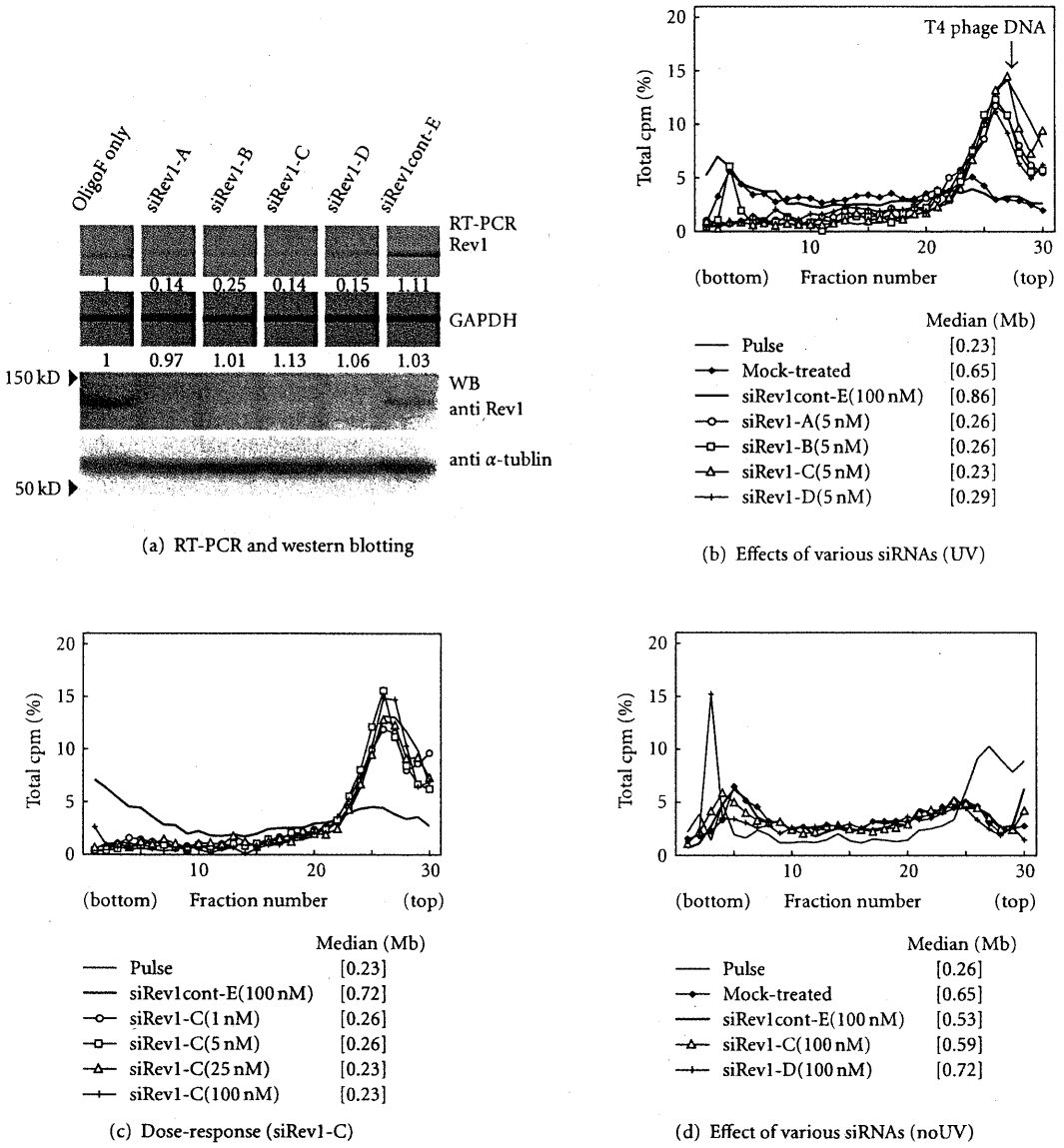


FIGURE 3: Efficient knockdown by Rev1 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev1 expression (RT-PCR analysis and western blot analysis); (b) Effects of various Rev1 siRNAs on UV-TLS; (c) Dose-response of siRev1-C on UV-TLS; (d) Effect of various siRNAs (no UV control). Twenty-four hours after Rev1 siRNA transfection, total RNA was isolated and Rev1 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev1 siRNA transfection, whole cell extracts were prepared and Rev1 protein was quantified by western blot analysis (a). Forty hours after Rev1 siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (b, c). Forty hours after Rev1 siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (d). Some of these profiles overlap (b, c). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5×10^7 Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

Choi et al. [38], and the target sequence of siPolι-A was 4 nt downstream from the one reported by Machida et al. [39] (human Polι mRNA sequence: NM_007195).

We prepared 3 siRNAs for knockdown of Polκ mRNA (NM_016218) (Figure 6(a)). In contrast to siPolι, these Polκ siRNAs (5 nM) delayed UV-TLS in HeLa cells (Figure 6(b)). The siPolκcont-B, from siPolκ-A with 3 nt mismatches, had little to no effect. The dose-response profile of siPolκ-A shows that the molecule sufficiently inhibited UV-TLS at 1 nM (Figure 6(c)). The Polκ siRNAs had no effect on normal replication (Figure 6(d)).

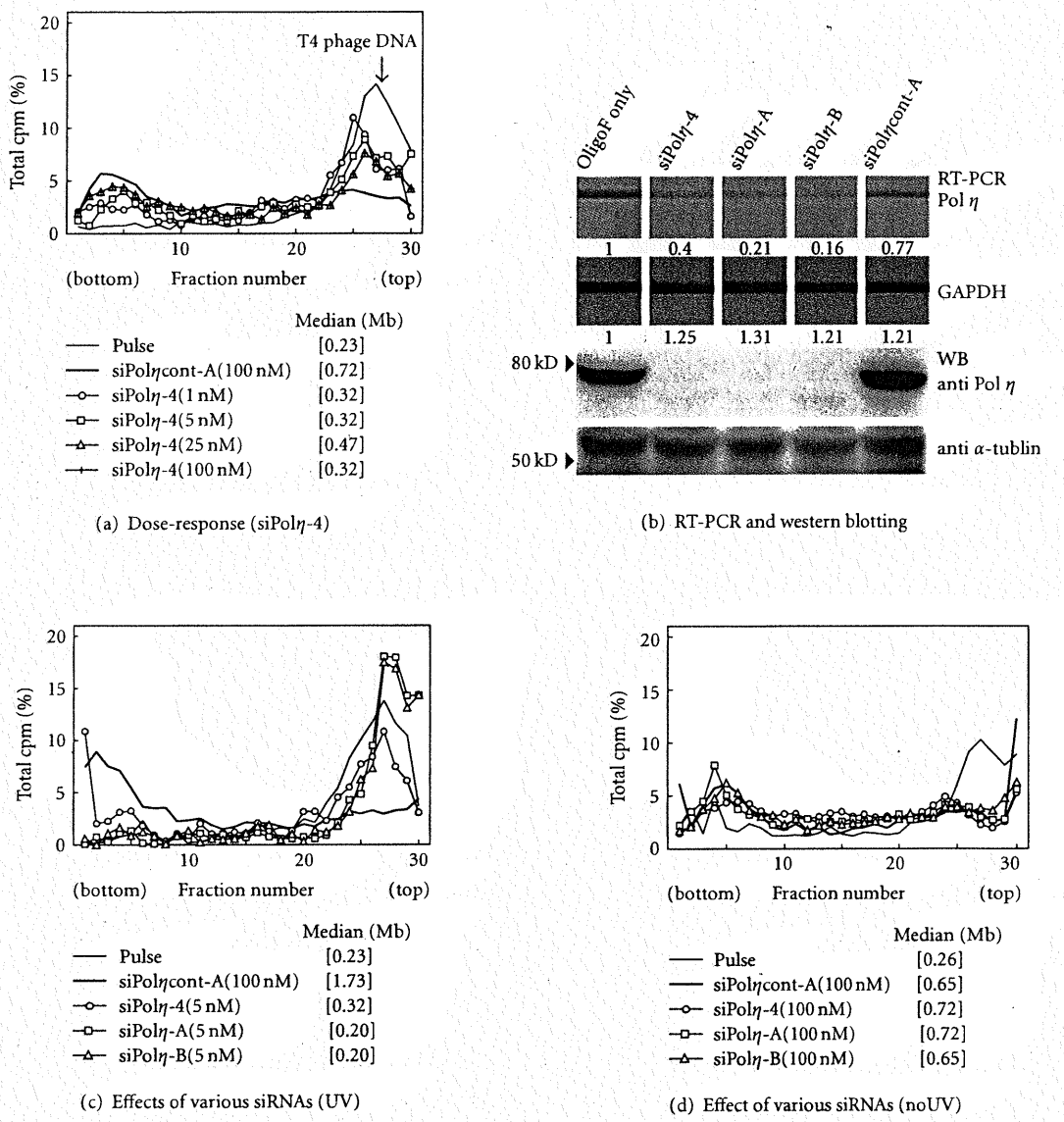
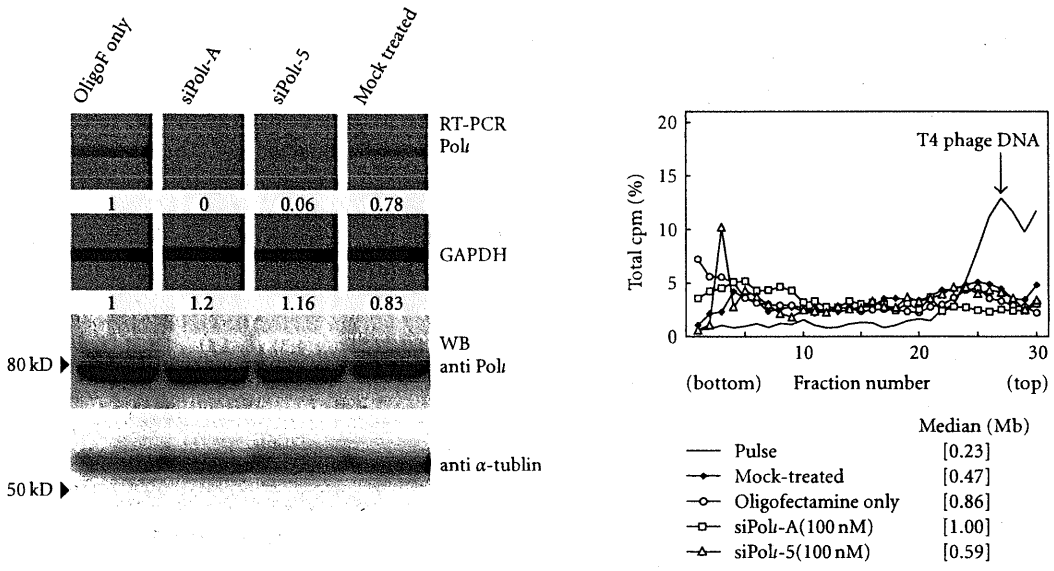


FIGURE 4: Efficient knockdown by Polη siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Dose-response of siPolη-4 on UV-TLS, Efficiency of knockdown on Polη expression; (b) RT-PCR analysis and western blot analysis, (c) Effects of various Polη siRNAs on UV-TLS, (d) Effect of various Polη siRNAs (no UV control). Twenty-four hours after Polη siRNA transfection, total RNA was isolated and Polη RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (b). Forty hours after Polη siRNA transfection, whole cell extracts were prepared and Polη protein was quantified by western blot analysis (b). Forty hours after Polη siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (a, c). Forty hours after Polη siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (d). Some of these profiles overlap (d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5×10^7 Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

4. Discussion

We verified the involvement of multiple bypass polymerases in UV-TLS in HeLa cells using original siRNAs and ASDG technique, which is consistent with the recent model of 2 polymerase mechanisms [40, 41]. Rev3 and Rev7, which comprise Polζ, were confirmed to participate in mutagenic UV-TLS. Also, Rev1 was suggested to play an important role



(a) RT-PCR and western blotting

(b) Effects of various siRNAs (UV)

FIGURE 5: Efficient knockdown by Polι siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Polι expression (RT-PCR analysis and western blot analysis); (b) Effects of two Polι siRNAs on UV-TLS. Twenty-four hours after Polι siRNA transfection, total RNA was isolated and Polι RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Polι siRNA transfection, whole cell extracts were prepared and Polι protein was quantified by western blot analysis (a). Forty hours after Polι siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (b). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5 × 10^7 Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

in human TLS, although in avian DT40 cells, Rev1 may have a distinct role [42]. We were surprised to find that siRNAs against Polη prevented TLS to a great extent. TLS was delayed in Polκ siRNA-transfected cells, but not in siPolι-transfected cells.

We anticipated a limited participation of Polη, because UV-TLS in HeLa cells is very slow (i.e., inefficient) and caffeine-sensitive [35]. However, siRNAs against Polη, particularly siPolη-A and siPolη-B, prevented TLS to a great extent. Since both Rev3 and Rev7 siRNAs also significantly abolished UV-TLS, these results suggest that the Polζ-dependent TLS pathway and the Polη-dependent process are not mutually exclusive but overlapped.

Enzymology of yeast Polζ revealed that this polymerase is too faithful to insert nucleotides opposite a CPD, although it efficiently extends from a matched or mismatched 3′ end [5, 6]. Therefore, we assumed that mutagenic (error-prone) TLS proceeded through the insertion by Polι or Polκ of mismatched nucleotides opposite UV photoproducts, followed by extension by Polζ. Our data showed, however, that siPolι had no effect, and siPolκ partially prevented TLS. These results suggest that in some cases, Polη, and to a lesser extent Polκ, may insert nucleotide(s) opposite UV photoproducts, followed by extension by Polζ.

Polη is capable of bypassing a CPD without aid of other TLS polymerases. Both yeast and human Polη, however, incorporate wrong nucleotide at a fairly high rate and can extend these mismatched primer termini with only a frequency of ~10^{-2} to 10^{-3} relative to extension from matched primer termini [6, 43]. Plausibly, Polη dissociates from there and the proof-reading exonuclease of Polδ removes the wrong nucleotide [44]. To the primer termini, Polη is recruited again and incorporates a new nucleotide. This cycle is repeated until Polη incorporates a correct nucleotide. We suppose that disruption or malfunction of this cooperation renders mismatched primer termini accessible to Polζ.

Recently, Yoon et al. published 2 papers describing the effects of siRNA knockdown on the efficiency of TLS at TT-CPD [45] or (6-4)TT PP [46] on duplex plasmids in human cells. They also reported the effects of siRNA knockdown on mutation frequencies in the λ phage *cII* gene lysogenized in mouse cells expressing a (6-4)PP photolyase [45] or CPD photolyase [46]. The results of this tremendous and detailed study demonstrated that Pols η, κ, and ζ contribute to CPD bypass, wherein Pols κ and ζ promote mutagenic TLS and Polη executes error-free bypasses (Polι siRNA had no effect) [45]. As for (6-4)PP bypass, Pols η and ι provide alternate pathways for mutagenic TLS, and Polζ acts in a predominantly error-free manner (Polκ siRNA had no effect) [46].

The participation of Pols κ or ι in CPD bypass was similarly demonstrated by our results and those of Yoon et al. [45]. Because (6-4)PP is a minor photoproduct, which is removed predominantly by NER, and because HeLa cells

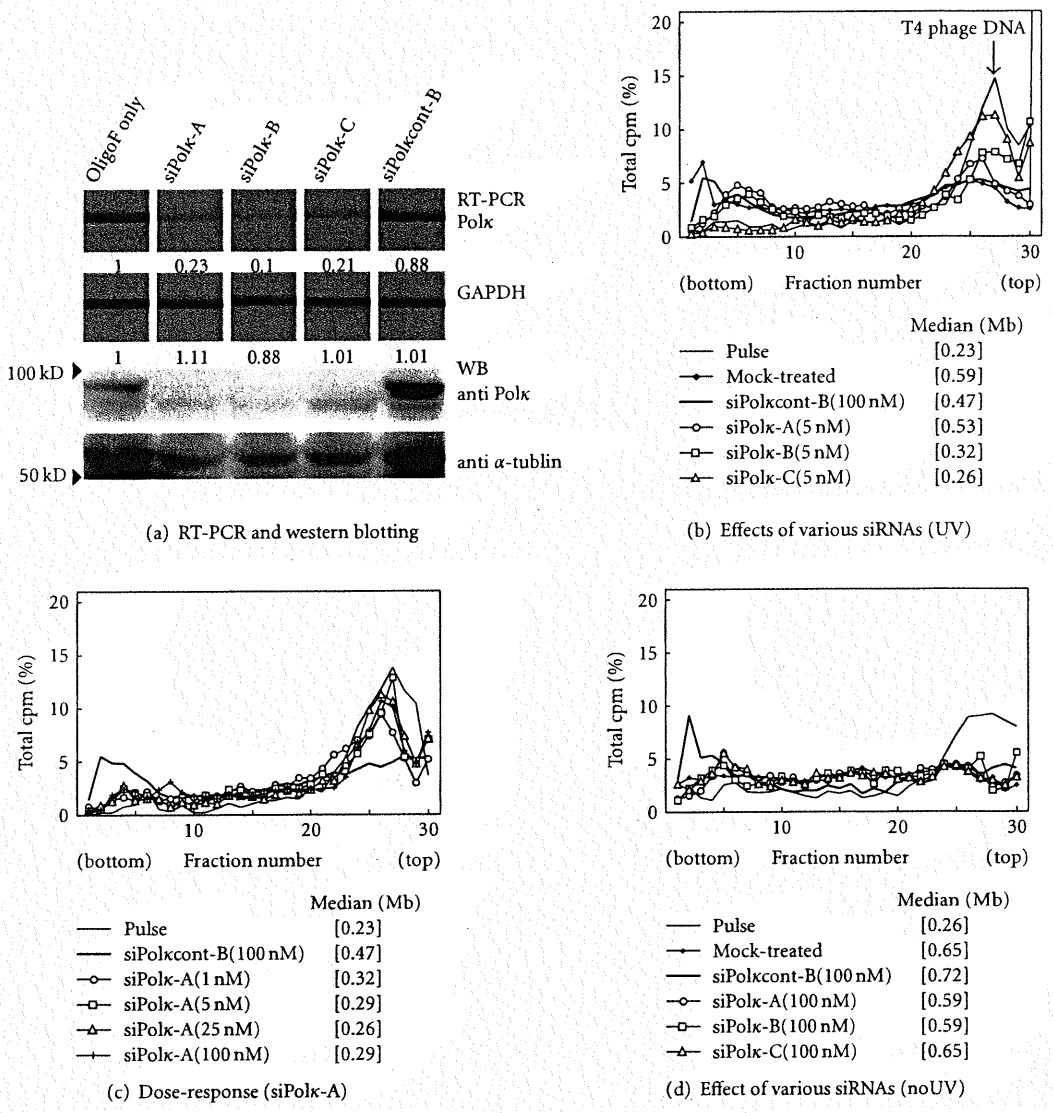


FIGURE 6: Efficient knockdown by Polκ siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Polκ expression (RT-PCR analysis and western blot analysis), (b) Effects of various Polκ siRNAs on UV-TLS, (c) Dose-response of siPolκ-A on UV-TLS, (d) Effect of various Polκ siRNAs (no UV control). Twenty-four hours after Polκ siRNA transfection, total RNA was isolated and Polκ RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Polκ siRNA transfection, whole cell extracts were prepared, and Polκ protein was quantified by western blot analysis (a). Forty hours after Polκ siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (b, c). Forty hours after Polκ siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (d). Some of these profiles overlap (c, d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5×10^7 Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

possess high NER activity (unpublished observation), it is reasonable to conclude that our phenomena observed in HeLa cells by ASDG are largely attributable to CPD, although we have not yet determined the extent of remaining (6-4)PP.

We may also conclude that Rev1 is indispensable for TLS across CPD. Thus far, it is unknown if Rev1 is equally involved in TLS across CPD and (6-4)PP, or if it exhibits some preference. Nelson et al. [25] demonstrated that Rev1p participates in UV-TLS across (6-4)PP, based on yeast transfected with a (6-4)PP-carrying plasmid; only slight differences were observed with a CPD-carrying plasmid.

In vitro lesion-bypass assay showed that Polη alone accomplishes bypass across TT-CPD as above (i.e., both insertion and extension) [8, 9]. However, Yoon et al.

presented complex results showing involvement of multiple bypass polymerases. They used SV-untransformed XP-A and XP-V cells, but did not include SV-untransformed normal fibroblasts, wherein we detected quick and caffeine-insensitive UV-TLS [35, 36]. It is possible that the kind of damage, as well as cell status (normal, transformed, or cancerous) may determine the participation of bypass polymerase(s).

We have presented the first apparent evidence that Polκ participates in UV-TLS. Polκ knockout mouse embryonic cells are known to be UV sensitive [47], but the mechanism had not yet been determined. Polκ is also thought to play a part in the repair-synthesis step of NER [48, 49]. From the results of lesion-bypass assays, human Polκ was suggested to be unable to bypass CPD or (6-4)PP. Because the outcomes of such *in vitro* assays depend on the assay conditions [12], these results must be validated *in vivo*, such as by ASDG analysis.

5. Conclusions

Using siRNAs originally designed and ASDG technique, we verified the participation of multiple bypass polymerases in UV-induced TLS in HeLa cells, which is the consistent with recent model of 2 polymerase mechanisms. UV-TLS was largely abolished by siRNAs to Rev3 or Rev7, suggesting that these 2 proteins, which constitute Polζ, play a primary role in mutagenic TLS. Rev1-targeted siRNAs also significantly abolished UV-TLS, consistent with prior suggestions that Rev1 is indispensable in mammalian mutagenic TLS. Unexpectedly, siRNAs to Polη prevented TLS to a great extent, implying that the Polη- and Polζ-dependent processes do not alternate but overlap. Polκ siRNAs, but not siRNAs to Polι, delayed TLS; this is the first apparent evidence for the participation of Polκ in UV-TLS.

Acknowledgments

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Original Article

Effect of soy isoflavone extract supplements on bone mineral density in menopausal women: meta-analysis of randomized controlled trials

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This study was conducted to clarify the effect of ingesting soy isoflavone extracts (not soy protein or foods containing isoflavones) on bone mineral density (BMD) in menopausal women. PubMed, CENTRAL, ICHUSHI, CNKI, Wanfang Data, CQVIP, and NSTL were searched for randomized controlled trials published in English, Japanese, or Chinese reporting the effects of soy isoflavone extracts on lumbar spine or hip BMD in menopausal women. Trials were identified and reviewed for inclusion and exclusion eligibility. Data on study design, participants, interventions, and outcomes were extracted. Eleven, seven, five, and five trials were finally selected for estimation of the effects on spine, femoral neck, hip total, and trochanter BMD, respectively. Meta-analysis including data from1240 menopausal women revealed that daily ingestion of an average of 82 (47–150) mg soy isoflavones (aglycone equivalent) for 6–12 months significantly increased spine BMD by 22.25 mg/cm^2 (95% CI: 7.62, 32.89; p=0.002), or by 2.38% (95% CI: 0.93, 3.83; p=0.001) compared with controls (random-effects model). Subgroup analyses indicated that the varying effects of isoflavones on spine BMD across trials might be associated with study characteristics of intervention duration (6 vs. 12 months), region of participant (Asian vs. Western), and basal BMD (normal bone mass vs. osteopenia or osteoporosis). No significant effects on femoral neck, hip total, and trochanter BMD were found. Soy isoflavone extract supplements increased lumbar spine BMD in menopausal women. Further studies are needed to address factors affecting the magnitudes of effect on spine and to verify the effect on hip.

Key Words: meta-analysis, isoflavones, dietary supplements, menopause, bone density

INTRODUCTION

Osteopenia and osteoporosis are major health problems in postmenopausal women, who experience a sharp decrease in estrogen concentration that leads to an increased rate of bone remodeling.[1,2] The yearly decline in bone mineral density (BMD) of the lumbar spine and hip in postmenopausal women is reported to be at least 1% and up to 2.4%.[1,3] Although hormone replacement therapy (HRT) has positive effects in increasing BMD in postmenopausal women with low bone mass,[1,4] it is associated with a higher risk of hormone-related cancer[5-7] and other unfavorable adverse events.[8,9]

Epidemiological studies indicate that women who have high soy intake have a lower risk of osteoporosis than women who consume a typical Western diet.[10-12] Consequently, many menopausal women use phytoestrogens to maintain their BMD because they are unlikely to cause the undesirable effects associated with steroid hormones.[8,13] The primary dietary phytoestrogens ingested are soy isoflavones, which have structures similar to that of estrogen.[14]

A meta-analysis of randomized controlled trials (RCTs) has estimated the effect of ingesting soy isoflavones on lumbar spine BMD.[15] This included 10 RCTs of both soy isoflavone tablets and isolated soy protein containing isoflavones, and revealed a significant increase of BMD by 20.6 mg/cm^2 (magnitude in term of percentage and effect on hip not presented) resulting from soy isoflavones. Given the result in units of mg/cm^2, whether the magnitude of increase can prevent the naturally occurring postmenopausal bone loss remains unclear. Subgroup analysis of three trials testing isoflavone tablet revealed no significant effect, however one trial testing soy isoflavone extract was mistakenly included in the isolated soy

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protein subgroup.[16] In addition, two[17] and three[18] comparisons from the same trial respectively with two and three soy isoflavone groups compared to the same control group were included simultaneously as separate studies in the meta-analysis. This is not recommended because it is considered to induce a serious unit-of-analysis problem.[19] Another recently published meta-analysis included 10 RCTs of soy isoflavones supplementation of at least one year duration (four RCTs testing isoflavones extracts), and did not find significantly beneficial effects of soy isoflavones on spine and hip BMD.[20]

Supplements of soy isoflavone extracts were easily ingested by the people who want to benefit from soy isoflavones, but are unable to usually consume and/or do not like to intake products of soy protein or soy foods containing isoflavones. In addition, the beneficial effects of soy protein might require synergistic reactions between isoflavones and other soy components.[15] Thus, clarifying the effects of extracted soy isoflavones (not as a constituent part in soy protein) is of more clinically important. However, both the two meta-analyses failed to reveal significant effects of soy isoflavone extracts in subgroup analysis, which might be due to the fact that only data from four RCTs were included.[15,20] We have identified 12 RCTs of soy isoflavone extracts (not of soy protein or foods containing isoflavones) that reported effects on spine BMD in menopausal women,[8,16-18,21-29] and performed the present meta-analysis to clarify the effects of soy isoflavone extract both in terms of change (mg/cm^2) and percentage change (%) from baseline for lumbar spine and hip BMD, without influence on the same parameters by soy protein per se or other components in soy protein.

MATERIALS AND METHODS

PubMed (1966–2008), CENTRAL (1966–2008), ICHUSHI (1983–2008), and CNKI (1979–2008) were searched for relevant studies that had been published by September 2008. We also searched Wanfang Data, CQVIP and NSTL, which are other major search engines in China. Reference lists of relevant studies were manually searched. Studies were eligible for inclusion if they met all of the following criteria: (1) randomized parallel-group controlled trials published in English, Japanese, or Chinese; (2) trials with a crossover design that contained data for the first period;[19,30] (3) tested the effects of ingesting supplements of soy isoflavone extracts (not of soy protein or foods containing isoflavones) on lumbar spine or hip (femoral neck, total hip, or trochanter) BMD in menopausal women; and (4) BMD data were measured by dual X-ray absorptiometry. When duplicate data were reported for the same study subjects, only the article with the largest sample was included.[19] Two reviewers independently reviewed and evaluated the studies, and consensus was reached by discussion when there were disagreements.

Data on study design, number of participants, interventions, and outcomes for BMD were also independently extracted by two reviewers and confirmed by each other. When necessary, data on outcomes for BMD were obtained from graphs. If possible, we obtained necessary data not reported in the articles by contacting to the authors. We calculated mean change (follow-up − baseline) and percentage change [(follow-up − baseline) ÷ baseline × 100%] from baseline in BMD, when the data were not directly available. We primarily determined missing SD of the changes if statistical analyses comparing the changes themselves were presented (e.g., confidence intervals, standard errors, t values, p values, F values). Alternatively, we imputed them by computing mean correlations between the baseline and final values from included trials in which SD for change, as well as for baseline and final measurements were available.[19] Standard deviation for percentage change was calculated by dividing SD for change with mean baseline value.

We used the Jadad scale to assess the quality of included RCTs, a score of < 3 indicating low quality.[31] We also used a 3-category grading system (A, B, C) to denote the methodological quality of each study.[32] Category A studies have the least bias and results are considered valid; B studies are susceptible to some bias, but not sufficient to invalidate the results; and C studies have significant bias that may invalidate the results. We arbitrarily defined category C as of low quality. Concealment of treatment allocation in RCTs was assessed as adequate, inadequate or unclear.[33] Two reviewers independently assessed the studies, and consensus was reached by discussion when there were disagreements.

We performed meta-analysis to determine the overall treatment effect of soy isoflavones on BMD, using the weighted mean difference method in Review Manager (version 5.0.20; Nordic Cochrane Center, Oxford, England). Treatment effect of each trial was estimated as the mean difference between changes (or percentage changes) from baseline in BMD for each comparison group (i.e., the change from the baseline for participants ingesting soy isoflavones minus that for controls). When data of more than one time points for the same trial were reported in one article or reported separately in two articles, we primarily used the data set for the short duration in order not to induce unit-of-analysis error. The data set for other time points were used for sensitivity analysis to prevent reporting bias. For trials had more than one isoflavone group compared with one control group, we combined the multiple isoflavones groups into a single group for each of these trials without inducing unit-of-analysis error.[34]

We used both a fixed effect model or a random effects model to calculate weighted mean differences (WMD), 95% CIs for each comparison, a combined overall effect with p-value, and the p-value for testing heterogeneity ($p<0.1$ was considered significant); when there was significant heterogeneity across included trials, the results based on the random effects model were shown.[19,30,35]

We conducted sensitivity analyses to evaluate the effects of degree of correlation between baseline and final values, time point of measurement (using data for long duration instead of data for short duration in trials with multiple time points of evaluation), study design (selecting only placebo-controlled trials), and study quality (eliminating low-quality trials). If at least 10 trials were available, subgroup analyses and meta-regressions were performed to investigate possible factors that might related to varying effects of soy isoflavones on BMD across trials, on the basis of pre-specified factors of intervention

duration, isoflavone dosage, region of participants, and basal BMD.[15,20] We used a cut-off point of 75 mg/day in subgroup analysis for isoflavone dosage, because daily isoflavone intake of up to 75 mg (aglycone form) is considered safe by the Japan Food Safety Commission. Significant tests based on test for heterogeneity, chi-squared statistics, were performed to investigate differences between two subgroups.[19,34] We examined potential publication bias by using funnel plots and by performing Egger's test to assess the asymmetry of funnel plots. Meta-regressions and Egger's test were respectively performed with the use of user-written "metareg" and "metabias" commands for Stata 10.1 for Windows (StataCorp LP, College Station, Tex).

RESULTS

The search strategy (Figure 1) yielded 16 potentially appropriate reports of RCTs to be included in the meta-analysis. After excluding one article[36] reporting only duplicate femoral data that had appeared in another article,[25] and two articles[37,38] describing a smaller sample than that analyzed in another article,[23,17] 13 articles on 12 trials were included for meta-analysis.[8,16-18,21-29] Two articles reported outcomes for durations of six months[27] and one year[28] for the same trial participants.

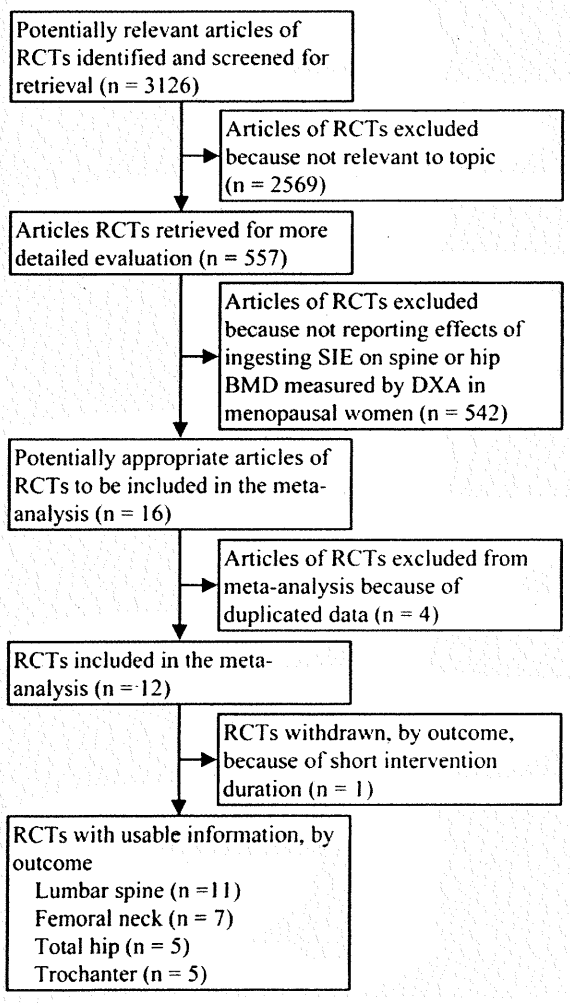


Figure 1. Search and selection of trials. Abbreviations: RCTs, randomized controlled trials; BMD, bone mineral density; SIE, soy isoflavone extracts, DXA, dual X-ray absorptiometry.

The characteristics of 12 trials are summarized in Table 1. Two articles for each trial contained data for two time points.[21,25] Three trials tested two isoflavone groups[17,22,24] and one tested three isoflavone groups[18] compared with one identical control group. One trial did not address the form and composition of soy isoflavones tested,[18] we assumed the dose as aglycone equivalent to calculate the mean dosage. Four, six, and two trials included participants of normal bone mass (T-score > –1 SD, corresponds to BMD > 0.937g/cm^2), low bone mass or osteopenia (–1 SD ≥ –2.5, corresponds to 0.937 g/cm^2 ≥ BMD ≥ 0.772 g/cm^2), and osteoporosis (T-score < –2.5 SD, corresponds to BMD < 0.772 g/cm^2) on the basis of averaged basal spine BMD, respectively.[39] Only one trial was assessed as "adequate" for concealment of treatment allocation,[22] and the remaining trials were assessed as "unclear" due to insufficient information. Participants in the comparison groups had similar dietary intakes of soy isoflavones, calcium, and vitamin D and physical activities. Most of the studies were designed to maintain the participants' usual diets, lifestyle and body weight. Adverse events were generally similar for both the isoflavone and control groups and no serious adverse events were noted in the included trials, although they were not well addressed in several trials.

Because bone is a slowly responding organ, a complete bone remodeling cycle takes up to 6 months, and therefore a study duration of less than 6 months is not sufficient to evaluate the effect of any intervention on bone BMD.[28] Thus, one 3-month trial of low-quality that reported negative effect of soy isoflavones on spine BMD was then withdrawn.[26] From 3126 relevant articles identified, 11,[8,16-18,21-25,27-29] 7,[8,17,22-25,27,28] 5,[16,17,22,27-29] and 5[17,22-24,27,28] trials were finally selected for estimating the effects on lumbar spine, femoral neck, total hip, and trochanter BMD, respectively (Figure 1, Table 1). Fourteen correlation coefficients between baseline and follow-up values were calculated from 5 reports of 4 trials,[17,23,24,27,28] which were consistent and resulted in a mean value of 0.98 (0.96–1).

Meta-analysis of the 11 trials with 1240 participants using the fixed effect model resulted in significant heterogeneity (p<0.001), and revealed that daily ingestion of an average of 82 (47–150) mg (aglycone equivalent) soy isoflavones for 6 months to one year significantly increased lumbar spine BMD by 12.08 mg/cm^2 (95% CI: 9.83, 14.33 mg/cm^2; p<0.001), or by 1.47% (95% CI: 1.21, 1.74%; p<0.001) compared with controls. Meta-analysis using the random effects model, revealed an significant overall effect of soy isoflavones in increasing spine BMD by 20.25 mg/cm^2 (95% CI: 7.62, 32.89 mg/cm^2, p=0.002), or by 2.38% (95% CI: 0.93, 3.83%, p=0.001; Figure 2). Of the11 selected trials, 7 trials revealed significant positive mean difference between changes or percentage change from baseline in spine BMD for isoflavone and control groups (favors isoflavone). The mean difference was negative at 27-week time point and was positive at 53-week time point in one trial,[21] the mean difference at 2-year duration was about two times of that at 1-year time point;[25] whereas, the

Table 1. Characteristics of included randomized controlled trials

Study	Design[†]	Follow-up	Participants[‡]	Intervention[§]	Baseline mean BMD outcomes (g/cm^2)[¶]	Jadad scale	Quality category
Brink 2008[21]	P; R, DB+, WD	27, 53 wk	N: 300/237 (21%) PoW; mean age: 53 y; TSM = 33 (12–60) mo; non-osteoporotic (spine Z-score ≥ 2); Netherlands, Italy, France	110 mg IAE [25–35% De, 60–75% Ge, 1–5% Gle] vs. placebo	L1–4: 0.990, mean (SD) T-score = −0.0 ± 1.1	4	C (dropout > 20%)
Chen 2003[22]	P; R+, DB+, WD	1 y	N: 203/175 (14%) PoW; mean age: 54.2 y; TSM = 4.1 (1–10) y; Hong Kong	40 and 80 mg IAE [46% De, 15% Ge, 39% Gle] vs. placebo	L1–4: 0.860; FN: 0.682; TH: 0.819; Tr: 0.605	5	A
Dong 2008[23]	P; R, WD	12 mo	N: 60/52 (13%) PoW; mean age: 54.7 y; TSM = 6.2 (≥ 1) y; T-score < −1.5 China	100 mg IC [66 mg IAE: 39% De, 61% Ge, 1% Gle] + calcium vs. calcium only (control)	L2–4: 0.756; FN: 0.719; Tr: 0.552	2	B
Gao 2006[18]	P; R	24 wk	N: 50/50 PoW; age: 48–62 y; TSM ≥ 1y; China	60, 90, and 150 mg IF vs. no-treatment (control)	L1–4: 0.974	1	B
Harkness 2004[16]	CO; R+, DB, WD	6 mo × 2	N: 20/19 (5%) PoW; mean age: 70.6 y; TSM = 19.1 (> 8) y; T-score < 2.5; USA	110 mg IAE [40% De, 52% Ge, 9% Gle] vs. placebo	L1–4: 0.881; TH: 0.800	4	B
Huang 2006[24]	P; R, OL, WD	1 y	N: 43/42 (2%) PoW; mean age: 52.4 y; TSM = 4.4 (1–13) y; Taiwan	100 and 200 mg IAE [29% De, 71% Ge] vs. regular diet only (control)	L1–4: 0.881; FN: 0.812; Tr: 0.715	2	B
Marini 2007[25]	P; R+, DB+, WD	12, 24 mo	N: 389/389 (10, 22%) PoW; mean age: 54.5 y; TSM = 63 mo (≥ 1 y); femoral neck BMD < 0.795 g/cm^2 (−1.0 T-score); Italy	54 mg pure Ge vs. placebo	L: 0.840; FN: 0.670	5	A, C (dropout > 20%)
Morabito 2002[8]	P; R, DB+	1 y	N: 90/90 PoW; mean age: 51.5 y; TSM = 6.5 (≥ 1) y; femoral neck BMD < 0.795 g/cm^2 (−1.0 T-score); Italy	54 mg pure Ge vs. placebo	L: 0.925; FN: 0.688	3	A
Uesugi 2003[26]	P; R, WD	3 mo	N: 22/21 (4%) PoW; mean age: 53.7 y; TSM = 6 (5–10) y; non-osteoporosis; Japan	62 mg IC [38 mg IAE: 52% De, 11% Ge, 37% Gle] vs. placebo	L2–4: 1.040	2	C (unclear analyzed N)
Wu 2006a[27], b[28]	P; R, DB+, WD	6, 12 mo	N: 136/128, 108 (6, 21%); mean age: 54.4 y; TSM = 3.2 (1–5) y; Japan	75 mg IC [47 mg IAE: 54% De, 13% Ge, 34% Gle] vs. placebo	L2–4: 0.899; FN: 0.672; TH: 0.782; Tr: 0.595	4	A, C (dropout > 20%)
Xin 2006[29]	P; R, DB	6 mo	N: 76 MW; age: 45–55 y; TSM ≤ 5 y; China	50 mg pure De + calcium vs. calcium only (control)	L2–4: 0.715; TH: 0.643	2	C (unclear analyzed N)
Ye 2006[17]	P; R+, SB, WD	6 mo	N: 90/84 (7%) PoW; mean age: 52.3 (1–5) y; TSM = 2.6 (1–5) y; China	84 and 126 mg IAE [52% D(e), 15% G(e), 33% Gl(e)] vs. placebo	L1–4: 0.864; FN: 0.702; TH: 0.800; Tr: 0.588	3	B

[†]CO, crossover; DB, double-blinded (gives 1 point to Jadad scale); DB+, double-blinded by appropriate method (gives 2 point); OL, open-labeled; P, Parallel; R, randomized (give 1 point); R+, randomized by appropriate method (gives 2 point); SB, single-blinded; WD, withdrawals and dropouts described (gives 1 point).

[‡]BMD, bone mineral density; N, randomize/analyzed number (dropout rate) of participants; MW, menopausal women; PoW, postmenopausal women; TSM, averaged time since menopause.

[§]IAE, isoflavone aglycone equivalents; IC, isoflavone conjugate containing glycoside and aglycone forms; IF, isoflavones (form and composition unknown); D(e), daidz(e)in; De, daidzein; Ge, genistein; G(e), genist(e)in; Gl(e), glycit(e)in; Gle, glycitein.

[¶]FN, femoral neck; L, lumbar spine; TH, total hip; Tr, trochanter.

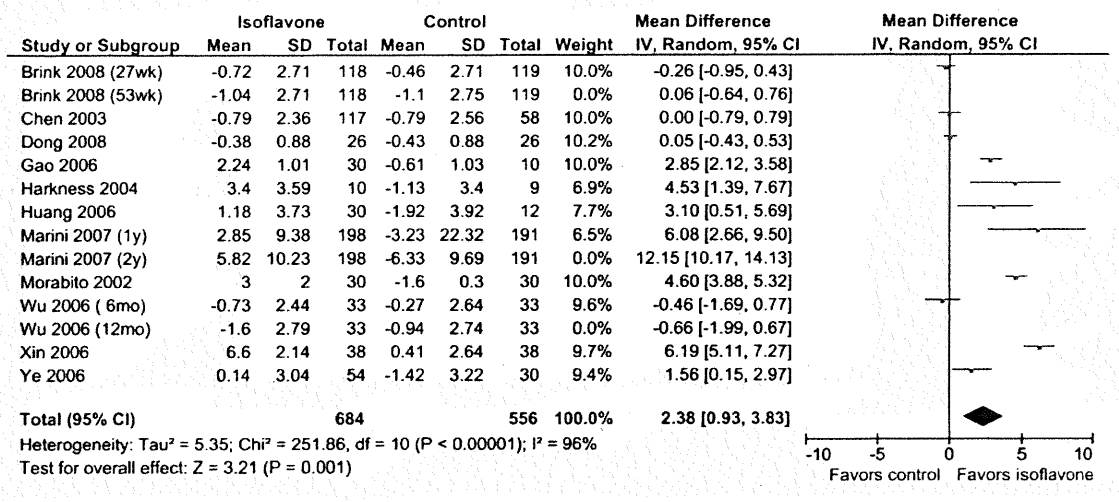


Figure 2. Effects of soy isoflavones on spine BMD (%). Mean Difference, weighted mean difference between percentage changes (%) of spine bone mineral density (BMD) from baseline for isoflavone and control groups; random, random effects model. Horizontal lines denote the 95% CI. Data sets for long duration not included in the meta-analysis were signed 0.0% Weight. ■ Point estimate (size of the square corresponds to its weight); ♦ Combined overall effect.

mean difference for 6 months duration[27] was similarly negative to that for 1 year duration.[28]

Sensitivity analyses assuming the level of correlation coefficient between baseline and follow-up values to be 0.75 and 0.5, using data sets of longer duration instead of short duration for trials with two time points of measurements, selecting only placebo-controlled trials, and eliminating low-quality trials (Jadad scale < 3 or Category C) did not result in significantly different overall effects of soy isoflavones on spine BMD.

Results of subgroup analyses of the effects of soy isoflavones on spine BMD were shown in Table 2. Each subgroup analysis resulted in significant heterogeneity and revealed significant effect of soy isoflavones in increasing spine BMD compared with controls using the fixed effect model. Results based on fixed effect model revealed that effects of soy isoflavones on spine BMD in subgroups of 6 months duration and of Asian region were significantly different with the effects in subgroup of 1 year duration and of Western region, respectively. Two subgroups of each subgroup analysis using the random effects model, show similarly significant effects of soy isoflavones in increasing spine BMD, except for a subgroup of participants with normal bone mass at baseline. Meta-regressions analyzing each of or all of the four pre-specified categorical study characteristics (intervention duration, isoflavone dosage, region of participants, and basal spine BMD), did not reveal that these pre-specified factors were significantly associated with the varying effects of soy isoflavones on spine BMD across trials. The funnel plots (Figure 3) and Egger's test of effects of soy isoflavones on spine BMD among the 11 trials (p=0.251 and p=0.267 for effects in terms of change and percentage change, respectively) did not indicate any obvious publication bias.

Meta-analysis of the 7 trials with 868 participants using the fixed effect model resulted in significant heterogeneity (p<0.001). Meta-analysis using the random effects model, revealed that daily ingestion of an average of 76 (47–150) mg (aglycone equivalent) soy isoflavones for 6 months to one year non-significantly increased femoral neck BMD by 10.24 mg/cm^2 (95% CI: −3.73, 24.20 mg/cm^2, p=0.15), or by 1.48% (95% CI: −0.54, 3.50%, p=0.15) compared with controls. Sensitivity analysis assuming the level of correlation coefficient between baseline and follow-up values to be 0.75 and 0.5, did not result in significantly different overall effects of soy isoflavones on femoral neck BMD. Whereas, sensitivity analysis using data sets of longer duration for trials with two time points of measurements, found that ingestion of soy isoflavones for 6 months to 2 years tended to increase femoral neck BMD by 16.89 mg/cm^2 (95% CI: −2.34, 36.11 mg/cm^2, p=0.09), or by 2.45% (95% CI: −0.31, 5.21, p=0.08; Figure 4) compared with controls (random effects model). Sensitivity analyses selecting only placebo-controlled trials and eliminating low-quality trials were not performed because of the small number of available trials.

Meta-analysis of the 5 trials with 420 participants using the fixed effect model resulted in non-significant heterogeneity (p≥0.1), revealed that daily ingestion of an average of 74 (47–110) mg (aglycone equivalent) soy isoflavones for 6 months to one year non-significantly change total hip BMD by 2.45 mg/cm^2 (95% CI: −1.41, 6.30 mg/cm^2, p=0.21), or by 0.05% (95% CI: −0.53, 0.63%, p=0.86) compared with controls. Sensitivity analyses assuming the level of correlation coefficient between baseline and follow-up values to be 0.75 and 0.5 and using data sets of longer duration for trials with two time points of measurements, did not result in significantly different overall effects of soy isoflavones on total hip BMD.

Meta-analysis of the 5 trials with 419 participants revealed that daily ingestion of an average of 85 (47–150) mg (aglycone equivalent) soy isoflavones for 6 months to one year non-significantly change trochanter BMD by −0.40 mg/cm^2 (95% CI: −6.58, 5.78 mg/cm^2, p=0.90), or by −0.07% (95% CI: −1.15, 1.02%, p=0.91) compared with controls (random effects model). Sensitivity analyses assuming the level of correlation coefficient between

Table 2. Subgroup analyses of the effects of soy isoflavones on spine BMD[†]

Variables	No. of trials	Sample size	*p* for heterogeneity	Fixed effect model			Random effects model	
				WMD (95% CI)	*p*-value	*p*-value (diff)	WMD (95% CI)	*p*-value
Intervention duration								
6 months	6[16-18, 21, 27, 29]	522	< 0.00001	17.72 (14.03, 21.41) mg/cm^2	< 0.00001	= 0.0002	18.74 (1.25, 36.23) mg/cm^2	0.04
			< 0.00001	1.81 (1.40, 2.21) %	< 0.00001	= 0.03	2.31 (0.16, 4.47) %	0.04
1 year	5[8, 22-25]	718	< 0.00001	8.74 (5.90, 11.58) mg/cm^2	< 0.00001		22.64 (1.54, 43.74) mg/cm^2	0.04
			< 0.00001	1.23 (0.88, 1.58) %	< 0.00001		2.52 (0.17, 4.87) %	0.04
Isoflavone dose								
≤ 75 mg/d	6[8, 22, 23, 25, 27, 29]	818	< 0.00001	11.70 (9.10, 14.30) mg/cm^2	< 0.00001	= 0.57	20.79 (1.48, 40.09) mg/cm^2	0.03
			< 0.00001	1.53 (1.20, 1.85) %	< 0.00001	= 0.59	2.59 (0.26, 4.92) %	0.03
> 75 mg/d	5[16-18, 21, 24]	422	< 0.00001	13.21 (8.73, 17.69) mg/cm^2	< 0.00001		19.49 (2.64, 36.34) mg/cm^2	0.02
			< 0.00001	1.37 (0.91, 1.83) %	< 0.00001		2.10 (0.31, 3.90) %	0.02
Region of participants								
Asian	7[17, 18, 22-24, 27, 29]	535	< 0.00001	9.01 (6.44, 11.59) mg/cm^2	< 0.00001	< 0.00001	15.06 (0.89, 29.23) mg/cm^2	0.04
			< 0.00001	1.17 (0.86, 1.49) %	< 0.00001	= 0.0006	1.85 (0.16, 3.54) %	0.03
Western	5[8, 16, 21, 25]	705	< 0.00001	21.97 (17.34, 26.60) mg/cm^2	< 0.00001		31.46 (0.56, 62.37) mg/cm^2	0.05
			< 0.00001	2.20 (1.71, 2.68) %	< 0.00001		3.56 (0.13, 6.99) %	0.04
Basal spine BMD								
Normal bone mass	3[18, 21, 24]	319	< 0.00001	12.31 (7.42, 17.20) mg/cm^2	< 0.00001	= 0.92	17.06 (−7.55, 41.66) mg/cm^2	0.17
			< 0.00001	1.27 (0.78, 1.76) %	< 0.00001	= 0.33	1.78 (−0.74, 4.29) %	0.17
Osteopinia or osteoporosis	8[8, 16, 17, 22, 23, 25, 27, 29]	921	< 0.00001	12.02 (9.48, 14.55) mg/cm^2	< 0.00001		21.70 (5.43, 37.97) mg/cm^2	0.009
			< 0.00001	1.56 (1.24, 1,87) %	< 0.00001		2.64 (0.69, 4.60) %	0.008

[†]BMD, bone mineral density; WMD, weighted mean difference; *p*-value, test for overall effect of each subgroup; *p*-value (diff), test for subgroup differences.

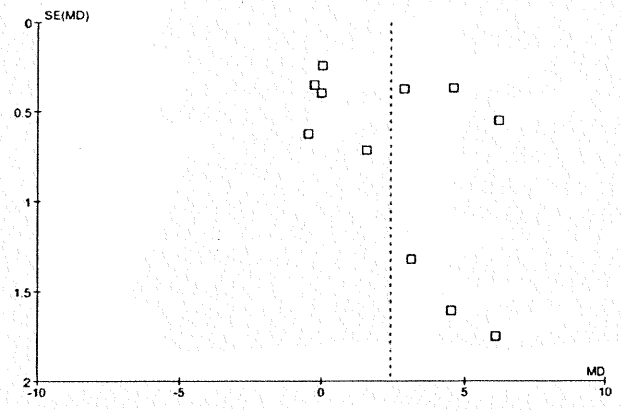
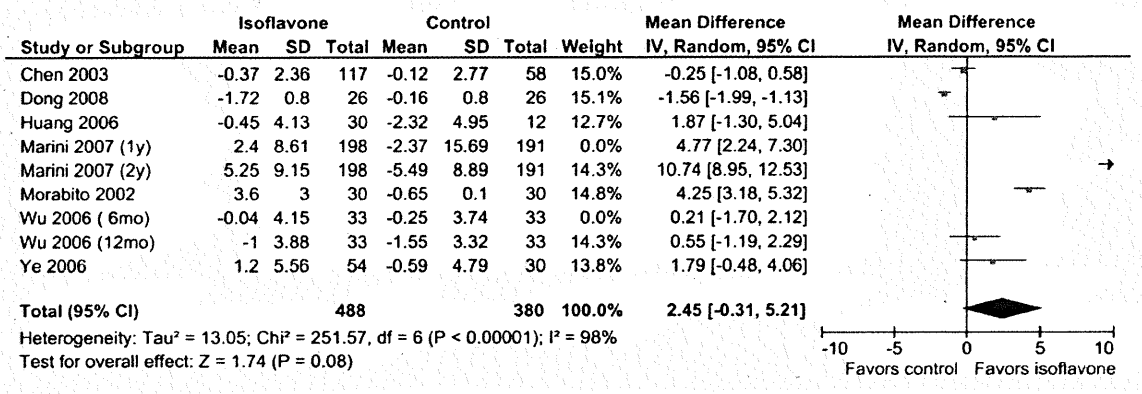


Figure 3. Funnel plots of effects of soy isoflavones on spine BMD (%). MD, weighted mean difference between percentage changes (%) of spine bone mineral density (BMD) from baseline for isoflavone and control groups; SE (MD), standard error of MD; fixed, fixed effect model.



Study or Subgroup	Isoflavone Mean	SD	Total	Control Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Chen 2003	-0.37	2.36	117	-0.12	2.77	58	15.0%	-0.25 [-1.08, 0.58]
Dong 2008	-1.72	0.8	26	-0.16	0.8	26	15.1%	-1.56 [-1.99, -1.13]
Huang 2006	-0.45	4.13	30	-2.32	4.95	12	12.7%	1.87 [-1.30, 5.04]
Marini 2007 (1y)	2.4	8.61	198	-2.37	15.69	191	0.0%	4.77 [2.24, 7.30]
Marini 2007 (2y)	5.25	9.15	198	-5.49	8.89	191	14.3%	10.74 [8.95, 12.53]
Morabito 2002	3.6	3	30	-0.65	0.1	30	14.8%	4.25 [3.18, 5.32]
Wu 2006 (6mo)	-0.04	4.15	33	-0.25	3.74	33	0.0%	0.21 [-1.70, 2.12]
Wu 2006 (12mo)	-1	3.88	33	-1.55	3.32	33	14.3%	0.55 [-1.19, 2.29]
Ye 2006	1.2	5.56	54	-0.59	4.79	30	13.8%	1.79 [-0.48, 4.06]
Total (95% CI)			**488**			**380**	**100.0%**	**2.45 [-0.31, 5.21]**

Heterogeneity: Tau² = 13.05; Chi² = 251.57, df = 6 (P < 0.00001); I² = 98%
Test for overall effect: Z = 1.74 (P = 0.08)

Figure 4. Effects of soy isoflavones on femoral neck BMD (%). Mean Difference, weighted mean difference between percentage changes (%) of femoral neck bone mineral density (BMD) from baseline for isoflavone and control groups; random, random effects model. Horizontal lines denote the 95% CI. Data sets for long duration not included in the meta-analysis were signed 0.0% weight. ■ Point estimate (size of the square corresponds to its weight); ♦ Combined overall effect.

baseline and follow-up values to be 0.75 and 0.5 and using data set of longer duration for trials with two time points of measurements, did not result in significantly different overall effects of soy isoflavones on trochanter BMD.

DISCUSSION

The present meta-analysis found that ingestion of about 82 mg of extracted soy isoflavones (in the aglycone form) per day for 6 months to 1 year significantly increased lumbar spine BMD by 2.38% compared with controls without isoflavones, in menopausal women. Results of sensitivity analyses indicated that the effect of soy isoflavone extracts in increasing lumbar spine BMD was robust. This magnitude of beneficial effect of soy isoflavones appears to almost completely offset naturally occurring postmenopausal bone loss. Effect of soy isoflavones in increasing femoral neck BMD seems to take more time than spine BMD. Our meta-analysis did not reveal significant effects on total hip and trochanter BMD, which might be due to the limited number of five trials.

An intake of 82 mg soy isoflavones/day (in the aglycone form) is approximately equivalent to 1.7 times the amount consumed habitually in Japan (mean: 47.2 mg/day).[40] The mechanism mediating the improvement of BMD at these skeletal sites by soy isoflavones is not well understood, but it may be a result of their chemical and biological similarity to mammalian estrogens, which are known to increase BMD in menopausal women.[1,4]

Results of subgroup analyses indicated that the varying effects of soy isoflavone extracts on spine BMD across the 11 trials were associated with study characteristics of intervention duration, region of participants, and basal BMD. The heterogeneity of effects of soy isoflavones on spine BMD across the 11 trials might also be induced by differences in habitual dietary intake of soy isoflavones,[28] time since menopause,[3] intervention duration,[25] isoflavone dosage,[17,41] chemical forms and proportions of individual soy isoflavones,[42-44] and participants' ethnicity. Isoflavone glycosides are not absorbed intact across the enterocytes of healthy adults, and their bioavailability requires initial hydrolysis by intestinal β-glucosidases for uptake into the peripheral circulation.[44] Asian and Western populations are reported to have differences in the capacity of intestinal flora to convert daidzein to its metabolite, equol.[45] Equol is easily absorbed and possesses substantial estrogenic activity because of its affinity for both the estrogen α and β receptors.[43] Equol is suggested to be the single most important factor that influences the clinical efficacy of soy isoflavones in preventing bone

loss.[46] Because of the limited number of trials and insufficient data available, our meta-analysis was also unable to evaluate possible influences on the varying effects of soy isoflavones on spine BMD across trials of dietary intake of soy isoflavones, time since menopause, chemical forms and proportions of individual soy isoflavones, blood isoflavone concentration, urinary isoflavone excretion, and equol producer status.

Since there was significant heterogeneity in effects of soy isoflavones on spine BMD, we preferably presented the results by incorporating heterogeneity into the random effects model in this meta-analysis. A random effects meta-analysis model involves an assumption that the effects being estimated in the different studies are not identical, but follow some distribution. The model represents our lack of knowledge about why real, or apparent, treatment effects differ by considering the differences as if they were random.[19]

The magnitude of effect of soy isoflavone extracts in increasing spine BMD by 20.25 mg/cm^2 revealed in our present meta-analysis, were consistent with the results (by 20.6 mg/cm^2) from the previous meta-analysis that included 10 RCTs testing both extracted soy isoflavones and isolated soy protein containing isoflavones.[15] Thus, soy isoflavones ingested either alone in extracted form or as constituent part of isolated soy protein have been demonstrated to exert a mild but significant effect in increasing lumbar spine BMD in menopausal women. Our meta-analysis also revealed that ingestion of soy isoflavones for 6 months appears to be enough to exert beneficial effect on spine BMD in menopausal women. The present meta-analysis did not reveal influences of isoflavone dosage on the effect on spine BMD, possibly due to the fact that trials tested various forms and compositions of soy isoflavones likely possessing different bioavailability and effects on bone mass; other explanations might be the limited number of trials or of some other factors inducing the heterogeneity.

CONCLUSION

The effect of soy isoflavones in increasing spine BMD in menopausal women are not as strong as those of approved pharmacologic therapies involving estrogen or bisphosphonates.[1,4,47,48] However, the present meta-analysis revealed that soy isoflavone extract supplements did result in a significant improvement of lumbar spine BMD with good tolerance and no induction of notable adverse events. Our meta-analysis suggested that soy isoflavone supplements can be used not only to offset the bone loss that occurs naturally in women after menopause, but are also applicable for complementary or alternative use in patients with postmenopausal osteopenia or osteoporosis who are unable to tolerate the side effects of estrogen or/and bisphosphonate therapies. Furthers studies are needed to address factors affecting the magnitudes of the effect of soy isoflavones on spine BMD and to verify the effect on hip BMD.

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Kyoko Taku, Melissa K. Melby, Jun Takebayashi, Shoichi Mizuno, Yoshiko Ishimi, Toyonori Omori and Shaw Watanabe, disclose no conflicts of interest.

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Original Article

Effect of soy isoflavone extract supplements on bone mineral density in menopausal women: meta-analysis of randomized controlled trials

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大豆異黃酮抽取物的補充劑對停經後婦女骨質密度的效果：隨機對照試驗的後設分析

本研究旨在確認攝取大豆異黃酮抽取物(並非大豆蛋白或含有異黃酮的食品)對停經後婦女骨質密度(BMD)的效果。我們從 PubMed，CENTRAL，ICHUSHI，CNKI，Wanfang Data，CQVIP，和 NSTL 檢索，以英語，日語，或中文發表，並報告大豆異黃酮抽取物對停經後婦女腰椎或髖關節 BMD 效果的隨機對照試驗論文。依照納入和排除標準，對試驗論文進行鑑別和評閱來判定是否採用。有關研究設計，對象，介入，和結果的數據被抽取出進行分析。最終分別有 11、7、5、和 5 個試驗被採用來評估對腰椎、大腿骨頸部、髖關節全體、和股骨大轉子 BMD 的效果。包括 1240 名停經後婦女的後設分析(隨機效果模型)顯示，與對照組相比，每日平均攝取 82 (47-150) mg 的大豆異黃酮(苷元當量)持續 6-12 個月，顯著地提高腰椎 BMD 22.25 mg/cm^2 (95%信賴區間: 7.61，32.89；p=0.002)，或提高 2.38% (95%信賴區間: 0.93，3.83；p=0.001)。亞組分析顯示，不同試驗間大豆異黃酮對腰椎 BMD 的效果各異，可能與介入期間(6 或 12 個月)，對象的區域(亞洲或西方)，和基礎 BMD(正常骨質或骨質減少症或骨質疏鬆症)的研究特徵相關。我們的後設分析沒有發現對大腿骨頸部，髖關節全體，和股骨大轉子 BMD 的效果。大豆異黃酮抽取物的補充劑提高了停經後婦女的腰椎 BMD。需要更深入的研究去闡明影響其對腰椎效果程度的因素，以及驗證其對髖關節的效果。

關鍵字：後設分析、異黃酮、膳食補充劑、停經、骨密度

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Effects of soy isoflavone supplements on bone turnover markers in menopausal women: Systematic review and meta-analysis of randomized controlled trials

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ABSTRACT

Introduction: Effects of soy isoflavone supplements on bone turnover markers remain unclear. This up-to-date systematic review and meta-analysis of randomized controlled trials (RCTs) was performed primarily to more completely and precisely clarify the effects on urinary deoxypyridinoline (DPD) and serum bone alkaline phosphatase (BAP) and secondarily to evaluate the effects on other bone turnover markers, compared with placebo in menopausal women.

Methods: PubMed, CENTRAL, ICHUSHI, and CNKI were searched in June 2009 for relevant studies of RCTs. Data on study design, participants, interventions, and outcomes were extracted and methodological quality of each included trial was assessed.

Results: From 3740 identified relevant articles, 10 (887 participants), 10 (1210 participants), and 8 (380 participants) RCTs were selected for meta-analysis of effects on DPD, BAP, and serum osteocalcin (OC), respectively, using Review Manager 5.0.22. Daily ingestion of an average 56 mg soy isoflavones (aglycone equivalents) for 10 weeks to 12 months significantly decreased DPD by 14.1% (95% CI: −26.8% to −1.5%; $P=0.03$) compared to baseline (heterogeneity: $P<0.00001$; $I^2=93\%$; random effects model). The overall effect of soy isoflavones on DPD compared with placebo was a significant decrease of −18.0% (95% CI: −28.4% to −7.7%, $P=0.0007$; heterogeneity: $P=0.0001$; $I^2=73\%$; random effects model). Subgroup analyses and meta-regressions revealed that isoflavone dose and intervention duration did not significantly relate to the variable effects on DPD. Daily supplementation of about 84 mg and 73 mg of soy isoflavones for up to 12 months insignificantly increased BAP by 8.0% (95% CI: −4.2% to 20.2%, $P=0.20$; heterogeneity: $P<0.00001$; $I^2=98\%$) and OC by 10.3% (95% CI: −3.1% to 23.7%, $P=0.13$; heterogeneity: $P=0.002$; $I^2=69\%$) compared with placebo (random effects model), respectively.

Conclusions: Soy isoflavone supplements moderately decreased the bone resorption marker DPD, but did not affect bone formation markers BAP and OC in menopausal women. The effects varied between studies, and further studies are needed to address factors relating to the observed effects of soy isoflavones on DPD and to verify effects on other bone turnover markers.

Introduction

Osteoporosis is a major health problem in postmenopausal women, who experience a sharp decrease in estrogen concentration that leads to an increased rate of bone remodeling [1,2]. The increased bone remodeling is associated with both decreased bone mineral density (BMD) and increased risk of fracture [3]. Together with BMD, bone turnover markers (BTMs) have been considered to be biomarkers for fracture risk [4]. BTMs can be used for the diagnosis and evaluation of therapy effects on osteoporosis [5], and include bone resorption markers (e.g. urine deoxypyridinoline (DPD), urine and serum type I collagen crosslinked N-telopeptide (NTX) and type I collagen crosslinked C-telopeptide (CTX)) and bone formation markers (e.g. serum bone alkaline phosphatase (BAP), osteocalcin (OC; or bone gamma-carboxyglutamate protein, BGP), and type I procollagen-N-propeptide (PINP)).

BTMs change earlier and to a larger extent than changes in BMD or risk of fracture. The decrease in BTMs in the early stages of treatment may reflect a reduction in the long-term risk of fracture [6,7]. Thus, a proper assessment of changes in BTMs may provide the earliest indication of whether to continue a particular treatment. Therapeutic effects of anti-osteoporosis drugs (e.g. bisphosphonates, raloxifene,

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and estrogen) can be assessed by DPD, NTX, CTX, BAP, or PINP; and the therapeutic effects are indicated only if the minimum significant change (MSC, %) in BTMs from baseline occurs during the course of treatment [5].

Although estrogen is known to change BTMs, it is associated with a higher risk of hormone-related cancer [8–10] and other unfavorable adverse events [11,12]. As a result, many women are searching for alternatives to estrogen for reduction of risk of osteoporosis. Phyto-estrogens may to some degree inhibit osteoporosis in postmenopausal women, owing to their oestrogenic activity [13]. Epidemiological studies indicate that soy food consumption may reduce the risk of fracture in postmenopausal women, particularly among those in the early years following menopause [14]. Consequently, many menopausal women use phyto-estrogens to maintain their bone mass because they are unlikely to cause the undesirable effects associated with steroid hormones [11,15]. The main dietary phyto-estrogens ingested are soy isoflavones, which have structures similar to that of estrogen [16]. A meta-analysis revealed that soy isoflavone intake for 3–24 months significantly increased spine BMD, compared with placebo in menopausal women [17]. The favorable effect became more significant when >90 mg/day of isoflavones were consumed, and ingestion for 6 months seemed to be enough to exert its beneficial effect on BMD. However, a subsequent meta-analysis revealed that a mean dose of 87 mg soy isoflavones for at least one year (12–24 months) did not significantly affect spine and hip BMD [18].

One meta-analysis of nine randomized controlled trials (RCTs) found that ingestion of soy isoflavones significantly decreased DPD by 2.08 nmol/mmol and increased BAP by 1.48 μg/L compared to controls in menopausal women [19]. We have identified several RCTs that addressed effects of soy isoflavones on bone turnover markers, but were not included in the above meta-analysis. Different RCTs reported data of bone turnover markers in different units and the values of each marker differed substantially across trials. In addition, the meta-analysis seems to combine the data of DPD and BAP without adjustment for different units reported.

The primary aims of this up-to-date systematic review and meta-analysis of RCTs were to more completely and more precisely clarify the effects of soy isoflavone supplements on urinary DPD and serum BAP compared with placebo, by combining their outcomes in terms of percentage changes from baseline in menopausal (including peri- and postmenopausal) women. The effects of soy isoflavones on other bone turnover markers were secondarily evaluated.

Materials and methods

Literature search

PubMed, CENTRAL, ICHUSHI, and CNKI were searched for published randomized controlled trials (RCTs) in June 2009, with the use of dozens of complex search strategies containing index terms. The best search strategy for each database that resulted in the most relevant RCTs was used in the final analysis (Appendix). The search strategies for PubMed and CENTRAL excluding PubMed were developed on the basis of Designing search strategies (section 6.4) described in the Cochrane Handbook for Systematic Reviews of Interventions (Version 5.0.1, updated September 2008), which accompanies with Review Manager 5.0.22 (Nordic Cochrane Center, Oxford, England). Reference lists of relevant RCTs and systematic reviews and meta-analyses were manually searched [19–21].

Inclusion and exclusion criteria

Studies were included for systematic review if they met all of the following criteria: *1)* included participants of menopausal (peri- or/and postmenopausal) women; *2)* evaluated intervention of soy supplements containing isoflavones and clearly described isoflavone dose; *3)* contained at least one relevant pair-wise comparison of intervention arms (i.e., soy isoflavone supplements vs. placebo, or both plus a non-estrogen add-on), and placebo used did not contain isoflavone or estrogen and was identical or similar in appearance and taste to comparative soy isoflavone supplements; *4)* reported outcomes for the effects on at least one of the bone turnover markers mentioned above (DPD, BAP, OC, NTX, CTX, and PINP); and *5)* was a parallel-group or crossover RCT. Duplicated reports or subgroup analysis of the primary study were excluded.

Meta-analysis based on means requires that data are at least approximately normally distributed, and the appropriate analysis of continuous data from crossover trials requires that neither carry-over nor period effects are thought to be a problem [22]. Thus, meta-analysis was performed to clarify effects of soy isoflavone supplements on bone turnover markers for which mean change from baseline and the SD/SE for normally distributed data were available in at least five RCTs. In addition, to be included in the meta-analysis, crossover RCT had to contain a washout period and did not have carry-over or period effects; alternatively, data for the first period were used for meta-analysis if there was no washout period. If there was more than one possible placebo, data for these placebo arms were combined to create one placebo arm. Similarly, if there was more than one dose of soy isoflavones, data for these arms were combined to create one isoflavone arm to compare to placebo. When two or more reports were published for the same trial, only the report with the largest sample size was included in this systematic review and meta-analysis.

Two researchers independently reviewed and evaluated the inclusion and exclusion of relevant RCTs for the systematic review and meta-analysis, and consensus was reached by discussion when there were disagreements.

Data extraction

Data on study design, number of participants, intervention, and outcomes for bone turnover markers were also independently extracted by two reviewers and were confirmed by each other. When necessary, data on outcomes for bone markers were obtained from graphs reported. If possible, we obtained necessary data not reported by contacting the authors. We calculated mean change (follow-up − baseline) and percentage change [(follow-up − baseline) ÷ baseline × 100%] from baseline in bone turnover markers, when the data were not directly reported. When the SDs of the mean changes were unavailable, we estimated them using the reported statistics (e.g., confidence intervals, standard errors, *t* values, *P* values, *F* values) comparing the mean changes; otherwise we conservatively imputed them by using the lowest correlation coefficient between the baseline and follow-up values calculated from included trials, in which SD (or SE) for change as well as for baseline and follow-up measurements were available [22]. SD for percentage change was then calculated by dividing SD for change with mean baseline value.

Quality assessment

We used the Jadad scale to assess the quality of included RCTs, a score of <3 indicating low quality [23]. We also used a 3-category grading system (A, B, and C) to denote the methodological quality of each study, detail as described in the evidence report [20]. Category A studies have the least bias and results are considered valid; category B studies are susceptible to some bias, but not sufficient to invalidate the results; and category C studies have significant bias that may invalidate the results (e.g., dropout rate >20%, missing baseline data, or irreconcilable apparent differences between data in figures, tables, and text). We defined category C as low quality. Concealment of treatment allocation in RCTs was assessed as

adequate, inadequate or unclear [24]. Two reviewers independently assessed the studies, and consensus was reached by discussion when there were disagreements.

Meta-analysis and statistical analysis

We performed a meta-analysis to determine the overall treatment effect of soy isoflavones on bone turnover markers, using the weighted mean difference method in Review Manager. The treatment effect of each trial was estimated as the mean difference between percentage changes from baseline in bone marker for each comparison intervention arm (i.e., the percentage change from the baseline for participants ingesting soy isoflavones minus that for placebo). Because the data required to perform a paired analysis in meta-analysis were unavailable in crossover trials, we incorporated crossover trials with parallel-group trials in the meta-analysis by using data from isoflavone periods and placebo periods as if the trial were a parallel-group trial of isoflavones and placebo [22]. When trials contained repeated measurements of bone markers, we used the data from the longest follow-up as long as the data from various time points had the same dropout rates; otherwise, the data set with the lowest dropout was used. The data sets for other time points were used for sensitivity analyses. For trials using multiple dosages of isoflavones compared to placebo, we combined the multiple isoflavones arms into a single arm for each of these trials; similarly, the data reported for each center in a multicenter trial were combined as if the data were from one center [22].

We used both a fixed effect model and a random effects model to calculate weighted mean differences (WMD), 95% CIs for each comparison, a combined overall effect with P value, and the P value for testing heterogeneity ($P<0.1$ was considered significant); when there was significant heterogeneity across included trials, the results based on the random effects model were shown [22]. The I^2 statistic (0% to 40%: might not be important; 30% to 60%: may represent moderate heterogeneity; 50% to 90%: may represent substantial heterogeneity; 75% to 100%: considerable heterogeneity) was used for quantifying inconsistency across studies [22]. This describes the percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance).

We conducted sensitivity analyses to evaluate the effects of degree of correlation between baseline and follow-up values (using the average of correlation coefficients calculated from included trials), time point of measurement (using data sets for other follow-up durations reported in trials with repeated measurements on participants), study quality (eliminating low-quality trials), and study design (excluding crossover trials). Since it is recommended that levels of bone resorption and formation markers are measured after 3 and 6 months of treatment, respectively [5], sensitivity analyses for bone resorption and formation markers were also performed by excluding trials with <3 months and <6 months intervention duration, respectively.

If at least 10 trials were available, subgroup analyses and meta-regressions were performed to investigate possible factors that might relate to varying effects of soy isoflavones on each bone marker across trials, on the basis of five pre-specified factors: region of participants, menopausal status, supplement type, isoflavone dose, and intervention duration [19]. We used a cutoff point of 75 mg/day in the subgroup analysis for isoflavone dosage, because a daily isoflavone intake of up to 75 mg (aglycone equivalent) is considered safe by the Japan Food Safety Commission. Tests for heterogeneity based on chi-squared statistics were performed to investigate differences between the two subgroups [22].

We examined potential publication bias by using funnel plots and by performing Egger's and Begg's tests to assess the asymmetry of funnel plots. Meta-regressions and tests for asymmetry of funnel plots were respectively performed with the use of user-written "metareg" and "metabias" commands for Stata 10.1 for Windows (StataCorp LP, College Station, Tex); "metainf" command was used to investigate the influence of each trial on the overall meta-analysis estimate.

Results

Search and selection

The flow information of the search and selection of RCTs were shown in Fig. 1. Among 3740 identified relevant articles, 37 articles met the 1st, 2nd, 4th, and 5th inclusion criteria for systematic review [11,25–60]. Continuously, 13 [26–28,31,33,35,40,43,45,46,51,54,59] articles were excluded because they did not meet the 3rd inclusion criteria for systematic review. Subsequently, one article [58] was excluded by applying the exclusion criteria for systematic review. Finally, 21 RCTs (reported in 23 articles) that cleared all the inclusion and exclusion criteria for systematic review were included [11,25,29,30,32,34,36–39,41,42,44,47–50,52,53,55–57,60]. Two RCTs were each reported in two [41,60] and another two articles [52,53], respectively.

One RCT [29] provided non-normally distributed DPD data, and one crossover RCT [47] without a washout period did not provide urine DPD data for the first period and data for all periods were

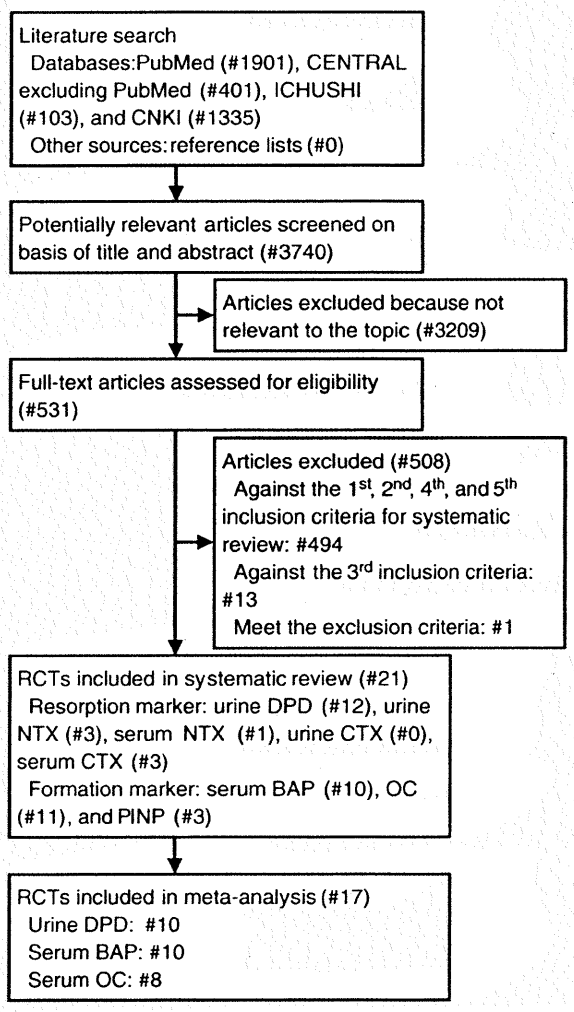

Fig. 1. Search and selection of randomized controlled trials (RCTs). #, number of records; BAP, bone alkaline phosphatase; CTX, type I collagen crosslinked C-telopeptide; DPD, deoxypyridinoline; NTX, type I collagen crosslinked N-telopeptide; OC, osteocalcin; PINP, type I procollagen-N-propeptide.