficial effects on fractures and colorectal cancer among women who adhere to treatment.

The fact that the trial was stopped early decreases the precision of estimates of long-term treatment effects. A longer intervention period might have shown more pronounced benefit for fractures and might have yielded a more precise test of the hypothesis that treatment reduces colorectal cancer. Nonetheless, it appears unlikely that benefit for CHD would have emerged by continuing the trial to its planned termination. The trial results indicate that treatment for up to 5.2 years is not beneficial overall and that there is early harm for CHD, continuing harm for stroke and VTE, and increasing harm for breast cancer with increasing duration of treatment. This risk-benefit profile is not consistent with the requirements for a viable intervention for the primary prevention of chronic diseases.

Implications

The WHI trial results provide the first definitive data on which to base treat-

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ment recommendations for healthy postmenopausal women with an intact uterus. This trial did not address the short-term risks and benefits of hormones given for the treatment of menopausal symptoms. On the basis of HERS and other secondary prevention trials, the American Heart Association recommended against initiating postmenopausal hormones for the secondary prevention of cardiovascular disease.[33] The American Heart Association made no firm recommendation for primary prevention while awaiting the results from randomized clinical trials such as WHI, and stated that continuation of the treatment should be considered on the basis of established noncoronary benefits and risks, possible coronary benefits and risks, and patient preference.

Results from WHI indicate that the combined postmenopausal hormones CEE, 0.625 mg/d, plus MPA, 2.5 mg/d, should not be initiated or continued for the primary prevention of CHD. In addition, the substantial risks for cardiovascular disease and breast cancer must be weighed against the benefit for fracture in selecting from the available agents to prevent osteoporosis.

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META-ANALYSIS OF THE EFFECTS OF SOY PROTEIN INTAKE ON SERUM LIPIDS

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Abstract *Background. In laboratory animals, the consumption of soy protein, rather than animal protein, decreases serum cholesterol concentrations, but studies in humans have been inconclusive. In this meta-analysis of 38 controlled clinical trials, we examined the relation between soy protein consumption and serum lipid concentrations in humans.*

Methods. We used a random-effects model to quantify the average effects of soy protein intake on serum lipids in the studies we examined and used hierarchical mixed-effects regression models to predict variation as a function of the characteristics of the studies.

Results. In most of the studies, the intake of energy, fat, saturated fat, and cholesterol was similar when the subjects ingested control and soy-containing diets; soy protein intake averaged 47 g per day. Ingestion of soy protein was associated with the following net changes in serum lipid concentrations from the concentrations reached with the control diet: total cholesterol, a decrease of 23.2 mg per deciliter (0.60 mmol per liter; 95 percent confidence interval, 13.5 to 32.9 mg per deciliter [0.35 to 0.85 mmol per liter]), or 9.3 percent; low-density lipoprotein (LDL) cholesterol, a decrease of 21.7 mg per deciliter (0.56 mmol per liter; 95 percent confidence interval, 11.2 to 31.7 mg per deciliter [0.30 to 0.82 mmol per liter]), or 12.9 percent; and triglycerides, a decrease of 13.3 mg per deciliter (0.15 mmol per liter; 95 percent confidence interval, 0.3 to 25.7 mg per deciliter [0.003 to 0.29 mmol per liter]), or 10.5 percent. The changes in serum cholesterol and LDL cholesterol concentrations were directly related to the initial serum cholesterol concentration ($P<0.001$). The ingestion of soy protein was associated with a nonsignificant 2.4 percent increase in serum concentrations of high-density lipoprotein (HDL) cholesterol.

Conclusions. We found that the consumption of soy protein rather than animal protein significantly decreased serum concentrations of total cholesterol, LDL cholesterol, and triglycerides. (N Engl J Med 1995;333:276-82.)

INGESTION of vegetable protein in place of animal protein appears to be associated with a lower risk of coronary heart disease[1,2]; this effect may reflect decreases in serum cholesterol concentrations.[3] The cholesterol-lowering effects of soy protein as compared with animal protein have been recognized in animals for more than 80 years.[4] Carroll reviewed the evidence that soy protein produced less hypercholesterolemia and less atherosclerosis in laboratory animals than animal protein.[5] Although many clinical investigators have examined the effects of soy protein on serum lipids in humans, the results have not been consistent[6]; consequently, the Nutrition Committee of the American Heart Association recently concluded that soy protein decreases serum cholesterol concentrations in rabbits but not in humans.[7]

Clinical investigators have used a variety of soy products, differing amounts of soy protein, differing criteria for selecting subjects, and a variety of protocols. We performed a meta-analysis of these studies, since combining the results of multiple studies of small or moderate size increases the statistical power brought to bear on the research question and thus greatly enhances the precision of estimates of effect. Our analysis indicated that the effects of soy protein in lowering serum cholesterol concentrations were significantly related to the initial serum cholesterol values. The substitution of soy protein for animal protein produced significant decreases in serum concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides without significantly affecting high-density lipoprotein (HDL) cholesterol concentrations.

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METHODS

Identification and Selection of Studies

We searched the medical literature for studies of the effects of soy protein on serum cholesterol concentrations in humans; 37 articles containing primary reports were identified.[8-11] Studies were selected for analysis if they had used isolated soy protein or textured soy protein; if they were controlled and had either a crossover or a parallel design; and if they provided initial (base line) values so that changes for each study group could be calculated. Studies were excluded if there was no control group[8,11,14,25,27]; if they used several sources of vegetable protein[29]; if whole soybeans rather than soy protein were used[30]; or if base-line values were not provided.[22] After these 8 articles were excluded, 29 articles remained in the analysis.

Subgroup Analyses

Changes in serum lipid concentrations were analyzed in relation to the initial serum lipid values, the types of soy protein used (isolated soy protein, textured soy protein, or a combination), the amount of soy protein ingested (in grams per day), the type of diet (usual Western diet or low-fat and low-cholesterol diet), the age group of the subjects (adults or children), and the similarity of the control diet and the soy-containing diet (specifically, regarding weight change in the subjects and dietary intake of fat, saturated fat, and cholesterol). Study diets were considered to be similar in terms of weight change if the subjects' change in weight during consumption of the two diets did not differ significantly. Study diets were considered to be similar in terms of the intake of total dietary fat, saturated fat, and cholesterol if the reported values for the soy-containing and control diets differed by less than 10 percent. When values were not reported for weight change, dietary fat, or cholesterol intake, these variables were assumed not to be similar.

Meta-Analysis

Summary results of each clinical trial and selected characteristics of the study were tabulated for analysis. The estimate of the principal effect was defined as the mean difference (in milligrams per deciliter) between the change in lipid concentrations when the subjects ingested the soy-containing diet (final value minus initial value) and the change when they ingested the control diet (final value minus initial value). This difference is referred to as the net change. In additional meta-analyses we used only the mean difference attributed to the in-

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gestion of soy protein. For the computation of pooled effects, each study was assigned a weight consisting of the reciprocal of its variance. When raw data were available, the variance for each study was calculated separately by computing the standard deviation of the differences between paired observations for the change during the soy-containing diet and the change during the control diet; the standard error of the differences was then calculated. When raw data were unavailable, the variances of the difference were based on the reported standard deviations for each measure and on either reported correlation coefficients or reported results of paired t-tests for the changes during the two diets.

Estimates of the average effect of soy protein on lipid values and 95 percent confidence intervals were calculated with models based on both fixed-effects and random-effects assumptions.[45,46] Because substantial variability between observations was indicated by preliminary tests for homogeneity, we have presented the results of random-effects models calculated according to the method of DerSimonian and Laird.[47] The assumption of heterogeneity implied by the use of random-effects models is plausible because of the diverse clinical settings and groups of subjects analyzed.

Predictive models were also developed to examine characteristics of the studies that were hypothesized to influence the observed treatment effects. For this purpose, two-stage mixed regression models (fixed-effects and random-effects) were used.[18,19] Predictive models were estimated by hierarchical linear modeling.[50] This approach models variation among studies as a function of the characteristics of the study that are hypothesized to affect the response to treatment and a two-stage random component. Both net and unadjusted effects of the substitution of soy protein for animal protein served as outcome variables in alternative models. The set of predictors used for testing hypotheses in regression models was defined at the outset and was based on a preliminary review of the literature. Several alternative coding strategies were evaluated in preliminary analyses. Our final models included the set of predictors specified above for subgroup analyses. To establish overall levels of variability in treatment effects, our regression analysis began with the estimation of unconditional random-effects regression models without predictors. In a second phase, predictors were entered into the models in bivariate and multiple-regression analyses. The degree of reduction in variance associated with each predictor was calculated by comparing the components of variance in unconditional models with those in conditional models containing predictors.[50]

Results

Characteristics of the Studies

Table 1 shows selected characteristics of the studies that met the criteria for analysis. The 29 articles chosen included the findings of 38 clinical studies; some articles reported data for different subgroups of subjects from one study (e.g., those with normal and those with high cholesterol concentrations); others reported on two different clinical studies. The 38 clinical studies were analyzed independently. In 4 studies the subjects were children, whereas in 34 they were adults. Most studies included both men and women, but the data necessary to analyze effects of soy protein according to sex were not available. Most studies used random assignment with crossover design. Twenty studies used isolated soy protein, 15 used textured soy protein, and 3 used a combination of the two. Soy protein intake averaged 47 g per day (range, 17 to 124); in 14 studies (37 percent) intake was ≤31 g per day.

In most studies the investigators attempted to provide similar amounts of total fat and saturated fat in the control and soy-containing diets. In 14 studies the diets were similar to conventional Western diets in fat and cholesterol content (these were termed "usual" diets), and in 18 studies the diets were low in fat content (≤30 percent of energy) and low in cholesterol content (≤200 mg per day). In 29 studies the amounts of total fat and saturated fat were similar in the control and soy-containing diets (i.e., they differed by less than 10 percent); 8 other studies were designed to provide similar total fat and saturated fat intake but the similarity of the diets was not documented. In 20 studies cholesterol intake was similar in the two diets; 9 other studies were designed to provide similar cholesterol intake but similarity was not documented.

All the studies except one[37] were designed to maintain weight; 34 studies reported similar weight changes for subjects ingesting the control and soy-containing diets. In all, 19 studies had control and soy-containing diets that were similar with respect to intake of dietary fat (total and saturated), intake of dietary cholesterol, and weight change. These 19 studies are listed as "similar" in each of the last three columns of Table 1.

Changes in Serum Lipid Concentrations

The ingestion of diets containing soy protein, as compared with the control diets, was accompanied by a significant reduction in serum concentrations of total cholesterol, LDL cholesterol, and triglycerides (Table 2). The net change (change during the soy diet minus change during the control diet) in serum cholesterol concentrations was a decrease of 23.2 mg per deciliter (0.60 mmol per liter; 95 percent confidence interval for the decrease, 13.5 to 32.9 mg per deciliter [0.35 to 0.85 mmol per liter]), or 9.3 percent. Of 38 studies, 34 (89 percent) reported a net decrease and 4 (11 percent) reported a net increase in serum cholesterol concentrations.

The net change in serum LDL cholesterol concentrations was a decrease of 21.7 mg per deciliter (0.56 mmol per liter; 95 percent confidence interval for the decrease, 11.2 to 31.7 mg per deciliter [0.30 to 0.82 mmol per liter]), or 12.9 percent. Figure 1 illustrates the net effects of the consumption of soy protein on serum LDL cholesterol concentrations as reported in 31 studies. Twenty-six studies (84 percent) reported a net reduction, four studies (13 percent) reported an increase, and one study (3 percent) reported no change.

Soy protein intake did not significantly affect serum HDL cholesterol concentrations, but the net change was an increase of 2.4 percent. Serum very-low-density lipoprotein (VLDL) cholesterol concentrations were not significantly altered by soy protein. The consumption of soy protein significantly decreased serum triglyceride concentrations, by 13.3 mg per deciliter (0.15 mmol per liter; 95 percent confidence interval for the decrease, 0.3 to 25.7 mg per deciliter [0.003 to 0.29 mmol per liter]), or 10.5 percent. Of 30 studies, 22 (73 percent) reported a net decrease in serum triglyceride concentrations, whereas 8 (27 percent) reported an increase.

Effect of Initial Serum Lipid Concentrations

Table 3 summarizes the effects of various factors on changes in serum cholesterol concentrations. In the complete regression model, the initial serum cholesterol concentration was the only significant predictor of the change in the serum cholesterol concentration

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*Table 1. Characteristics of the 38 Studies.**

Study	No. of Subjects	Male/Female (%)†	Characteristics of Subjects‡	Study Design§	Soy Preparation¶	Amount of Soy (g/day)	Type of Diet‖	Dietary Fat**	Dietary Cholesterol**	Weight Change**
Bakhit et al.[44]	21	100/0 100/0	Free-living, NC and HC	RC, MD	ISP	25	LFC	Similar	Similar	Similar
Bakhit et al.[44]	11		Free-living, HC	RC, MD	ISP	25	LFC	Similar	Similar	Similar
Carroll et al.[10]	6	0/100	Free-living, NC	FC, MD	ISP, TSP	47	Usual	Similar	Decreased	Decreased
Carroll et al.[10]	10	0/100	Free-living, NC	RC, MD	ISP, TSP	44	Usual	Similar	Similar	Similar
Descovich et al.[12]	127	53/47	Free-living, HC	FC	TSP	47	LFC	NA	NA	Similar
Fumagalli et al.[18]	4	75/25	HC	FC, RW	TSP	39	LFC	Similar	Similar	Similar
Fumagalli et al.[18]	3	67/33	HC	FC, RW	TSP	39	LFC	Similar	Similar	Similar
Gaddi et al.[33]	16	56/44	Children, free-living, HC	FC	TSP	56	LFC	NA	NA	Similar
Gaddi et al.[39]	20	40/60	Free-living, HC	FC	TSP	75	LFC	Similar	Decreased	Similar
Giovannetti et al.[31]	12	0/100	Free-living, NC	RC, MD	ISP	71	Usual or LFC	Similar	Similar	Similar
Goldberg et al.[19]	12	58/42	Free-living, HC	RC	ISP	90	Usual	Similar	Similar	Similar
Goldberg et al.[19]	4	75/25	Free-living, NC	RC	ISP	90	Usual	Similar	Similar	Similar
Holmes et al.[23]	12	75/25	Free-living, HC	FC	TSP	27	LFC	Similar	Similar	Similar
Holmes et al.[23]	10	60/40	Free-living, HC	RC	TSP	62	LFC	Similar	Similar	Similar
Huff et al.[24]	5	100/0	Free-living, HC	RC	TSP, ISP	41	LC	Similar	Increased	Similar
Jenkins et al.[37]	11	0/100	Free-living, obese	RC	ISP	28	Hypocaloric	Increased	Decreased	Decreased
Laurin et al.[40]	9	60/40	Children, free-living, HC	RC	ISP	31	LFC	—	—	—††
Lovati et al.[34]	12	42/58	Free-living, HC	RC	TSP	64	LFC	Similar	Similar	Similar
Meinertz et al.[36]	10	50/50	Free-living, NC	RC, LFD	ISP	113	LFC	Similar	Increased	Similar
Meinertz et al.[38]	11	45/55	Free-living, NC	RC, LFD	ISP	124	LF	Similar	Similar	Similar
Mercer et al.[35]	5	Unknown	Free-living, HC	RC	ISP	17	Usual	Similar	Similar	Similar
Potter et al.[42]	25	100/0	HC	RC, RW	ISP	50	LFC	Similar	Similar	Similar
Sacks et al.[23]	13	69/31	Free-living, NC, V	RC	ISP	27	Vegetarian	Similar	NA	NA
Shorey et al.[15]	24	100/0	Free-living, HC	RP	ISP	55	Usual	Similar	Similar	Similar
Sirtori et al.[9]	20	50/50	HC	RC, RW	TSP	47	LC	NA	NA	Similar
Sirtori et al.[28]	65	45/55	Free-living, HC	FC	TSP	47	LFC	Similar	Decreased	Similar
Sirtori et al.[28]	65	45/55	Free-living, HC	FC	TSP	23	LFC	Similar	Decreased	Similar
Steele[41]	14	NR	Free-living, NC and HC; O, LOV, and V	RC	ISP	21	Usual	NA	NA	Similar
Steele[41]	18	NR	Free-living, NC and HC; O, LOV, and V	RC	ISP	26	Usual	NA	NA	Similar
van Raaij et al.[16]	24	NR	Free-living, NC and HC	RP	ISP	54	Usual	Similar	Similar	Similar
van Raaij et al.[20]	20	NR	Free-living, NC and HC	RP	ISP	53	Usual	Similar	Similar	Similar
van Raaij et al.[20]	20	NR	Free-living , NC and HC	RP	TSP	55	Usual	Similar	Similar	Similar
Verrillo et al.[29]	19	42/58	Free-living, HC	RC	TSP	31	Usual	Similar	Similar	Similar
Verrillo et al.[29]	38	50/50	Free-living, HC	RC	TSP	31	Usual	Similar	Decreased	Similar
Vessby et al.[21]	6	67/33	HC	FC, RW	TSP	37	LC	Similar	Decreased	Similar
Widhalm[32]	11	64/36	Children, free-living, HC	FC	ISP	20	LFC	NA	NA	Similar
Widhalm et al.[43]	23	52/48	Children, free-living, HC	FC	ISP	18	LFC	NA	NA	NA
Wolfe et al.[17]	7	100/0	Free-living, HC	RC	ISP	47	LC	NA	NA	Similar

*The 38 studies were reported in 29 published articles. The pairs of studies reported together are listed separately in this table. The numbers of subjects shown are those for whom data were available for comparisons of total cholesterol concentrations.

†NR denotes not reported; both male and female subjects were included.

‡NC denotes subjects with normal cholesterol concentrations, HC those with hypercholesterolemia, V vegetarians, O omnivores, and LOV lacto-ovo-vegetarians.

§RC denotes randomized crossover, MD metabolic diet (i.e., with all food prepared in a metabolic kitchen), FC fixed-sequence crossover, RW research ward, LFD liquid formula diet, and RP randomized parallel.

¶ISP denotes isolated soy protein, and TSP textured soy protein.

‖LFC denotes low fat and cholesterol, LC low cholesterol, and LF low fat.

**Similar indicates that the soy-containing and control diets were similar in terms of dietary fat intake, dietary cholesterol intake, or weight change in the subjects. Increased indicates significantly higher in the soy-containing diet than the control diet, and decreased significantly lower in the soy-containing diet. NA denotes that the information was not available.

††Dietary fat and cholesterol and weight change in subjects consuming the two diets were similar. However, only eight of the subjects received both diets. An additional subject received only the soy-containing diet, and a different subject received only the control diet.

($P<0.001$). The relation between the initial serum cholesterol concentration and changes in serum cholesterol was modeled as a quadratic polynomial function. The proportion reduction in variance among studies between conditional and unconditional models indicated that the base-line cholesterol concentration accounted for approximately 77 percent of the overall variance. However, significant heterogeneity continued to be present in the model even after adjustment for hypothesized predictors of variation (variance component = 0.134, $P<0.001$).

Table 4 presents changes in serum cholesterol and LDL cholesterol concentrations, according to quartiles of the initial cholesterol concentration. Subjects with normal cholesterol levels, who had initial values below 200 mg per deciliter, had nonsignificant reductions of 3.3

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*Table 2. Net Change in Serum Lipids and Lipoprotein Concentrations in Subjects Ingesting the Soy-Containing Diets, as Compared with the Control Diets.**

Index	No. of Studies	No. of Subjects	Change (mg/dl)†	95% CI	Percent Change
Total cholesterol	38	730	−23.2	−32.9 to −13.5	−9.3
LDL cholesterol	31	564	−21.7	−31.7 to −11.2	−12.9
HDL cholesterol	30	551	+1.2	−3.1 to +5.4	+2.4
VLDL cholesterol	20	255	−0.4	−4.6 to +3.9	−2.6
Triglycerides	30	628	−13.3	−25.7 to −0.3	−10.5

*Net change is expressed as the change during the soy-containing diet minus the change during the control diet. VLDL denotes very-low-density lipoprotein, and CI confidence interval.

†To convert values for cholesterol to millimoles per liter, multiply by 0.02586; to convert values for triglycerides to millimoles per liter, multiply by 0.01129.

percent while receiving the soy protein diet. Those with mild hypercholesterolemia, who had initial values of 200 to 255 mg per deciliter (5.2 to 6.6 mmol per liter), had nonsignificant reductions of 4.4 percent. Subjects with moderate hypercholesterolemia, who had initial values of 259 to 333 mg per deciliter (6.70 to 8.61 mmol per liter), had significant decreases of 7.4 percent. Subjects with severe hypercholesterolemia, whose initial values were above 335 mg per deciliter (8.66 mmol per liter), had significant reductions of 19.6 percent.

The pattern of changes in serum LDL cholesterol concentrations, according to quartiles of the initial serum cholesterol values, was similar to the pattern for serum cholesterol concentrations: first quartile, a decrease of 7.7 percent; second quartile, a decrease of 6.8 percent; third quartile, a decrease of 9.8 percent; and fourth quartile, a decrease of 24.0 percent. Changes in serum HDL cholesterol concentrations were similar for all quartiles. Changes in serum triglyceride concentrations were significantly related to the initial serum triglyceride concentrations ($P<0.05$). However, changes in individual quartile groups were not statistically significant.

Effect of Other Variables

As shown in Table 3, the type of soy protein did not have a significant effect on the net change in serum cholesterol concentrations and accounted for only approximately 1.0 percent of the variance. The amount of soy protein in the diet was also not significant ($P=0.39$) when net changes were assessed. The type of diet, although not statistically significant, accounted for approximately 12.6 percent of the variance ($P=0.07$); larger changes tended to occur when the control diets were "usual" diets rather than low-fat and low-cholesterol diets. The results of studies of adult subjects did not differ significantly from those of the four studies of children; the age group of the subjects thus had a negligible effect on variance. The changes in the 19 studies with similar diets in terms of fat and cholesterol intake and weight change did not differ significantly from the changes in the remaining studies, in which the diets were not similar; this factor accounted for negligible variance.

To examine the effects of the type and amount of soy protein further, we performed a complete regression analysis using changes observed with the soy diet alone instead of net changes (soy diet minus control diet) as the outcome variable. In this model, significant effects were obtained for the initial serum cholesterol concentration ($P<0.001$; proportion of reduction accounted for, 0.69) and the amount of soy protein ($P=0.02$; proportion of reduction, 0.13). This model predicted that soy protein intake would be associated with the following decreases in serum cholesterol concentrations, after adjustment for the initial values and other variables: 25 g per day of soy protein, a decrease of 8.9 mg per deciliter (0.23 mmol per liter); 50 g per day of soy protein, a decrease of 17.4 mg per deciliter (0.45 mmol per liter); and 75 g per day of soy protein, a decrease of 26.3 mg per deciliter (0.68 mmol per liter). The type of soy

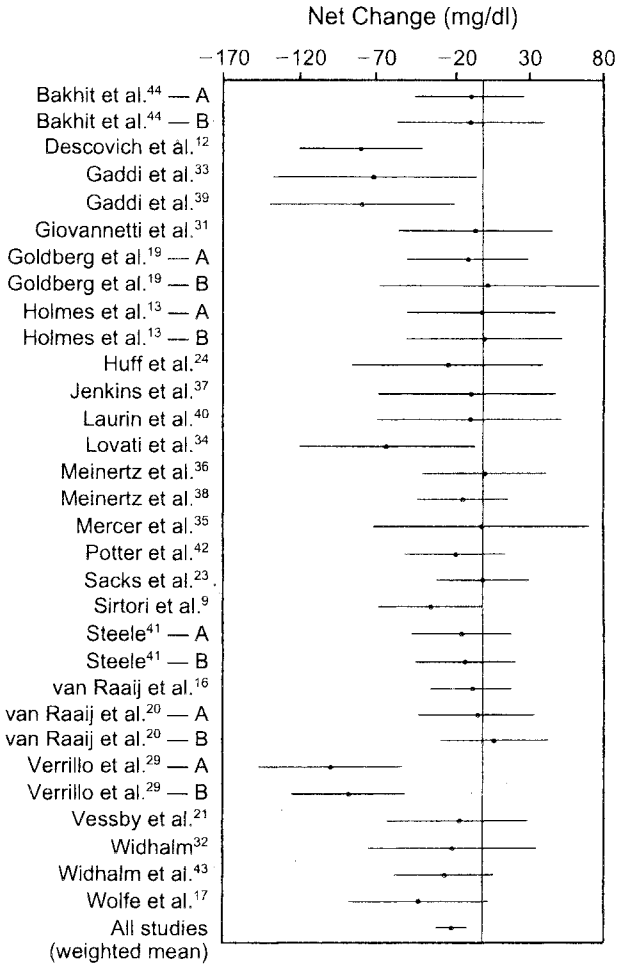

Figure 1. Net Changes in Serum LDL Cholesterol Concentrations in 31 Clinical Trials of the Effects of Soy Protein on Serum Lipids.

These 31 trials presented data on LDL cholesterol for a total of 564 subjects. The values shown are the mean changes in LDL cholesterol concentrations while subjects received the diet containing soy protein minus the changes during the control diet, with 95 percent confidence intervals. A and B indicate separate studies reported in a single published article, listed here in the same order as in Table 1. To convert values to millimoles per liter, multiply by 0.02586.

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protein (P=0.16), the type of diet (P=0.11), the age group of the subjects (adults or children) (P=0.39), and the similarity of the diets (P=0.28) did not have significant effects on this model.

*Table 4. Changes in Serum Cholesterol and LDL Cholesterol Concentrations According to Quartiles of the Study Group for Initial Cholesterol Concentration.**

Variable	Quartile 1	2	3	4
Cholesterol (mg/dl)				
Initial range	127.1 to 197.8	201.2 to 255.4	259.3 to 332.8	>335
Change	−5.2	−10.1	−22.2	−71.5
95% CI	−17.1 to +6.7	−21.8 to +1.7	−37.3 to −7.1	−86.6 to −56.5
% Change	−3.3	−4.4	−7.4	−19.6
LDL cholesterol (mg/dl)				
Change	−7.1	−10.7	−18.3	−68.1
95% CI	−20.0 to +6.0	−24.3 to +2.9	−35.3 to −1.3	−90.2 to −45.9
% Change	−7.7	−6.8	−9.8	−24.0

*Exact ranges are given for cholesterol and total cholesterol concentrations in the quartiles. To convert values for cholesterol to millimoles per liter, multiply by 0.02586. CI denotes confidence interval.

Discussion

This analysis of 38 controlled clinical studies reported in 29 scientific articles indicated that the replacement of animal protein in the diet with soy protein was associated with a significant decrease in serum cholesterol and LDL cholesterol concentrations. This was a fairly consistent finding, since decreases in serum cholesterol concentrations were reported in 34 of 38 studies; in the 4 studies[15,20,23,36] that did not report such reductions, the subjects had fairly low initial serum cholesterol values (average, 185 mg per deciliter [4.78 mmol per liter]). Changes in serum lipid concentrations were independent of changes in body weight and dietary intake of total fat, saturated fat, and cholesterol.

The strength and consistency of these observations are surprising in the light of the conclusion of the Nutrition Committee of the American Heart Association that the "consumption of vegetable proteins leads to lower cholesterol levels than consumption of animal proteins in rabbits but not in humans."[7] This comment was supported by only one study.[51]

Initial serum cholesterol concentrations had a powerful effect on changes in serum cholesterol and LDL cholesterol concentrations and accounted for approximately 77 percent of the variance among studies. The amount of soy protein ingested had a significant effect on serum cholesterol concentrations when the effects of the soy diet were examined alone, without the effects of the control diet. Soy protein intake averaged 47 g per day, and 37 percent of the studies used 31 g per day or less. These observations suggest that the daily consumption of 31 to 47 g of soy protein can significantly decrease serum cholesterol and LDL cholesterol concentrations. After adjustment for initial serum cholesterol concentrations and other variables, the ingestion of 25 or 50 g of soy protein per day was estimated to decrease serum cholesterol concentrations by 8.9 or 17.4 mg per deciliter, respectively. Persons with moderate or severe hypercholesterolemia (>250 mg per deciliter [6.46 mmol per liter]) should have even larger decreases in serum cholesterol concentrations when soy protein replaces animal protein in the diet.

*Table 3. Fixed-Effects Estimates from the Regression Model Predicting Net Changes in Serum Cholesterol Concentrations as a Function of Characteristics of the Study.**

Variable†	Regression Coefficient	Standard Error	Proportion Reduction in Variance	P Value
Intercept	0.194	0.381	—	—
Square of initial cholesterol value (mg/dl)‡	−0.020	0.005	0.773	<0.001
Type of soy protein	−0.115	0.133	0.010	0.27
Amount of soy protein (g/day)	−0.001	0.003	—	0.39
Type of diet	0.322	0.173	0.126	0.07
Age group of subjects	0.024	0.331	—	0.39
Similarity of diets	0.132	0.169	—	0.29

*Final estimate of random effects for net change: variance component=0.134; chi-square = 100.4; P<0.001.

†In the regression analysis, the following values were assigned to categorical variables: type of soy protein, 0=isolated soy protein, 1=isolated and textured soy proteins, and 2=textured soy protein; type of diet, 0=usual and 1=low fat and cholesterol; age group, 0=children and 1=adults; similarity, 0=not similar or data unavailable, and 1=similar.

‡Modeled as a quadratic polynomial function.

Soy protein products are widely available in supermarkets, and lower-fat soy products are easily obtainable. Persons with hypercholesterolemia can achieve an intake of more than 30 g of soy protein per day by consuming two to three servings of soy products daily. The amount of soy protein in a single serving of various soy products is as follows: 8 oz (226 g) of soy milk contains 4 to 10 g of soy protein; 4 oz (113 g) of tofu, 8 to 13 g; 1 oz (28 g) of soy flour, 10 to 13 g; 1 oz (28 g) of isolated soy protein, 23 g; 1/2 cup (113 g) of textured soy protein, 11 g; and 3.2 oz (91 g) of meat analogue, 18 g.[52] Thus, substituting two cups (473 ml) of soy milk for regular milk and consuming one serving of meat analogue would provide approximately 30 g of soy protein per day.

The mechanisms responsible for the effects of soy protein on serum lipoproteins are unknown[6,44] and were not addressed in this study. Carroll[6] recently reviewed and discussed various hypotheses. In experiments in animals, the amino acid composition of the diet affects serum cholesterol concentrations; increases in arginine are accompanied by decreases in serum cholesterol concentrations.[6] Although some studies suggest that alterations in bile acid or cholesterol absorption may contribute to altered cholesterol homeostasis,[6] Fumagalli et al.[18] found no differences in the fecal excretion of bile acids or sterols by human subjects. Some observers, as discussed by Carroll,[6] suggest that alterations in the ratio of serum glucagon to serum insulin may affect hepatic cholesterol synthesis; others[6,44] suggest that serum free thyroxine concentrations may

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be higher when the diet contains soy protein. Huff and colleagues[24] suggest that turnover of VLDL apoprotein B is increased in humans when soy protein is substituted for meat and dairy protein. Lovati and colleagues[34] report that the LDL-receptor activity of monocytes is eight times greater in human subjects receiving soy protein than in those eating control diets.

Setchell[53] suggests that soy estrogens may contribute to the cholesterol-lowering effects of soy protein. Most soy protein products contain soy estrogens (isoflavones or phytoestrogens),[54] which have weak estrogenic effects under certain circumstances and antiestrogenic effects under others.[55] The administration of oral estrogens[56] or the synthetic antiestrogen tamoxifen[57] decreases serum cholesterol and LDL cholesterol concentrations; soy estrogens may have similar actions. This suggestion is supported in studies by Anthony and colleagues.[58-60] In three studies using cynomolgus or rhesus monkeys, soy protein rich in soy estrogens favorably affected serum lipids, whereas soy protein from which the soy estrogens had been extracted had a minimal effect. These primate studies suggest that soy estrogens may account for 60 to 70 percent of the hypocholesterolemic effects of soy protein.

In summary, this meta-analysis of 38 studies indicates that the consumption of soy protein is associated with significant decreases in serum cholesterol, LDL cholesterol, and triglyceride concentrations and with a nonsignificant increase in serum HDL cholesterol concentrations. The decreases in serum cholesterol and LDL cholesterol concentrations were strongly related to the subjects' initial serum cholesterol concentrations. Soy estrogens may be responsible for most of the hypocholesterolemic effects of soy protein.

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Effects of the phytoestrogen genistein on hot flushes, endometrium, and vaginal epithelium in postmenopausal women: a 1-year randomized, double-blind, placebo-controlled study.

D'Anna R, Cannata ML, Atteritano M, Cancellieri F, Corrado F, Baviera G, Triolo O, Antico F, Gaudio A, Frisina N, Bitto A, Polito F, Minutoli L, Altavilla D, Marini H, Squadrito F.

Department of Obstetrical and Gynaecological Sciences, University of Messina, Messina, Italy.

OBJECTIVE: To evaluate in a 12-month, prospective, randomized, double-blind, placebo-controlled study whether pure administration of the phytoestrogen genistein (54 mg/d) might reduce the number and severity of hot flushes in postmenopausal women with no adverse effect on the endometrium. DESIGN: A total of 389 participants met the main study criteria and were randomly assigned to receive the phytoestrogen genistein (n=198) or placebo (n=191). About 40% of participants in both groups did not suffer from hot flushes, and the evaluation was performed in a subgroup of 247 participants (genistein, n=125; placebo, n=122). Reductions from baseline in the frequency and severity of hot flushes were the principal criteria of efficacy. Endometrial thickness was evaluated by ultrasonography. The maturation value was also used to determine hormonal action on the vaginal cells. RESULTS: There were no significant differences in age, time since menopause, body mass index, and vasomotor symptoms between groups at baseline (4.4 +/- 0.33 hot flushes per day in the genistein group and 4.2 +/- 0.35 hot flushes per day in the control group). The effect was already evident in the first month and reached its peak after 12 months of genistein therapy (-56.4% reduction in the mean number of hot flushes). Furthermore, there was a significant difference between the two groups at each evaluation time (1, 3, 6, and 12 months). No significant difference was found in mean endometrial thickness and maturation value score between the two groups, either at baseline or after 12 months. CONCLUSIONS: The phytoestrogen genistein has been shown to be effective on

vasomotor symptoms without an adverse effect on endometrium.

PMID: 17251874 [PubMed - indexed for MEDLINE]

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Effects of the phytoestrogen genistein on hot flushes, endometrial thickness, and vaginal epithelium in postmenopausal women: a 2-year randomized, double-blind, placebo-controlled study. [Menopause. 2009]

Effects of genistein on hot flushes in early postmenopausal women: a randomized, double-blind EPT- and placebo-controlled study. [Menopause. 2004]

Efficacy and safety of a soy isoflavone extract in postmenopausal women: a randomized, double-blind, and placebo-controlled study. [Menopause. 2007]

Review Phytoestrogens for vasomotor menopausal symptoms. [Cochrane Database Syst Rev. 2007]

Review Phytoestrogens for treatment of menopausal symptoms: a systematic review. [Obstet Gynecol Surv. 2004]

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Effect of soy-derived isoflavones on hot flushes, endometrial thickness, and the pulsatility index of the uterine and cerebral arteries.

Penotti M, Fabio E, Modena AB, Rinaldi M, Omodei U, Viganó P.

Second Department of Obstetrics and Gynecology of the University of Milan, Milan, Italy. mauropen@tiscalinet.it

OBJECTIVE: To determine the effect of soy-derived isoflavones on hot flushes, endometrial thickness, and the vascular reactivity of uterine and cerebral arteries. DESIGN: Double-blind, randomized, placebo-controlled trial. SETTING: Healthy volunteers in an academic research environment. PATIENT(S): Sixty-two postmenopausal women aged 45-60 years attending the Outpatient Menopause Clinic of our gynecological departments. INTERVENTION(S): The patients were administered 72 mg of soy-derived isoflavones or placebo under double-blind conditions. The daily number of hot flushes was recorded in a diary. Endometrial thickness was measured by means of transvaginal ultrasound; the uterine, internal carotid, and middle cerebral arteries were evaluated using Doppler ultrasound. MAIN OUTCOME MEASURE(S): The daily number of hot flushes, endometrial thickness, and arterial pulsatility index (PI). RESULT(S): Both treatments led to a 40% reduction in the number of hot flushes. Soy-derived isoflavones had no effect on endometrial thickness or the PI of the uterine and cerebral arteries. CONCLUSION(S): The daily administration of 72 mg of soy-derived isoflavones is no more effective than placebo in reducing hot flushes in postmenopausal women. It also has no effect on endometrial thickness or the PI of the uterine and cerebral arteries.

PMID: 12738504 [PubMed - indexed for MEDLINE]

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A pilot study of the effects of phytoestrogen supplementation on postmenopausal endometrium.

Balk JL, Whiteside DA, Naus G, DeFerrari E, Roberts JM.

Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee-Womens Hospital, University of Pittsburgh, Pennsylvania 15213, USA. rsijlb@mail.magee.edu

OBJECTIVE: This study was designed to assess endometrial histology in postmenopausal women not taking hormone replacement therapy, to evaluate side effects and efficacy of phytoestrogens in treating menopause-associated symptoms, and to determine whether 6 months of phytoestrogen supplementation altered endometrial histology.METHODS: We performed a prospective, double-blinded, randomized, placebo-controlled trial comparing the effects of 6 months of dietary phytoestrogen supplementation versus placebo in postmenopausal women. Baseline endometrial biopsies were performed and, if adequate, nonhyperplastic, noncancerous, and nonovulatory, subjects were randomly assigned to receive daily placebo or soy cereal supplementation for 6 months. Study subjects completed baseline and weekly dietary, symptom, and side effect logs. Repeat endometrial biopsies were obtained at 6 months.RESULTS: Subjects were recruited from January 1998 through June 2000. Twenty-seven subjects were randomized, and 19 completed the study. One (3.7%) baseline endometrial sample was weakly proliferative. All other baseline and final biopsies were consistent with atrophic, inactive endometrium. The maximum risk of endometrial stimulation with phytoestrogens is 35%. Hot flushes, night sweats, and vaginal dryness were significantly less severe at the final week of the study compared with baseline in the placebo group. Insomnia was more common in the treated group. There were no other statistically significant differences in symptoms or side effects.CONCLUSION: Phytoestrogens did not cause stimulation of the endometrium. Insomnia was more

frequent over the 6-month study in the soy group, whereas hot flushes, night sweats, and vaginal dryness improved from baseline in the placebo group but not in the soy group.

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Lack of effect of isoflavonoids on the vagina and endometrium in postmenopausal women.

Nikander E, Rutanen EM, Nieminen P, Wahlström T, Ylikorkala O, Tiitinen A.

Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland. eini.nikander@pp.fimnet.fi

OBJECTIVE: To determine the effects of soy-derived isoflavones on vaginal epithelium and the endometrium. DESIGN: Double-blind, randomized, placebo-controlled crossover trial. SETTING: Outpatient clinic of a university hospital. PATIENT(S): Sixty-four postmenopausal women with a history of breast cancer. INTERVENTION(S): The women took (in a randomized order) 114 mg of isolated isoflavonoids or placebo in tablets daily for 3 months; the treatment regimens were crossed over after a 2-month washout period. The subjects were studied before and on the last day of each treatment period. MAIN OUTCOME MEASURE(S): Vaginal dryness, maturation index (MI) of vaginal epithelium, endometrial thickness, histology, and expression of estrogen (E) and progesterone (P) receptors and the proliferation marker Ki-67 in the endometrium. RESULT(S): Isolated isoflavones did not relieve vaginal dryness. Maturation index values remained unchanged during the isoflavone regimen, but decreased during the placebo regimen. No changes were found in any of the variables measured in the endometrium. CONCLUSION(S): Daily administration of 114 mg of isolated isoflavones for 3 months had no effect on the subjective perception of vaginal dryness or on objective findings in the vagina or endometrium. This implies safety with regard to the endometrium.

PMID: 15652899 [PubMed - indexed for MEDLINE]

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A double-blind randomized study on the effects of red clover isoflavones on the endometrium. [Maturitas. 2001]

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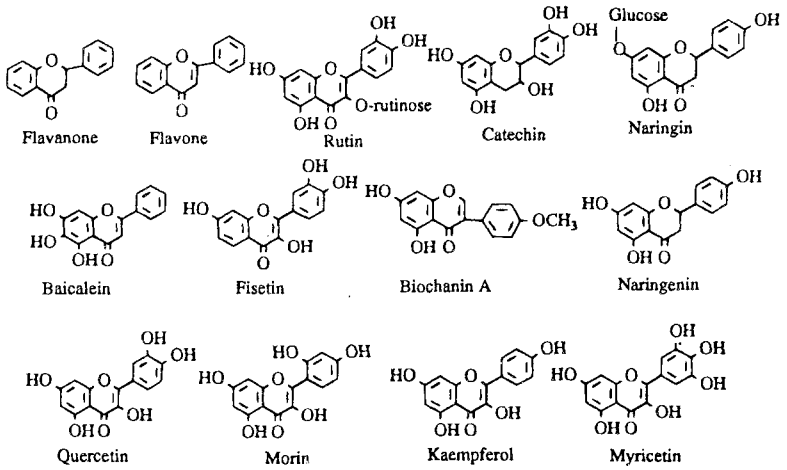

Figure 1. Flavonoid structures.

solved in Tris-HCl–KI buffer containing catalase and dialyzed against the same buffer. The dialyzed enzyme preparation was subjected to trypsin (0.02%) digestion for 1 h at room temperature. The digestion was stopped with soybean trypsin inhibitor (0.02%), the mixture was centrifuged at 100000*g* for 1 h, and the supernatant was fractionated again with ammonium sulfate as described above. The precipitate from ammonium sulfate fractionation was dissolved in and dialyzed against 0.02 M phosphate buffer (pH 6.8) containing 0.1 mM KI and catalase (500 units/L). The enzyme solution was concentrated by ultrafiltration (MWCO = 30 kDa) and subjected to gel filtration on a Bio-Gel A 1.5 m column (2.0 × 40 cm). The column was equilibrated and eluted with 0.02 M phosphate buffer (pH 6.8). The fractions containing guaiacol oxidation activity were pooled and rechromatographed on a hydroxylapatite column (1.8 × 20 cm) equilibrated with 0.02 M phosphate buffer (pH 6.8) containing 0.1 mM KI. The enzyme was eluted with a linear gradient of 0.02–0.25 M phosphate buffer (pH 6.8) containing 0.1 mM KI. The inclusion of catalase in the buffer during homogenization and initial purification, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic surfactant, for solubilization greatly increased the yield (ca. 6 mg of purified enzyme/ kg thyroid slices) of the purified enzyme. The R_f value of the enzyme preparations used in the current studies ranged from 0.32 to 0.42. The concentration of the enzyme was measured spectrophotometrically by using the extinction coefficient 1.12×10^5 $M^{-1}cm^{-1}$ at 412 nm (*17*). Guaiacol oxidation activity was determined at pH 7.0 as described previously (*18*).

Inhibition Assays. TPO (2.0 nM) was incubated with tyrosine (150 μM), iodide ion (150 μM), and hydrogen peroxide (50 μM) with different fixed concentrations of flavonoid, and iodination to 3-iodotyrosine (MIT) was monitored spectrophotometrically at 289 nm. MIT was quantified using HPLC for those flavonoids with absorbance maxima that interfered with spectrophotometric monitoring. It was determined that MIT formation rates were identical within experimental error whether determined by the UV or HPLC methods, although the UV method was more precise. HPLC analysis was performed immediately using a Radial Compression Module equipped with a NovaPak C18 cartridge (5 × 100 mm, 4-μm particle size, Waters Associates, Milford, MA) using UV detection at 289 nm essentially as described earlier (*18*).

Inactivation Experiments. TPO or lactoperoxidase (LPO, 1.0 μM) was preincubated with the flavonoid (100–150 μM) for 1 min prior to initiation of the reaction by the addition of hydrogen peroxide (200 μM). After 4 min of incubation, aliquots were removed and diluted 1000- to 2000-fold, and the residual enzyme activity was measured using the tyrosine iodination assay as described previously (*19*). It was determined that, following dilution, the concentration of flavonoid remaining had a minimal effect on the enzyme activity assay. A minimum of triplicate analyses was performed.

Time Course of Inactivation of TPO. Those flavonoids that inactivated TPO by more than 50% in the above experiment were studied further. TPO (1.0–5.0 μM) was incubated with kaempferol, 8–40 μM; morin, 10–50 μM; naringenin, 6–30 μM; or quercetin, 10–50 μM at 25 ± 0.1 °C in 0.1M phosphate buffer (pH 7.4), and hydrogen peroxide (200 μM) was added to initiate the reaction. Aliquots were withdrawn at various time points and diluted 1000- to 2000-fold, and the remaining enzyme activity was determined. The half-times for inactivation were estimated graphically from plots of activity remaining vs time (cf. Figure 3 inset and ref 20). The inactivation of TPO by the solvent ethanol (final concentration <2%, v/v) and H_2O_2 was minimal during the incubation period.

Hydrogen Peroxide Concentration Dependence of Inactivation. TPO (1.0 μM) was incubated with excess naringenin (150 μM), and enzyme activity was titrated by addition of varying but limiting concentrations of hydrogen peroxide. After 4 min of incubation, aliquots were withdrawn and diluted, and residual activity was measured. Soret spectral changes were monitored by second derivative UV–vis spectrophotometry to maximize sensitivity using a Hewlett-Packard 8452A diode array spectrophotometer as previously described (*19*).

Steady-State Kinetics of Inhibition of TPO-Catalyzed Tyrosine Iodination by Myricetin and Naringin. The inhibition of TPO (1.25–2.5 nM) by myricetin (0–2.0 μM) or naringin (0–6.0 μM) was studied in the presence of varying concentrations of either iodide ion (0–100 μM) or H_2O_2 (0–160 μM) at fixed concentrations of H_2O_2 (25 μM) and iodide ion (150 μM), respectively. MIT formation was monitored spectrophotometrically at 289 nm.

Iodination of Biochanin A. TPO (2.5 nM) was incubated with varying concentrations of biochanin A (0–50 μM) at a fixed concentration of iodide ion (250 μM) in 0.1 M phosphate buffer (pH 7.4) at 25 ± 0.1 °C, and the reaction was started by addition of hydrogen peroxide (50 μM). The increase in absorbance at 290 nm was monitored for 1–2 min spectrophotometrically.

Competitive iodination kinetics were determined in the presence of an enzymatic hydrogen peroxide-generating system. Biochanin A, TPO (2.5 nM), iodide ion (150 μM), tyrosine (150 μM), glucose (1.25 mM), glucose oxidase (10 nM), and biochanin A (0–20 μM) were incubated in 0.1 M phosphate buffer (pH 7.4) at 25 ± 0.1 °C. The reaction was followed by monitoring MIT formation.

For determination of reaction products, biochanin A (50 μM) was incubated with TPO (2.5 nM), iodide ion (0.4 mM), and H_2O_2 (50 μM) at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4). After 3 min of incubation, biochanin A and its products were extracted with 2 volumes of ethyl acetate. This was repeated twice before concentrating the combined aliquots of the ethyl acetate extract by Speed Vac (Savant Instruments Inc., Farmingdale, NY). The concentrate was reconstituted with 400 μL of HPLC mobile phase. HPLC analysis of 20–40 μL aliquots was performed

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Table 1. Effect of Flavonoids on TPO Activity[a]

compound	IC_{50} (μM)[b]	% inactivation[c]
flavanone	>1500	2.1 ± 0.13
flavone	>2000	6.8 ± 0.34
rutin	40.6 ± 3.88	36.5 ± 4.26
catechin	36.4 ± 3.86	42.9 ± 6.32
naringin	12.6 ± 1.56	2.3 ± 1.52
baicalein	8.3 ± 0.93	8.3 ± 1.76
fisetin	6.3 ± 0.63	31.1 ± 3.97
biochanin A	6.2 ± 0.84	14.6 ± 2.01
naringenin	2.7 ± 0.99	72.4 ± 5.89
quercetin	2.4 ± 0.64	63.9 ±12.59
morin	2.1 ± 0.82	52.2 ± 6.43
kaempferol	1.2 ± 0.48	76.9 ± 10.67
myricetin	0.6 ± 0.18	8.9 ± 1.02

[a] The assay system containing TPO (2.0 nM), tyrosine (0.15 mM), iodide ion (0.15 mM), and the appropriate concentration of test compound was preincubated in phosphate buffer (pH 7.4) for 2 min. The conditions for HPLC analysis are described in Materials and Methods. [b] IC_{50} values (mean ± SD) are based on 2–5 experiments in which the compounds were tested at 8–12 concentrations in duplicate. [c] Inactivation experiments were carried out in the presence of TPO (1.0 μM), test compound (150 μM), and H_2O_2 (200 μM) at 25 °C in 0.1 M phosphate buffer (pH 7.4). After 2–3 min of incubation, aliquots of the reaction mixture were withdrawn and diluted 1000 to 2000-fold, and tyrosine iodination activity was measured spectrophotometrically.

using a reversed phase column (3 cm × 2 mm, 3 μm particle size, Perkin Elmer, Norwalk, CT) using 60% acetonitrile in H_2O with a flow rate of 1.0 mL/min. The detection was carried out at 230 nm, and UV spectra (220–350 nm) were obtained using a Spectra Focus rapid scanning detector (Spectra Physics, San Jose, CA). Individual product peaks were collected for further analysis (see below).

The products of the biochanin A iodination were investigated by using ES/MS with a VG Platform mass spectrometer (Fisons Instruments, Altrincham, U.K.). The mass spectrometer was operated at the following conditions: capillary voltage 2.63 kV, HV lens voltage 0.57 kV, cone voltage 45–75 V, and source temperature 60 °C; and scanning was carried out at an m/z range of 100–600 at a rate of 1.49 s/scan for both positive and negative ion acquisitions. A 10 μL sample containing ca. 150 ng of the products (separated off-line by LC as described above) was introduced by flow injection analysis using a syringe pump delivering 50% acetonitrile in water at 10 μL/min.

The major product of biochanin A described above was further characterized by ^{1}H-NMR (Bruker AM1 spectrometer operated at 500.13 MHz) in $CDCl_3$.

Results

All of the flavonoids tested except flavanone and flavone inhibited tyrosine iodination by TPO, but with markedly different potencies (see Table 1). IC_{50} values ranged from 0.6 to 40.6 μM with the following order of potency: myricetin > kaempferol > morin > quercetin > naringenin > biochanin A > fisetin > baicalein > naringin > catechin > rutin. Subsequent studies were undertaken to determine the mechanisms for the observed inhibitory effects.

Time-Dependent Inactivation of TPO by Flavonoids. Incubation of TPO with kaempferol, morin, naringenin, or quercetin in the presence of H_2O_2 showed time-dependent loss of enzyme activity in an apparent first-order process. No loss of activity was observed in the absence of H_2O_2, and the rate of inactivation was dependent on the concentration of the flavonoid. Inactivation was associated with spectral changes in the Soret absorbance band (Figure 2). Native TPO had an absorption maximum at 413 nm, whereas naringenin- or kaempferol-inactivated TPO had an absorption maximum at 428 nm (a red shift of 15 nm) and about 60–70% decrease in the Soret absorbance. Similar changes were observed during inactivation of the enzyme in the presence of catechin, fisetin, morin, or quercetin. In contrast, analogous incubations with flavanone, flavone, myricetin, or naringin resulted in no spectral changes. Control experiments in which the flavonoid was omitted from the incubation mixture resulted in no change in absorbance. It is likely that the 390/393 nm peaks observed were due to the presence of oxidized flavonoids. The levels formed were low in these cases because of the enzyme inactivation that occurred; however, with phenol and pyrogallol, which are good substrates, prominent absorptions in this spectral region are observed (data not shown). As predicted for mechanism-based inhibitiors (*20*), a linear relationship was observed between the concentration of kaempferol, morin, naringenin, or quercetin versus the product of inactivation half-times and the flavonoid concentration. Values for k_i and K_i ranged from 1.4 to 6.7 and 6.2 to 12.9, respectively. A typical plot obtained with naringenin is shown in Figure 3. The saturation kinetics observed are consistent with formation of a reversible complex between enzyme and the flavonoid prior to inactivation. The apparent enzyme–inhibitor dissociation constants (K_i, Table 2) for the flavonoids are approximately one-half to one-third the apparent K_m value for iodide ion (22.4 μM, tyrosine iodination) determined under similar conditions for TPO. The apparent maximal inactivation rate constants (k_i) for these flavonoids are ca. 700- to 3000-fold lower than the turnover number for the normal enzyme reaction with iodide ion (tyrosine iodination, ~4800 min^{-1}).

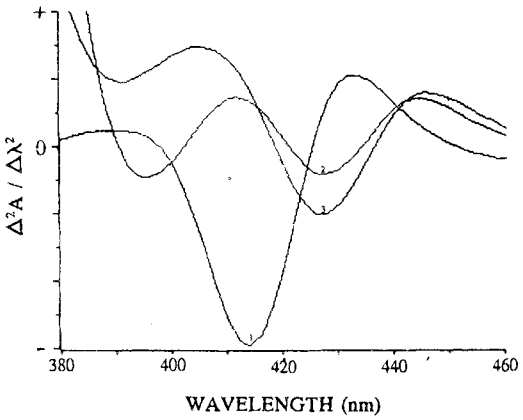

Figure 2. Second-derivative Soret spectra of native TPO and flavonoid-inactivated TPO. TPO (1.0 μM) was incubated with or without kaempferol (150 μM)/naringenin (150 μM) and H_2O_2 (200 μM). After 4 min of incubation, spectra were obtained between 380 and 460 nm. Spectra 1, 2, and 3 are native TPO, kaempferol-inactivated TPO, and naringenin-inactivated TPO, respectively.

Figure 4 shows the titration of TPO activity by varying limiting concentrations of H_2O_2 in the presence of naringenin under aerobic or minimal oxygen conditions. Inactivation was increased by hypoxia at all H_2O_2 concentrations tested. Extrapolation of the linear portion of the curves yielded approximate partition ratio values of 8 and 42, respectively, in hypoxic and aerobic conditions. Addition of other TPO substrates (e.g., pyrogallol, iodide ion) reduced the inactivation of TPO by kaempferol (see Table 3). The presence of pyrogallol, a potent substrate for TPO, in the reaction mixture at ≥150 μM completely protected the enzyme from inactivation by kaempferol. The presence of iodide ion (5.0 mM) in the

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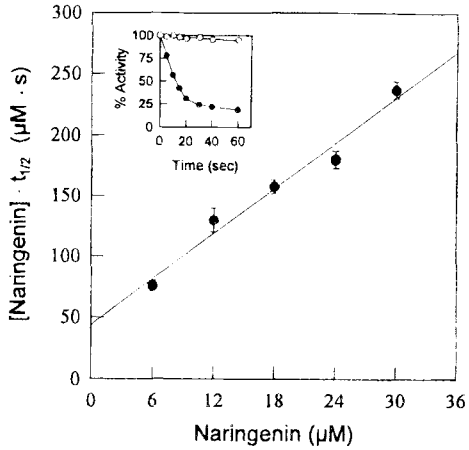

Figure 3. Kinetics of inactivation of TPO by naringenin. Inset: TPO was incubated with (●) naringenin (6 μM) or without (○) naringenin and hydrogen peroxide (200 μM) at 25 ± 0.1 °C in phosphate buffer (pH 7.4), and the activity remaining at various time points was determined (n = 6). Half-times for inactivation of TPO (1.0–5.0 μM) by naringenin (6.0–30.0 μM) were determined graphically from the time course of inactivation.

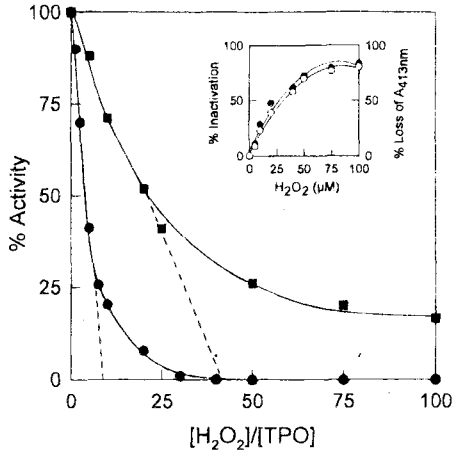

Figure 4. 4. H_2O_2 dependence of inactivation of TPO by naringenin under aerobic and hypoxic conditions. TPO (1.0 μM) was incubated with naringenin (150 μM) in 0.1 M phosphate buffer (pH 7.4) at 25 ± 0.1 °C, and the enzyme activity was titrated by addition of limiting amounts of hydrogen peroxide (■). Minimal oxygen conditions (●) were maintained by flushing with nitrogen and keeping the solutions under nitrogen atmosphere during the reaction. Partition ratios were estimated by extrapolating the linear portion of the curves to the abscissa (42 and 8 μM). Inset: Correlation between H_2O_2 added and the inactivation of TPO by naringenin (●) and loss of Soret absorbance (○).

Table 2. Kinetic Constants for the Inactivation of TPO by Flavonoids[a]

compound	K_i (μM)	k_i (min^{-1})
kaempferol	6.23	2.64
morin	12.87	1.36
naringenin	7.06	6.68
quercetin	7.09	1.69

[a] Reaction mixtures consisting of TPO (1.0–5.0 μM), kaempferol (8.0–40.0 μM)/morin (10.0–50.0 μM)/naringenin (6.0–30.0 μM)/quercetin (12.0–60.0 μM), and H_2O_2 (0.2 mM) were incubated at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4), and aliquots were withdrawn at various time points and residual activity of TPO was measured. The ratio of TPO/flavonoid was maintained at 0.125, 0.1, 0.167, and 0.083, respectively, for kaempferol, morin, naringenin, and quercetin. Half-time for inactivation was determined from the time course plots as previously described (*19, 20*).

Table 3. Inactivation of TPO by Kaempferol in the Presence and Absence of Alternate Substrates[a]

reaction conditions	activity remaining (% of control)
TPO	99.2 ± 3.17
TPO + H_2O_2	97.4 ± 5.86
TPO + KAE	96.3 ± 4.89
TPO + KAE + H_2O_2	18.2 ± 8.96
TPO + I^-	96.8 ± 4.59
TPO + PYG	99.6 ± 3.92
TPO + KAE + I^- + H_2O_2	63.8 ± 8.46
TPO + KAE + PYG + H_2O_2	94.3 ± 7.29

[a] TPO (1.0 μM) was incubated with 5.0 mM iodide ion, 150 μM kaempferol (KAE), 200 μM pyrogallol (PYG), and 200 μM hydrogen peroxide (H_2O_2) in the combinations indicated at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4) for 4 min, aliquots were withdrawn and diluted 1000- to 2000-fold, and the tyrosine iodination activity was measured. The values are mean ± SD (n = 6).

Table 4. Inactivation of Various Peroxidases by Naringenin/Quercetin[a]

peroxidase	% inactivation (naringenin)	% inactivation (quercetin)
LPO	73.2 ± 2.36	61.4 ± 6.32
TPO	82.7 ± 3.18	73.3 ± 4.96
MPO	48.5 ± 1.45	51.8 ± 6.32
CPO	24.8 ± 9.83	17.1 ± 10.53
CcP	24.6 ± 15.43	61.0 ± 8.67
HRP	2.0 ± 8.25	43.7 ± 7.93

[a] Peroxidases (1.0 μM) except CcP were incubated with naringenin or quercetin (150 μM) and H_2O_2 (200 μM) for 3 min at 25 °C in 0.1 M phosphate buffer (pH 7.4). In the case of CcP, 5.0 μM of enzyme was incubated with 250 μM test compound. Aliquots were withdrawn and diluted 1000-fold, and residual guaiacol oxidation activity was determined. The values given are mean ± SD (n = 4). The activity in the absence of test compound, but containing 5% ethanol, was taken as 100%.

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reaction mixture partially protected the enzyme against inactivation.

Dialysis of inactivated TPO was performed in order to distinguish between covalent or reversible binding of naringenin or kaempferol. Dialysis of enzyme alone resulted in 2–8% loss of enzyme activity with little or no change in the spectral properties. Incubation of TPO with the flavonoids in the absence of H_2O_2 caused no significant changes in either activity or spectra compared to control incubation. However, in the presence of H_2O_2, kaempferol or naringenin produced ~70% inactivation of TPO and ~65% decrease in the Soret absorbance that remained after dialysis.

Inactivation of Different Peroxidases by Naringenin/Quercetin. Table 4 lists the activities of different enzymes remaining after incubation of these enzymes with naringenin or quercetin in the presence of H_2O_2. TPO, LPO, and CcP were found to be more susceptible to inactivation by quercetin compared to other enzymes, although significant inactivation of MPO and HRP was observed.

Effect of Myricetin and Naringin on the Kinetics of TPO-Catalyzed Tyrosine Iodination. Myricetin and naringin, the glycoside of naringenin, did not inactivate TPO, but low concentrations did inhibit tyrosine iodination. The iodide ion concentration dependence of TPO-catalyzed tyrosine iodination was measured in incubations with myricetin and naringin. For both compounds, the inhibition was noncompetitive with respect to iodide ion concentration since V_{max}, but not K_m, was affected. The Dixon plots shown in Figure 5A,C

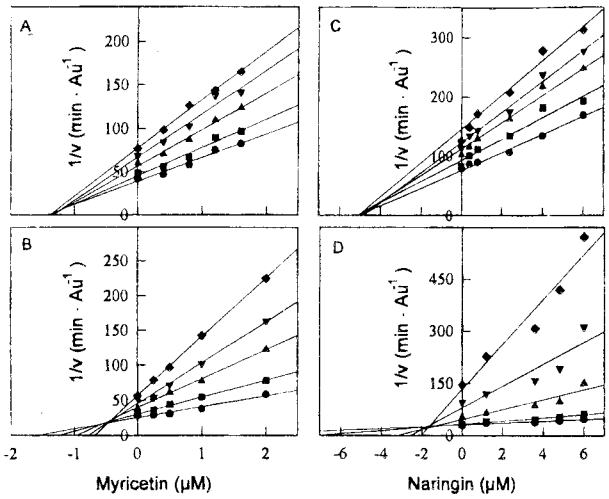

Figure 5. 5. Dixon plots showing the inhibition of TPO-catalyzed tyrosine iodination by myricetin and naringin. (A) The incubation system contained 2.5 nM TPO, 150 μM tyrosine, 12.5–100 μM (♦–●) iodide ion, 25 μM H_2O_2, and 0–1.6 μM myricetin. In (B) 2.5 nM TPO, 150 μM tyrosine, 150 μM iodide ion, 10–160 μM H_2O_2 (♦–●) and 0–2.5 μM myricetin were present. (C) 1.25 nM TPO, 150 μM tyrosine, 25 μM H_2O_2, 12.5–166.7 μM (♦–●), iodide ion, and 0–6.0 μM naringin were present in the incubation mixture. In (D), 2.5 nM TPO, 150 μM iodide ion, 150 μM tyrosine, 5–80 μM H_2O_2 (♦–●), and 0–6.0 μM naringin were present.

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yielded an apparent value for the enzyme–inhibitor dissociation constant, K_i, of 1.4 and 4.8 μM, respectively, for myricetin and naringin when measured in incubations containing 25 μM H_2O_2. These K_i values are ca. 5- to 16-fold lower than the respective K_m for iodide ion.

The H_2O_2 concentration dependence of TPO-catalyzed tyrosine iodination was also measured in incubations with myricetin and naringin. The Dixon plots (Figure 5B,D) of the reciprocal rate of tyrosine iodination as a function of flavonoid concentration yielded families of curves intersecting above the *x*-axis. This pattern is consistent with either competivive or linear mixed-type inhibition (*21*), and an apparent K_i value of 0.54 and 1.7 μM, respectively, for myricetin and naringin was obtained when measured at 150 μM iodide ion. Replots of the Dixon plot slopes revealed that myricetin was a mixed-type inhibitor and naringin was a competitive inhibitor with respect to H_2O_2 concentration. The K_i values are 20- to 63-fold lower than the K_m determined for H_2O_2 (34 μM).

Inhibition of TPO-Catalyzed Tyrosine Iodination by Biochanin A. Figure 6 shows the time course of iodination of tyrosine in the presence of different concentrations of biochanin A when glucose/glucose oxidase was used to produce a steady state concentration of H_2O_2. The presence of biochanin A in the reaction mixture resulted in initial blockade of iodination followed by a linear increase in MIT formation after the lag phase. The length of the lag phase was dependent on the concentration of biochanin A. However, the rate of MIT formation following lag phase was unchanged from the control rate. TPO-catalyzed iodination of bioichanin A (0–50 μM) was measured at fixed concentrations of iodide ion (250 μM) and H_2O_2 (50 μM). Reactions were started by the addition of H_2O_2 to the reaction mixture and monitored spectrophotometrically at 290 nm. Reciprocal velocities ($1/\Delta A$ per minute) were plotted against the reciprocal of biochanin A concentration, and K_m and V_{max} were determined using the Michaelis–Menten relationship to be 17.30 μM and 0.0283 AU/min at a fixed concentration of iodide ion and H_2O_2.

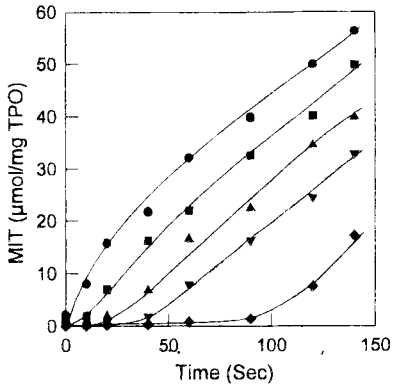
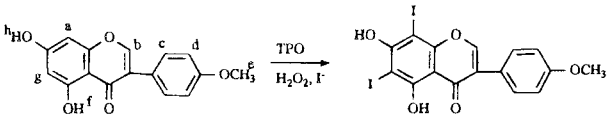


Figure 6. Time course of tyrosine iodination in the presence of biochanin A. The reaction was carried out in the presence of 2.5 nM TPO, 150 μM iodide ion, 150 μM tyrosine, 1.25 mM D-glucose, 10 nM glucose oxidase, and 0 μM (●), 4.0 μM (■), 8.0 μM (▲), 12.0 μM (▼), or 20.0 μM (♦) biochanin A at 25 ± 0.1 °C. The reaction was started by addition of glucose oxidase to the reaction mixture. MIT formed was monitored by LC as described in Materials and Methods.

Scheme 1. Iodination of Biochanin A by TPO



Characterization of Biochanin A Products Using ES/MS and NMR. TPO catalyzed the conversion of biochanin A into at least three products as determined by HPLC-UV. The peaks were collected off-line and analyzed by ES/MS in positive and negative ion modes. The major product (retention time 11.9 min) gave a deprotonated molecule at nominal mass 535 Da with prominent fragment ions at m/z 521 (M − CH_2) and 127 (I^-). In positive ion mode, the protonated molecule had a nominal mass of 537 Da, consistent with a diiodinated derivative. Both minor products were similarly analyzed and were consistent with isomeric monoiodinated [(M − H) , m/z 409; $(M + H)^+$, m/z 411]. Insufficient amounts of purified monoiodinated products precluded additional means of structure proof.

The structure of diiododinated product was further identified using ^{1}H-NMR. All proton resonances for biochanin A (see Scheme 1) were tentatively assigned as follows: 3.83 (s, 3H, H_e); 5.45 (bs, 1H, H_f); 6.28 (d, 1H, H_a, $J_{Ha,Hg}$ = 2.2 Hz); 6.35 (d, 1H, H_g); 6.96 (d, 2H, H_d, $J_{Hc,Hd}$ = 6.7 Hz); 7.43 (d, 2H, H_c); 7.84 (s, 1H, H_b); 12.90 (s, 1H, H_h). The assignments for H_c and H_d were confirmed by selective NOE irradiation experiments: NOE was observed for H_c upon irradiation of H_b and for H_d upon irradiation of H_e; the resonances for H_a and H_g were assigned based on known effects of *o*-hydroxyl groups and the observed 2.2 Hz coupling. The assignment of H_f was based on the observed line broadening expected for a hydrogen bonded proton. H_h was assigned based on the expected acidity and its resulting extreme downfield resonance. The diiodinated product had similar proton resonances except that the two protons in the resorcinol moiety (H_a and H_g), and no others, were eliminated (data not shown).

Discussion

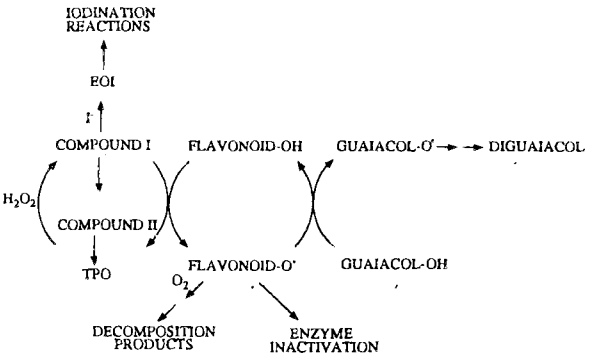
The flavonoids investigated in this study fit into several broad groups based on IC_{50} values for inhibition of TPO-catalyzed tyrosine iodination (see Table 1). While it is not valid to compare IC_{50} values for compounds with unknown inhibition mechanisms, the ability to differentiate grossly flavonoid potency was a useful screening procedure in the initial phase of this study. In previous work, we have demonstrated several mechanisms can be responsible for inhibition of TPO- and LPO-catalyzed reactions: suicide inactivation (*19*), rapid equilibrium (reversible) binding (*22*), and alternate substrate competition for the enzymatic iodinating intermediate (*18, 23*). These experimental approaches were used to determine inhibition mechanisms and structure–activity relationships for these typical dietary flavonoids.

When the more potent inhibitors were investigated further, it was seen that naringenin, quercetin, morin, and kaempferol caused irreversible inactivation of TPO (see Table 1, column 3). The kinetics of irreversible enzyme inactivation (see Table 2 and Figure 3), the altered visible absorbance spectrum of inactivated TPO (Figure 2), and the correspondence of spectral changes with loss of enzymatic activity (Figure 4) were consistent with mechanism-based inhibition as previously described for the action of resorcinol and derivatives on TPO and LPO (*19*). In the previously proposed mechanism, reactive resorcinol radicals are formed in the active site by compound I-mediated oxidation of the phenolic suicide substrate, and inactivation occurs by covalent binding of these radicals to catalytic amino acid radical(s) on compound II (*19*). This conclusion was supported by the observed binding of 10 mol of resorcinol/mol of LPO inactivated. The spectral changes observed for flavonoid-inactivated TPO (see Figure 2) are similar to those seen in resorcinol-inactivated TPO and LPO, suggesting a common mechanism of inactivation. At the present time, the cause for the observed spectral changes is not clear but may result from heme modification in addition to active site amino acid residues. The lack of commercial sources for radiolabeled flavonoids precluded further investigation of the covalent binding of flavonoids to TPO.

In agreement with our previous study, a free resorcinol moiety is present in all of the flavonoids (naringenin, quercetin, morin, kaempferol) that cause substantial inactivation (>40%) under the conditions (see Table 1). The smaller amounts of inactivation, observed for flavonoids that do not contain a resorcinol moiety (flavone, flavanone, baicalein, fisetin), were similar to the low level inactivation caused by the action of monophenolic radicals (e.g., guaiacol), as was observed in the previous study (*19*).

However, the presence of a resorcinol moiety in flavonoids was not sufficient to cause substantial TPO inactivation, as rutin, biochanin A, and myricetin were weak inactivators. Possible modifying factors for these compounds are as follows: the presence of a sugar moiety on the resorcinol hydroxyl group in naringin completely blocked the inactivation observed for the aglycon, naringenin; the presence of a sugar moiety in the pyranone ring of rutin; the presence of a pyrogallol moiety in the 2-aryl substituent of myricetin appears to make this flavonoid a potent substrate and competitive inhibitor of TPO-catalyzed reactions; biochanin A, in which the aryl substituent is attached to the 3-position of the pyranone ring, is an alternate substrate inhibitor of iodination (see below); the change in going from a hydroxypyranone ring in kaempferol to a hydroxydihydropyran in catechin produced a marked reduction in IC_{50} and TPO inactivation; however, the same change from the hydroxypyranone in kaempferol to a dihydropyranone in naringenin produced a minimal change in inactivation potency.

Scheme 2. Proposed Formation and Reactions of Flavonoid Radicals



Reversible (rapid equilibrium) inhibition of TPO-catalyzed tyrosine iodination was observed in the presence of myricetin and naringin. Figure 5 shows the Dixon plots for inhibition of tyrosine iodination in the presence of varying amounts of the two substrates, iodide ion (A, C) or H_2O_2 (B, D). The inhibition by myricetin and naringin was consistent with a noncompetitive mechanism with respect to iodide ion and linear mixed-type with respect to H_2O_2 (*21*). In both noncompetitive and linear mixed-type mechanisms, inhibition results from the binding of inhibitor and substrate to different sites or enzyme forms. A minimal mechanism consistent with these kinetics and the known properties of peroxidases is shown in Scheme 2. In this proposed mechanism, myricetin and naringin interact with TPO compounds I and II but not EOI (the enzymatic iodinating species) or native TPO. Further interpretation of the inhibition kinetics in this multisubstrate ping-pong system is beyond the scope of this discussion.

Figure 6 shows the effects of increasing amounts of biochanin A on TPO-catalyzed tyrosine iodination in the presence of a continuous source of H_2O_2 produced by glucose/glucose oxidase. As the concentration of biochanin A was increased, the length of the lag phase increased. Following the lag, tyrosine iodination resumed at the control rate. During the lag phase, or in the absence of tyrosine, biochanin A (λ_{max} 262, 322 nm) was converted to products with red-shifted UV absorption maxima (λ_{max} 280, 340 nm), and HPLC analysis demonstrated the formation of at least three products with altered UV spectra (data not shown). The identity of the major product was established by ES/MS and ^{1}H-NMR as the diiodinated resorcinol derivative shown in Scheme 1. The two minor products observed by HPLC were found to be monoiodinated, presumably resulting from iodination of either position on the resorcinol ring. These data are consistent with alternate substrate inhibition of iodination as previously reported for inhibition of TPO-catalyzed iodination reactions by ethylenethiourea and N,N'-disubstituted benzimidazole-2-thiones (*18, 23*). In this mechanism, competition between tyrosine and the alternate substrate for the enzymatic iodinating species (EOI, see Scheme 2) results initially in complete blockade of tyrosine iodination because of the higher affinity for biochanin A. However, after the alternate substrate is

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consumed, tyrosine iodination resumes at an unchanged rate.

Resorcinol-mediated inactivation was previously investigated for a series of peroxidases, and it was determined that TPO, LPO, and CcP were highly susceptible, but HRP and other peroxidases were resistant (*19*). These observations were consistent with the presence of catalytic amino acid radicals in the susceptible peroxidases but not in HRP. A similar trend was seen in the present study for naringenin, except that MPO was also significantly inactivated (see Table 4). This latter observation could be consistent with the suggestion that MPO also forms protein-centered radicals following reaction with H_2O_2 (*24*). Inactivation by quercetin was highest for TPO, LPO, and CcP, but significant inactivation of all peroxidases tested occurred, including HRP.

Most compounds tested inhibited the TPO-catalyzed oxidation of guaiacol with IC_{50}s of a magnitude similar to that seen for tyrosine iodination. However, low concentrations of myricetin and naringin (<10 μM) caused a stimulation of guaiacol oxidation, and the kinetics showed an increase in V_{max} (data not shown). These observations are consistent with preferential TPO-mediated oxidation, by either compound I or compound II, of the flavonoid to a phenoxyl radical that abstracts a hydrogen atom from guaiacol to form the guaiacol radical which produces the colored oxidation product, presumably a dimeric species (see Scheme 2, ref 25). Regeneration of the flavonoid completes a catalytic process that drives guaiacol oxidation at an increased rate due to higher enzymatic turnover of the flavonoid relative to guaiacol.

Other evidence for the participation of flavonoid radicals in TPO-mediated reactions is seen in Figure 4. It was observed that TPO inactivation by naringenin was enhanced when O_2 was removed from the incubation medium. Excluding O_2 decreased the partition ratio, the ratio between rate constants for suicide substrate turnover and inactivation (*20*), by ca. 5-fold from 42 to 8 μM. Similar results were observed with resorcinol (data not shown). These observations are consistent with a reaction between O_2 and the phenoxyl radicals formed by TPO-catalyzed oxidation that diverts the inactivating radical to a pathway leading to turnover of the suicide substrate and not enzyme inactivation (see Scheme 2). Similar reactions have been proposed for light emission that accompanies peroxidase-mediated metabolism of tetracycline and oxicams under aerobic conditions (*26*).

The inhibitory effects on TPO demonstrated for these dietary flavonoids are sufficient to explain the antithyroid effects reported in humans and experimental animals (*12*, *13*). The different mechanisms identified here for TPO inhibition do, however, suggest differences in the potential hazards to humans consuming these compounds. In vivo, flavonoids that are TPO suicide substrates are likely to exert a long-lasting depression of thyroid hormone synthesis because de novo enzyme synthesis is required to restore lost activity. However, the inhibitory effects of flavonoids that are alternate substrate iodination inhibitors would be attenuated by TPO-catalyzed iodination to inactive products, and the effects of reversibly binding inhibitors would be attenuated by extrathyroidal metabolism and excretion. Therefore, intermittent or low-dose exposure to suicide substrates in the diet, but not alternate iodination substrates or reversible inhibitors, could have significant impact on thyroid hormone status. However, chronic exposure to any of these flavonoid inhibitors, especially at high doses, could elicit prolonged blockade of thyroid hormone synthesis. Any compound that causes chronic inhibition of thyroid hormone synthesis is a *potential* thyroid carcinogen that acts by long-term stimulation of thyroid growth induced by elevated levels of thyroid stimulating hormone (TSH) (*27*). Although there is considerably less evidence of a role for TSH stimulation in the etiology of thyroid cancer in humans as opposed to experimental animals, three case-control studies in the U.S. consistently showed thyroid cancer was strongly associated with preexisting goiter and thyroid nodules (for a review, see ref 27). However, studies relating iodide intake levels and human thyroid cancer in endemic goiter regions of the world are equivocal (*27*). Nevertheless, our results do suggest that the consumption of foods rich in flavonoids, especially those that *inactivate* TPO, has the potential to induce goiter and in this manner may be involved in the etiology of human thyroid cancer.

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Research Section

Inhibition of thyroid type 1 deiodinase activity by flavonoids

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Abstract

Some dietary flavonoids inhibit thyroperoxidase and hepatic deiodinase activity, indicating that these compounds could be classified as anti-thyroid agents. In this study, we evaluated the in vitro effect of various flavonoids on thyroid type 1 iodothyronine deiodinase activity (D1). D1 activity was measured in murine thyroid microsome fractions by the release of ^{125}I from ^{125}I-reverse T3. D1 activity was significantly inhibited by all the flavonoids tested; however, the inhibitory potencies on thyroid D1 activity differed greatly among them. A 50% inhibition of D1 activity (IC_{50}) was obtained at 11 μM baicalein, 13 μM quercetin, 17 μM catechin, 55 μM morin, 68 μM rutin, 70 μM fisetin, 72 μM kaempferol and 77 μM biochanin A. Our data reinforce the concept that dietary flavonoids might behave as antithyroid agents, and possibly their chronic consumption could alter thyroid function. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Deiodinase; Flavonoids; Thyroid

1. Introduction

The enzyme 5′-iodothyronine deiodinase is responsible for converting thyroxine (T4) to triiodothyronine (T3), the biologically active hormone. Based on several functional criteria and on different protein sequences, 5′-deiodinases is classified into two isoenzymes: type 1 (D1) and type 2 (D2). The type 1 enzyme is predominantly found in the liver, kidney and thyroid and is responsible for generating most of the circulating T3 (Leonard and Korhle, 1996). In humans, T3 production by the thyroid is increased in patients with Basedow-Graves disease, due to stimulated intrathyroidal T4 deiodination (Salvatore et al., 1996). In addition, under physiological conditions in rats, as reported by Chanoine et al. (1993), the thyroid gland is an important source of serum T3.

Flavonoids are widely distributed in plant-derived foods, as fruits and vegetables, and have a variety of biological activities, including antioxidant (Middleton, 1984) and antithyroid effects (Cody et al., 1989; Lindsay et al., 1989; Doerge and Divi, 1995; Divi and Doerge, 1996). Previously, in experimental animals, it has been described that the high consumption of flavonoids and other xenobiotics diminishes thyroid iodide uptake and thyroperoxidase (TPO) activity, inducing enlargement and histological changes in the thyroid (Moudgal et al., 1958; Cody et al., 1989; Lindsay et al., 1989; Gaitan, 1996). Recently, it was demonstrated that *Kalanchoe brasiliensis* aqueous extract, a Brazilian medicinal herb that is known to contain flavonoids (Rossi-Bergmann et al., 1997), causes in vitro TPO activity inhibition and scavenges H_2O_2, an essential TPO cofactor (Ferreira et al., 2000).

Synthetic flavonoids such as EMD21388, and natural plant derived flavonoids seem to inhibit T4 5′-deiodinase activity in vivo (Schroder-van der Elst et al., 1991) and are potent inhibitors of hepatic deiodinase activity in vitro (Koehrle, 2000). However, flavonoids are less potent inhibitors of pituitary and brain D2 (Koehrle, 2000). Hence, the studies about the effects of flavonoids on thyroid hormones metabolism are restricted to their peripheral metabolism and there are no data about a possible direct effect of flavonoids on intrathyroidal deiodination. Thus, the aim of the present study was to investigate the in vitro role of some dietary flavonoids on thyroid D1 activity.

Abbreviations: D1, deiodinase activity; PTU, propylthiouracil; T3, triiodothyronine; T4, thyroxine; TPO, thyroperoxidase.

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2. Materials and methods

Adult male Wistar rats weighing 200–250 g were sacrificed by decapitation and their thyroids were excised and processed for D1 activity measurement, as previously described (Curty et al., 2000). Briefly, thyroids were homogenized in 50 mM Tris–HCl buffer pH 6.8, containing 10 mM dithiotreitol and 1 mM EDTA, using an Ultra-Turrax (Staufen, Germany) homogenizer. The homogenates were centrifuged at 1500 g, 4 °C for 30 min. Supernatants were centrifuged at 190,000 g, 4 °C for 90 min and the pellets, containing microsomal fraction, were resuspended in the homogenizing buffer and stored at −70 °C (Berry et al., 1991; Curty et al., 2000).

D1 activity was measured using ^{125}I-rT3 as substrate, based on methods previously described, with slight modifications (Visser et al., 1979; Leonard and Rosenberg, 1980; Berry et al., 1991; Curty et al., 2000). Flavonoids (Sigma Chemical Co., St Louis, MO, USA) were dissolved in propylene glycol and diluted in phosphate buffer. A screening of inhibitory flavonoids was done measuring a known D1 activity in the presence of 100 μM of each flavonoid; then, lower concentrations of flavonoids were used in order to evaluate their inhibitory potencies. Equal aliquots of purified ^{125}I-rT3 (1.07 mCi/μg; New England Nuclear-Dupont, Boston, MA, USA) were added to each assay tube. The mixture was incubated on a shaking-bath at 37 °C, and the reaction was stopped after 30 min by the addition of 8% bovine serum albumin and 10 mM propylthiouracil (PTU), followed by cold 20% trichloroacetic acid. Samples were centrifuged (1500 g, 4 °C, 5 min), and the supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen form BioRad, Richmond, CA, USA). Free ^{125}I was eluted from the column with 10% acetic acid and the radioactivity was measured in a gamma-counter. Specific enzyme activity was expressed as pmoles of rT3 deiodinated.h^{-1}./mg^{-1} protein.

Protein concentrations in the microsomal fraction were measured by the Bradford method (Bradford, 1976).

The experiments were done at least three times and the results are expressed as mean±standard error and were analyzed by the Mann–Whitney non-parametric *t*-test for assessment of significance of data. Inhibitory curves with each flavonoid were done in triplicate and were analyzed by the statistical curve-fitting package Enzfitter (Elsevier-Biosoft, Cambridge, UK).

3. Results

Thyroid D1 activity (control activity ranged from 127,100±12,200 to 395,000±31,810 pmol ^{125}I.h^{-1}./mg^{-1} protein) was significantly inhibited by all the flavonoids tested at the concentration of 100 μM (P <0.05); propylene glycol alone did not inhibit D1 activity (Fig. 1).

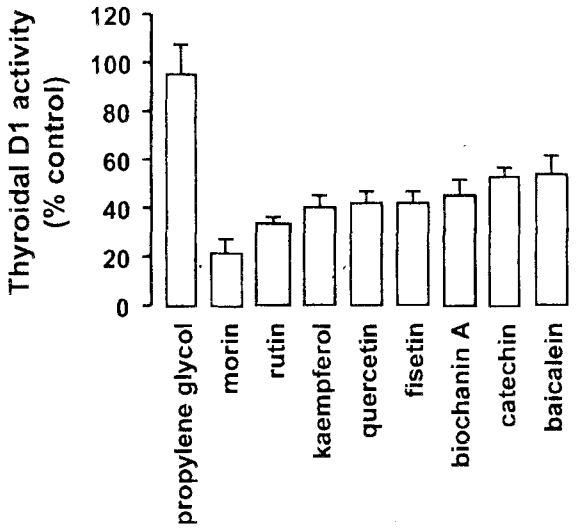

Fig. 1. Effect of flavonoids on thyroid D1 activity. D1 activity in rat thyroid microsomal fractions was evaluated by the deiodination of ^{125}I-rT3 and ^{125}I release. Enzyme activity was measured in the absence (control = 100%, ranged from 127,100±12,200 to 395,000±31,810 pmol ^{125}I.h^{-1}./mg^{-1} protein) and in the presence of 100 μM flavonoids or the diluent propylene glycol. All the flavonoids tested significantly inhibited thyroid D1 activity (P <0.05).

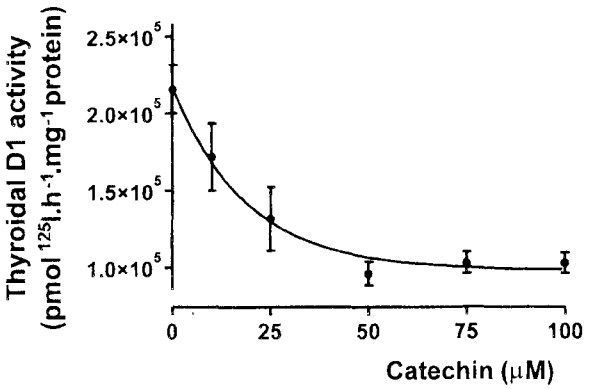

Fig. 2. Effect of catechin on thyroid D1 activity. In rat thyroid microsomal fractions D1 activity was evaluated by the deiodination of ^{125}I-rT3 and ^{125}I release. Enzyme activity was measured in the presence of increasing concentrations of catechin. Results are expressed as mean±S.E.M.

In order to compare inhibitory potencies (IC_{50}), D1 activity was assayed in the presence of lower concentrations of the flavonoid. Inhibitory kinetics were different among the flavonoids tested. Catechin, quercetin and baicalein inhibitory curves showed residual velocity, demonstrating that the enzyme could not be completely inhibited by these flavonoids in our experimental conditions (a representative curve of catechin is shown on Fig. 2). Besides, D1 inhibition by rutin and biochanin A

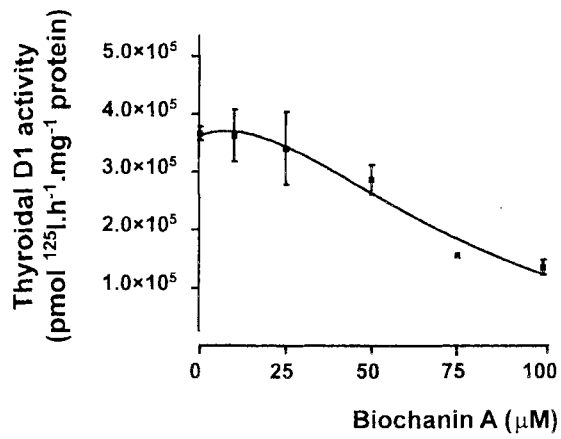

Fig. 3. Effect of biochanin A on thyroid D1 activity. In rat thyroid microsomal fractions D1 activity was evaluated by the deiodination of ^{125}I-rT3 and ^{125}I release. Enzyme activity was measured in the presence of increasing concentrations of biochanin A. Results are expressed as mean±S.E.M.

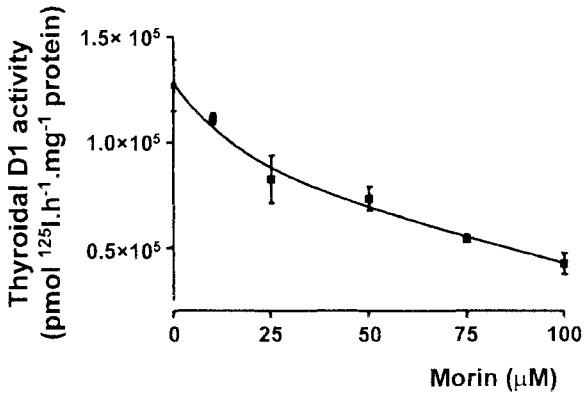

Fig. 4. Effect of morin on thyroid D1 activity. In rat thyroid microsomal fractions D1 activity was evaluated by the deiodination of ^{125}I-rT3 and ^{125}I release. Enzyme activity was measured in the presence of increasing concentrations of biochanin A. Results are expressed as mean±S.E.M.

Table 1
Effect of flavonoids on thyroid type 1 5′-deiodinase (D1) activity. Thyroid D1 activity was evaluated in the presence of increasing concentrations of flavonoid. Concentrations necessary to inhibit D1 activity by 50% (IC_{50}) are shown

Flavonoid	IC_{50} (μM)
Baicalein	10.64±0.04
Quercetin	13.23±1.51
Catechin	17.47±6.41
Morin	55.05±0.12
Rutin	67.95±1.02
Fisetin	70.36±2.58
Kaempferol	71.81±12.8
Biochanin A	77.05±1.01

Results are expressed as mean±S.E.M.

showed some co-operative activity demonstrated by the kinetics of inhibition (a representative curve of biochanin A is shown on Fig. 3), as their inhibitory curves show that at lower concentrations of flavonoids the activity is maintained and then abruptly decreases. The curve-fitting analysis of D1 inhibition by kaempferol, morin and fisetin presumed no residual activity (a representative curve of morin is shown on Fig. 4).

Inhibitory potencies also differed among the flavonoids tested as 50% inhibition of D1 activity (IC_{50}) was obtained at different concentrations of flavonoids (Table 1). Baicalein and quercetin were the most potent inhibitors and biochanin A the less potent D1 inhibitory substance.

4. Discussion

Flavonoids are universally present in vascular plants and in fruits, grains and vegetables. They are present in high concentrations in various staple foods such as millet, soy, beans and nuts. Following ingestion, flavonoid glycosides are hydrolyzed by intestinal microbial glycosidases to flavonoids aglycones. These may be absorbed and undergo further metabolism by many tissues. It has been reported that each metabolic step increases their antithyroid effects (Gaitan, 1996).

The inhibition of hepatic T4 5′-deiodinase by flavonoids has already been demonstrated in vitro (Spanka et al., 1990). In the present study we used microsomal preparations; however, we do not believe that our findings are due to an artifact of disrupted cells. In vivo inhibition of T4 5′-deiodinase has also been suggested, since T3 is decreased in many tissues after long-term administration of a synthetic flavonoid, despite normal T3 serum levels (Schroder-van der Elst et al., 1991). It is also interesting to point out that all the flavonoids tested here were active; however, the inhibitory potencies were very different among them, again indicating that inhibition was not an artifact.

Divi and Doerge (1996) demonstrated that some flavonoids are potent inhibitors of thyroperoxidase iodination activity in vitro; however, their mechanism of action and inhibitory potencies differed. In their study, the most potent TPO inhibitory flavonoids were kaempferol, morin and quercetin. In our study, catechin showed a lower IC_{50} for thyroid D1 inhibition than that described for TPO by Divi and Doerge (1996), suggesting that catechin might be a more potent inhibitor of D1 than TPO activity. Baicalein and rutin showed similar inhibitory potencies on TPO and thyroid D1 activity. All the other flavonoids tested were at least five times more potent on TPO than on thyroid D1 inhibition. We have no data on whether every flavonoid tested inhibits D1 by the same mechanism. However, since there is no

parallelism in the dose–response curve on D1 inhibition we can speculate that these flavonoids might also inhibit this enzyme by different mechanisms.

The well-known antithyroid agent propylthiouracil, which produces TPO inhibition in vitro and in vivo, is also a potent D1 inhibitor. So, flavonoids that can inhibit both thyroperoxidase and D1 activities could also behave as antithyroid agents in vivo.

Recently, for the first time in humans, the pharmacokinetic behavior of naturally occurring isoflavones has been reported (Setchell et al., 2001). This study confirmed the extensive distribution of these substances after absorption.

The present paper refers to in vitro findings that can be relevant under real exposure conditions, since there is some previous reports showing that orally administered flavonoids are distributed and accumulated in various endocrine organs, including the thyroid (Chang et al., 2000). Furthermore, flavonoids can cross the placenta and accumulate in fetal tissues (Schroder-van der Elst et al., 1998; Doerge et al., 2001). It has already been reported that the concentrations of genistein (the principal soy isoflavone) achieved in plasma from humans consuming soy products can reach approximately 1 μM (Divi et al., 1997), a concentration similar to the IC_{50} for TPO inhibition in vitro. Some recent findings have demonstrated that TPO inhibition occurs in vivo, as predicted by the in vitro studies (Chang and Doerge, 2000). However, further studies on the bioavailability of these compounds are clearly still required.

This is the first report on the in vitro inhibition of flavonoids on thyroid D1 activity. Intrathyroidal T4 5′-deiodination is relevant for T3 production and secretion by the thyroid. Thus, apart from diminished local T3 production, chronic consumption of flavonoids might also induce decreased T3 serum levels, mainly baicalein, quercetin and catechin, the most potent inhibitors. However, it has to be established whether the in vitro effects are also relevant in vivo.

In conclusion, we demonstrate the inhibitory effect of some flavonoids on thyroid type 1 deiodinase activity in vitro. This information, added to previous studies, suggest that flavonoids can impair thyroid function and might act as goitrogenic agents.

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Dietary Genistein Inactivates Rat Thyroid Peroxidase *in Vivo* without an Apparent Hypothyroid Effect

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Dietary Genistein Inactivates Rat Thyroid Peroxidase *in Vivo* without an Apparent Hypothyroid Effect. Chang, H. C., and Doerge, D. R. (2000). *Toxicol. Appl. Pharmacol.* 168, 244–252.

Biological effects of genistein are currently under investigation by the National Toxicology Program because of widespread and increasing soy consumption by humans and evidence for modulation of endocrine function. Rats were exposed to genistein aglycone in soy-free feed fortified at 0, 5, 100, and 500 ppm starting *in utero* through 20 weeks. Thyroid glands and serum were analyzed for total genistein (aglycone + conjugates) using HPLC with electrospray mass spectrometric detection. Microsomal thyroid peroxidase (TPO) activity was measured spectrophotometrically. The total genistein content in rat serum was as high as 8 μM, and significant dose-dependent increases of genistein in thyroid tissue up to 1 pmol/mg were found in male and female rats. The activity of TPO in male and female rats was found to be reduced by up to 80% in a dose-dependent manner. Male and female rats consuming a standard soy-based rodent diet (NIH 31) had TPO activity approximately 50% lower than rats consuming a soy-free diet and this loss was commensurate with measured serum levels of isoflavones. Suicide inactivation of rat, porcine, and human TPO was observed *in vitro* at concentrations of genistein aglycone comparable to those measured in rat thyroids. Thyroid hormone levels (T3, T4, TSH) in serum, thyroid weights, and histopathology showed no differences between treated and untreated groups. These findings suggest that, even though substantial amounts of TPO activity are lost concomitant to soy isoflavone consumption by normal rats, the remaining enzymatic activity is sufficient to maintain thyroid homeostasis in the absence of additional perturbations.

Key Words: genistein; soy; thyroid peroxidase; mass spectrometry.

A long-standing association between soy consumption and goiter in animals and humans is documented in the scientific literature. Beginning over 70 years ago, many studies have shown the goitrogenic effects of soy in rats and the protective effect of dietary iodide supplementation (McCarrison, 1933; Block *et al.*, 1961; Nordsiek, 1962; Konijn *et al.*, 1972; Kimura *et al.*, 1976; Kay *et al.*, 1988). Other studies have documented the goitrogenic effects of soy formula in infants and subsequent reversal upon switching to cow milk or iodine-supplemented diets (Van Wyk *et al.*, 1959; Hydovitz, 1960; Shephard *et al.*, 1960; Ripp, 1961; Pinchera *et al.*, 1965; Labib *et al.*, 1989; Chorazy *et al.*, 1995; Jabbar *et al.*, 1997). Antithyroid effects were also reported in a study of 37 healthy, iodine-sufficient adults without known thyroid disease (Ishizuki *et al.* 1991). These workers observed goiter and elevated individual TSH[2] levels, although still within the normal range, in 37 healthy adults fed 30 g of pickled soy beans per day for as little as 1 month. Changes in serum thyroid hormone (T3 and T4) levels were not observed. One month after stopping soy consumption, the individual TSH values had decreased to the original levels and goiters were reduced in size. Duncan *et al.* (1999) described a statistically significant decrease in T3 levels in 14 premenopausal but not 18 postmenopausal women consuming up to 2 mg total of soy isoflavones per kg body weight per day for about 3 months (Duncan *et al.*, 1999a,b). The data regarding possible antithyroid effects of soy isoflavones and related compounds in humans were summarized in a recent review article (Fitzpatrick, 2000).

A previous report from this laboratory identified genistein and daidzein as the components in soy that inhibited TPO (Divi *et al.*, 1997) and described the suicide inactivation of bovine lactoperoxidase (bLPO) and porcine thyroid peroxidase (pTPO). These findings, coupled with the history of soy's antithyroid effects in humans and animals, prompted an evaluation *in vivo* of possible antithyroid effects of the principal soy isoflavone, genistein. As part of a multigeneration study of the endocrine-modifying properties of genistein, several thyroid endpoints were evaluated in male and female Sprague–Dawley rats, including rTPO activity.

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[2] Abbreviations: ANOVA, analysis of variance; ES, electrospray; LOD, limit of detection with signal/noise ratio of 3; LPO, lactoperoxidase; PND, postnatal day; MMI, methimazole; PTU, 6-*n*-propyl-4-thiouracil; SIM, selected ion monitoring; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; T4, thyroxine; T3, triiodothyronine.

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MATERIALS AND METHODS

Reagents

Genistein, with purity greater than 99% from ^{1}H- and ^{13}C-NMR, EI/MS, melting point, and TLC analysis, was obtained from Toronto Research Chemicals (Ontario, Canada); daidzein, catalase, crude glucuronidase/sulfatase from *Helix pomatia* containing 10^5 units/ml glucuronidase activity + 5×10^3 units/ml sulfatase activity, genistin, and guaiacol were obtained from Sigma Chemical (St. Louis, MO). Human goiter TPO was obtained from CalBiochem (Torrey Pines, CA). Deuterated daidzein (6,3′,5′-d3, 95%) and genistein (6,8,3′,5′-d4, 95%) were purchased from Cambridge Isotope Laboratories (Andover, MA). All solvents were HPLC grade and Milli-Q water was used throughout.

Animal Feeding Procedures

All procedures involving care and handling of animals were reviewed and approved by the NCTR Laboratory Animal Care and Use Committee. CD (Sprague–Dawley) rats were from the NCTR colony. Pups were taken from dams that had been consuming soy- and alfalfa-free diet fortified with genistein aglycone at various levels (0, 5, 100, or 500 μg/g) continuously since weaning. After weaning, 48 pups were placed on the same genistein-supplemented chow received originally by the mother and were maintained on this food source until the time of termination at postnatal day (PND) 140. The basal diet was irradiated 5K96 meal (Purina Mills, St. Louis, MO). This diet is similar to the standard NIH 31 rat chow (Knapka, 1983), except that the soymeal and alfalfa components are replaced by casein, soy oil is replaced by corn oil, and the vitamin content is adjusted to compensate for irradiation effects. The genistein (0.54 μg/g) and daidzein (0.48 μg/g) contents in this feed were determined using LC-ES/MS/MS analysis after complete hydrolysis of glucoside conjugates (not shown). The genistein-fortified feed concentrations were verified using LC-UV (260 nm detection). Rats in the diet comparison study were fed the standard NIH 31 feed from weaning until 6 months of age.

Sample collection procedures. Animals (PND 140) were removed from feed early in the morning (7 a.m.) in a randomized manner with respect to dose group. Blood (~100 μl) was removed from the tail vein immediately following removal from food and transferred to Microtainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) for preparation of serum by centrifugation after clotting, and the serum was stored frozen at −60°C. Animals were then allowed access to food overnight before termination from carbon dioxide asphyxiation on the following morning within 3 h after removal from food. Blood was removed by cardiac puncture. Thyroid glands were surgically dissected and frozen in liquid nitrogen before storage at −70°C.

In the diet comparison study, animals were maintained on either NIH 31 or 5K96 diet until termination at 190 days (n = 6). Blood was removed by cardiac puncture, allowed to clot at 4°C, and centrifuged at 3000g for 25 min; serum was then collected and stored at −70°C until analysis. Thyroid glands were collected as described earlier.

Intrathyroidal and serum genistein analysis. The procedures previously described by Chang *et al.* (2000) were used. Briefly, rat thyroid tissue was weighed (~10 mg from one lobe) and homogenized in citrate buffer (200 μl of 25 mM, pH 5.0). Deuterated genistein (1 pmol/mg tissue) and 800 μl methanol were added and the suspension was sonicated. The sample mixtures were then diluted with 2 ml citrate buffer and divided into two equal portions for determination of total genistein (after enzyme deconjugation) and aglycone (without deconjugation). Deconjugation was performed by adding 100 μg of an enzyme mixture from *Helix pomatia* (40–60 units glucuronidase activity and 1.5–4 units sulfatase activity; Sigma) and incubating at 37°C for 60 min. Samples were centrifuged and then cleaned up by using solid-phase extraction (SPE) prior to LC/MS analysis. Total genistein levels in serum were measured using on-line SPE coupled with LC-ES/MS as described previously (Chang *et al.*, 2000; Doerge *et al.*, 2000).

Mass Spectrometry

A Platform II single quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an ES interface was used with an ion source temperature of 150°C. Positive ions were acquired in selected ion monitoring mode (SIM) for ESI/MS (dwell time = 0.3 s, span = 0.02 Da, and interchannel delay time = 0.03 s) for the protonated molecule $(M+H)^+$ ions for genistein (m/z 271) and genistein-d4 (m/z 275), which were monitored at a sampling cone-skimmer potential of 30 V. Validation of the isotope dilution MS technique for analysis of genistein and characterization of the deuterated genistein were previously reported in detail (Holder *et al.*, 1999).

The sensitivity of ES/MS detection allowed the use of small volumes of plasma (50 μl) or tissue (10 mg) and the detection limit (LOD, s/n = 3) for genistein in serum was approximately 20 nM, and in thyroid was approximately 0.04 pmol/mg tissue.

Preparation and assay of microsomal rTPO. Thyroid samples (~10 mg from a single lobe) were homogenized at 4°C in 2 ml of 5 mM potassium phosphate buffer (pH 7.4) containing 200 mM sucrose, 500 units/ml catalase, and 1 mM EDTA. The homogenate was centrifuged at 600 rpm for 10 min at 4°C. The supernatant was collected and then centrifuged at 105,000g for 45 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 250 μl of 200 mM potassium phosphate buffer (pH 7.4) then stored at −80°C. Protein concentration was measured by using the Lowry method. Microsomal rTPO was incubated with 35 mM guaiacol and 300 μM H_2O_2 in 100 mM phosphate buffer (pH 7.4) at 37°C, and the oxidation of guaiacol was monitored spectrophotometrically (470 nm, 8432A; Hewlett Packard, Palo Alto, CA). The TPO isolation and assay procedures were validated with respect to linearity of response vs protein and thyroid tissue weight using frozen hog thyroids and untreated rat thyroids (not shown).

Inactivation of rTPO by genistein in vitro. Microsomal rTPO, prepared from untreated animals (80 μg protein with a specific activity of 2 Au/min/mg protein), was incubated for 12 min at room temperature with various concentrations of genistein (10–1000 nM) in the presence of 100 nM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0. The reaction was initiated by addition of H_2O_2, and at various times, 100-μl aliquots were assayed spectrophotometrically for the remaining guaiacol oxidation activity. The guaiacol assay contained 5 mM guaiacol and 500 μM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0. Inactivation half-times were determined graphically and then plotted against the reciprocal of genistein. The inhibitor-enzyme dissociation constant K_i was determined from the negative reciprocal of the intercept on the abscissa and the maximal inactivation rate constant k_{inact} was determined from the ordinate.

Statistical analysis. Two-way analysis of variance (ANOVA) was used to test for main effects of dose and sex and dose/sex interactions. Comparisons of each dose group against the controls within each sex were made using Dunnett's test.

RESULTS

Genistein Concentration in Serum and Thyroid

The serum (see Table 1) and thyroid (see Fig. 1) levels of genistein, determined using isotope dilution LC-ES/MS, increased in a dose-dependent manner (Chang *et al.*, 2000). The higher blood levels of genistein observed in female rats as opposed to males were reflected by the higher thyroid levels observed in females (Chang *et al.*, 2000). The serum levels shown represent total genistein obtained after enzymatic deconjugation because the glucuronide conjugate is the predominant form present in rat serum (~98%) and the remainder is mostly aglycone (Holder *et al.*, 1999). In thyroid tissue, the

TABLE 1
Total Serum Genistein in Adult Male and Female Rats Fed Genistein-Fortified Diets

Sex	Control	Genistein 5 ppm	Genistein 100 ppm	Genistein 500 ppm
Male	<0.02	0.06 ± 0.006	0.59 ± 0.03	6.00 ± 0.65
Female	<0.02	0.10 ± 0.008	0.94 ± 0.21	7.94 ± 2.47

Note. Total genistein (conjugates + aglycone) was measured using LC-ES/MS or MS/MS in serum from rats terminated at PND 140 as previously described (Chang *et al.*, 2000). The values shown represent (in μM) the means ± SEM ($n = 6$). The total genistein in serum of male and female rats fed the soy-free chow contained <0.02 μM ($n = 11$).

percentage of aglycone form was substantially increased over that in serum (18–28% of total; see Fig. 1). As previously reported, one-way ANOVA showed a significant treatment effect ($p < 0.05$) on total thyroidal genistein in males and females and aglycone genistein in females (Chang *et al.*, 2000). Dunnett's test showed significant differences between the respective control values as indicated in Fig. 1.

Previous work described the *in vitro* formation of mono-, di-, and triiodinated genistein derivatives upon incubation of genistein with TPO, H_2O_2, and iodide by an alternate substrate mechanism (Divi *et al.*, 1997). Using the chromatographic and mass spectrometric conditions previously reported, no iodinated genistein derivatives were observed in rat serum or thyroid using LC-ES/MS following enzymatic deconjugation (estimated LOD <50 nM in serum or 0.1 pmol/mg in thyroid tissue).

Inactivation of rTPO in Vivo

During the isolation of microsomal rTPO, catalase was added to the buffers to minimize possible losses of activity resulting from H_2O_2-dependent inactivation in the presence of genistein (Divi and Doerge, 1996). However, an additional control experiment was conducted to demonstrate that artifactual losses in TPO activity resulting from interactions with intrathyroidal genistein were not occurring during the isolation procedures. Thyroid tissue from untreated rats ($n = 3$) was divided into two portions and to one was added genistein aglycone at a concentration greater than those actually measured in thyroids (1 μM; see Fig. 1). Both portions were then processed identically. Measurement of guaiacol oxidation activity showed no difference between the two treatment groups (not shown).

Figure 2 shows TPO activity determined in thyroids from the animals fed genistein-fortified feed (see Fig. 1 and Table 1). In both male and female rats, dose-dependent decreases in rTPO activity, to as much as 80% loss, were observed. Although control rTPO levels were comparable for male and female rats, two-way ANOVA showed a greater loss of activity in females relative to males ($p < 0.002$). This finding was consistent with the higher serum and thyroid levels of genistein measured in females. One-way ANOVA showed significant treatment effects for females ($p < 0.001$) and males ($p < 0.001$), and Dunnett's test indicated that all groups were different from controls ($p < 0.03$).

Additional feeding studies were conducted to determine whether the stage of development during genistein exposure had an effect on the extent of TPO inactivation. Genistein concentrations were measured in serum and thyroid along with TPO activity in male and female rats from dose-range-finding studies (0, 25, 250, 1250 ppm genistein in the diet), in which the F0 females ($n = 4$) were exposed for 63 days and for F1 males and females ($n = 4$), in which exposure was 14 days *in utero* through weaning to PND 63 (77 days total). In all cases, significant decreases in TPO activity and elevated concentra-

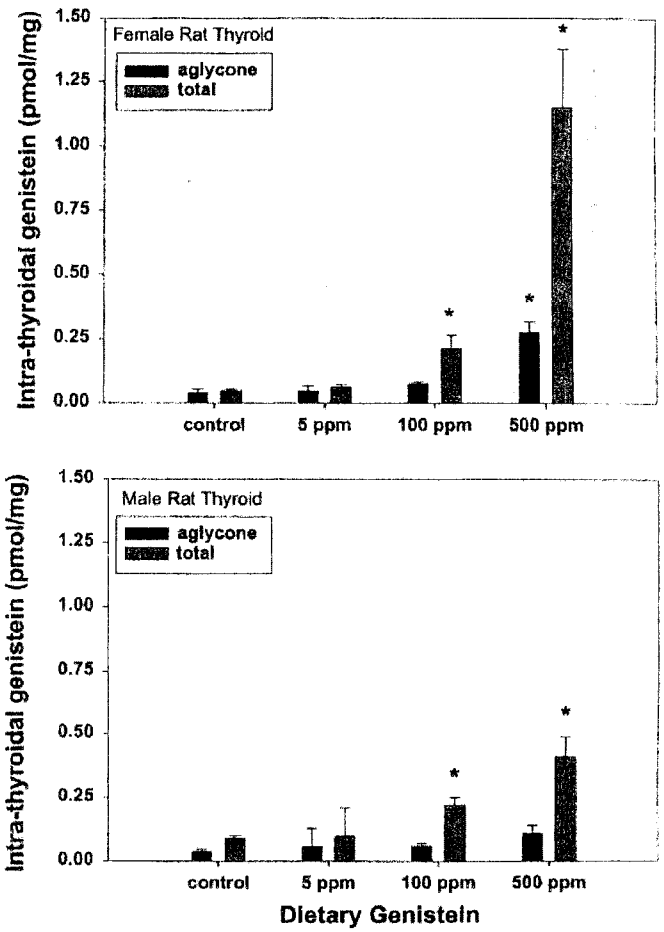

FIG. 1. Dietary consumption of genistein increases intrathyroidal concentrations of genistein. The amounts of genistein present in thyroid tissue, both total and genistein aglycone, were determined with and without enzymatic hydrolysis of conjugates using LC-ES/MS. One-way ANOVA demonstrated a significant treatment effect ($p < 0.05$) for aglycone and total genistein in females and for total genistein in males. Statistical significance relative to the respective control (*) was determined using Dunnett's test ($p < 0.05$).

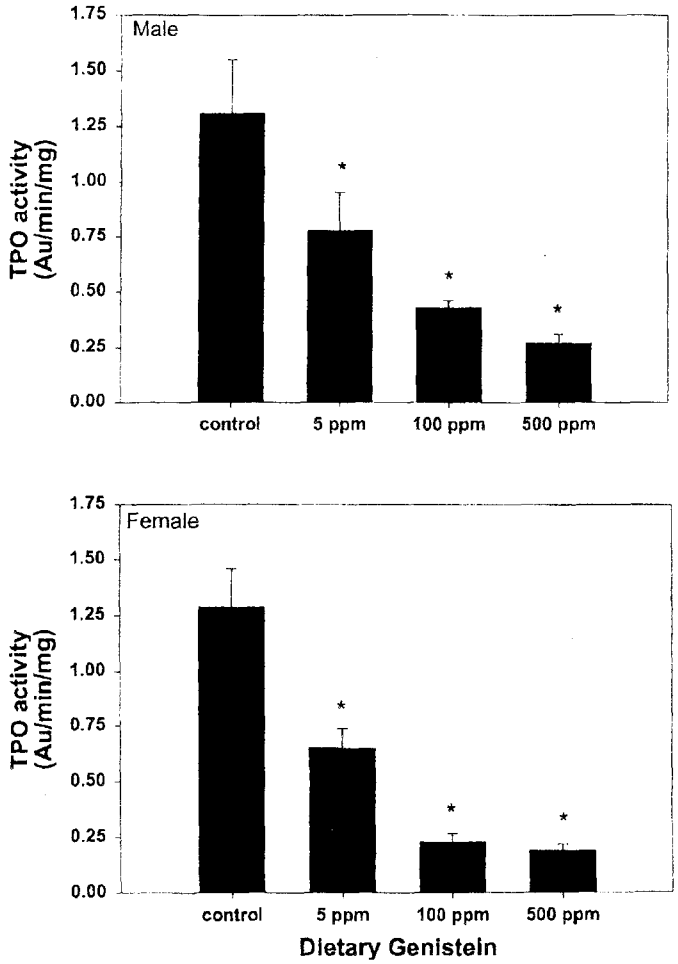

FIG. 2. Dietary consumption of genistein reduces rat TPO activity. Microsomal rTPO activity was measured using a sensitive spectrophotometric guaiacol oxidation assay. One-way ANOVA demonstrated a significant treatment effect ($p < 0.05$) for both males and females and statistical significance relative to the respective control (*) was determined using Dunnett's test ($p < 0.05$). Two-way ANOVA showed a significant sex difference ($p < 0.002$).

tions of genistein in the thyroids were observed relative to control animals (data not shown).

Diet Comparison Study

An additional study was performed in which male and female rats were fed either the standard soy-containing NIH 31 feed that contains approximately 30 ppm each of genistein and daidzein (see Thigpen *et al.*, 1999), or the soy-free 5K96 feed that contains approximately 0.5 ppm of each isoflavone. This was done to determine whether the form of genistein in the diet (i.e., either as aglycone or glucoside conjugates) could affect the degree of TPO inactivation. Figure 3 shows the serum levels of genistein and daidzein measured after continuous feeding of the respective diet into adulthood. The average total genistein concentration observed in females was 0.62 μM and in males was 0.35 μM (Doerge *et al.*, 2000). These values are about one-third lower than those measured in the serum of male and female rats consuming the diet fortified with genistein at 100 ppm (see Table 1). Additionally, the average total daidzein content in serum was 0.25 and 0.20 μM, respectively. Intrathyroidal isoflavone concentrations were not measured in this study.

Figure 3 also shows the TPO activity measured in rats from the diet comparison study. For both male and female rats, a mean loss of more than 50% of the TPO activity was observed and the activity differences between the two diet groups were statistically significant using the two-tailed t test ($p < 0.001$ and 0.03, respectively). These losses in enzymatic activity are comparable to those observed for male and female rats consuming the 100 ppm genistein feed (67 and 82% loss, respectively), especially when the respective serum isoflavone concentrations are considered.

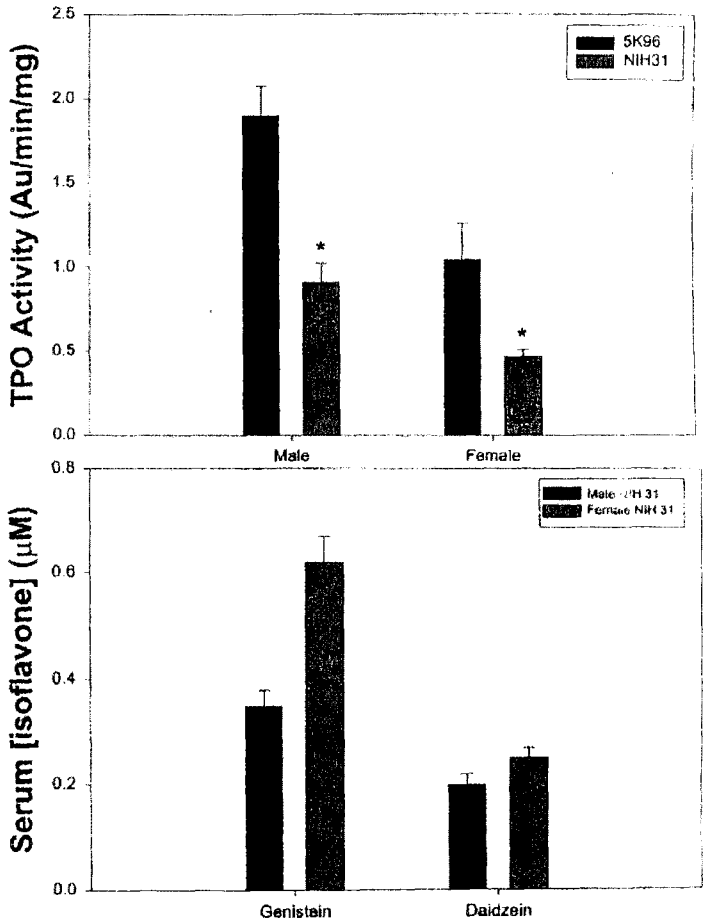

FIG. 3. Measurement of serum isoflavones and TPO activity in rats consuming either a standard soy-containing feed or a soy-free feed. Bottom: Total isoflavone concentrations (conjugates + aglycones) in serum were measured using LC-ES/MS as described under Materials and Methods. Top: Rats were fed from postnatal day (PND) 21 through PND 190 with NIH 31 feed, which contained about 30 ppm each of genistein and daidzein, or from birth through PND 190 with 5K96 feed, which contained about 0.5 ppm each. Microsomal TPO was assayed as described under Materials and Methods and in Fig. 2.

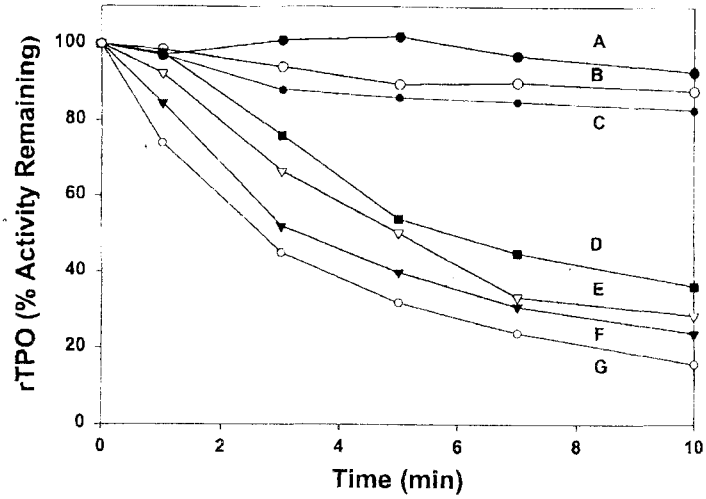

FIG. 4. Inactivation of rat TPO by genistein *in vitro*. Microsomal rat TPO was prepared from untreated animals (activity = 2 Au/min/mg protein) and incubated with the indicated concentrations of genistein in the presence of H_2O_2 (100 nM) at room temperature in 0.1 M phosphate buffer, pH 7.0. At various times, aliquots were removed and remaining activity determined using the guaiacol oxidation assay. The kinetics of inactivation were analyzed graphically (K_i = 50 nM, k_{inact} = 0.28 min^{-1}, not shown). A, control rTPO alone; B, rTPO + H_2O_2; C, rTPO + 1000 nM genistein; D, rTPO + H_2O_2 + 10 nM genistein; E, rTPO + H_2O_2 + 50 nM genistein; F, rTPO + H_2O_2 + 100 nM genistein; G, rTPO + H_2O_2 + 1000 nM genistein.

Inactivation of Mammalian Peroxidases in Vitro

Rat microsomal TPO was prepared from untreated animals and genistein-mediated inactivation was measured *in vitro*. Figure 4 shows the time-dependent loss of rTPO activity in the presence of genistein concentrations comparable to those actually observed in thyroid tissues (see Fig. 2). The control experiments shown demonstrate that neither H_2O_2 nor genistein had an effect on enzymatic activity, which is consistent with the suicide mechanism proposed previously (Divi *et al.*, 1997). Not shown is the kinetic analysis for inactivation of rTPO by genistein from which were determined the apparent inhibition constant K_i and the maximal inactivation rate constant k_{inact}, of 50 nM and 0.28 min^{-1}, respectively. Daidzein similarly inactivated rTPO (not shown) with the corresponding kinetic constants of 143 nM and 0.31 min^{-1}. These kinetic parameters were of the same order as those calculated for bLPO (not shown).

The sensitivity of microsomal rTPO to inactivation by genistein was compared with several related purified soluble mammalian peroxidases under similar conditions. Incubation of purified bLPO, pTPO, hTPO, and microsomal rTPO with genistein (10 μM) and H_2O_2 (100 μM) for 5 min produced activity losses of 53 ± 3, 40 ± 6, 62 ± 3, and 66 ± 7%, respectively (means ± SD). This study shows that the inactivation of microsomal rTPO was comparable to that produced by genistein with other peroxidases, including hTPO.

DISCUSSION

TPO Inactivation by Genistein in Vitro

Previous results showed that genistein and daidzein were suicide substrates for bLPO and pTPO *in vitro*. The results presented here extend these findings to rTPO by showing that it is similarly susceptible to suicide inactivation by low doses of isoflavones *in vitro* (see above and Fig. 4). Furthermore, a commercial preparation of hTPO was similarly inactivated by genistein (see above). These findings *in vitro* suggest that isoflavone-mediated inactivation of TPO is a general phenomenon across mammalian species.

Intrathyroidal Genistein Concentration

The genistein concentration in rat thyroid increased as a function of dietary consumption in a statistically significant and dose-dependent manner (see Fig. 1). Both conjugated and aglycone forms were present in the thyroid, although the proportion of aglycone in the thyroid was elevated relative to that in blood. This suggests that the nonpolar aglycone form, present at low concentrations in blood, partitions preferentially into lipophilic tissues such as thyroid. A previous study from this laboratory showed that this occurred in other endocrine-responsive tissues to an even greater extent (Chang *et al.*, 2000). Using previously published estimates for the aqueous content of thyroid follicular cells (60%) (Chow and Woodbury, 1965) it was possible to estimate intracellular concentrations of total genistein at up to 1920 nM and up to 350 nM for the aglycone. These concentrations of genistein aglycone are of the same order as those shown to inactivate rTPO *in vitro* (10–1000 nM; see Fig. 4). We previously showed that both conjugated and aglycone forms of genistein inhibit TPO, a though the aglycone was about 20-fold more potent at blocking the TPO-catalyzed coupling reaction (Divi *et al.*, 1997). In the present study it was demonstrated that genistein, the glucoside conjugate of genistein, also inactivated LPO, although it was about 100-fold less potent (not shown). This result suggests that intrathyroidal genistein glucuronides can contribute to the observed TPO inactivation, especially in the highest dose groups.

Although all possible blood was removed from each rat by cardiac puncture after anesthetization, the effect of residual blood in the thyroid was considered as a possible contributor to the measured genistein content and dismissed for the following reasons. The distribution of genistein between unconjugated and conjugated forms in the thyroid was different from that in blood; 18–28% aglycone in the thyroid (Chang *et al.*, 2000) vs 2% aglycone in serum of adult male and female rats (Holder *et al.*, 1999). All tissues examined (brain, liver, mammary, ovary, prostate, testes, uterus) showed a similar trend with even larger proportions of aglycone. Additionally, a large sex difference in liver content of total and aglycone genistein was observed

Zhang *et al.*, 2000). It is deemed unlikely that a sex-based difference in blood retention in the highly perfused liver is possible, especially in light of the similar relative liver weights and the lack of changes from genistein ingestion that were observed.

Inactivation of TPO by Genistein in Vivo

The consumption of dietary genistein by male and female rats also led to substantial losses in TPO activity measured in microsomes prepared from isolated glands (see Fig. 2). In the highest dose group, 75–80% of control TPO activity was lost, and the effect was greater in females, presumably because of the higher serum and intrathyroidal concentrations (see Fig. 1 and Table 1). The results of the control experiment described under Results gave confidence that the measured TPO activities were accurate indications of those present *in vivo*. Therefore, given that the concentrations of genistein measured in rat thyroids were sufficient for inactivation of rTPO *in vitro*, it is reasonable to conclude that the reductions observed in TPO activity were the result of enzyme inactivation *in vivo*.

In a previous study, TPO inhibition by isoflavones *in vitro* was described and conditions were identified under which suicide inactivation or alternate substrate inhibition occurred (Divi *et al.*, 1997). The presence of iodide, at concentrations expected in the normal thyroid gland,[3] attenuated the inactivation pathway *in vitro*. In the present study, no evidence was obtained from LC-ES/MS analysis of serum or thyroids to indicate formation of any of the iodinated genistein species previously characterized (Divi *et al.*, 1997). This finding, coupled with the observation of extensive TPO inactivation, suggests that the inactivation by isoflavones is favored in the rat thyroid gland over competitive substrate iodination.

These findings, obtained from a diet fortified with genistein aglycone, were confirmed in a diet comparison study where rats were fed either a standard soy-containing feed (NIH 31) or a special soy-depleted feed (5K96), which contained approximately 60 or 1 ppm total isoflavones, respectively. Soy contains isoflavones as glucoside conjugates (Barnes *et al.*, 1994), so this study addressed the issue of whether biological effects can be altered by the form of isoflavone administered. Reductions in TPO activity were observed in the rats consuming the standard soy diet, and the magnitude of reduction was commensurate with the measured blood levels of isoflavones (see Fig. 3). This observation shows that the form of genistein present in the diet, either aglycone or glucoside conjugates, does not affect the total serum isoflavone concentrations reached at termination or the dose-dependent reduction in TPO content. A previous study concluded that administration of genistein, either as the aglycone or the mixture of glucoside conjugates present in soy, had a minimal effect on total absorption, although small differences were observed in the peak concentrations (King *et al.*, 1996). Thigpen *et al.* (1999) have shown that the isoflavone content of typical rodent diets can vary from less than 5 ppm up to about 500 ppm. These findings suggest the possibility for additive effects on TPO activity between soy in the diet and exogenous chemicals being tested for carcinogenicity or other toxicities.

The two preceding studies were performed by feeding rats the respective diets from gestation through weaning until termination as an adult. Additional studies were performed to understand the effect of timing of genistein exposure on the inactivation of TPO. In dose-range-finding studies, exposure to genistein aglycone in the diet of 63–77 days duration produced dose-dependent decreases in TPO activity, regardless of whether it was administered only in adulthood (F0 females) or from gestation Day 5 through weaning and adulthood (F1 males and females). This suggests that the developmental timing of genistein exposure does not affect the extent of TPO activity loss measured in adulthood.

This study with genistein appears to be the first in which chemically induced loss of TPO activity has been demonstrated both *in vitro* and *in vivo*. The mechanism for inhibition of TPO-catalyzed reactions by the antihyperthyroid drugs propylthiourea (PTU) and methimazole (MMI) has been studied previously. Shiroozu *et al.* (1983) showed that injection of PTU or MMI into Sprague–Dawley rats produced no changes in total TPO activity following either a single- or multiple-injection regimen. This observation was paradoxical, considering the fact that PTU and MMI are potent inactivators of porcine TPO *in vitro*. A reevaluation of this phenomenon led to the conclusion that the inhibition of intrathyroidal iodination produced by PTU *in vivo* could be accounted for solely by the persistence of unmetabolized PTU in the thyroid and competition for oxidized TPO catalytic species (i.e., competitive substrate activity; see Taurog and Dorris, 1989). The lack of persistent effects on iodination *in vivo* by MMI were attributed to its rapid clearance from the gland. These findings, coupled with the present study of isoflavones, emphasize the lack of correlation between *in vitro* suicide inactivation of pTPO and *in vivo* inactivation of TPO in rats.

Absence of Hypothyroid Indicators

Given the substantial reduction of TPO activity in rats consuming genistein-fortified and standard soy-containing diets, decreased levels of T3/T4 and higher levels of TSH were predicted. However, analysis of sera from all rats in both these studies showed no significant differences in the levels of these hormones relative to untreated rats (data not shown). Furthermore, gland weights and histopathological examination of thyroid sections showed no differences between untreated and 500

[3] The concentration of free iodide is reported to be low, and using estimates for the percentage iodide (0.25%) (Taurog, 1996) and the total iodine content (Abrams and Larsen, 1973), the concentration in rat thyroid tissue is estimated to be 17 pmol/mg gland (approximately 28 μM).

μm genistein-dose rats treated identically in a parallel study (Dr. John Latendresse, personal communication). A recent study showed only minor irregularities of the thyroid follicles following administration of a 20% soy diet to rats (Ikeda *et al.*, 2000). These findings were quite paradoxical in light of the dramatic effects on TPO activity.

Thyroid hormone synthesis requires that TPO activity be colocated with its substrates thyroglobulin, iodide, and H_2O_2 (Taurog, 1996), which is produced by the NADPH oxidase (Dupuy *et al.*, 1991). The best evidence suggests that this occurs at the interface between the apical membrane of the follicular cell, which contains TPO and the NADPH oxidase (Capen, 1996), and the lumen in which iodide is accumulated by a sodium iodide symporter present in the basolateral membrane (Castro *et al.*, 1999) and into which thyroglobulin is secreted (Capen, 1996). However, histochemical studies have shown that TPO can be more widely distributed in the cell (Tice and Wollman, 1972). This leaves open the possibility that an additional pool of TPO could be sequestered, such that contact with isoflavones and H_2O_2, but not iodide, is possible. One possible explanation is the vesicles, shown by histochemical analysis to contain TPO (Capen, 1996); however, it is not clear how isoflavones and H_2O_2, but not iodide, would have access to this pool of TPO. Another possible explanation for the paradoxical findings is that there is a large excess of TPO present in the apical membrane, such that even substantial losses have a minimal effect on hormone homeostasis. An observation that is inconsistent with this idea is the coordinated stimulation of TPO gene expression along with that for sodium iodide symporter and thyroglobulin in response to TSH stimulation of thyroid follicular cells (Rapoport and Spaulding, 1996).

Effect of Iodine Deficiency on Soy-Induced Hypothyroid Effects

In all studies described here, the rats received normal amounts of iodide in the diet. Previous reports have shown that the additional factor of iodine deficiency is crucial in the genesis of adverse effects of soy in the thyroid gland. Kay *et al.* (1988) showed that normal iodide intake could prevent goiter in rats consuming a soy bean diet. Kimura *et al.* (1976) showed that only rats on an iodine-deficient diet containing defatted soybeans developed goiter that rapidly progressed to metastatic carcinoma. Block *et al.* (1961) showed that iodide could reverse soybean-induced goiter in rats. A recent study showed that consumption for 10 weeks by female rats of a diet containing 20% soy had synergistic effects on antithyroid effects caused by iodine deficiency (Ikeda *et al.*, 2000). Based on goiter formation, hormone measurements (e.g., T3, T4, TSH), and microscopic analysis, these authors concluded that direct effects of soy on the pituitary gland, in addition to thyroid-specific actions, were the likely mechanism. Citing only unpublished results, these authors also stated that high isoflavone-containing diet did not exacerbate the goitrogenic effects of iodine deficiency. Taken together, the observations from these studies suggest that, under abnormal conditions like iodine deficiency, normal homeostatic mechanisms in the rat were overwhelmed and that loss of TPO activity that occurred upon exposure to soy isoflavones could be involved in the goitrogenic responses.

Extrapolation to Human Populations

Based on the concentrations of total genistein present in serum (see Table 1), rats fed the control and low-dose (5 ppm) diets are similar to human adults consuming a typical Western diet (<0.1 μM). Rats fed the 100 ppm genistein diet are similar to human adults consuming a typical Asian diet (0.1–1.2 μM) (Adlercreutz *et al.*, 1994) or soy nutritional supplements (0.5–0.9 μM) (Doerge *et al.*, 2000b). Rats fed the 500 ppm genistein diet are similar to infants on soy formulas (2–7 μM) (Setchell *et al.*, 1997). These comparisons of blood levels make it reasonable to assume that human consumption of soy isoflavones under the aforementioned conditions could result in isoflavone transfer into the thyroid gland (see Fig. 1) and inactivation of hTPO.

These studies suggest that, especially in the presence of additional risk factors for thyroid dysfunction, the added effects of long-term soy consumption could be sufficient to induce antithyroid effects in humans and animals. These factors include biochemical impairment of hormone synthesis and metabolism, dietary iodine deficiency, and exposure to environmental goitrogens (e.g., sulfonamides, glucosinolates, flavonoids, and persistent halogenated aromatic compounds).

This potential antithyroid effect of soy isoflavones may be particularly relevant to elderly women, the population subgroup in which overt hypothyrodism and a subclinical hypothyroid state (Woeber, 1997) are most likely to occur (rates up to 4 and 10%, respectively) (Vanderpump and Tunbridge, 1996; Canaris *et al.*, 2000). Soy products are currently marketed in the United States for relief from the symptoms of menopause, despite the absence of clinical data demonstrating any hormonal effects beyond the placebo (Duncan *et al.*, 1999b). This connection suggests that elderly women should be cognizant of and monitored for possible thyroid complications arising from consumption of such products. This is especially important given the potential for self-administration in large doses of soy nutritional supplements, which are essentially unregulated products.

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Gutachten über das Nahrungsergänzungsmittel NADH

Nicotinsäureamid-Adenin-Dinukleotid (abgekürzt NAD) ist ein Hydridionen (Zwei-Elektronen/Ein-Proton) übertragendes Koenzym, das an zahlreichen Redoxreaktionen des Stoffwechsels der Zelle beteiligt ist. Das H in der Formel NADH kennzeichnet den Wasserstoff, der in geordneter Form an Sauerstoff in einer kaskadenförmigen Reaktion bindet, wodurch in der Zelle ATP, die energetische Grundwährung der Zelle entsteht. Dieses Koenzym ist an mehr als tausend Stoffwechselreaktionen beteiligt, weshalb es auch Coenzym 1 bezeichnet wird (1)

Jede Zelle ist in der Lage, sich NADH selbst herzustellen, allerdings sinkt diese Fähigkeit mit zunehmendem Alter (2), was für altersassoziierten Problemen in Zusammenhang gebracht wird (3). NAD wird in unterschiedlichem Ausmaß auch mit der Nahrung zugeführt, weshalb es seit Jahrzehnten Bemühungen gab, ein NADH angereichertes Nahrungsergänzungsmittel auf den Markt zu bringen.

Dem FemmeWell zugrunde liegende Zubereitungsverfahren des NADH als Nahrungsergänzungsmittel wurden 1995 internationale Patente erteilt.

1. Wirksamkeit

Stabilität und Resorption des Wirkstoffes sind Grundvoraussetzungen der zu erwartenden Wirksamkeit und wurden durch den Zusatz von Mannit und Natrium - Hydrogencarbonat in einer definierten Konzentration, wodurch NADH für mehr als 2 Jahre stabil gemacht werden konnte, erreicht. Für diese spezielle Formulierung von NADH mit Mannit und Backpulver, wurde ebenfalls eine Reihe von internationalen Patenten erteilt.(4, 5).

Ursprünglich war man der Meinung, dass NADH als treibende Kraft der ATP-Produktion nur in der Zelle gebildet werden kann. An der Universität Graz konnte jedoch gezeigt werden, dass NADH, wenn es von außen Zellen zugesetzt wird, die ATPKonzentration in den Zellen erhöht. (5a.)

In einer an der Freien Universität Berlin durchgeführten Untersuchung wurde nachgewiesen, dass oral eingenommenes NADH die Darmschleimhaut mittels passiver Diffusion penetriert und auf diese Weise in die Blutzirkulation gelangt.(6).

Weitere Arbeite zur Pharmakokinetik von NADH in vivo haben frühere Absorptionsexperimente bestätigt. NADH wird vom Darm resorbiert (7).

NADH kann auch die Blut-Hirnschranke passieren:
Die Bioverfügbarkeit von NADH nach sublingualer Verabreichung wurde unter Anwendung der laserinduzierten Fluoreszenzspektroskopie von NADH dokumentiert.
Legt man im Tierversuch eine NADH Tablette unter die Zunge, kommt es innerhalb von 15 Minuten zu einem signifikanten Anstieg der NADH - Fluoreszenz in der Hirnrinde (8).

Pathophysiologische Erklärung der Wirkung von NADH:

L-DOPA ist ein Neurotransmitter der im Laufe des Lebens geringer wird und im schwersten Fall zum M. Parkionson führt. Die endogene Synthese von L-DOPA wird durch die Tyrosin Hydroxylase vorgenommen, dessen Cofaktor das Tetra-Hydro-Biopterin ist. Zur Bildung von Tetra-Hydro-Biopterin benötigt die Zelle NADH, das damit die Aktivität der Tyrosin – Hydroxylase anregt. In Kulturen von Nervenzellen steigert NADH die Produktion von Dopamin bis auf das 6-fache (9).

Klinische Beobachtungen:

In der Zeit von 1990 bis 1992 wurden 205 Patienten mit depressiver Verstimmung und Leistungsabfall in Form einer offenen Studie mit NADH behandelt. Sie erhielten NADH entweder als intravenöse oder als intramuskuläre Injektion oder in Form von NADH-Tabletten. Die Dosis war 10 mg NADH pro Tag. Die Dauer der Therapie betrug 6 Monate. Bei 93 % der Patienten kam es zu einer Verbesserung ihrer Beschwerden um bis zu 44 % nach der für diese Studie verwendeten validierten Beurteilungsskala. Nebenwirkungen wurden von keinem der Patienten angegeben. (10)

In einer weiteren, in den USA durchgeführten und genehmigten Doppelblind Placebo kontrollierte Cross-over Studie, wurde an der Georgetown Universität in Washington die Wirksamkeit von NADH beim chronic fatigue Syndrom (CSF) überprüft.
Die Patienten erhielten 2 Tabletten 5mg NADH (total 10 mg) pro Tag für 4 Wochen, danach war eine gleich lange Auswaschperiode, in der beide Gruppen keine Tabletten erhielten. Nach diesen 4 Wochen erhielt die NADHGruppe nun Placebo Tabletten und die andere Gruppe NADH.
31 % der CFS Patienten zeigten nach 4 Wochen ein Verbesserung ihrer Symptome. Nach 6 Monaten NADH-Einnahme (10 mg NADH pro Tag) fühlten sich 82 % der Patienten wesentlich besser und hatten mehr Energie. Die Ergebnisse dieser Studie wurden von der Georgetown Universität veröffentlicht.(11)

In einer sportmedizinischen, am Institut für Sportmedizin der Universität Freiburg durchgeführten doppel geblindeten, placebokontrollierten Cross over Studie wurde GCP-konform der Einfluß von NADH of objektive Respirationsparameter evaluiert. Eine Gruppe von Sportlern erhielt 3 Tabletten NADH, also insgesamt 30 mg NADH pro Tag für 4 Wochen. Danach war eine 6-wöchige Pause, „eine Auswaschphase", in der kein NADH eingenommen wurde. Anschließend wurden der 1. Gruppe für 4 Wochen 3 Placebo Tabletten pro Tag verordnet. Die 2. Gruppe, die in den ersten 4 Wochen Placebo Tabletten einnahm, bekam nach der Auswaschphase 3 Tabletten NADH pro Tag ebenfalls für 4 Wochen.
Untersucht wurden folgende Parameter:
die maximale aerobe Kapazität (Spiroergometrie)
Sauerstoff Aufnahme
Kohledioxyd-Abgabe

Laktat-Spiegel im Blut
Katecholamine im Plasma.

Diese Tests wurden jeweils am Beginn und am Ende der Interventionsphase also insgesamt 4 Mal, am Beginn und Ende der 1.Phase und am Beginn und Ende der 2. Phase, durchgeführt.
Unter Einnahme von 30 mg NADH / Tag zeigten sich eine Reduzierung des O2 - Verbrauches und eine Erhöhung des respiratorischen Quotienten bei definierter Arbeit (12).

Am Forschungsinstitut der Universität Jyvaskyla in Finnland wurde von Professor Komi die Wirksamkeit des NADH ebenfalls an Sportlern überprüft. Die Ergebnisse bestätigten die vorhin erhobenen Befunde. Unter NADH war der Laktat-Spiegel im Blut nach einem Lauftest unter anaeroben Bedingungen deutlich niedriger als nach Einnahme von Placebo. Auch die Sprungkraft war unter NADH besser und die Reaktionszeit nach einem Lichtsignal unter NADH signifikant kürzer als unter Placebo (13)

Der Cog Screen ist eine validierte Methode, die auch von der Vereinigung der amerikanischen Airline Piloten (American Assoziation of Airline Pilotes) und von der NASA zur Überprüfung der kognitiven Fähigkeiten von Piloten und Astronauten eingesetzt wird. In einer prospektiven Untersuchung wurde der Einfluß des NADH auf Vigilanz and Müdigkeit bei Passagieren, die Zeitzonen überfliegen, untersucht. Wie erwartet, führte Jet lag zu einer erhöhten Schläfrigkeit und zu einer Verschlechterung bestimmter Hirnleistungen. Die häufigsten Fehler wurden bei der Aufmerksamkeit, beim Gedächtnis und der Geschwindigkeit der visuellen Erfassung von bestimmten Signalen gemacht.
Die NADH-Probanden zeigten hingegen in den Hirnleistungstests und im Schläfrigkeitsgrad signifikant bessere Werte als die Probanden, die Placebo erhielten.(14, 15)

2. Sicherheit von NADH

Als Voraussetzung zur Durchführung von klinischen Prüfungen verlangten die Amerikanische Gesundheitsbehörde eine umfassende wissenschaftliche Dokumentation über die Chemie, Pharmakologie und Toxikologie des zu prüfenden Produktes und des darin enthaltenen Wirkstoffes. Dabei wird der Sicherheit des Wirkstoffes besondere Bedeutung beigemessen.
Aus diesem Grund wurden für das Nahrungsergänzungsmittel und die darin enthaltene Wirksubstanz die behördlich vorgeschriebenen Untersuchungen über die akute, subakute und chronische Toxizität veranlasst und von einer unabhängigen, international Firma in Großbritannien durchgeführt.

Die maximal tolerierbare akute Dosis von NADH:

Zur Bestimmung der maximal tolerierbaren Dosis,(MTD) von NADH wurden Ratten steigende Dosen injiziert und evaluiert, ab welcher NADH - Konzentration die Tiere starke Veränderungen in ihrer Befindlichkeit zeigten.

Als maximal tolerierbare intravenöse Dosis von NADH wurden 500 mg pro Kilogramm Körpergewicht gefunden. Rechnet man diesen Wert für einen 70 kg schweren Menschen um, so ergibt sich eine Menge von 35.000 mg oder 35 Gramm NADH. Das entspricht in etwa dem Volumen von 5 Esslöffeln NADH, die man ohne Beschwerden einnehmen könnte. Die Ergebnisse dieser Untersuchungen ist publiziert (16)

Die Ermittlung der *subakuten Toxizität* von NADH wurde, wie von der FDA und den Europäischen Zulassungsbehörden gefordert, an Hunden vorgenommen. Dabei verabreichte man wieder NADH in steigenden Konzentrationen bis zu der vorher ermittelten maximal tolerierbaren Dosis in Form von Tabletten. 14 Tage nach Verabreichung wurden die Organe aller Tiere auf pathologische Veränderungen untersucht. Es konnten weder bei den männlichen noch bei den weiblichen Tieren Organ- oder Gewebeschäden festgestellt werden.

Langzeitanwendung von höheren Dosen von NADH:
Zusätzlich zur Bestimmung der maximal tolerierbaren Dosis verlangten die Gesundheitsbehörden auch die Prüfung möglicher Nebenwirkungen eines Oral zugeführten Wirkstoffes unter Langzeitbeobachtungüber mindestens 6 Monate , wobei der Wirkstoff täglich apliziert werden musste (Nachweis der chronischen Toxizität).
Nachdem dieErgebnisse der subakuten Toxizität ausgewertet waren, führte das gleiche Institut in Großbritannien auch Studien zur chronischen Toxizität durch. Dazu wurde Ratten 26 Wochen lang je eine Tablette mit 5 mg NADH pro Tag verabreicht. Einer gleichen Zahl von Ratten wurde je eine Placebo - Tablette pro Tag verfüttert. 6 Monate nach Einnahme von täglich 5 mg NADH sind alle Organe der beiden Tiergruppen makroskopisch und mikroskopisch auf mögliche Veränderungen untersucht worden und zeigten weder makroskopische noch mikroskopische Veränderungen. Sie unterschieden sich auch nicht von den Organen der Tiere, die Placebo verabreicht bekamen. (17) Rechnet man die NADH - Menge von 5 mg für eine Ratte mit 330 Gramm Körpergewicht auf die äquivalente NADH - Dosis für einen Menschen mit ca. 70 kg Körpergewicht um, ergibt sich ein Wert von 1050 mg . Dividiert man diese Zahl durch den NADH - Gehalt von 5mg, erhält man die Anzahl der NADH -Tabletten, die ein Mensch pro Tag für 26 Wochen einnehmen kann, ohne dabei Schaden zu nehmen. Dies ergibt einen Wert von 210 Tabletten. Man könnte demnach 210 Tabletten mit 5mg NADH oder 140 Tabletten mit 7.5 mg NADH oder 105 Tabletten mit 10 mg NADH ein halbes Jahr täglich einnehmen ohne Organschäden zu erleiden (18).

Bisher sind mit NADH an folgenden Universitäten
Studien mit Menschen nach GCP (Good Clinical Practise) - Richtlinien durchgeführt worden,
Georgetown Universität in Washington,
Cornell Universität in New York
Lennox Hill Hospital (NISMAT Institute) in New York Neurologische Klinik der Universität Zagreb
Institut für Sportmedizin der Universität Freiburg
Birkmayer Institut für Parkinson Therapie, Wien
1st Military University, Guangzhou Hospital, China.
Institut für Medizinische Chemie der Universität Graz
Institut für Physiologie der Universität Graz

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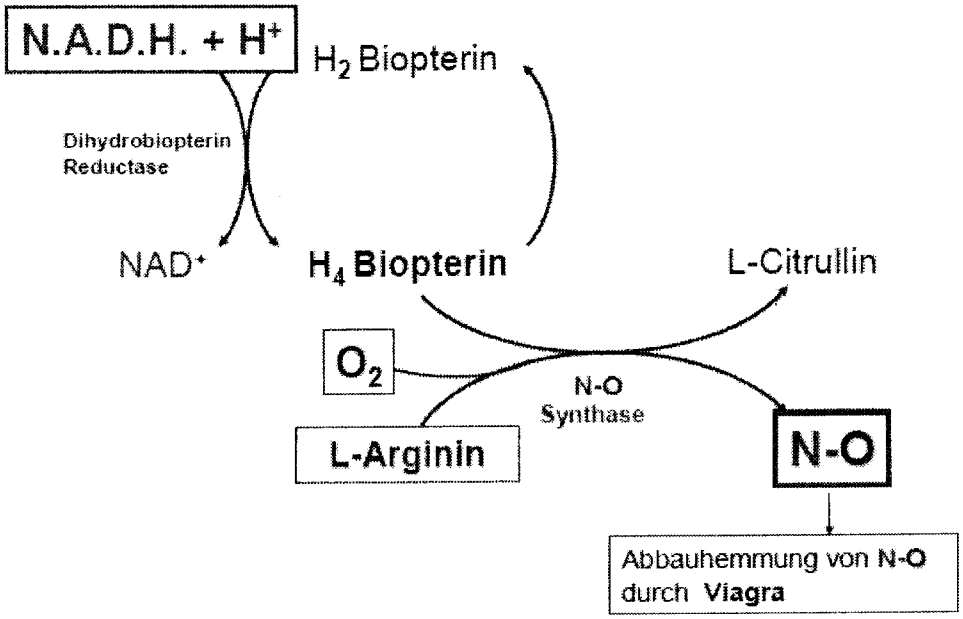
N.A.D.H. + H+
H2 Biopterin
Dihydrobiopterin
Reductase
NAD+
H4 Biopterin
L-Citrullin
O2
N-O
Synthase
L-Arginin
N-O
Abbauhemmung von N-O
durch Viagra

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NADH supplementation decreases pinacidil-primed $I_{K(ATP)}$ in ventricular cardiomyocytes by increasing intracellular ATP

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1 The aim of this study was to investigate the effect of nicotinamide-adenine dinucleotide (NADH) supplementation on the metabolic condition of isolated guinea-pig ventricular cardiomyocytes. The pinacidil-primed ATP-dependent potassium current $I_{K(ATP)}$ was used as an indicator of subsarcolemmal ATP concentration and intracellular adenine nucleotide contents were measured.

2 Membrane currents were studied using the patch-clamp technique in the whole-cell recording mode at 36–37°C. Adenine nucleotides were determined by HPLC.

3 Under physiological conditions (4.3 mM ATP in the pipette solution, ATP_i) $I_{K(ATP)}$ did not contribute to basal electrical activity.

4 The ATP-dependent potassium ($K_{(ATP)}$) channel opener pinacidil activated $I_{K(ATP)}$ dependent on $[ATP]_i$ showing a significantly more pronounced activation at lower (1 mM) $[ATP]_i$.

5 Supplementation of cardiomyocytes with 300 $\mu g\,ml^{-1}$ NADH (4–6 h) resulted in a significantly reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells. The current density was 13.8 ± 3.78 ($n=6$) *versus* 28.9 ± 3.38 pA pF^{-1} ($n=19$; $P<0.05$).

6 Equimolar amounts of the related compounds nicotinamide and NAD^+ did not achieve a similar effect like NADH.

7 Measurement of adenine nucleotides by HPLC revealed a significant increase in intracellular ATP (NADH supplementation: 45.6 ± 1.88 nmol mg^{-1} protein *versus* control: 35.4 ± 2.57 nmol mg^{-1} protein, $P<0.000005$).

8 These data show that supplementation of guinea-pig ventricular cardiomyocytes with NADH results in a decreased activation of $I_{K(ATP)}$ by pinacidil compared to control myocytes, indicating a higher subsarcolemmal ATP concentration.

9 Analysis of intracellular adenine nucleotides by HPLC confirmed the significant increase in ATP.
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Keywords: NADH; ATP-dependent potassium current; $I_{K(ATP)}$; pinacidil; patch clamp; whole-cell clamp; intracellular ATP; guinea-pig ventricular myocytes

Abbreviations: ($I_{K(ATP)}$), ATP-dependent potassium current

Introduction

The reduced nicotinamide-adenine dinucleotide (NADH) plays a central role for the energetic state of a cell. NADH carries electrons derived from catabolic reactions to their entry into the respiratory chain leading to the synthesis of ATP. This electron transfer results in the formation of NAD^+. The sum of NADH and NAD^+ is thought to be rather constant in a cell; thus the NADH/NAD^+ ratio is a crucial factor for its energetic state. Therefore, it is conceivable that oral application of NADH might positively affect the cellular energetic condition of humans.

Moreover, NADH serves as a cofactor for various enzyme reactions, further emphasizing the crucial role of NADH for numerous cell functions. Recent clinical studies have already demonstrated a positive effect of NADH treatment on patients suffering from Morbus Parkinson, chronic fatigue syndrome and depression (Birkmayer & Birkmayer, 1991; Birkmayer *et al.*, 1993; Kuhn *et al.*, 1996; Forsyth *et al.*, 1999). In addition, changes in NADH levels seem to correspond with stimulation and inhibition of neuronal metabolism, respectively (Rex *et al.*, 1999). NADH has also been identified as sensor of blood flow requirement in brain, muscle and other tissues (Ido *et al.*, 2001).

To test whether extracellular application of NADH affects the energetic state of a cell, we studied NADH-incubated cardiac ventricular myocytes by electrophysiological techniques.

The electrophysiological properties of a cardiomyocyte are strongly affected by its energetic condition. Especially, the ATP-dependent potassium current ($I_{K(ATP)}$) is known to link bioenergetic metabolism with membrane excitability by sensing intracellular concentrations of ATP and ADP. Under

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physiological conditions, $K_{(ATP)}$ channels are predominantly closed because of inhibition by intracellular ATP (ATP_i) (Noma, 1983; Heidbüchel *et al.*, 1990; Schaffer *et al.*, 1999). However, when the ATP_i concentration falls below certain values (like in pathological states such as acute myocardial ischemia), the $K_{(ATP)}$ channels open. Certain drugs known as potassium channel openers (PCOs) are able to shift the ATP sensitivity of $K_{(ATP)}$ channels resulting in channel opening even at physiological levels of ATP_i (Arena & Kass, 1989; Nakayama *et al.*, 1990; Tseng & Hoffman, 1990; Pelzmann *et al.*, 2001). Thus, the amount of $I_{K(ATP)}$ activation induced by these drugs serves as an indicator of the cellular ATP content (Sasaki *et al.*, 2001).

Studying the PCO-primed $I_{K(ATP)}$, we report that supplementation of cardiomyocytes with NADH but not with the related compounds nicotinamide and NAD^+ results in a decrease of $I_{K(ATP)}$ activation by pinacidil. The NADH-induced decrease of $I_{K(ATP)}$ activation fits to the increase of intracellular ATP content measured by HPLC.

Methods

Myocyte isolation

Guinea-pig ventricular myocytes were isolated by Langendorff perfusion using collagenase as described previously (Piper *et al.*, 1982). The isolated myocytes were stored in Medium 199 (M2154, Sigma, St Louis, MO, U.S.A.), supplemented with $5\,\mu g\,ml^{-1}$ penicillin, $5\,IU\,ml^{-1}$ streptomycin, 0.5% glutamine and 5% fetal calf serum and were kept in an incubator at 37°C. Experiments were performed within 24 h after isolation.

Incubation procedure

The isolated myocytes were incubated with NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt) and the related compounds nicotinamide and NAD^+ (β-nicotinamide adenine dinucleotide, oxidized form) in equimolar amounts 4 – 6 h before electrophysiological parameters and ATP content were evaluated.

Electrophysiological recordings and data analysis

Membrane currents were recorded using the whole-cell single-electrode voltage-clamp configuration of the patch-clamp technique (Hamill *et al.*, 1981) using a List L/M-EPC 7 amplifier (List, Darmstadt, Germany) as previously described (Pelzmann *et al.*, 2001). Myocytes were placed in an experimental chamber mounted on the stage of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) and were superfused with standard extracellular solution (composition in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 2.2, NaH_2PO_4 0.4, HEPES/Na^+ 10, D(+)-glucose 5.6, adjusted to a pH of 7.4 with NaOH) at 36 – 37°C with a flow rate of about $1.5\,ml\,min^{-1}$. When filled with standard internal solution (composition in mM: KCl 110, ATP/K^+ 4.3, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES/K^+ 10, adjusted to a pH of 7.4 with KOH) and placed into standard external solution, patch-pipette tip resistances were 1 – 3 MΩ. Only quiescent rod-shaped cells with clear cross striation were used for voltage-clamp experiments. Cell membrane capacitance (C_m) was determined by integration of the capacitive transient elicited by a 10 mV hyperpolarizing pulse from −50 mV. C_m (up to 100 pF) and series resistance (R_s, by at least 50%) were compensated. Voltage-clamp pulses were generated with a personal computer connected to a D/A and A/D converter (Digidata 1200, Axon Instruments, Foster City, U.S.A.). Data acquisition and analyses were performed using pCLAMP 5.7.1 software (Axon Instruments). In order to allow equilibration of the pipette solution with the cytosol, current recordings were started 5 min after rupture of the membrane patch.

Two experimental protocols were used. Modulation of $I_{K(ATP)}$ by drugs was evaluated as the change of outward current density (current amplitudes divided by C_m ($pA\,pF^{-1}$) in order to compensate for variations in cell size) at +30 mV in response to a 2 s ramp from −100 to +60 mV (I_{ramp}). Time course of $I_{K(ATP)}$ activation and blockade was studied by recording the holding current at −40 mV ($I_{hold(-40\,mV)}$).

Determination of adenine nucleotides

The analytical method to determine adenine nucleotides has been reported previously (Hallström *et al.*, 2002). Alterations of this method are in brief: separation was performed on a Hypersil ODS column (5 μm, 250 mm × 4 mm ID) using a Waters™ 717 plus Autosampler (Waters, Milford, MA, U.S.A.), two constaMetric III pumps, a gradient controller (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and a Waters 969 photodiode array detector. Detector signals (absorbance at 254 nm) were recorded with an AGC Personal Computer. The program Millennium (Waters) was used for data acquisition and analysis. Cardiomyocytes were deproteinized with 250 μl of 0.4 M perchloric acid. After centrifugation (12,000 × *g*), 100 μl of the acid extract were neutralized with 10 μl of 2 M potassium carbonate (4°C). The supernatant (10 μl) obtained after centrifugation was used for HPLC analysis. The pellets of the acid extract were dissolved in 1 ml of 0.1 M sodium hydroxide and further diluted 1 : 10 with physiologic saline for protein determination (BCA Protein Assay, PIERCE, Rockford, IL, U.S.A.). Percentage of living cells was estimated by determining the ratio of rod-shaped to total myocytes. The percentage of rod-shaped myocytes varied between 28 and 32% in different cell preparations. At least 600 myocytes were counted in each preparation.

Statistics

Data are expressed as means ± s.e.m., n = number of cells. Error bars in figures represent s.e.m. Statistical significance was determined by a two-tailed Student's t-test or, if more than two conditions were compared, by one-way analysis of variance (ANOVA) with the LSD *post hoc* test. Differences were considered significant at $P<0.05$.

Drugs

Stock solutions of $K_{(ATP)}$ channel blocker glibenclamide and of $K_{(ATP)}$ channel opener pinacidil were prepared daily in DMSO. Both drugs were purchased from Sigma.

NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt) was purchased from Roche (Mannheim, Germany), nicotinamide and NAD^+ (β-nicotinamide adenine dinucleotide, oxidized form) from Sigma.

Results

Activation of $I_{K(ATP)}$ by pinacidil

Mean membrane capacitance (C_m) of isolated guinea-pig ventricular myocytes used in this study was 113.6±3.13 pF (n=102). Under physiological conditions (4.3 mM ATP in the pipette solution, ATP_i), outward current density of I_{ramp} was 2.19±0.26 pA pF^{-1} at +30 mV (n=19). $I_{K(ATP)}$ did not contribute to the basal electrical activity since glibenclamide (50 μM), a $K_{(ATP)}$ channel blocker, did not affect I_{ramp} (data not shown). Figure 1 shows the effect of pinacidil on $I_{hold(-40\,mV)}$ and I_{ramp}. Exposure to 30 μM pinacidil caused $I_{K(ATP)}$ activation, shown as a strong increase in $I_{hold(-40\,mV)}$. Superfusion of the cell with the sulfonylurea glibenclamide (1 μM) completely inhibited $I_{K(ATP)}$ almost immediately, and the holding current returned to control level (Figure 1a). Figure 1b shows the original current traces elicited by a voltage ramp applied at different stages of the experimental protocol (indicated by letters in (a)). The current–voltage relation recorded under control conditions revealed the shape typical for ventricular myocytes. During application of pinacidil, a large increase in membrane current was observed. Addition of 1 μM glibenclamide completely reversed this effect. The glibenclamide-sensitive current (inset of Figure 1b) obtained by digital subtraction represents $I_{K(ATP)}$ showing an almost linear current–voltage relation. The reversal potential of about −80 mV indicates high potassium selectivity. Under exposure of 30 μM pinacidil, the outward current density at +30 mV was 28.9±3.38 pA pF^{-1} (n=19). After a washout period of 5 min, the current density returned to control values (1.81±0.22 pA pF^{-1}, n=12). Figure 2 shows the pinacidil (30 μM)-induced activation of $I_{K(ATP)}$, demonstrated as the increase in I_{ramp} density at +30 mV in the presence of a physiological (4.3 mM) and a low (1 mM) ATP_i concentration. Under control conditions (measured 5 min after rupture of the membrane patch), $I_{ramp(+30\,mV)}$ density was not statistically different at physiological and low ATP_i (2.19±0.26 pA pF^{-1} (n=19) *versus* 3.02±0.51 pA pF^{-1} (n=8) using 4.3 and 1 mM ATP_i, respectively). However, using 1 mM ATP_i, the pinacidil-induced increase in outward current density was significantly higher compared to 4.3 mM ATP_i (P<0.05). The current density was 28.3±3.26 (n=19) and 52.2±13.8 pA pF^{-1} (n=8) at 4.3 and 1 mM ATP_i, respectively. Thus, the pinacidil-primed $I_{K(ATP)}$ is an indirect indicator of subsarcolemmal ATP concentration.

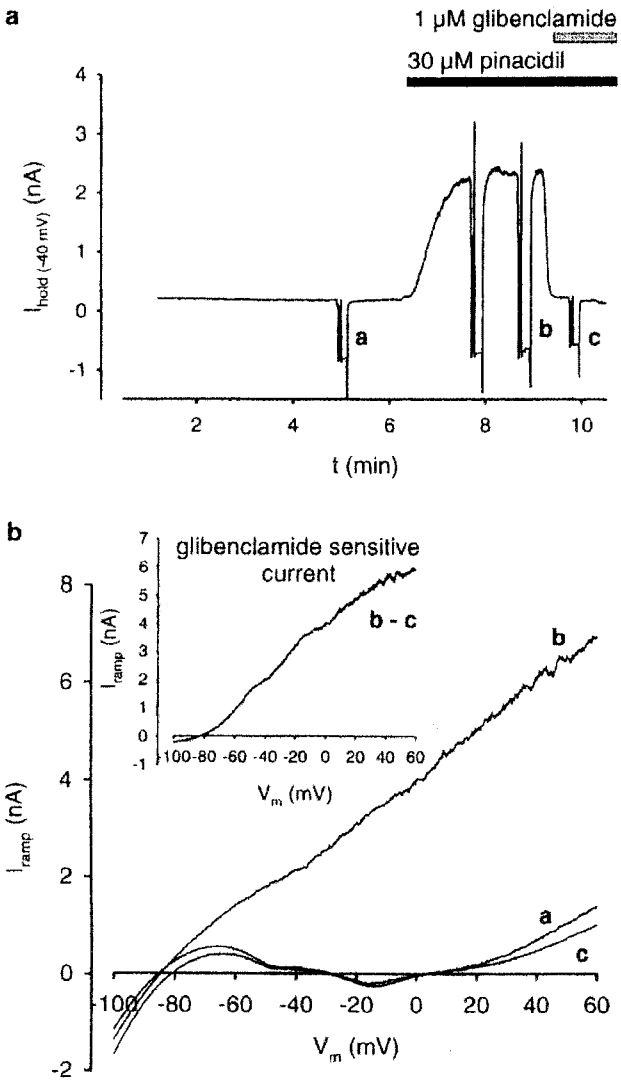

Figure 1 (a) Time course of $I_{K(ATP)}$ activation by pinacidil (30 μM) and subsequent blockade by glibenclamide (1 μM) in a guinea-pig ventricular myocyte. Sharp vertical deviations represent the voltage ramp-elicited current traces. (b) Original current traces, elicited by a 2-s voltage ramp, recorded at different stages of the experimental protocol indicated by letters in (a). The inset shows the glibenclamide-sensitive current, that is $I_{K(ATP)}$, evaluated by digital subtraction.

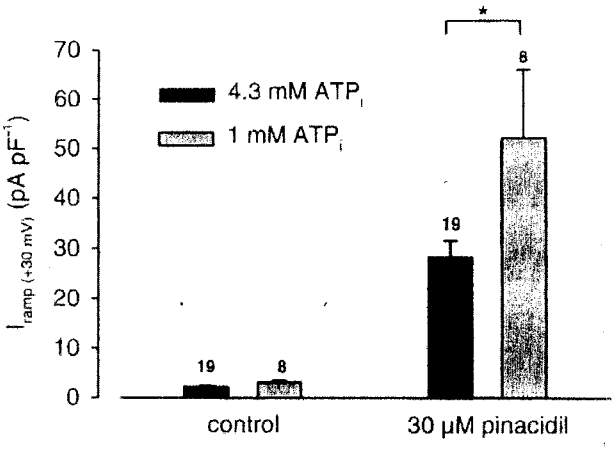

Figure 2 Outward current density (at +30 mV) under control conditions and in response to 30 μM pinacidil at different concentrations of ATP_i (1 and 4.3 mM). Numbers given represent n. *P<0.05.

Effects of incubation with NADH and related compounds

Figure 3 shows the concentration-dependent effect of NADH on $I_{K(ATP)}$ activation by 30 μM pinacidil under physiological conditions (4.3 mM ATP_i). Guinea-pig ventricular myocytes were incubated with different concentrations of NADH (200, 300, 400, 800, 1600 μg ml^{-1} cell culture medium) for 4–6 h before electrophysiological experiments were performed. For

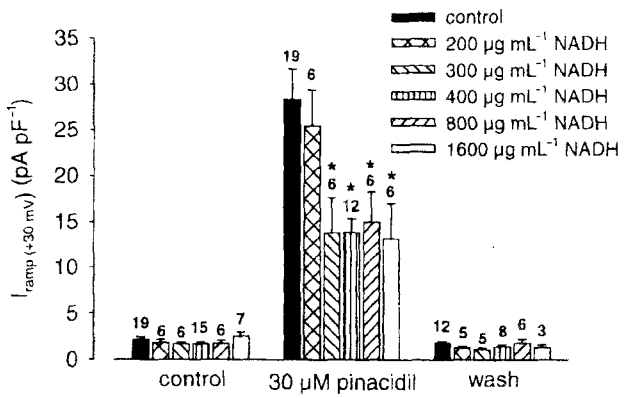

Figure 3 Concentration-dependent effects of NADH incubation on $I_{K(ATP)}$ activation by 30 μM pinacidil. Numbers given represent n. $^*P<0.05$ compared to control myocytes.

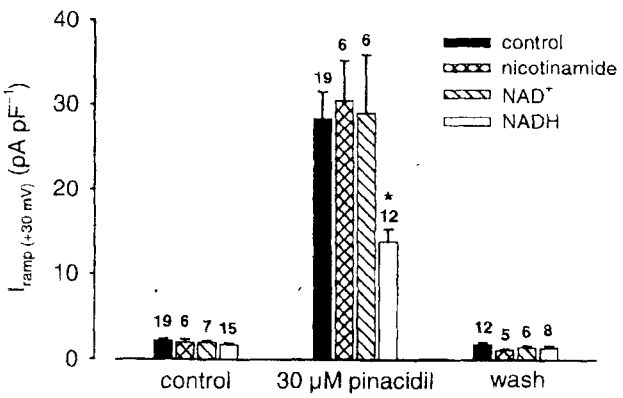

Figure 4 $I_{K(ATP)}$ activation by 30 μM pinacidil in myocytes incubated with 400 μg ml^{-1} NADH and equimolar amounts of nicotinamide and NAD$^+$. Numbers given represent n. $^*P<0.05$ compared to all groups under 30 μM pinacidil.

control experiments, an identical time schedule was used. Under control conditions, outward current density at +30 mV was not different between control and NADH-incubated cells. The current density was 2.19±0.26 (n=19), 1.82±0.33 (n=6), 1.72±0.19 (n=6), 1.70±0.18 (n=15), 1.76±0.36 (n=6) and 2.53±0.49 pA pF^{-1} (n=7) in control and after incubation with 200, 300, 400, 800 and 1600 μg ml^{-1} NADH, respectively. Incubation of the myocytes with 200 μg ml^{-1} NADH resulted in a reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells, but this effect did not reach statistical significance (28.3±3.26 (n=19) *versus* 25.4±3.97 pA pF^{-1} (n=6) at +30 mV). However, using 300 μg ml^{-1}, NADH outward current density was significantly reduced (13.8±3.78 pA pF^{-1}, n=6; $P<0.05$). A further increase of NADH concentrations did not further diminish the effect of pinacidil on $I_{K(ATP)}$ activation. The outward current density was 13.9±1.49 (n=12), 15.0±3.19 (n=6) and 13.2±3.74 pA pF^{-1} (n=6) in myocytes incubated with 400, 800 and 1600 μg ml^{-1} NADH, respectively. The effect of the $K_{(ATP)}$ channel opener pinacidil could be washed out completely. The outward current density returned almost to the initial value and was 1.81±0.21 (n=12), 1.24±0.24 (n=5), 1.09±0.14 (n=5), 1.37±0.25 (n=8), 1.75±0.48 (n=6) and 1.29±0.31 pA pF^{-1} (n=3) in control and after incubation with 200, 300, 400, 800 and 1600 μg ml^{-1} NADH, respectively.

To investigate whether this decreased activation of $I_{K(ATP)}$ by pinacidil is a specific effect of NADH or can also be caused by related compounds, the results with 400 μg ml^{-1} NADH were compared with equimolar amounts of nicotinamide and NAD$^+$ (Figure 4). Under control conditions, no difference in outward current density was observed (2.19±0.26 (n=19), 2.01±0.36 (n=6), 1.96±0.20 (n=7) and 1.70±0.18 pA pF^{-1} (n=15) in control and after incubation with nicotinamide, NAD$^+$ and NADH, respectively). $I_{K(ATP)}$ activation by pinacidil, however, could neither be reduced by nicotinamide nor by NAD$^+$. There was no statistically significant difference between currents in control myocytes and myocytes incubated with nicotinamide or NAD$^+$. Outward current density at +30 mV after addition of 30 μM pinacidil was 28.3±3.26 pA pF^{-1} (n=19) in control myocytes, 30.5±4.83 (n=6) and 29.0±7.01 pA pF^{-1} (n=6) in nicotinamide and NAD$^+$-incubated cells, respectively. After a washout period, the outward current density returned nearly to control values (1.81±0.22 (n=12), 1.13±0.15 (n=5), 1.44±0.25 (n=6) and 1.37±0.25 pA pF^{-1} (n=8) in control and after incubation with nicotinamide, NAD$^+$ and NADH, respectively).

Since $I_{K(ATP)}$ serves as an indicator of subsarcolemmal ATP concentration, the most likely explanation for the observed effects is an NADH-induced increase in intracellular ATP content. A direct effect of extracellular NADH on pinacidil-activated $I_{K(ATP)}$ can be excluded. Simultaneous superfusion of myocytes with 400 μg ml^{-1} NADH and 30 μM pinacidil did not change I_{ramp} (data not shown).

Determination of intracellular adenine nucleotides

To confirm an NADH supplementation-induced increase in ATP_i concentration, we measured the intracellular adenine nucleotide content in cardiomyocytes with and without NADH supplementation by HPLC. Figure 5 shows the summarized results. The increase in ATP content in cardiomyocytes after 4 h supplementation with NADH was highly significant (45.59±1.88 nmol mg^{-1} protein of living cells *versus* control, 35.35±2.57 nmol mg^{-1} protein of living cells, $P<0.000005$, n=7), whereas ADP and AMP showed no significant alteration compared to control.

Discussion

Almost all of the cardiac ATP is regenerated by respiratory chain-linked phosphorylation, whereby the reduction potential for the respiratory chain is mainly supplied as the reduced coenzyme NADH which is oxidized by complex I of the respiratory chain. The cellular NADH content can be influenced by extracellular supply of metabolic substrates. Recently, Williams *et al.* (2001) showed that because of the presence of glutamate during the isolation procedure, the intracellular glutamate concentration in single isolated rat myocytes is raised; this in turn increased metabolic flux as indicated by a higher NADH/NAD$^+$ ratio and ATP content as well as improved recovery from simulated hypoxia. The NADH/NAD$^+$ ratio can also be increased by the addition of other metabolic substrates like pyruvate (White & Wittenberg,

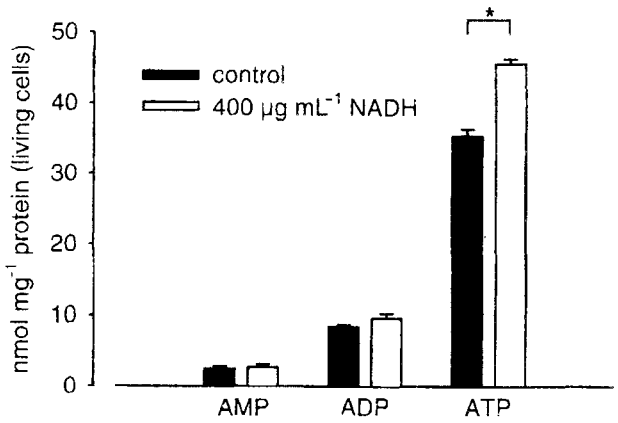

Figure 5 Adenine nucleotide content of cardiomyocytes incubated with 400 μg ml^{-1} NADH for 4 h *versus* control. The values were calculated as 100% living cells. $n=7$ in each group. $^*P<0.000005$.

2000; Williams *et al.*, 2001). The activity of cardiac K_{ATP} channels is controlled by a cytosolic ATP pool for which oxidative phosphorylation is the predominat ATP source (Knopp *et al.*, 2001). Since the respiratory chain is fuelled mainly with NADH, it was the aim of this study to investigate, whether NADH supplementation *per se* leads to an improved metabolic state of cardiomyocytes. The pinacidil-primed $I_{K(ATP)}$ was used as sensor of the subsarcolemmal ATP concentration.

Under physiological conditions, $K_{(ATP)}$ channels are predominantly in the closed state because of a strong inhibition of channel activity at an ATP_i concentration in the millimolar range (Noma, 1983; Heidbüchel *et al.*, 1990; Koumi *et al.*, 1997). These data are consistent with our observations in guinea-pig ventricular myocytes revealing no contribution of $I_{K(ATP)}$ to the basal electrical activity, since under physiological conditions the sulfonylurea glibenclamide (50 μM), a $K_{(ATP)}$ channel blocker, did not affect current–voltage relation elicited by a voltage ramp (data not shown).

In the present study, pinacidil, a $K_{(ATP)}$ channel opener (Smallwood & Steinberg, 1988; Arena & Kass, 1989), which is known to increase the open probability of the $K_{(ATP)}$ channel (Isomoto & Kurachi, 1997), activated $I_{K(ATP)}$ showing similar characteristics as described in other studies (Noma & Shibasaki, 1985; Arena & Kass, 1989; Koumi *et al.*, 1997).

Using a physiological (4.3 mM) and a low (1 mM) ATP_i concentration, we could show that pinacidil causes a significantly more pronounced activation of $I_{K(ATP)}$ at a low ATP_i concentration. These results are in line with previous observations reporting the pinacidil action to be dependent on ATP_i with increasing sensitivity to openers at lower ATP_i (Arena & Kass, 1989; Nakayama *et al.*, 1990; Tseng & Hoffman, 1990; Pelzmann *et al.*, 2001). Thus, the pinacidil-primed $I_{K(ATP)}$ serves as an indicator of subsarcolemmal ATP concentration as demonstrated by Sasaki *et al.* (2001).

The results of this study show that supplementation of guinea-pig ventricular myocytes with 300 μg ml^{-1} NADH (4–6 h) causes a significantly reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells, indicating an increased subsarcolemmal ATP concentration. These findings could be confirmed by measuring the intracellular adenine nucleotide content using HPLC, revealing a highly significant increase in ATP content in cardiomyocytes supplemented with NADH, whereas ADP and AMP did not significantly differ from control. Any direct effects of extracellular NADH on $I_{K(ATP)}$ could be excluded. Clinical studies have demonstrated a positive effect of NADH treatment on patients suffering from Morbus Parkinson, chronic fatigue syndrome and depression (Birkmayer & Birkmayer, 1991; Birkmayer *et al.*, 1993; Kuhn *et al.*, 1996; Forsyth *et al.*, 1999). Vrecko *et al.* (1997) showed that NADH supplementation of PC12 cells leads to increased dopamine production being of interest for the treatment of Morbus Parkinson which is characterized by a dopamine deficit. NADH-induced increase of dopamine release could also be shown in rat striatal slices (Pearl *et al.*, 2000). These data indicate that extracellular application of NADH results in cellular functional alterations, whereby the mechanisms are not completely understood. In nigrostriatal dopaminergic terminals release of dopamine is modulated by $K_{(ATP)}$ channels, whereby the extracellular concentration of dopamine is significantly decreased by the PCO cromakalim (Zhu *et al.*, 1999). Thus, modulation of $K_{(ATP)}$ channels obviously plays an important role in dopamine release, whereby a modulation of $K_{(ATP)}$ channel activity by NADH, as described in this work, could be of importance.

In summary, our data show that NADH supplementation, but not of the related compounds nicotinamide and NAD^+, improves the metabolic state of isolated ventricular myocytes indicated by a decreased pinacidil-primed $I_{K(ATP)}$. Measurement of adenine nucleotides confirmed a significant elevation of ATP levels in cardiomyocytes treated with NADH. The mechanism of this increase (elevated $NADH/NAD^+$ ratio, enhanced mitochondrial ATP production, enhanced intracellular reduction potential or NADH induced enzymatic alterations) is at present under investigation. NADH supplementation could be a useful pharmacological and therapeutical tool producing beneficial effects upon cell metabolism of cardiomyocytes and other high-energy-demanding cells *in vivo*.

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Bioavailability of Reduced Nicotinamide-adenin-dinucleotide (NADH) in the Central Nervous System of the Anaesthetized Rat Measured by Laser-Induced Fluorescence Spectroscopy

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Abstract: Drugs intended to increase wellness or quality of life ("lifestyle drugs") have gained popularity and/or importance over recent years. Biogenic substances like nicotinamide adenine dinucleotide (NADH) are supposed to increase the physical and intellectual performance without side-effects. NADH is an energy-delivering co-substrate in the respiratory chain. Clinical studies showed positive effects of peripherally given NADH in Morbus Parkinson and major depression. NADH can be measured by its fluorescence. In this study a pulsed N_2-laser combined with a fibre-optic probe and photomultipliers was used to induce and measure NADH fluorescence in the rat cortex. The aims of the study were to assess the suitability of the laser-induced spectroscopy for *in vivo* and on-line measurement of NADH in neuroscience and the assessment of the central availability of NADH after peripheral administration. NADH (50 mg/kg) but not the precursor nicotinamide caused a significant rise of the NADH fluorescence intensity indicating an increase of the NADH concentration in the rat cortex. In conclusion, the results suggest that NADH given orally or intraperitoneally increases the amounts of NADH in the brain. The results may thus help to explain the clinical effects reported.

Nicotinamide adenine dinucleotide (NADH) plays a central role as a co-substrate for energy transfer in the mitochondrial oxidative phosphorylation, which supplies more than 95% of the total energy requirement in the cells (Erecinska & Wilson 1982). In most cells NADH is the main source of reducing equivalents and NADH is therefore an important factor of cellular metabolism. The vitamin B3 nicotinamide is the main precursor for the synthesis of NADH molecules in eucariotic cells.

There is evidence that an additionally supply of co-factors or substrates could have beneficial effects in the diseases of the central nervous system. NADH as an energy-supplying substrate could also support enzymatic reactions. Clinical studies demonstrate the positive effect of peripherally administered NADH on serious diseases such as Parkinson's disease, chronic fatigue syndrom and major depression (Birkmayer & Birkmayer 1991; Birkmayer *et al.* 1993; Forsyth *et al.* 1999).

In connection with these clinical studies which show central effects after peripheral administration, we wanted to investigate whether peripherally administered NADH might change the central concentration of NADH.

Selective determination of NADH *in vivo* is possible due to its spectroscopic properties. NADH and the corresponding oxidized molecule NAD+ are natural fluorophores, which start to fluoresce after stimulation. The reduced and energy-rich form NADH has two absorption peaks at (λ= 260 nm and 340 nm), the oxidized form has only one absorption peak at (λ=260 nm). Stimulation of the molecules with light at wavelengths around 340 nm (ultraviolet-spectrum) leads to generation of fluorescence signals (maximum at 465 nm) that start only from the reduced molecules. The strength of the NADH fluorescence signal is proportional to the concentration of the NADH molecules. Various fluorometric methods have been developed with high powered light sources such as xenon lamps, tungsten-halogen lamps, mercury arc lamps or lasers (Renault *et al.* 1984; Ince *et al.* 1992). In this study a nitrogen-laser-fluorescence detector with spectral filters has been used to induce and measure NADH fluorescence in the cortex of rats. Inclusion of a time-gate allows the suppression of long-lifetime fluorescence in the detection range (e.g. collagen fibres). The concentrations of NADPH with NADH-like fluorescence-properties are very low and do not interfere with the determination of NADH.

The aim of our study was application of the laser-induced fluorescence spectroscopy for *in vivo* and on-line measurement of NADH in neuroscience and determination of the central availability of NADH after peripheral administration.

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Materials and Methods

Animals. Male Wistar rats (Schönwalde, Germany) 220±25 g, group-housed under a 12 hr light-dark schedule were used. All ani-

mal experiments were carried out following the principles of laboratory animal care and the German Law on Protection of Animals and were approved by the animal protection board of the State of Berlin "Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, Fachgruppe Veterinärwesen, Lebensmittelwesen und Gentechnik".

Drugs. The following substances were used: Nicotinamide-adenin-dinucleotide reduced (NADH), nicotinamide-adenin-dinucleotide oxidized (NAD+), nicotinamide (all obtained from Sigma-Aldrich) and vehicle (sodium bicarbonate buffer as stabilizer for NADH). All drugs were dissolved in the vehicle prior to use. The injection volume was 1 ml/kg body weight. For oral application NADH, nicotinamide and vehicle in tablet form were used, all delivered from Birkmayer Laboratories (Austria).

Apparatus. For the determination of changes in NADH concentrations a fluorescence detector (Laser Fluoroscope 302, IOM, Berlin, Germany) in combination with data acquisition software and a standard personal computer were used (Rex *et al.* 1999a). The detector uses a pulsed nitrogen laser (λ=337 nm, P=30 μJ, t=350 ps) as light source. The laser beam is coupled into an optical fibre (fused silica, $\varnothing$=100 μm) and applied to the cortex. A second fibre of the same diameter, positioned directly beside the 'stimulating' fibre, collects part of the fluorescence and randomly back-scattered light to lead it back to the detection unit. Temporal and spectral gating isolates the NADH fluorescence. Changes of the NADH fluorescence were determined with a high spatial solution, at least for an *in vivo* method, by the small diameter of the optical fibre and the penetrating depth of the laser light (0.5 mm) into the brain.

Testing procedure. Oral application of the tablets was performed immediately before anaesthesia with an adapted applicator for small animals (Kruuse, Denmark) about 60 min. before the start of the NADH fluorescence measurement.

Then the rats were anaesthetized with chloralhydrate (Sigma-Aldrich, 400 mg/kg intraperitoneally) and afterwards a catheter was placed in the vena femoralis. The drugs were injected at a rate of 50 μl/min.

The head of the anaesthetized animal was fixed in a stereotaxic frame (David Kopf, USA), the skull trepanated and the optic probe slowly inserted (4 mm posterior and 2 mm lateral from the intersection of the coronal and sagittal sutures of the skull (bregma), 2 mm deep, according to the stereotaxic brain atlas by Paxinos & Watson 1998). After the insertion of the optical probe the fluorescence was measured continuously. Due to the operative trauma of the brain tissue fluorescence data were used for the evaluation after reaching relatively stable levels (approximately after 120 min.). To secure steady experimental conditions, a continuous humidification of the cortical surface with oxygenised artificial cerebrospinal-fluid (CSF, pH 7.4) containing: 5 mM glucose, 125 mM NaCl, 27 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 1.2 mM Na_2HPO_4, 0.5 mM $NaSO_4$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ was maintained.

At the end of the experiment the animals were killed quickly by an overdose of the anaesthetic administered intravenously. Following death, the utilization of NADH in the respiratory chain stopped and the intensity of NADH fluorescence increased rapidly (within the next 2 min.) and reached a stable plateau for 15 min. (fig. 1). The mean (n=10 animals) of this maximal increase in the NADH fluorescence was used as the reference point for the pharmacological manipulations and set to 100 per cent.

All animals, which did not show this typical *post mortem* increase in the NADH fluorescence (Mottin *et al.* 1997), were excluded from the study.

Data handling. The fluorescence data represent the changes in per cent of the maximally observed changes in the fluorescence intensity (overdose of the anaesthetic). The basal level of fluorescence measured before a pharmacological manipulation was set to zero.

For the minimization of haemodynamic or tissue-modulated effects on the fluorescence measured, a compensation method based on the measurement of the back-scattered light can be used to detect changes in the NADH fluorescence closely related to metabolic changes. We used a compensation method gained by subtraction of the scattered light from the NADH fluorescence (F): ($F^{450\ nm}$ - $SL^{337\ nm}$) (Harbig *et al.* 1976; Mayevsky 1984).

The intensity of the fluorescence depends on the intensity of the excitation light. To exclude artefacts induced by changes in the excitation intensity by the laser energy, the fluorescence measured was set in relation to the power of the laser.

Statistics. The data were analysed using an one-way ANOVA on repeated measures. A difference of the means with an error probability of $P<0.05$ was considered significant. Data are presented as mean±S.E.M. To ensure clarity in fig. 2 and 4 data are presented as mean and the range of the S.E.M. is stated in the legends to figures.

Results

Following insertion of the optical probe into the cortex the intensity of the NADH fluorescence declined for the first 120 min. and then reached a stable level with a variation of the fluorescence less than two percent in untreated controls.

Application of NADH (50 mg/kg) intraperitoneally induced an increase in the intensity of the cortical NADH fluorescence of about 18±3.8% for approximately 30 min. compared to the fluorescence intensity in the control group, while a lower dose of NADH (10 mg/kg intraperitoneally) did not show any effect (fig. 2).

Neither NAD+ (10 mg/kg, 50 mg/kg) nor nicotinamide (10 mg/kg, 50 mg/kg) had a significant effect on the NADH fluorescence in the cortex during the entire measurement (120 min. after the last manipulation) (fig. 2).

Following oral application of NADH (2 tablets ENADA of 5 mg NADH=51±1.1 mg/kg) the cortical fluorescence intensity at the beginning of the measurements (60 min. after application) was increased compared to the NADH

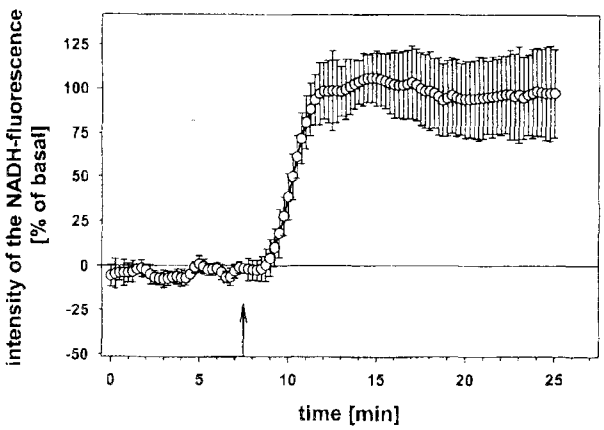

Fig. 1. Change in the intensity of the cortical NADH fluorescence following administration of an overdose of chloralhydrate compared to basal levels. The maximal increase of NADH fluorescence was set to 100 per cent. Data are corrected for scattered light and expressed as means±S.E.M. (n=10).

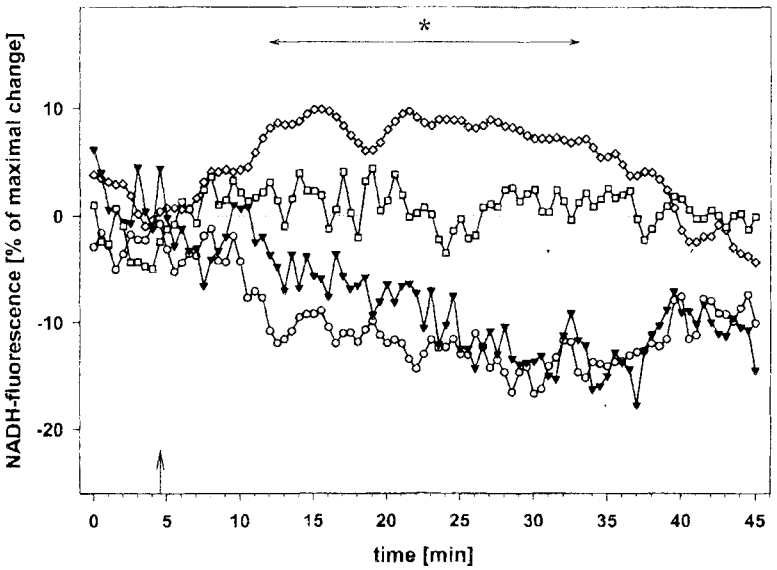

Fig. 2. Effects of NADH (◆, 50 mg/kg, intraperitoneally, S.E.M.±4.1%), NAD+ (▽, 50 mg/kg, intraperitoneally, S.E.M.±7.8%) and nicotinamide (□, 50 mg/kg, intraperitoneally, S.E.M.± 4.6%) on the cortical NADH fluorescence. Data are expressed as mean (n=10). (ONE WAY-ANOVA plus Dunnett's test, *P<0.05, compared to controls, ○, S.E.M.±8.2%).

fluorescence in the vehicle-treated controls (fig. 3). Administration of nicotinamide had no effect on the intensity of the measured NADH fluorescence (fig. 3), however, there was no alteration in the general course of the fluorescence intensity over the time the NADH fluorescence was measured, comparing the different treatments.

Intravenous application of NADH (10 mg/kg) induced an immediate rise (17±4.6% of the maximal increase) in the intensity of the cortical NADH fluorescence for about 2.5 min. Following application of a higher dose NADH (50 mg/kg) this increase in the fluorescence intensity (19±5.1% of the maximal increase) was prolonged to about 9 min. (fig. 4).

After intravenous application of NAD+ (10 mg/kg) a rise in the intensity of NADH fluorescence (16±3.9% of the maximal increase) could be observed for about 3 min. Application of 50 mg/kg NAD+ induced an increase of NADH fluorescence (20±4.8% of the maximal increase) for about 9 min. comparable to the effect of NADH (fig. 4).

Discussion

Biochemical and histochemical methods for the determination of NADH *in vitro* are routine (Bergmeyer & Bernt 1974). In addition to the already established techniques, spectroscopic procedures for measurement of NADH concentrations *in vivo* were developed (Chance *et al.* 1962; Ince *et al.* 1992; Mayevski *et al.* 1996). For selective determination of the NADH fluorescence in our study, laser-induced fluorescence spectroscopy was used (Beuthan *et al.* 1990; Fink *et al.* 1993).

In a previous study we have shown that the measurement of changes in NADH fluorescence with laser technique allows determination of changes in CNS metabolism with high regional selectivity and time resolution *in vivo* (Rex *et al.* 1999a). In the study sodium cyanide, a potent blocker of the respiratory chain, induced an increase in the cortical NADH fluorescence comparable to increased intensity following administration of an overdose of the anaesthetic chloralhydrate. Similar changes could be demonstrated in *in vitro* experiments showing inhibition of NADH consumption after treatment with potassium cyanide (Gille & Nohl 2000). Pathophysiological manipulations as the induction of a cortical spreading depression (Rex *et al.* 1999a) or pharmacological treatment with the serotonin agonist 8-

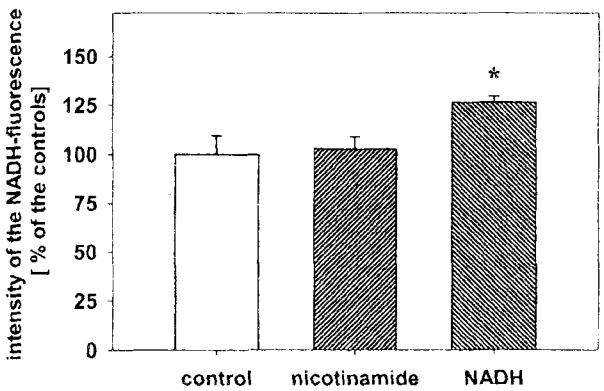

Fig. 3. Effects of NADH (50 mg/kg orally) and nicotinamide (50 mg/kg orally) on the cortical NADH fluorescence directly after implantation of the optical probe into the cortex (60 min. after drug application) compared to the NADH fluorescence of the controls. Data are expressed as mean±S.E.M. (n=10) (*P<0.05, compared to controls).

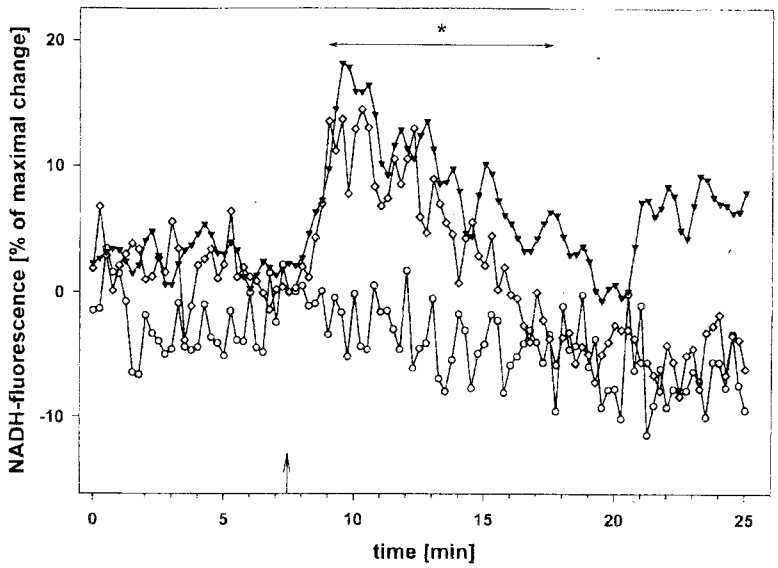

Fig. 4. Effects of NADH (◆, 50 mg/kg, intravenously, S.E.M.±5.6) and NAD+ (▽, 50 mg/kg, intravenously, S.E.M.±6.3) on the cortical NADH fluorescence. Data are expressed as mean (n=10) (*P<0.05, compared to controls, ○, S.E.M.±5.2).

OH-DPAT induces smaller changes in the intensity of the NADH fluorescence (Rex *et al.* 1999b). However, these relatively small changes may be important for the metabolism and function of tissues.

Assessment of the bioavailability of NADH shows a possible application of the laser fluorescence spectroscopy in neuropharmacological studies.

The results of the present study show a significant rise of the measured NADH fluorescence intensity in the cortex of rats after peripheral application of NADH.

To reach the central nervous system, the NADH molecules have to pass two membranes in the processes of absorption and distribution: The membranes in the gastrointestinal tract and the blood-brain barrier between the cerebral vessels and the brain tissue. To our knowledge there are no experimental results on the passage of NADH through the blood-brain barrier.

To show possible central effects of a drug, intravenous administration offers one advantage. The distribution of the drug into the brain is hindered only by the blood-brain barrier. An expected effect of the substance in the brain is not affected by reduced absorption and/or breakdown in the periphery.

The rapid increase in NADH fluorescence as observed after intravenous administration of NADH and as shown in fig. 4 suggests that NADH leaves the blood circulation and reaches the brain tissue.

It could be argued, however, that the NADH measured remains in the blood vessels in the brain, but the following arguments are in favour for passage through the blood-brain barrier.

It is known that haemoglobin absorbs both the excitation light as well as the emitted NADH fluorescence, hence NADH fluorescence measurement in blood vessels is not possible. Measurement of NADH fluorescence can be done only in vessel-free or vessel-poor areas. In addition, blood-vessel restricted rise of the NADH concentration can be excluded since an increase in the dosage given intravenously with the same injection volume leads to prolonged increase in fluorescence, but has no effect on the intensity of the fluorescence. If the increase of NADH is limited to the vessels, only the strength of the fluorescence signal would increase.

An increase in the NADH fluorescence following intraperitoneal and oral administration suggests that NADH molecules might be also absorbed from peritoneum and to a smaller degree also from the intestine as shown in the fig. 2 and 3, respectively.

The intraperitoneal injection is a standard administration of drugs to rodents in experimental pharmacology. The big surface and the good vascularization of the peritoneum ensure a good absorption of substances through the peritoneal membrane (Scheler 1989). Encouraged by the intestine movements, NADH injected into the peritoneal cavity spreads fast in the peritoneum and thus might be absorbed quickly. Intraperitoneal injection avoids degradation of the NADH through enzymes located in the digestion tract.

Absorption of NADH after oral application could be assumed by the clearly raised NADH fluorescence at the beginning of the experiment, as seen in fig. 3. The long period between the application and the measurement of the NADH fluorescence can be responsible for the relatively small changes in the fluorescence intensity of NADH. This interval however, is mandatory due to the duration of the surgery (approximately 60 min.).

The slow absorption from the gastrointestinal tract and subsequently lower concentrations in the portal vein (same amount of drug over a longer timespan) could also enhance

the effects of metabolizing enzymes in the liver leading to a relatively small bioavailability of NADH after oral application.

However, it has not been verified that the NADH molecules that cause the increased fluorescence in the cortex are identical with the injected NADH molecules. The occurrence of NADH in the periphery could also induce a change in the lactate/pyruvate ratio, since the Krebs cycle is under only minor control by NADH (Lance *et al.* 1985). Pyruvate and lactate can pass the blood-brain barrier and induce an increase in the NADH formation in the CNS.

The oxidized pyridine nucleotide NAD+ is converted within cells from nicotinamide. The pyrimidine ring in the NAD+ molecule accepts a hydrogen atom, and is able to stabilize the accepted electron. Hence, NAD+ can be reduced to NADH, which can proceed in reactions by carrying an electron and a hydrogen ion.

Intravenous administration of NAD+ causes a rise in the intensity of the NADH fluorescence. This increase in NADH fluorescence after the intravenous injection may point to a steady-state existing in idle status between the low-energy form NAD+ and the high-energy form NADH in the tissue so that a rapid and one-sided supply of NAD+ might lead to a partial reduction of NAD+ to NADH.

After intraperitoneal injection of the oxidized form NAD+, no modification of the NADH fluorescence in the cortex could be observed. This may be attributed to an impaired absorption of the positively loaded molecule.

Nicotinamide (vitamine B3) is a precursor of NADH in the biochemical pathway and is an integral component of the NADH molecule.

Since we know that nicotinamide is absorbed rapidly and completely following oral application (Stratford *et al.* 1996), intravenous administration of nicotinamide was not needed. Nicotinamide did not have any effect on the cortical NADH fluorescence. One possible explanation for the lack of effect of nicotinamide in our investigations could be either the pretreatment time and/or the single administration given. This interpretation would be in line with a study where a single injection of nicotinamide (500 mg/kg intraperitoneally) did not increase the cortical concentration of NADH in the CNS of rats (Klaidman *et al.* 2001). It is possible that chronic treatment with nicotinamide could increase the synthesis of NADH, but to our knowledge no data are available.

Our results stand in accordance with clinical studies, in which beneficial central effects of oral NADH in patients with Parkinson's disease and major depression were verified (Birkmayer *et al.* 1993; Forsyth *et al.* 1999).

The fact that the fluorescence measurements were carried out in anaesthetized animals with reduced metabolic activity should not be neglected. These animals have smaller NADH consumption and higher NADH pool. In principle measurement of NADH in awake and freely moving animals is possible and should be further investigated.

In conclusion, the results prove that the applied method for *in vivo* measurement of NADH fluorescence by laser-induced fluorescence spectroscopy is a useful method for *in vivo* and on-line measurement of NADH in neuroscience and could be a suitable tool for pharmacological and pharmaceutical studies.

Acknowledgements

This study was supported by Birkmayer Laboratories Vienna.

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Stimulation of dopamine biosynthesis in cultured PC 12 phaeochromocytoma cells by the coenzyme nicotinamide adeninedinucleotide (NADH).

Vrecko K, Birkmayer JG, Krainz J.

Institute for Medical Chemistry, University of Graz, Austria.

The activity of the tyrosine hydroxylase, the enzyme which is diminished in the brains of Parkinson patients, has been measured in cultured PC 12 rat phaeochromocytoma cells. In the same way dopamine content in the medium after incubating these cells with or without NADH was assayed. The experiment shows that NADH is able to increase the activity of the tyrosine hydroxylase and dopamine - production in PC 12 cells up to 6 times. The results provide evidence that NADH is able to stimulate dopamine - biosynthesis directly.

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Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. [J Neurochem Res. 1998]

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Therapeutic effects of oral NADH on the symptoms of patients with chronic fatigue syndrome.

Forsyth LM, Preuss HG, MacDowell AL, Chiazze L Jr, Birkmayer GD, Bellanti JA.

Department of Pediatrics, Georgetown University School of Medicine, Washington, D.C., USA.

BACKGROUND: Chronic fatigue syndrome (CFS) is a disorder of unknown etiology, consisting of prolonged, debilitating fatigue, and a multitude of symptoms including neurocognitive dysfunction, flu-like symptoms, myalgia, weakness, arthralgia, low-grade fever, sore throat, headache, sleep disturbances, and swelling and tenderness of lymph nodes. No effective treatment for CFS is known. OBJECTIVE: The purpose of the study was to evaluate the efficacy of the reduced form of nicotinamide adenine dinucleotide (NADH) i.e., ENADA the stabilized oral absorbable form, in a randomized, double-blind, placebo-controlled crossover study in patients with CFS. Nicotinamide adenine dinucleotide is known to trigger energy production through ATP generation which may form the basis of its potential effects. METHODS: Twenty-six eligible patients who fulfilled the Center for Disease Control and Prevention criteria for CFS completed the study. Medical history, physical examination, laboratory studies, and questionnaire were obtained at baseline, 4, 8, and 12 weeks. Subjects were randomly assigned to receive either 10 mg of NADH or placebo for a 4-week period. Following a 4-week washout period, subjects were crossed to the alternate regimen for a final 4-week period. RESULTS: No severe adverse effects were observed related to the study drug. Within this cohort of 26 patients, 8 of 26 (31%) responded favorably to NADH in contrast to 2 of 26 (8%) to placebo. Based upon these encouraging results we have decided to conduct an open-label study in a larger cohort of patients. CONCLUSION: Collectively, the results of this pilot study indicate that NADH may be a valuable adjunctive therapy in the management of the chronic fatigue syndrome and suggest

that further clinical trials be performed to establish its efficacy in this clinically perplexing disorder.

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EINFLUSS EINER NADH-SUPPLEMENTATION AUF DIE MUSKULÄRE ENERGIEBEREITSTELLUNG BEIM MENSCHEN

D Grathwohl, M Klann, HM Müller, H Schlachter und A Berg

Universitätsklinikum Freiburg, Abt. für Rehabilitative und Präventive Sportmedizin

Hintergrund: NADH ist einer der wichtigsten Elektronen-Transporter bei der Oxidation von Nährstoffen und als essentielles Coenzym in jeder Zelle vorhanden. Biologisch ist NADH damit unersetzbar für die ATP-Produktion und Zellregulation, die DNA-Reparartur, das zelluläre Immunsystem sowie die antioxidative Regulation. Die Leistungsfähigkeit der Zelle und ihre zellspezifische Kompetenz ist also mit ihrem Gehalt an NADH korreliert. Die von uns untersuchte Substanz ist eine stabilisierte Form des NADH (reg.Patent, Birkmayer, Wien), die oral aufgenommen und resorbiert am zellulären Wirkort NADH-verbrauchende Vorgänge unterstützen und das ATP-Angebot begünstigen soll. Es wird angenommen, daß der Wirkstoffs einerseits über die Steigerung der Transmitter-Produktion, andererseits über die Verbesserung des aeroben Zellstoffwechsels und der Energieausbeute wirkt. Zur Sicherung dieser Hypothese wurden in einem kontrollierten Design gesunde Sportlern auf mögliche metabolisch-energetische Einflüsse von stabilisiertem NADH über Parameter der muskulären Leistungsfähigkeit und systemischen Stressregulation untersucht.

Methoden

In einer placebo-kontrollierten Cross-over-Studie wurden 14 Ausdauersportler (18-49 J., VO_{2max} > 55ml/kg/min) mit stabilem Trainings-, Ernährungs- und Gewichtsverhalten untersucht; die Einnahme von Placebo- bzw. Verumkompretten (Tagesdosierung 30mg als 2x3 Tab.) erfolgte über jeweils 4 Wochen bei 6 wöchiger Wash-out-Phase. Untersuchungen wurden jeweils zu Beginn und nach der Interventionsphase mit jeweils 2 Untersuchungen vor und nach Cross-over durchgeführt; zu jeder Untersuchung wurde die maximale aerobe Kapazität (Spiroergometrie) mit begleitender Leistungsdiagnostik (Herzfrequenz, Laktat, Catecholamine) bestimmt und eine Beurteilung der Muskel- und Ganzkörperantwort im standardisierten Ausdauertest als Intensitäts-gesteuerte-Ausdauer-Laufbandbelastung über validierte und definierte Prüfparameter vorgenommen. Trainingsverhalten, Wohlbefinden und Gesundheitszustand wurden protokolliert.

Ergebnisse

Im Studienverlauf wurden keine unerwünschte Nebenwirkungen oder Beeinflussungen von Sicherheitsparametern beobachtet. Drop-outs lagen ebenfalls nicht vor. Folgende Veränderungen, die in der vergleichenden Statistik (zwei-seitiger Test für gepaarte Stichproben) im Trend liegende ($p<0.1$) oder gesicherte ($p<0.05$) statistische Unterschiede als Treatment-Effekt aufwiesen, können im Sinne der oben genannten Wirkhypothese herausgestellt werden:

Im metabolisch-energetischen Bereich: Die Reduzierung des O_2-Verbrauchs und Erhöhung des RQ bei definierter Arbeit, die Reduzierung der CO_2-Abgabe und der Blut-Laktatspiegel bei Ausdauerarbeit sowie die Erhöhung der freien Fettsäuren unter Nüchtern-Ruhe-Bedingungen.

Im metabolisch-regulativ Bereich: Die Reduzierung des belastungsinduzierten Plasma-Kaliumspiegels bei definierter und auch Ausdauerarbeit, die Reduzierung der Plasma-Kreatininspiegel und des Serum-CK-Anstiegs unter Ausdauerarbeit.

Im Bereich der systemischen Stressindikatoren: Die Reduzierung der Plasma-ACTH- und Noradrenalinspiegel sowie des Leukozytenanstiegs unter Ausdauerarbeit.

Trägt man zusätzlich die Einzelwerte für VCO_2 und VO_2 pro Atemzug in einem Scattergramm auf (Abb.1) und fittet diese über eine Gleichung 3.Grades, so läßt sich über die Koeffizienten der jeweiligen Gleichungen mit der Behandlung ein gesicherter Treatmenteffekt von 7% für die Energiebereitstellung aufzeigen.

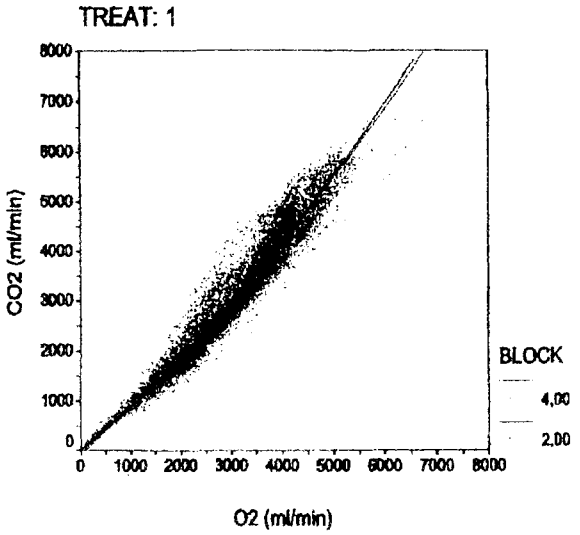

Abb.1: Scattergramm für die im Stufentest gemessenen VCO_2 und VO_2 Einzelwerte (ml/min) mit Übergang von Placebo zu Verum; in jeder der Punktwolken liegt eine signifikant andere Kurve

Schlussfolgerung

Die Reduzierung des O_2-Verbrauchs und die Verschiebung des CO_2/O_2-Scattergramms deuten auf eine verbesserte zelluläre O2-Utilisation hin; diese wird möglicherweise über eine vermehrte zelluläre Verfügbarkeit von NADH und ein darüber erhöhtes ATP-Angebot erreicht; als Fazit nimmt dadurch die aerobe Arbeitsökonomie unter Körperarbeit zu. Dies wird durch begleitende Beobachtungen wie die Reduzierung der Blut-Laktatspiegel bei Ausdauerarbeit sinngemäß unterstützt. Auch indirekte Parameter zur Beurteilung der zellulären ATP-Bereitstellung (belastungs-induzierter Anstieg für Kalium, Kreatinin, CK) deuten auf eine mögliche Verbesserung des ATP-Angebotes bzw. der Membranstabilitat hin.

Da die hier vorgestellten Ergebnisse allerdings explorativ erhoben wurden, wäre jetzt die Bestätigung der postulierten Wirkhypothese in einem konfirmatorischen Ansatz sinnvoll. Ebenso sollten basierend auf den hier vorliegenden Ergebnissen Studien initiiert werden, um den mitochondrialen Wirkansatz und dessen klinische Bedeutung experimentell zu überprüfen.

Effects of nicotinamide adenine dinucleotide hydride on physical and mental performance

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Abstract

The aim of this study was to assess the effects of nicotinamide adenine dinucleotide hydride (NADH) on maximal oxygen uptake ($[Vdot]O_{2max}$), maximal anaerobic running time, and mental performance. Eight men were exposed to a supplement treatment (30 mg NADH as ENACHI tablets per day) and to a placebo treatment, each of 4 weeks' duration, in a balanced, double-blind, and cross-over design. The two treatments were separated by a 14-week wash-out period. The results indicated that $[Vdot]O_{2max}$, maximal anaerobic running time, and the ability to concentrate were similar in the NADH and placebo conditions. There were also no differences in blood lactate, creatine kinase, reaction time or feelings of fatigue between the treatments. A counter-movement jump performed at rest and 2 min after the aerobic test differed significantly ($P \leq 0.05$) between the treatment conditions and was higher in the NADH group. In conclusion, the NADH supplementation for 4 weeks had no effects on $[Vdot]O_{2max}$, maximal anaerobic running time or mental performance.

Keywords: NADH; endurance; strength; reaction time; lactate concentration

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G. D. Birkmayer, G. G. Kay und *E. Viirre*

Schlüsselwörter: Jetlag – Hirnleistungstest – NADH – Schlafdefizit.

Keywords: Jet lag – cognitive testing – NADH – fatigue countermeasures.

Zusammenfassung: Jetlag ist ein Zustand, der eintritt, wenn man mehrere Zeitzonen, insbesondere gegen die Uhr, überfliegt. Die Symptome sind generelle Müdigkeit, Abgeschlagenheit, gestörter Schlafrhythmus und reduzierte Hirnleistung. Mittel gegen Jetlag wie Melatonin, Koffein, Sedativa oder Phototherapie sind von begrenzter Wirksamkeit oder Praktikabilität. Die Wirksamkeit der stabilisierten sublingualen Form von reduziertem Nikotinamid-Adenin-Dinukleotid (NADH, ENADAlert®, Menuco Corp.) wurde als Gegenmittel für Jetlag-Symptome in einer doppelblinden, placebokontrollierten wissenschaftlichen Studie geprüft. 35 gesunde Personen wurden von der Westküste der USA über Nacht an die Ostküste geflogen, was zu einem Zeitunterschied von drei Stunden führt. Am Abend vor dem Abflug und in der Früh nach der Landung wurden alle Probanden einem Hirnleistungstest unterzogen. Eine Gruppe erhielt 20 mg ENADAlert®, die andere Gruppe identische Placebotabletten. Alle Teilnehmer der Studie beendeten die computergesteuerten Hirnleistungstests (Cog Screen), die die Hirnfunktion, die Stimmung, den Schläfrigkeitsgrad am Morgen und zu Mittag nach dem Flug messen. Jetlag führte zu einer erhöhten Schläfrigkeit bei mehr als der Hälfte der Teilnehmer und zu einer Verschlechterung der Hirnleistungsfunktionen. Am Morgen nach dem Nachtflug kam es zu Fehlern im Aufmerksamkeitstest sowie zu Störungen im Arbeitsgedächtnis, der geteilten Aufmerksamkeit und der Geschwindigkeit der visuellen Perzeption. Probanden, die NADH erhielten, hatten in vier Hirnleistungstests signifikant bessere Ergebnisse ($P \leq .05$) und berichteten eine geringere Schläfrigkeit im Vergleich zu den Probanden, die Placebo einnahmen. Die Gruppe, die NADH-Tabletten bekam, hat keine Nebenwirkungen beobachtet.
Zusammenfassend kann gesagt werden, daß stabilisiertes sublingual absorbierbares NADH (ENADAlert®) die durch Jetlag induzierten negativen Wirkungen auf die Hirnleistung und die Schläfrigkeit signifikant verbessert.
(Wien. Med. Wschr. 2002;152;450–454)

Stabilized NADH as a Counter Measure for Jet Lag

Summary: Current remedies for jet lag (phototherapy, melatonin, stimulant, and sedative medications) are limited in efficacy and practicality. The efficacy of a stabilized, sublingual form of reduced nicotinamide adenine dinucleotide (NADH, ENADAlert®, Menuco Corp.) as a countermeasure for jet lag was examined. Because NADH increases cellular production of ATP and facilitates dopamine synthesis, it may counteract the effects of jet lag on cognitive functioning and sleepiness. Thirty-five healthy, employed subjects participated in this double-blind, placebo-controlled study. Training and baseline testing were conducted on the West Coast before subjects flew overnight to the East Coast, where they would experience a 3-hour time difference. Upon arrival, individuals were randomly assigned to received either 20 mg of sublingual stabilized NADH ($n = 18$) or identical placebo tablets ($n = 17$). All participants completed computer-administered tests (including Cog Screen) to assess changes in cognitive functioning, mood, and sleepiness in the morning and afternoon. Jet lag resulted in increased sleepiness for over half the participants and deterioration of cognitive functioning for approximately one third. The morning following the flight, subjects experienced lapses of attention in addition to disruptions in working memory, divided attention, and visual perceptual speed. Individuals who received NADH performed significantly better on 4 cognitive test measures ($P \leq .05$) and reported less sleepiness compared with those who received placebo. No adverse effects were observed with NADH treatment. Stabilized NADH significantly reduced jet lag-induced negative cognitive effects and sleepiness, was easily administered, and was found to have no side effects.

Einleitung

Jetlag ist ein Zustand, der auftritt, wenn man mehrere Zeitzonen, insbesondere gegen die Uhr, in kurzer Zeit überfliegt. Es beeinflußt in großer Zahl Vielreisende, Flugbegleiter wie Piloten. Die Symptome von Jetlag sind allgemeine Müdigkeit und Niedergeschlagenheit, gestörter Schlafrhythmus, gastrointestinale Beschwerden und verminderte Hirnleistungen. *Rosekind* schätzt, daß Jetlag die Fähigkeit, Entscheidungen zu treffen, was Kommunikation und Gedächtnis betrifft, bis zu 70 % reduziert (14). Die Störung des 24-Stunden-Rhythmus, der Schlafinitiation und anderer Aktivitäten des Tages- und Nachtrhythmus ist der Auslöser für Jetlag (6, 17). Zu den Vielfliegern zählen neben den Piloten und den Flugbegleitern Soldaten, Athleten und Manager. Sie müssen in den meisten Fällen in bester Form ihre Aufgaben am Ankunftsort erfüllen. Die Probleme und die Menschen, die an Jetlag leiden, haben in den letzten Jahren deutlich zugenommen, weil viele Berufsreisende viel öfter fliegen, aber weniger Tage an ihrem Zielort bleiben, um sich dort umstellen zu können. Die Forschung auf dem Gebiet des Jetlags hat sich auf Methoden konzentriert, den zirkadianen Rhythmus auf die neuen Zeitzonen zu adaptieren. Diese Methoden inkludieren Veränderungen des Schlafrhythmus, Phototherapie, Einnahme von Sedativa und/oder Stimulantien wie Koffein (10, 13, 18). Diese Methoden haben Nebenwirkungen und einige sind äußerst unpraktisch in der Anwendung (5). Ein alternativer Zugang, die Symptome des Jetlags zu vermindern, ist, Vielflieger mit zusätzlicher Energie zu versorgen, während die Person sich an die neue Zeitzone adaptiert. Eine Methode, um diesen Menschen Energie zuzuführen, ist das energieproduzierende Co-Enzym NADH (auf Beta-Nikotinamid-Adenin-Dinukleotid reduzierte Form, auch Co-Enzym 1 genannt). In dieser Studie wurde die Wirkung von stabilisiertem, sublingual absorbierbaren NADH auf die Hirnleistung von gesunden Probanden am Tag nach einem Flug über ganz Amerika untersucht. Die sublinguale Form von NADH (ENADAlert®, Menuco Corp., New York) wurde ausgewählt, um die maximale Absorption, optimale Bioverfügbarkeit und den raschen Wirkungseintritt dieses Co-Enzyms zu gewährleisten. In früheren Studien konnte gezeigt

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werden, daß NADH die Produktion von Dopamin und Noradrenalin in Nervenzellen stimuliert und die zelluläre Energieproduktion durch Bildung von ATP verstärkt (1, 12). Außerdem spielt NADH eine Schlüsselrolle bei der Reparatur von geschädigter DNA und stellt aufgrund seiner biochemischen Eigenschaften ein potentes Antioxidans dar (19). Alle diese Eigenschaften von NADH haben uns verleitet, die Wirkung dieser Substanz als Gegenmittel für Jetlag zu untersuchen. Klinische Studien haben gezeigt, daß oral absorbierbares NADH sehr wirksam ist, die Symptome des chronischen Müdigkeitssyndroms, der Parkinson-Krankheit, der Alzheimer-Krankheit und der Depression zu reduzieren (2, 3, 4, 7). In diesen Studien wurde NADH oral bis zu 6 Monate verabreicht. Dabei wurde klinisch eine Verbesserung des Energieniveaus und der Hirnleistungen festgestellt, ohne daß Nebenwirkungen beobachtet werden konnten.

Methoden

36 Probanden zwischen 35 und 55 Jahren in gutem Gesundheitszustand wurden in die Studie aufgenommen. Ausschlußkriterien waren Alkohol- oder Drogenabusus, BMI >30 kg/m^2, Flugkrankheit, Schwangerschaft, Rauchen oder Schlafstörungen. Probanden war es nicht erlaubt, Antidepressiva, ZNS-Stimulantien, Neuroleptika, Ginseng, Ginkgo biloba, Melatonin, Phosphatylcholine, N-Acetyl-Carnitin oder andere Medikamente oder Nahrungsergänzungsmittel, die die Hirnleistung verstärken können, innerhalb der 90 Tage vor der Studie einzunehmen. Während der Studie war es den Probanden nicht erlaubt, Kaffee, Alkohol oder irgendwelche Medikamente einzunehmen, die die Funktion des Zentralnervensystems verstärkten oder reduzierten.

Ablauf der Studie

Die Probanden kamen um 12 Uhr mittags am Tag des Abflugs nach San Diego, California, USA. Die Hirnleistungstests wurden den Probanden erklärt. Jeder erhielt einen Laptop Computer (IBM Thinkpad Model 760) und wurde in die Hirnleistungstests eingeführt. Um 3 Uhr nachmittag führten alle Probanden die Hirnleistungstestbatterie durch. Das Ergebnis wurde als Ausgangswert festgehalten. Die Probanden wurden dann zum Flughafen San Diego transportiert und nach Phoenix/Arizona geflogen. Dort erhielten sie ein Abendessen und um 20:30 Uhr wurde der Computer-Hirnleistungstest von ihnen nochmals durchgeführt. Danach wurden sie zum Flugplatz gebracht und verließen Phoenix um 22:30 Uhr Richtung Baltimore/Maryland. 30 Minuten nach dem Start wurden die Probanden gebeten, einen Teil der Hirnleistungstestbatterie durchzuführen. Der Flug von Phoenix nach Baltimore dauerte etwa vier Stunden. Die Zeitdifferenz zwischen San Diego und Baltimore beträgt drei Stunden. Die Lokalzeit in Baltimore bei der Ankunft war 06:00 Uhr morgens. Dort erhielten die Probanden ein Frühstück und wurden mit einem Bus nach Washington gebracht, wo sie etwa um 8 Uhr morgens ankamen. Dort erhielt eine Gruppe der Probanden sublinguales NADH 20 mg (ENADAlert®), die andere Gruppe erhielt die gleiche Anzahl von identischen Placebotabletten. 90 Minuten nach der Einnahme der Tabletten, also um 09:30 Uhr Ortszeit, mußten die Probanden den Hirnleistungstest, den sie in San Diego um 3 Uhr nachmittag durchgeführt hatten, wiederholen. Um 12:30 Uhr Ortszeit wurde der gleiche Hirnleistungstest noch einmal von allen Probanden wiederholt. Das Ende der Studie war um 14 Uhr Ortszeit.

Hirnleistungstests

Kay Continous Performance Test (KCPT)

Dieser Test mißt die Daueraufmerksamkeit und die Vigilanz. Bei diesem Test beobachten die Probanden den Computermonitor und reagieren nur, wenn ein bestimmtes Symbol mit sehr niedriger Frequenz (etwa 5%) am Bildschirm erscheint (9). Die Fehler wurden vom Computer registriert und danach ausgewertet.

Cog Screen

Folgende Untertests der Cog-Screen-Testbatterie wurden von den Probanden durchgeführt:

- Shifting attention ist ein Test zur Messung des arbeitenden Gedächtnis. Der Proband liest am Computer eine Zwei-Worte-Instruktion und wendet diese Instruktion auf dem nächstfolgenden Bildschirm an. Dieser Test mißt die Genauigkeit, die Zahl der korrekten Antworten pro Minute und die mittlere Reaktionszeit für die korrekten Antworten.
- Test der früheren Nummer: Dieser Test mißt das arbeitende Gedächtnis. Der Proband trägt die früher erscheinenden Nummern, z. B. 1, 2 oder 3 ein, wenn die nächste Nummer erscheint. Die Genauigkeit der Antwort und die Zahl der korrekten Antworten pro Minute werden aufgezeichnet.
- Vergleichstest (matching to samples test): Dieser Test mißt die Geschwindigkeit, mit der die visuelle Perzeption und das Arbeitsgedächtnis arbeiten. Probanden sehen ein vier mal vier schachbrettartiges Muster und am folgenden Bildschirm mehrere ähnliche schachbrettartige Muster, wobei eines mit dem vorigen ident ist. Die Genauigkeit der Antworten und die Zahl der korrekten Antworten pro Minute sowie die mittlere Reaktionszeit der korrekten Antworten werden registriert.
- Vergleich der visuellen Abfolge (visual sequence comparison): Dieser Test mißt die visuelle Prozessierung von Ziffern- und Buchstabenfolgen. Die Genauigkeit der Antworten und die Zahl der korrekten Antworten pro Minute und die mittlere Reaktionszeit für korrekte Antworten werden registriert.
- Zweifachaufgabentest (dual task test): Er dient zur Messung der psychomotorischen Funktionsfähigkeit. Die Aufgabe des Probanden ist, den Cursor eines Computers, der auf einer horizontalen Linie nach links und rechts oszilliert, durch die Bedienung der linken und rechten Cursortaste in eine zentrale Position zu bringen. Der mittlere absolute Fehler, Abweichung vom Mittelpunkt und die Zahl der Fehler werden registriert.
- Markierte-Nummern-Test (complex cognitive assessment battery) (CCAB): Dieser Computertest mißt das Arbeitsgedächtnis und die geteilte Aufmerksamkeit (15). Der Proband identifiziert und markiert eine Ziffer in einem Diagramm entsprechend den Instruktionen (z. B. „Markiere alle geraden Nummern zwischen 20 und 46"). Während dieser Aufgabe wird der Proband unterbrochen und instruiert, die größere oder kleinere von zwei blinkenden Ziffern festzustellen und zu markieren. Nach dieser zweiten Aufgabe setzt der Proband mit der ersten Aufgabe fort. Das Gesamtergebnis, das sich zusammensetzt aus der Gesamtzahl der korrekten Markierungen, der Geschwindigkeit, die Aufgabe zu beenden und der Leistung der zweiten Aufgabe sowie die mittlere Reaktionszeit, in der auf die zweite Aufgabe reagiert wird, werden registriert.

Automatisierte neuropsychologische Assessment Matrix (ANAM) (9)

- Running Memory Test: Dieser Test mißt das Arbeitsgedächtnis. Die Probanden werden instruiert, ob ein Buchstabe, der auf dem Bildschirm gezeigt wird, der gleiche ist wie der auf dem vorigen Bildschirm. Die Genauigkeit der Antworten und die Zahl der richtigen Antworten pro Minute sowie die mittlere Reaktionszeit der korrekten Antworten werden registriert.
- Mathematischer Test: Dieser Test mißt das arbeitende Gedächtnis und die Fähigkeit, mathematische Schlußfolgerungen zu ziehen. Den Probanden werden drei Nummern gezeigt und zwei Operationszeichen (z. B. 3 + 5 – 2) und er wird instruiert zu entscheiden, ob das Ergebnis dieser Rechenoperation größer oder kleiner als 5 ist. Die Genauigkeit der Antworten sowie die Zahl der korrekten Antworten pro Minute und die mittlere Reaktionszeit der korrekten Antworten werden registriert.

Selbstbeurteilungsskalen der Stimmung (Walter Reed Mood Scale)

Die Probanden bekommen am Bildschirm eine Abfolge von Eigenschaftswörtern aufgelistet und sollen bejahen oder verneinen, ob diese ihre gegenwärtige Stimmung wiedergibt (9). Die Eigenschaftswörter reflektieren auch Müdigkeit und Aktivität.

Schläfrigkeitsskala (Stanford Sleepyness Scale)

Dabei handelt es sich um eine siebenstufige Selbstbeurteilungsskala über die gegenwärtige Schläfrigkeit mit 1 = am wenigsten schläfrig und 7 = am schläfrigsten (8).

Statistische Analyse

Für die statistische Analyse wurden die Varianzanalyse (SPSSS-PC, Version 10.7) sowie der CHI-Square-Test verwendet.

Ergebnisse

Probandendaten

36 Probanden (19 Männer und 17 Frauen im Alter zwischen 35 und 55 Jahren) wurden in die Studie aufgenommen. Ein männlicher Proband, der den Aufnahmetest bestanden hatte, erschien nicht zur ersten Testserie. Die Probanden wurden randomisiert der NADH- und der Placebogruppe zugeteilt. Beide Gruppen unterschieden sich nicht in Alter oder Geschlechtszusammensetzung.

Nebenwirkungen

14 Probanden berichteten über Kopfschmerzen während der Studiendauer. Die Kopfschmerzen traten jedoch vor der Administration von NADH oder Placebo auf. Daher waren die Kopfschmerzen in keinem Zusammenhang mit NADH.

Vigilanz

Beim Basistest, also vor dem Abflug von San Diego, fanden sich in dem primären Vigilanztest keine Unterschiede zwischen der NADH- und der Placebogruppe. Ein Drittel der Probanden von beiden Gruppen hatte beim Test in der Früh in Washington eine Zunahme der Auslassungsfehler. Beim Mittagstest hatten 85,7 % der NADH-Probanden, die 0 bis 1 Auslassungsfehler beim Basistest hatten, das gleiche Niveau wieder erreicht. Im Vergleich dazu haben nur 68,8 % der Placeboprobanden im Mittagstest ihr Leistungsniveau des Basistests erreicht. Der Zustand des Jetlags hat auf die Auslassungsfehler einen geringeren Einfluß. Bei den Probanden der Placebogruppe machten 82,4 % 0 bis 1 Auslassungsfehler bei jeder Testsitzung. Im Gegensatz dazu zeigten Probanden der NADH-Gruppe eine progressive Verbesserung der Vigilanz, indem sie weniger Fehler beim Test am Morgen nach dem Flug und am Mittag nach dem Flug machten als beim Basistest am Abend vor dem Abflug. Am Abend davor machten 77,8 % der Probanden der NADH-Gruppe 0 bis 1 Fehler. Dieser Wert verbesserte sich auf 88,9 % um 9 Uhr in der Früh und um 12:30 Uhr mittags.

Arbeitendes Gedächtnis

Die NADH-Gruppe und die Placebogruppe unterschieden sich beim Basistest nicht. Am Morgen nach dem Nachtflug konnten die Probanden der NADH-Gruppe 13,2 mehr Probleme pro Minute korrekt beantworten im Vergleich zu dem Basistest am Abend vor dem Abflug. Die Probanden der Placebogruppe konnten nur 6,8 Probleme mehr korrekt pro Minute beantworten. Bei der Placebogruppe fiel die Genauigkeit von 93 % beim Basistest auf 91 % beim Test am Morgen nach dem Flug. Für die Probanden der NADH-Gruppe verbesserte sich die Performance von 92,5 % beim Basistest auf 95 % bei der Morgensitzung nach dem Flug. Beim ANAM running memory test fiel die Genauigkeit bei den Probanden der Placebogruppe von 95 % auf 91 % in der Früh nach dem Nachtflug. Sie blieb auch beim Mittagstest um 12:30 Uhr auf dem niedrigeren Niveau von 91 %. Probanden der NADH-Gruppe blieben über den ganzen Testbereich in der Früh nach dem Nachtflug und mittags nach dem Nachtflug beim selben Wert wie am Abend vor dem Abflug, nämlich 96 %.

Geteilte Aufmerksamkeit

Die Reaktionszeit, mit der auf die zweite Aufgabe reagiert wurde, nahm bei den Probanden der NADH-Gruppe um 0,15 Sekunden ab. Das heißt, die Probanden, die NADH einnahmen, reagierten schneller. Bei den Probanden der Placebogruppe verlangsamte sich die Reaktionszeit um 0,44 Sekunden (P = 0,016). Der Gesamtscore erhöhte sich bei den Probanden der NADH-Gruppe um 77,5 Punkte, wohingegen sich dieser Wert bei den Probanden der Placebogruppe nur um 19,2 erhöhte (P = 0,011).

Geschwindigkeit und Genauigkeit der visuellen Perzeption

Die Probanden der NADH-Gruppe komplettierten korrekt 5,4 Aufgaben pro Minute mehr bei dem Mittagstest nach dem Nachtflug verglichen mit dem Basistest vor dem Abflug. Zum Vergleich: Die Probanden der Placebogruppe komplettierten korrekt nur 1,4 mehr Objekte pro Minute (P = 0,029) (Abb. 1). Beim matching-to-sample-test konnten keine signifikanten Unterschiede zwischen der NADH- und der Placebogruppe festgestellt werden. Jedoch zeigte der Trend (P = 0,078) eine Verbesserung in der Durchführung bei den Probanden der NADH-Gruppe. 4,9 mehr korrekte Antworten pro Minute von den Probanden der NADH-Gruppe im Vergleich zu 1,0 korrekt beantworteten für die Probanden der Placebogruppe.

Probanden sehen eine aus 4 schachbrettartigen Figuren. Danach sehen sie auf den folgenden Bildschirmen die gleiche oder ähnliche Figur und müssen feststellen, ob sie wirklich ident mit der zuerst gezeigten Figur ist.

Die Genauigkeit der Antworten, die Anzahl der korrekten Antworten pro Minute und die mittlere Reaktionszeit werden dabei registriert. Probanden unter NADH komplettierten 5,4 Figuren mehr pro Minute in der Früh nach dem Nachtflug gegenüber dem Test am Vorabend, wogegen die Placebo-Probanden nur 1,4 Figuren mehr pro Minute komplettierten.

Psychomotorische Leistung

Die Probanden der NADH-Gruppe hatten 31 % Fehler beim Basistest am Abend vor dem Abflug und 33 % Fehler am nächsten Tag in der Früh nach dem Nachtflug. Beim Mittagstest um 12:30 Uhr hatten die Probanden der NADH-Gruppe jedoch nur 11 % Fehler. Im Gegensatz dazu hatten die Probanden der Placebogruppe 29 % Fehler beim Basistest am Abend und 41 % Fehler beim Test am Morgen nach dem Nachtflug und 29 % Fehler beim Mittagstest um 12:30 Uhr nach dem Nachtflug (Abb. 2).

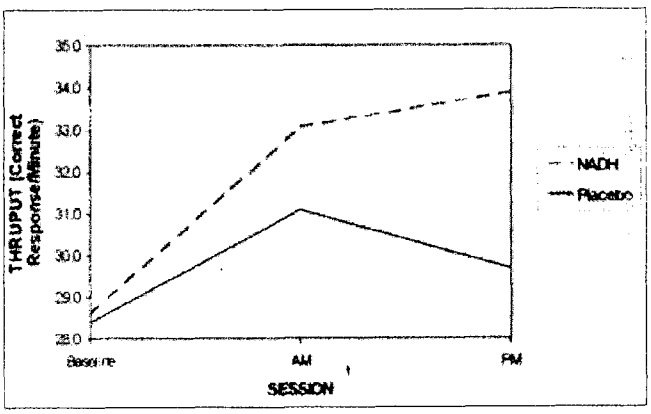

Abb. 1. Visuelle Perzeption: Geschwindigkeit und Genauigkeit. Visueller Vergleich der Sequenz: korrekte Antworten pro Minute.

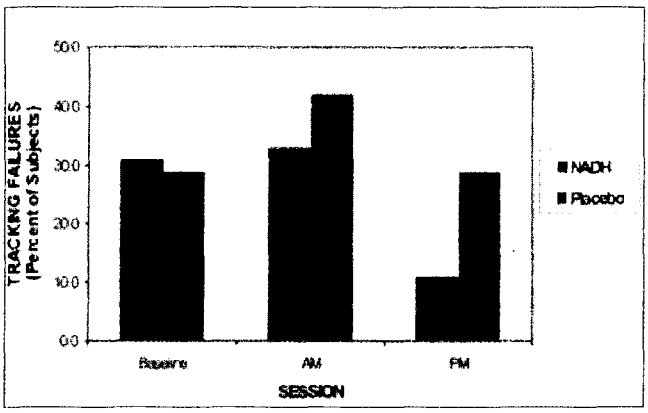

Abb. 2. Psychomotorische Leistung. Doppelfunktionstest (visuelle Perzeption und Arbeiten des Gedächtnisses).

Cog-Screen-Doppelfunktionstest (Ablauf)

Die Aufgabe besteht darin, einen instabilen Cursor, der sich nach rechts und links in einer horizontalen Linie bewegt, und die linken und rechten Cursortasten in einer zentralen Position zu halten. Die mittleren absoluten Spurfehler und die Anzahl der Spurfehler werden registriert. Die Probanden, die NADH einnahmen, hatten am Mittag nach dem Nachtflug 11 % Fehler gegenüber 31 % Fehler am Abend vor dem Flug. Die Probanden der Placebogruppe hatten am Mittag nach dem Nachtflug 29 % Fehler, da die Fehlerquote am Abend vor dem Abflug ebenfalls bei 29 % lag, konnte keine Verbesserung der Aufmerksamkeit registriert werden.

Schläfrigkeit und Stimmung

Am Abend vor dem Abflug berichteten 14 Probanden (82 % der NADH-Gruppe) eine Schläfrigkeit von 1 oder 2 auf der 7stufigen Stanford Sleepyness Skala (SSS) und 3 Probanden beurteilten ihre Schläfrigkeit mit 3. 16 Probanden der Placebogruppe (94 %) beurteilten ihre Schläfrigkeit mit 1 oder 2 auf der Basis SSS und ein Proband beurteilte seine Schläfrigkeit mit 3. Am Morgen nach dem Nachtflug hatten beide Gruppen identische Schläfrigkeitsbeurteilung. Bei der Mittagssitzung um 12:30 Uhr wurde hingegen ein Trend zu geringerer Schläfrigkeit bei den Probanden der NADH-Gruppe ($P = 0{,}08$) gefunden.

Diskussion

Die Studie wurde durchgeführt, um den Einfluß von Jetlag auf gesunde Menschen festzustellen und die Effekte eines Mittels gegen Jetlag zu prüfen. Die Ergebnisse der Studie zeigen, daß ein Flug gegen die Uhr, der vier Zeitzonen überspannt, Symptome und Jetlag hervorruft, inklusive Müdigkeit, Schläfrigkeit und verminderte Hirnleistung sowie verlangsamte Reaktionszeit. Die Hirnleistungstestbatterie, die in dieser Studie angewandt wurde (Cog Screen), konnte eine Verschlechterung der kognitiven Leistungen nach dem Nachtflug feststellen. Stabilisiertes, sublingual absorbierbares NADH führt zu einer Verbesserung der kognitiven Hirnleistung und verringert die Schläfrigkeit, wenn es nach der Landung angewendet wird. Von den Probanden, die NADH-Tabletten einnahmen, wurden keine wie immer gearteten Nebenwirkungen berichtet. Das Nichtauftreten von Nebenwirkungen bestätigt die Ergebnisse von früheren Studien, daß nach der Verabreichung von NADH auch über einen längeren Zeitraum von mehreren Monaten keine Nebenwirkungen beobachtet werden (2, 3, 7).

Jetlag hat, wie diese Echtzeit (real time)-Studie gezeigt hat, eine verstärkende Wirkung auf die Schläfrigkeit, die durch die Stanford Sleepyness Skala gemessen wird. Während des Morgentests nach dem Flug hatten 57,1 % der Probanden der NADH-Gruppe und 62,5 % der Probanden der Placebogruppe über eine erhöhte Schläfrigkeit im Vergleich zum Basistest am Abend vor dem Abflug berichtet. In der Nachmittagssitzung um 12:30 Uhr berichten 57,67 % der Probanden der NADH-Gruppe über keine Verschlechterung der Schläfrigkeit relativ zu ihrem Wert am Abend vor dem Abflug. Zum Unterschied dazu berichten nur 25 % der Probanden der Placebogruppe über keine erhöhte Schläfrigkeit. Diese Befunde weisen, insbesondere in Kombination mit den Ergebnissen des Vigilanz-Tests darauf hin, daß NADH die durch Jetlag verursachte Benommenheit und verminderte Aufmerksamkeit und Alertness signifikant verbessern kann.

Der Einfluß von Jetlag auf die Gesundheit und die Wirtschaft wird meist unterschätzt (8). Die Zahl der Passagiere auf interkontinentalen Flügen nimmt deutlich zu. Diese Reisenden erfahren die negativen Wirkungen von Jetlag, die in dieser Studie demonstriert worden sind. Reisende, die unter Jetlag stehen, machen mehr Aufmerksamkeitsfehler, haben Schwierigkeiten, sich zu konzentrieren und können Aufgaben der geteilten Aufmerksamkeit weniger effizient durchführen. Weiters sind Vielflieger mit Jetlag weniger aufmerksam, weniger aktiv und viel müder. Für Athleten führt die Wirkung von Jetlag sehr wahrscheinlich zu einer reduzierten Leistung. Piloten und Flugbegleiter, die diese Passagiere transportieren, sollten über die Wirkungen und Konsequenzen von Jetlag mehr aufgeklärt werden. Sie sollten ebenso über Wirksamkeit und Risken verschiedener Jetlag-Gegenmittel informiert werden. Manager, Angestellte, Vielflieger sowie Piloten und Flugbegleiter müssen ihre Erwartungen an diese Ergebnisse anpassen. Es sollte ihnen auch ausreichende und adäquate Gelegenheit gegeben werden, sich von den Wirkungen des Jetlags zu erholen. Die Implikationen dieser Studie sind weitreichend. Die Fähigkeit, Entscheidungen zu treffen, die athletische Leistungsfähigkeit, erfordert Aufmerksamkeit bei vielfältigen Aufgaben kontinuierliche Konzentrationsfähigkeit und rasche Interpretation von visuellen Eindrücken. Diese werden durch Flüge gegen die Zeitzonen stark reduziert. Stabilisiertes, sublingual absorbierbares NADH (ENADAlert®) zeigt eine signifikante Verbesserung der durch Jetlag hervorgerufenen verminderten Hirnleistung. Für Piloten besteht eine kritische Abhängigkeit von ihrer Vigilanz, ihrem Kurzzeitgedächtnis und der visuellen Perzeption. Nachdem diese Eigenschaften durch NADH verbessert werden, ist diese natürlich in jeder Zelle vorkommende Substanz besonders nützlich für Piloten oder Flugbegleiter, die unter Jetlag oder Müdigkeit leiden.

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Mitteilungen

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- **On the Safety of Reduced Nicotinamide Adenine Dinucleotide (NADH)**

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ABSTRACT

The objective of the study was to determine both the toxicity of the stabilized orally absorbable form of nicotinamide adenine dinucleotide (NADH) (ENADA® the maximum tolerated intravenous dose (MTD) of βNADH (the reduced form of NADH) in beagle dogs. The administration of the stabilized orally absorbable of NADH to beagle dogs at dose levels of 20, 100, and 150 mg/kg for 14 days elicited no signs of a toxicological effect. A transitory change in stool formatior observed with the intermediate and high dose in males. There were also apparent increases in adrenal, heart, kidney, liver, brain, and thyroid weights, particularly in males, but none of these changes were considered to be toxicologically significant. In addition, four dogs (two of each sex) received intraveno infusions of 100 mg NADH/kg/day for 4 days, followed by 200 mg NADH/kg/day for 3 days, followed by 500 mg NADH/kg/day for 4 days, and 1000 mg NADH/kg/day on the final day. At the end of the MTD phase, the control animals that had received saline solution in the MTD phase were used to evaluate th potential toxicity of the established MTD. These animals received 500 mg NADH/kg/day for 14 days (fixed dose phase). There were no deaths. At dose levels between 100 and 1000 mg/kg/day, effects on the cardiovascular system and also some evidence of an effect on the central nervous system and on the adre were observed. At doses of 500 mg/kg/day and above, food consumption and body weight were reduced. On the basis of the observed changes, the maximu intravenous dose of NADH tolerated by beagle dogs was considered to be 500 mg/kg/day. There were no gross histological findings indicative of toxicity in th organs of tissues examined. Based on these findings, the stabilized orally absorbable form of NADH (ENADA) can be regarded as safe.

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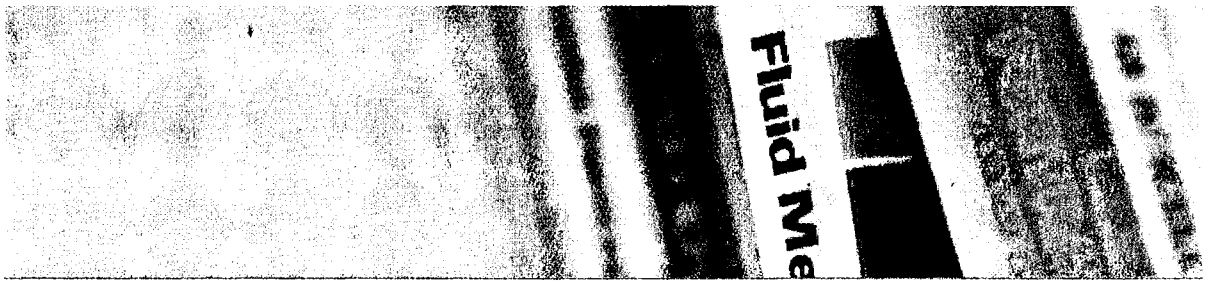

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Safety of stabilized, orally absorbable, reduced nicotinamide adenine dinucleotide (NADH): A 26-week oral tablet administration of ENADA®/NADH for chronic toxicity study in rats

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Résumé / Abstract
The safety of the stabilized, orally absorbable form of reduced nicotinamide adenine dinucleotide (NADH), known under the brand name ENADA, was investigated over a period of 26 weeks. Eighty healthy rats (40 males and 40 females) were divided into two groups. One tablet ENADA®/NADH 5 mg per day was administered orally to one group while identical-looking white tablets not containing NADH (placebo) were given to the other group. The following parameters were statistically analyzed: body weight, body weight gain, food consumption, hematology, clinical chemistry, organ weight and organ histology Clinical signs and mortality were recorded. There were no deaths associated with the study drug and no treatment-releated clinical signs. No differences in body weight between the placebo and the ENADA®-treated males were observed. In the second half of the treatment period (weeks 13-26) females treated with NADH gained significantly ($p < 0.05$) more body weight than the controls. Food consumption in the treated males was similar to that in controls. From approximately week 15, the treated females consumed up to 10% more food than the controls. No differences were observed between the control and the treated groups in terms of hematology or clinical chemistry parameters. There was no apparent treatment-related effect on urine analysis parameters or on either the absolute or the relative organ weight. Furthermore, no macroscopic evidence of specific target organ toxicity associated with the test drug was observed. Histological findings in the treated rats were generally similar to those in control rats. A daily dose of 5 mg in a rat corresponds to a dose of 175 mg per day in a 70-kg human. This is 175 times the recommended daily dosage of 1 ENADA® tablet per day Hence ENADA®/NADH 5 mg tablets can be generally regarded as safe.

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Zusammenfassende Beurteilung

Auf Grund wissenschaftlicher Arbeiten der Evidenzklasse I und auf Grund von Metaanalysen kann der Schluß gezogen werden, dass Isoflavone bei leichten und mittleren Wechselbeschwerden in der frühen Postmenopause effektiv sind. Gleichzeitig findet sich bei den zahlreichen an Menschen vorgenommenen klinischen Studien kein Hinweis auf eine Risikoerhöhung für hormonabhängige Malignome oder Schilddrüsenerkrankungen.

Das NADH ist eine körpereigene Substanz, die in der vorgelegten Galenik resorbiert wird. Tierexperimentelle und toxikologische Untersuchungen zeigen, dass NADH in der vorgelegten Dosierung keine gesundheitlichen Schäden hervorruft. Die vorliegenden klinischen Arbeiten erlauben den Schluß, dass NADH in der vorliegenden Form gegen Ermüdungserscheinungen wirksam ist.