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ORIGINAL ARTICLE

Soybean isoflavone can protect against osteoarthritis in ovariectomized rats

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Abstract Osteoarthritis (OA) is a chronic and incurable disease and a leading cause of significant pain and disability that is closely associated with aging and obesity. An appropriate long-term therapy regimen is presently unknown. An estrogen deficiency after menopause increases the incidence and severity of OA in women. Soybean isoflavone have weak estrogenic effects in several organs and have been considered as a potentially safe natural selective estrogen receptor modulator. The present study aimed to determine the effects of isoflavone on cartilage degradation in ovariectomized rats. Six-month-old female Sprague-Dawley (SD) rats (n = 40) were randomly assigned to sham operation (n = 10), ovariectomy (OVX) (n = 15) or OVX + isoflavone (OVXI) (n = 15) groups. The OVXI group was fed with soybean isoflavone (51.0 mg/kg/day) for nine weeks, then knee joints were excised. Cartilage degradation was evaluated by toluidine blue staining joint specimens, and by comparing values for serum C-telopeptides of Type II collagen (CTX-II) and cartilage oligomeric matrix protein (COMP) between baseline and the end of the study. Cartilage damage scored by Toluidine blue staining was significantly lower in the OXVI, than the OVX group ($P < 0.016$). The CTX-II values before the surgical procedure and the end of experiment, did not significantly differ among the groups. Values for COMP in all samples were below detection limits in all samples. Soy bean isoflavone limited the degeneration of cartilage induced by OVX in rats.

Keywords Isoflavone · Osteoarthritis · Cartilage degradation · Ovariectomized rats · Selective estrogen receptor modulator

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Introduction

Osteoarthritis (OA) is chronic, incurable, and the leading cause of age-related pain and disability. Global estimates show that 9.6% and 18.0% of men and women aged > 60 years have symptomatic OA, that approximately 80% of persons with OA will develop movement limitations and 25% will have limited major activities of daily living (ADL) (World Health Organization 2012). In addition, an appropriate long-term treatment regimen for OA has not been established.

Osteoarthritis is associated not only with age, but also with many other risk factors, namely, obesity, previous trauma, exercise and bone density (Blagojevic et al. 2010). Estrogen might also play important roles in the pathogenesis of OA. Estrogen receptors have been identified in the articular cartilage, bone, synovial tissue and ligaments of joints (Sciore et al. 1998; Ushiyama et al. 1999; Dietrich et al. 2006). Therefore, these tissues are responsive to estrogen. In fact, the prevalence of OA is higher among men than in women aged < 50 years, but higher in women

than in men aged > 50 years (Oliveria et al. 1995). An estrogen deficiency is associated with an increase in the incidence and severity of OA among post-menopausal women.

Ovariectomy leads to OA-like changes in joints, and estrogen replacement therapy reduces cartilage degradation (Ham et al. 2002; Høegh-Andersen et al. 2004). A systematic review of the effects of ovariectomy and estrogen therapy on cartilage in animal models (Sniekers et al. 2008) has revealed that 11 of 14 studies found that an estrogen deficiency has detrimental effects, with a considerable amount of evidence supporting a relationship between cartilage degeneration and ovariectomy in mature experimental animals. However, that review also found that the effects of estrogen were inconclusive. Only 11 of 22 experimental animal studies identified a beneficial effect on cartilage, whereas all of six studies of selective estrogen receptor modulators (SERM) administered after ovariectomy found protective effects.

On the other hand, soybeans, which are widely consumed in east Asia, is a rich source of the isoflavone including daidzein, genistein, glycitein, and their glycoside derivatives. All of these compounds have weak estrogenic effects on several organs (Adlercreutz et al. 1992). Osteoporosis with bone loss during the first post-menopausal decade in women is associated with a sharp loss of estrogen around the age of 50 years. Many studies of postmenopausal osteoporosis in experimental animal models support the notion that isoflavone exert significant protective effects on bone (Setchell and Lydeking-Olsen 2003). Isoflavone dependently inhibit bone loss in ovariectomized mice without conferring adverse effects on reproductive organs (Ishimi et al. 1999, 2000). Therefore, soybean isoflavone are of interest as a safe, natural source of SERM that might be tolerated over the long term without side effects compared with extended hormone replacement therapy that poses a risk of breast cancer and might increase risk for heart attacks and stroke (Anderson et al. 2003).

We also confirmed that the oral administration of isoflavone glycosides prevents bone loss in Sprague-Dawley (SD) rats that become hormone deficient after ovariectomy (Toda et al. 1999; Uesugi et al. 2001) and we postulated that isoflavone might influence not only bone loss, but also OA induced by ovariectomy. The association between OA and an estrogen deficiency and the weak estrogenic action of isoflavone prompted the present study.

Here, we aimed to determine the effects of soybean isoflavone extracts on cartilage degradation and turnover in 6-month-old ovariectomized SD rats to understand the potential role of isoflavone in treating postmenopausal OA.

Materials and methods

Soybean isoflavone extract

Hot-water extracts of soybean hypocotyls were eluted with water and ethanol through columns containing Sepabeads® SP-207 (Mitsubishi Chemical Corporation, Tokyo, Japan). The ethanol eluate was evaporated, dried, precipitated in water, then separated and dried again. Isoflavone were quantified by high-performance liquid chromatography (HPLC) using a 250 × 4.6-mm YMC-pack ODS-AM-303 column, and a linear gradient of acetonitrile from 15 to 30% containing 0.1% acetic acid for 50 min. The solvent flow rate was 1 mL/min and absorption was at 254 nm. Figure 1 and Table 1 show the extraction scheme and the isoflavone content and composition of the extract, respectively.

Animals

Forty female SD rats (Japan SLC Inc., Shizuoka, Japan) aged 6 months (mean weight ± standard deviation [SD], 332.8 ± 27.3 g) were housed individually in cages at 23 °C ± 2 °C on a 12-h light/dark cycle. They were acclimated for one week on a normal diet with access to water and food ad libitum, then randomly assigned to OVX (n = 15), OVX + isoflavone (OVXI; n = 15) and sham-operated (sham; n = 10) groups. All surgical

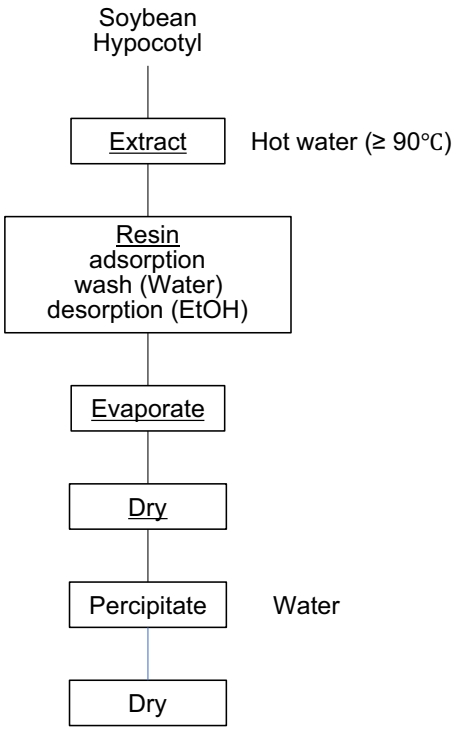

Fig. 1 Scheme of soybean isoflavone extraction

Table 1 Isoflavone content and composition of the soybean extract

Isoflavone	Content (mg/g)
Daidzin	516.7
Glycitin	305.3
Genistin	57.7
6″-*O*-Malonyldaidzin	1.3
6″-*O*-Malonylglycitin	3.2
6″-*O*-Malonylgenistin	0.0
6″-*O*-Acetyldaidzin	14.8
6″-*O*-Acetylglycitin	6.6
6″-*O*-Acetylgenistin	1.4
Daidzein	4.4
Glycitein	2.0
Genistein	0.0
Total	913.4

procedures on SD rats anesthetized with ether proceeded via the dorsal approach to the ovaries under sterile conditions. Both ovaries were identified and removed from the rats in the OVX and OVXI groups. The sham-operated rats underwent the same procedure, except the ovaries remained in situ.

All rats were fed with the AIN-93G diet comprising 20% protein, 7% fat, 64% carbohydrate, and a total calorie content of 400 kcal/100 g (soybean oil was exchanged for corn oil. Oriental Yeast, Tokyo, Japan). The OVXI group were then fed with the test diet (AIN-93G + 0.14% soybean isoflavone extract). All rats were maintained on their allotted diets for nine weeks, then we weighed the rats and measured their food intake at 2, 3, 4, 5, 7 and 9 weeks.

Baseline blood samples were sampled from the tail veins of the rats. The rats were anesthetized with ethanol, then blood samples were collected from the abdominal aorta at the end of the study. Serum was separated by centrifugation at 1,500 rpm for 15 min, then immediately stored at − 20 °C. The animals were then euthanized by cutting the abdominal aorta and knee joints were collected. Ovariectomy was verified from atrophic changes in the uterus caused by an estrogen deficiency. The Ethics Committee at Osaka City University approved the study (No. 12,021.) All animal experiments were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and our institutional Guidelines for Animal Experimentation, which are identical to those in the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Histological evaluation

Excised knee joints were carefully peeled out, fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and histologically stained with toluidine blue to determine cartilage proteoglycan depletion. Degrees of cartilage destruction represented as staining intensity were scored as from 0 to 3, with intensely stained cartilage scoring 0 and $> 75\%$, 50% and $< 50\%$ staining intensity scoring as 1, 2 and 3 (no staining, or completely lost articular cartilage), respectively (Joosten et al. 1999). Histological changes in knee joints were scored in the patella/femur region on five semi-serial joint sections. The evaluator (Y.S.) was blinded to the groups of specimens. All microscopic images were acquired using the DP70 micro digital filing system (Olympus Corporation, Tokyo, Japan).

Cartilage metabolic markers

Serum C-telopeptide of type II collagen (CTX-II) was measured using Rat C-telopeptide of type II collagen ELISA kits (Wuhan Huamei Biotech Co. Ltd., Wuhan, China) as described by the manufacturer. The detection range was 0.312–20 ng/mL. Serum COMP levels were measured using rat COMP ELISA kits, (Wuhan Huamei Biotech Co. Ltd.).

Statistical analysis

Differences in chronological changes in body weight and food intake among groups were analyzed by repeated measures one-way analysis of variance (ANOVA). Differences among groups at each time point, as well as CTX-II and cartilage damage scores among groups were compared using ANOVA and post-hoc pair-wise comparisons of means using Tukey–Kramer tests, with values of $P < 0.05$ being considered significant. Food intake was analyzed post-hoc using Games-Howell tests. Data were statistically analyzed using the Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA).

Results and discussion

Changes in body weight and food intake

One rat each in the sham and OVX groups died during the surgical procedure. One rat in the OVXI group died due to hypersensitivity to general anesthesia, two died during ovariectomy, and ovariectomy was incomplete in one rat. None of the rats died during the feeding period. Thus, data

from nine, 14 and 11 rats in the sham, OVX, and OVXI groups were statistically assessed.

Rats gained significantly more weight in the OVX, than the sham groups throughout the experimental period (Fig. 2A). This might have been due to more food intake by the OVX group (Fig. 2B). Mean body weight was significantly lower in the OVXI, than in the OVX group until week 5, but did not significantly differ between them during the late phase of the study.

Significantly more food was consumed by the OVX group than by the other groups at weeks 3 and 4, than at weeks 7 and 9, and the amounts of food intake were similar among the groups (Fig. 2B).

Isoflavone might modulate estrogen receptor expression and improve cartilage through a mechanism similar to that of SERM. However, little is understood about the effects of isoflavone on cartilage. One study found that long-term supplementation with soy isoflavone did not significantly impact either the articular cartilage lesions of osteoarthritic knees, or the levels of any cartilage components in a monkey model (Ham et al. 2002). However, soy protein isolate was the source of isoflavone in that study.

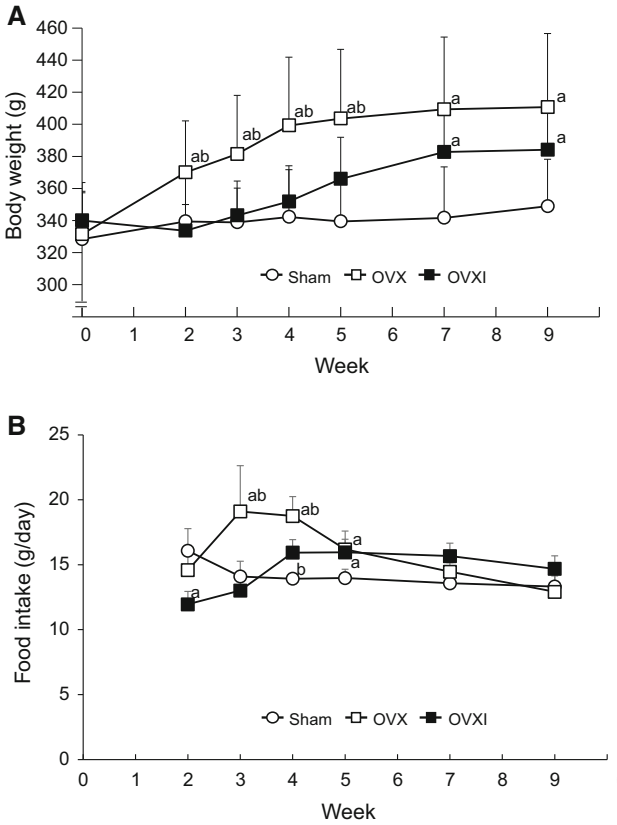

Fig. 2 Changes in body weight and food intake. Six-month-old SD rats underwent ovariectomy (OVX) (n = 25) or sham operation (sham; n = 9). Soybean isoflavone extract (51.0 mg/kg/day) was fed to rats in OVXI group for 9 weeks [■] (n = 11). Sham [◯] (n = 9) and OVX [□] (n = 14) groups were fed with regular diet for nine weeks. Values are shown as means ± SD. **A** Body weight. Chronological changes among three groups were compared using repeated measures ANOVA. Profiles of body weight changes among three groups significantly differed: (a) versus sham and (b) versus OVXI; both $P < 0.001$ (ANOVA, Tukey–Kramer tests). **B** Food intake. (a) versus sham and (b) versus OVXI; both $P < 0.05$ (ANOVA and Games-Howell tests). OVXI, ovariectomized and fed with isoflavone extract

Because we previously found that 50 mg/kg/day of daidzin, genistin and glycitin significantly prevented bone loss in ovariectomized rats (Toda et al. 1999; Uesugi et al. 2001), we calculated that isoflavone intake of 51.0 mg/kg/day would be appropriate for the rats in the present study. Although the OVXI group consumed slightly less food for 3 weeks, this increased thereafter and reached a plateau. Thus, the mean isoflavone intake during the present study was 50 mg/kg/day.

Cartilage degeneration

Knees were excised after nine weeks and cartilage degradation was histologically assessed by staining with toluidine blue. Staining was intense for the sham group (Fig. 3a) and faint in the OVX group, indicating a loss of matrix proteoglycans (Fig. 3b). Isoflavone supplementation ameliorated the loss of toluidine blue staining induced by ovariectomy (Fig. 3c). Defects at the tidemark of cartilage were not evident in any samples. Cartilage proteoglycan depletion was scored based on the intensity of toluidine blue staining. Damage scores were significantly higher for the OVX, than the sham group. In contrast, damage scores were significantly lower for the OVXI, than the OVX group (Fig. 4).

Cartilage metabolism might have been influenced by the estrogen deficiency induced by ovariectomy, because chondrocytes in articular cartilage possess estrogen receptors. Soybean isoflavone extract significantly reduced cartilage damage scores compared with the OVX group. The isoflavone administered in this animal model might have bound to and functioned with estrogen receptors in cartilage.

Ovariectomy induces more severe surface erosion in the knee cartilage of 5- and 7-month-old SD rats than in that of sham operated SD rats of the same age (Høegh-Andersen et al. 2004). That study also found that both 17-ethinylestradiol and SERM (-)-cis-3, 4-7-hydroxy-3-phenyl-4-(4-(2-pyrrolidinoethoxy) phenyl) chromane significantly suppressed cartilage degradation and erosion in ovariectomized rats treated with vehicle.

CTX-II and COMP in serum

Joint components including matrix and cartilage are degraded by proteases as osteoarthritis progresses. Degraded products such as CTX-II are released into serum and

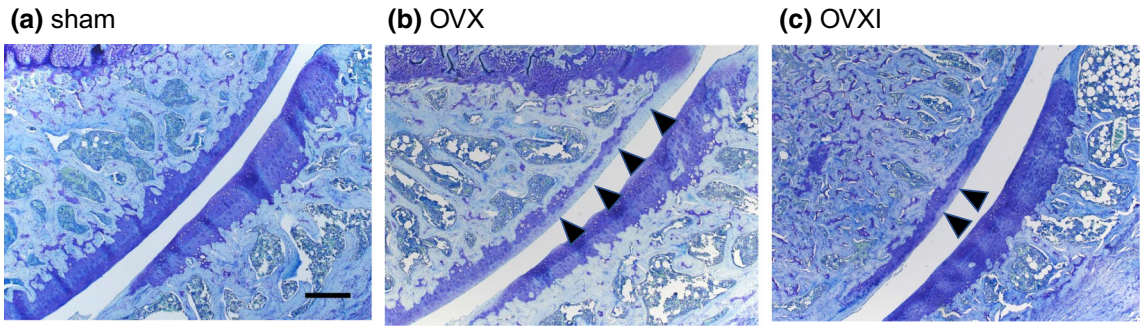

Fig. 3 Effects of ingested isoflavone extract on proteoglycan loss in knee joints. Experimental conditions were as described in Fig. 2. Sham operation (**a**) ovariectomy (OVX; **b**); OVXI (**c**). Intensity of toluidine blue staining for proteoglycans is obviously reduced in b and partially reduced in c (arrowhead). Bar = 400 µm. OVXI, ovariectomized and fed with isoflavone extract

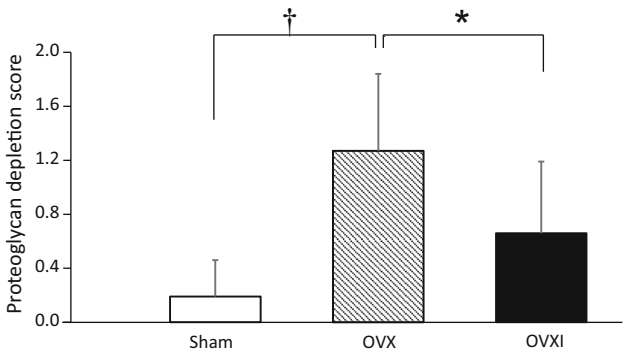

Fig. 4 Proteoglycan depletion of knee joint cartilage. Experimental conditions were as described in Fig. 2. Proteoglycan depletion in cartilage was scored from 0 (intense staining) to 3 (no staining). One-way ANOVA was adapted to clarify significant differences in cartilage depletion scores among groups. Post-hoc analysis proceeded using Steel–Dwass tests. Unfilled, shaded and black columns represent sham, ovariectomized (OVX) and OVXI groups, respectively. OVXI, ovariectomized and fed with isoflavone extract. Columns and bars represent means ± SD. $^{*}P < 0.05$; $^{\dagger}P < 0.01$

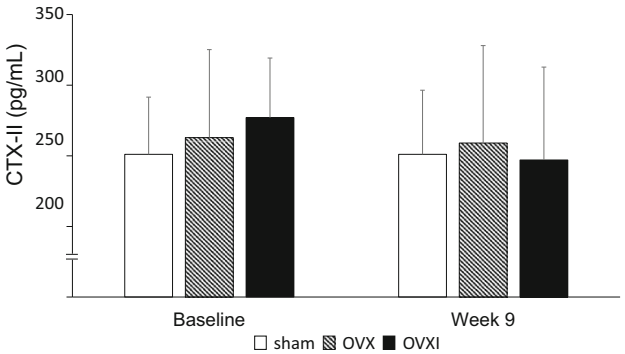

Fig. 5 Effects of OVX and oral soybean isoflavone on CTX-II values. Experimental conditions were as described in Fig. 2. Values at baseline and after nine weeks did not significantly differ among three groups (one-way ANOVA). Unfilled, shaded and black columns represent sham, ovariectomized (OVX) and OVXI groups, respectively. Columns and bars indicate means ± SD. OVXI, ovariectomized and fed with isoflavone extract

urine, and thus CTX-II concentration reflects the status of OA progression. However, serum CTX-II values did not significantly differ among the three groups either at baseline, or at the end of the study period (Fig. 5). Rates at which serum CTX-II decreased between before ovariectomy and the end of the study period, were lower in the OVXI, than in the sham and OVX groups (− 11.6% ± 15.9% vs. 3.18% ± 29.4% and − 0.75% ± 16.3%). However, the differences among the groups did not reach statistical significance (ANOVA, $P = 0.246$). Concentrations of serum COMP, which is a non-collagenous extracellular matrix protein and potential biological marker of cartilage turnover, in all samples were below the limits of detection (data not shown). The effects of ovariectomy on both CTX-II and COMP apparently were negligible.

Two studies have found increased cartilage turnover assessed by CTX-II assays in 5- to 7-month-old ovariectomized, compared with sham-operated rats (Høegh-Andersen et al. 2004; Oestergaard et al. 2006). The difference was most pronounced at 4 weeks after ovariectomy, tended to decrease thereafter, and was essentially minimal after 9 weeks between the OVX and sham operated group. The authors considered that these findings indicated a transient increase in cartilage turnover induced by the loss of endogenous estrogen production in the ovariectomized model, and might reflect the activation of mechanisms that antedated cartilage damage. We measured CTX-II levels in 6-month-old SD rats immediately before, and nine weeks after ovariectomy, during which the rats were fed with soybean isoflavone extract. Although we should have collected blood samples and measured CTX- II over time, the present results were not in conflict with those of Høegh-Andersen et al. (2004) and Oestergaard et al. (2006).

Conclusion

We postulated that isoflavone would influence OA induced by ovariectomy and thus investigated the effects of a highly concentrated extract of soybean isoflavone on cartilage degradation and turnover in 6-month-old ovariectomized SD rats. The histological findings showed that oral soybean isoflavone extract prevented increases in cartilage damage in a 6-month-old SD rat models of ovarian hormone deficiency. Consumption of soybean isoflavone might offer an alternative strategy for treating cartilage degeneration in post-menopausal women who are deficient in ovarian hormones. However, the present findings did not strongly support the notion that soy isoflavone are effective against OA. The main limitation of the present study is that we did not collect blood samples over time and could not assess relationships between cartilage damage and serum biomarkers. More biochemical analyses and dose dependent studies are required to establish the effects of soy isoflavone in ovariectomized animal models. Further studies are also needed to establish the effects of isoflavone in humans and to elucidate their modes of action in experimental animals.

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