Original Article

Combined effects of soy isoflavones and milk basic protein on bone mineral density in hind-limb unloaded mice

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We examined whether the combination of isoflavone and milk basic protein both are reported to be effective for bone metabolism, prevents bone loss induced by skeletal hind-limb unloading in mice. Female ddY strain mice, aged 8 weeks, were divided into six groups (n = 6–8 each): (1) normally housed group, (2) loading group, (3) hind-limb unloading group fed a control diet, (4) hind-limb unloading group fed a 0.2% isoflavone conjugates diet, (5) hind-limb unloading group fed a 1.0% milk basic protein diet, and (6) hind-limb unloading group fed a 0.2% isoflavone conjugates and 1.0% milk basic protein diet. After 3 weeks, femoral bone mineral density was markedly reduced in unloading mice. The combination of isoflavone and milk basic protein showed cooperative effects in preventing bone loss and milk basic protein inhibited the increased expression of osteogenic genes in bone marrow cells in unloading mice. These results suggest that the combination of soy isoflavone and milk basic protein may be useful for bone health in subjects with disabling conditions as well as astronauts.

***Key Words*: hindlimb-unloading, bone mineral density, soy isoflavones, milk basic protein (MBP)**

Spaceflight induces rapid bone loss as a result of mechanical unloading by microgravity. Humans exposed to long periods of spaceflight experience bone density loss, estimated at 1–1.5% per month.[1,2] As well as microgravity, bedridden induces bone loss as a result of mechanical unloading by immobility. Several studies reported that the degree of reduction in bone mineral density (BMD) of weight-bearing bone is associated with the degree of immobility.[3] As a model to simulate microgravity, hind-limb unloading, in which rats or mice are suspended by their tails and placed in a head-down tilt position, has been well established.[4] Their hind limbs are subjected to unloading and show bone loss.

In order to prevent the bone loss induced by microgravity or immobility by food components, we have examined the effects of soy isoflavones (ISO) in tail-suspension, hind-limb unloading animal models.[5] Soy ISO has structural similarities to estradiol, exhibiting weak estrogenic actions by binding to estrogen receptors.[6] A number of studies have found that administration of soy ISO inhibits bone loss in osteoporotic animal models.[7–9] Milk basic protein (MBP), which consists of whey protein with basic properties isolated from milk by cation exchange chromatography, reported that prevents bone loss in animal model as well as humans.[10–13] Morita *et al.*[14] reported that MBP might have multiple effects on bone metabolism in ovariectomized rats, which are achieved by the numerous proteins present in MBP.

On the other hand, ISO and MBP are approved by the Japanese Consumer Affairs Agency as a "Food for Specified Health Uses" for bone health. Therefore, these components have been verified the efficacy, safety and mechanism. However, the combination efficacy and mechanism of ISO and MBP in hind-limb unloaded mice remain to be fully understood.

We hypothesized that the combination of ISO and MBP show the additive or cooperative effects on prevention of bone loss caused by skeletal unloading. In this study, we examined the combination effects of 0.5% ISO and 1.0% MBP on bone mineral density and mRNA expression of bone related genes in bone marrow cells in hind-limb unloaded mice.

Materials and Methods

Animals and design. ddY female mice (7 weeks old, SLC, Shizuoka, Japan) were housed in individual cages in a temperature and humidity controlled (23 ± 1°C and 60 ± 5% relative humidity) room with a 12-h light/dark cycle. After 1 week of acclimation, the mice were randomly divided into 6 groups matched for body weight: normally housed group (Normal: n = 8), loading group (Loading: n = 6), hind-limb unloading group fed a control diet (UL-C: n = 8), hind-limb unloading group fed a 0.5% Fujiflavone P40 (Fujicco, Kobe, Japan) diet (UL-ISO: n = 8), hind-limb unloading group fed a 1.0% MBP (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) diet (UL-MBP: n = 8), and hind-limb unloading group fed a 0.5% Fujiflavone P40 and 1.0% MBP diet (UL-I + M: n = 8). The ISO and MBP concentrations were determined by our previous studies. We reported that 0.5% isoflavone diet maintained the whole femur BMD in hind-limb unloading mice, but not 0.25% isoflavone diet.[5] We also examined that 1.0% MBP diet maintains proximal tibia BMD in hind-limb unloaded mice (unpublished data). The mice were fed an AIN-93G diet[15] with corn oil instead of soybean oil with or without Fujifulavone 0.5% P40 and/or 1.0% MBP for 3 weeks (Table 1).

Fujiflavone P40 (ISO content, 40%; 0.2% ISO conjugates) is an ISO conjugates, with the following aglycones in 100 mg of conjugates: 33 mg daidzein, 8.5 mg genistein, and 15 mg glycitein. MBP was kindly provided by the Milk Science Research Institute (Megmilk Snow Brand) as described by Toba *et al.*[13] The protein concentration of MBP was 98%. Most of this protein is lactoferrin and lactoperoxidase, which comprise approximately 56% and 42% of the total MBP protein, respectively.[16] Hind-limb unloading was achieved using a tail suspension model.[4] The

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Table 1. Composition of the experimental diets[a]

Ingredient	Control[b]	ISO[c]	MBP[d]	ISO + MBP[e]
	g/100 g diet			
α-Corn starch	52.95	52.95	52.95	52.95
Casein milk	20	20	19	19
Sucrose	10	9.5	10	9.5
Corn oil	7	7	7	7
Cellulose	5	5	5	5
Mineral mixture	3.5	3.5	3.5	3.5
Vitamin mixture	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25
tert-Butylhydroquinone	0.0014	0.0014	0.0014	0.0014
Fujiflavone P40	—	0.5	—	0.5
Milk basic protein (MBP)	—	—	1	1

[a]Prepared according to AIN-93G formulation. [b]Control diet. [c]0.2% isoflavone conjugates (ISO) containing diet. [d]1% Milk basic protein (MBP) containing diet. [e]0.2% ISO + 1% MBP containing diet.

Table 2. Sequence of primers used for real-time PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
β-actin	CCACAGCTGAGAGGGAAATC	AAGGAAGGCTGGAAAAGAGC
Osterix	CCCTTCTCAAGCACCAATGG	AGGGTGGGTAGTCATTTGCATAG
Alkaline phosphatase (ALP)	ACACCTTGACTGTGGTTACTGCTGA	CCTTGTAGCCAGGCCCGTTA

Loading group was equipped for tail suspension, however, the mice were allowed to place the limbs on the cage bottom. The mice were fasted overnight before the dissection. The dissection was started at AM 10:00. The mice were euthanized by exsanguination under anesthetized with pentobarbital sodium (40 mg/kg body weight). Blood was withdrawn from the heart with a heparin coated syringe and separated the plasma by centrifugation at 3,000 rpm for 15 min at 4°C, and then stored at –80°C until analysis. For BMD measurement, the left femur was removed and stored in 70% ethanol. The left tibiae were removed to extract total RNA from the bone marrow cells. All procedures were maintained in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals in Japan.

Radiographic analysis of the femur. The left femoral BMD was measured by dual-energy X-ray absorptiometry (DXA; DCS-600EX-IIIR; Aloka, Tokyo, Japan). The scanned area of each femur was divided into three equal regions (proximal, midshaft, and distal) to assess the regional differences.

Plasma biochemistry. Plasma genistein, daidzein and equol were analyzed the time-resoved fluoroimmunoassay (TR-FIA) methods of Wang,[17] Uehara[18] and Broewerset *et al.*,[19] respectively. Plasma was hydrolyzed by glucuronidase and sulfatase, and the plasma was extracted using diethyl ether. Plasma genistein, daidzein and equol concentrations were determined by fluorescence using a DELFIA Victor 1420 multilabel counter (Perkin Elmer, Wellesley, MA). The final results were calculated using the following formula: final results = concentration × 1/ recovery × dilution factor (nM). The limit of detection for genistein, daidzein and equol are 4.0 nM, 2.0 nM and 3.3 nM, respectively. We used a commercially available kit for plasma concentrations of albumin (Wako, Osaka, Japan) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qRT-PCR). Total RNA was extracted from bone marrow of the tibia using Isogen II (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using Prime Script RT Master Mix (Takara, Shiga, Japan). cDNA was quantified by real-time reverse transcription polymerase chain reaction (RT-qPCR) using the Mini-Opticon Real-time PCR System (Bio-Rad, CA) and SYBR Premix Ex Taq II (Takara, Shiga, Japan). Cycling conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The primer sequences are shown in Table 2.

Statistical analysis. Data are expressed as mean ± SEM. The significance of difference for BMD was determined by single-factor analysis of covariance (ANCOVA) and Fisher's protected least-significant difference test. Body weight was used as a covariance in the analysis of BMD to adjust for possible confounding effects. The remaining data were determined by one-way analysis of variance (ANOVA) and Fisher's protected least-significant difference test. Comparisons of the effects of dietary ISO, MBP, and the interaction of both interventions were conducted using a two-way ANOVA among the unloading groups. Statistical significance was defined as $p<0.05$. Statistical analysis was performed using SPSS ver. 19 (IBM, NY).

Results

Body weights, food intake and plasma albumin concentration. Initial body weight did not differ significantly among the 6 groups (Table 3). However, the final body weights in the UL-C, UL-ISO, UL-MBP and UL-I + M groups were lower than those in the Normal and Loading groups (Table 3). Body weight of the mice in the UL-C, UL-ISO, UL-MBP and UL-I + M groups were reduced, but the weight of Normal and Loading groups were increased. (Fig. 1) The food intakes in the UL-C, UL-ISO, UL-MBP and UL-I + M groups were significantly lower than those in the Normal and Loading groups (Table 3). Plasma albumin concentration in the UL-ISO group was significantly higher than those in the Normal, Loading, UL-MBP and UL-I + M groups, however, no significant difference between the UL-ISO and the UL-C groups was observed (Table 3).

Femur BMD by DXA. The DXA analysis indicated that the BMD of the whole and distal femur in the UL-C group was significantly lower than that in the Loading groups (Fig. 2A and B). Therefore, the attenuation in BMD caused by unloading was significantly inhibited by ISO and/or MBP treatment (Fig. 2A and B). Furthermore, the interaction between ISO and MBP on whole

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Table 3. Body weights, food intake, plasma albumin concentration, and isoflavone concentration

	Normal	Loading	UL-C	UL-ISO	UL-MBP	UL-I + M
Initial body weight (g)	29.98 ± 0.74	29.68 ± 0.53	30.51 ± 0.69	30.43 ± 0.65	30.40 ± 0.60	30.41 ± 0.55
Final body weight (g)	31.25 ± 1.87[a]	29.50 ± 0.89[a]	25.58 ± 0.44[b]	24.30 ± 0.73[b]	25.52 ± 0.81[b]	24.40 ± 0.57[b]
Food intake (g)	164.1 ± 1.44[a]	163.9 ± 1.51[a]	151.7 ± 2.05[b]	148.9 ± 1.93[b]	151.4 ± 1.46[b]	149.2 ± 1.96[b]
Plasma						
Albumin (g/dl)	2.68 ± 0.14[b]	2.51 ± 0.09[b]	2.77 ± 0.11[ab]	3.08 ± 0.08[a]	2.55 ± 0.14[b]	2.65 ± 0.17[b]
Genistein (nM)	nd[b]	nd[b]	nd[b]	1,735 ± 389[a]	nd[b]	795 ± 209[ab]
Daidzein (nM)	nd[b]	nd[b]	96 ± 36[b]	4,664 ± 1463[a]	nd[b]	2,126 ± 824[ab]
Equol (nM)	nd[b]	nd[b]	nd[b]	3,308 ± 685[a]	nd[b]	3,413 ± 869[a]

Values are expressed as mean ± SEM, n = 6–8; means with different letters differ, p<0.05. All differences were analyzed by multiple comparison with one-way ANOVA and Fisher's protected least significant difference test.
Normal, normal caged mice fed the control diet; Loading, loading mice fed the control diet; UL-C, unloading mice fed the control diet; UL-ISO, unloading mice fed the 0.2% isoflavone conjugates (ISO) diet; UL-MBP, unloading mice fed the 1.0% milk basic protein (MBP) diet; UL-I + M, unloading mice fed the 0.2% ISO and 1.0% MBP diet; nd, not ditect.

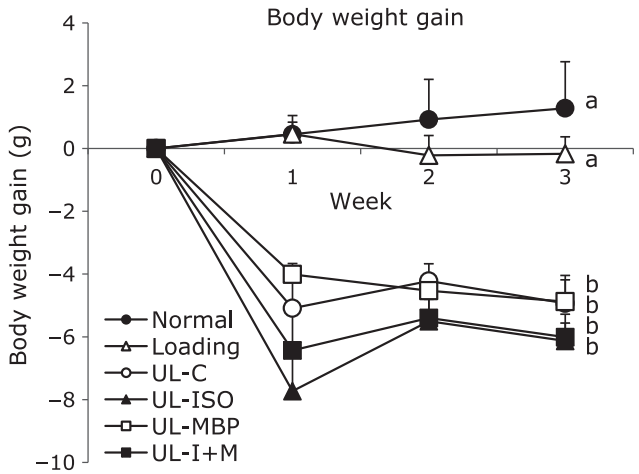


Fig. 1. Body weight gain in hind-limb unloading mice. Values are expressed as mean ± SEM, n = 6–8; means with different letters differ, p<0.05. All differences were analyzed by multiple comparison with one-way ANOVA and Fisher's protected least significant difference test. Normal, normal caged mice fed the control diet; Loading, loading mice fed the control diet; UL-C, unloading mice fed the control diet; UL-ISO, unloading mice fed the 0.2% isoflavone conjugates (ISO) diet; UL-MBP, unloading mice fed the 1.0% milk basic protein (MBP) diet; UL-I + M, unloading mice fed the 0.2% ISO and 1.0% MBP diet.

femur BMD was significant (Fig. 2A). Although there was no significant interaction between ISO and MBP on the distal femur BMD, a slight interaction was observed (p = 0.064) (Fig. 2B).

Quantitation of mRNA expression in bone marrow cells from the tibia. The mRNA expression of osterix and alkaline phosphatase (ALP), which are osteogenesis genes, were significantly higher in the UL-C group compared with Loading group (Fig. 3A and B). Significant reductions of those genes mRNA expressions by MBP were observed (Fig. 3A and B), whereas there were no significant effects of ISO, and an interaction between ISO and MBP was not observed (Fig. 3A and B).

Plasma genistein, daidzein and equol concentrations. Plasma daidzein, genistein and equol levels were higher with ISO intake compared with non-treated groups (Table 3). Plasma genistein concentration in the UL-I + M group was significantly lower than that in the UL-ISO group. Although plasma daidzein concentration in the UL-I + M group tended to be lower than that in the UL-ISO group, there was no significant difference in plasma equol concentration between the UL-I + M and the UL-ISO groups (Table 3).

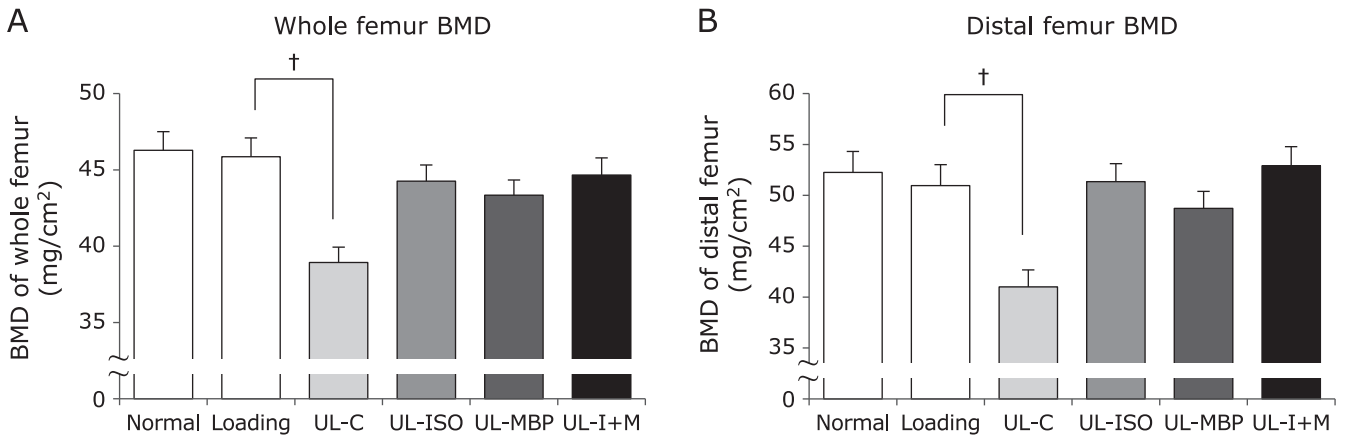
Discussion

The present study demonstrated that ISO or MBP treatment for 3 weeks prevents bone loss induced by skeletal unloading, and the combination of ISO and MBP have a cooperative effect on preventing unloading-induced bone loss. Furthermore, MBP inhibited the increased mRNA expression of osteogenic genes caused by unloading in bone marrow cells in unloading mice.

We observed the attenuation in BMD caused by unloading was significantly inhibited by ISO and/or MBP treatment (Fig. 2A), and there was significant interaction between ISO and MBP on whole femur BMD (Fig. 2A). Although there was no significant interaction between ISO and MBP on the distal femur BMD, a slight interaction was observed (p = 0.064) (Fig. 2B). It may be for this reason that the distal femur is rich in trabecular bone, which has a large surface area and is subject to bone resorption. In contrast, there was no significant effect of ISO and/or MBP treatment in the midshaft of the femur (data not shown), which is rich in cortical bone. Several studies support the bone-sparing effect of ISO or MBP in rodent models as well as humans.[5,10,13,20,21] In this study, we showed that bone loss induced by unloading was suppressed by ISO and/or MBP treatment, and an interactive effect between ISO and MBP was observed. These results suggest that ISO and MBP have a cooperative effect on preventing unloading-induced bone loss. Further studies are needed to determine synergetic or additive effect of ISO and MBP with the low level against unloading-induced bone loss in mice.

Hind-limb unloading resulted in a decrease in bone formation preceded by a transient increase in bone resorption; therefore the unloading model is considered to reflect imbalanced bone remodeling. We investigated the effect of ISO and/or MBP on the mRNA expression of osteogenesis (Osterix and ALP), which are critical genes for the regulation of osteoblasts in bone (Fig. 3A and B). The mRNA expression levels of these osteogenetic genes were significantly higher in the UL-C group compared with Loading group (Fig. 3A and B). Significant reduction of the expressions of Osterix and ALP mRNA by MBP were observed (Fig. 3A and B), whereas there were no significant effects of ISO, and an interaction between ISO and MBP was not observed (Fig. 3A and B).

In this study, unloading induced significant increase on the expression of Osterix and ALP mRNA. Bikle *et al.*[22] reported that hind-limb unloading increased ALP mRNA expression, which is consistent with our findings. These results indicate that osteogenic gene expression may be regulated by a compensatory response to the decline in osteoblast maturation. On the other hand, MBP significantly inhibited the elevation in the expression of these osteogenetic genes induced by unloading (Fig. 3A and B). A previous study reported that MBP might maintain the balance in bone remodeling because MBP contains several effective components for bone formation and resorption.[10] Thus, MBP may



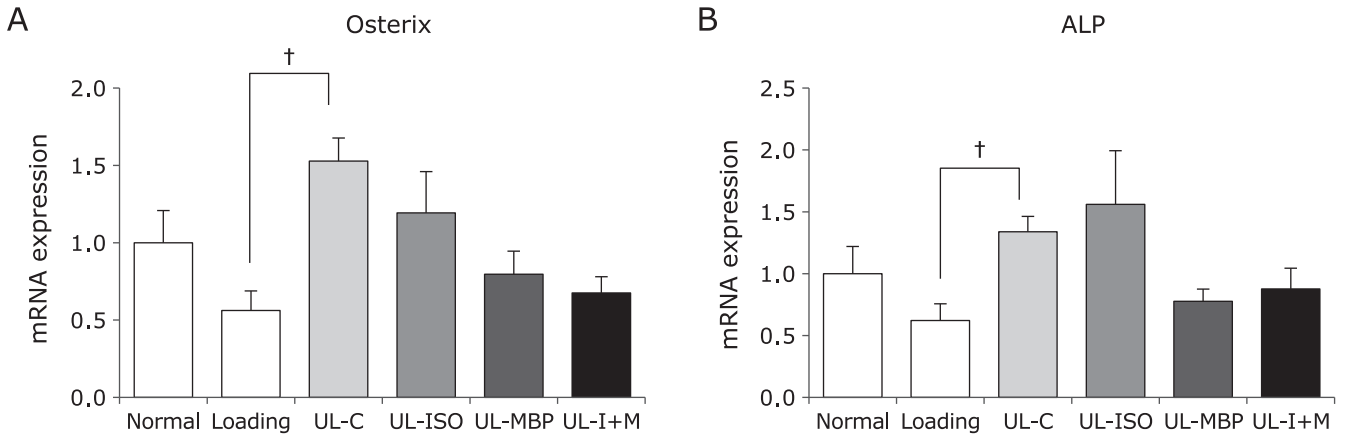
Two-way ANOVA

Factors		Interactions
ISO	MBP	ISO×MBP
$p<0.01$	$p<0.05$	$p<0.05$

Two-way ANOVA

Factors		Interactions
ISO	MBP	ISO×MBP
$p<0.01$	$p<0.01$	$p=0.064$

Fig. 2. Whole and distal femoral bone mineral densities in hind-limb unloading mice. Values are expressed as mean ± SEM, $n = 6–8$; †Significantly difference from the Loading group, the differences in BMD were determined by single-factor analysis of covariance (ANCOVA) and Fisher's protected least significant difference test ($p<0.05$). Body weight was used as a covariate in the analysis of BMD to adjust for possible confounding effect. Comparisons of the effects of dietary ISO, MBP, and the interaction of both interventions were conducted using a two-way ANOVA among the unloading groups. Normal, normal caged mice fed the control diet; Loading, loading mice fed the control diet; UL-C, unloading mice fed the control diet; UL-ISO, unloading mice fed the 0.2% isoflavone conjugates (ISO) diet; UL-MBP, unloading mice fed the 1.0% milk basic protein (MBP) diet; UL-I + M, unloading mice fed the 0.2% ISO and 1.0% MBP diet.



Two-way ANOVA

Factors		Interactions
ISO	MBP	ISO×MBP
$p=0.211$	$p<0.01$	$p=0.556$

Two-way ANOVA

Factors		Interactions
ISO	MBP	ISO×MBP
$p=0.514$	$p<0.05$	$p=0.804$

Fig. 3. Bone formation-related gene expression in hind-limb unloading mice. Values are expressed as mean ± SEM, $n = 6$; †Significantly difference from the Loading group, the differences was analyzed by multiple comparison with one-way ANOVA and Fisher's protected least significant difference test ($p<0.05$). Comparisons of the effects of dietary ISO, MBP, and the interaction of both interventions were conducted using a two-way ANOVA among the unloading groups. Osterix (A) and ALP (B) expressions in bone marrow cells of the tibia were normalized to β-actin. Normal, normal caged mice fed the control diet; Loading, loading mice fed the control diet; UL-C, unloading mice fed the control diet; UL-ISO, unloading mice fed the 0.2% isoflavone conjugates (ISO) diet; UL-MBP, unloading mice fed the 1.0% milk basic protein (MBP) diet; UL-I + M, unloading mice fed the 0.2% ISO and 1.0% MBP diet.

prevent bone loss caused by normalizing the bone remodeling balance through regulation of the osteogenic gene expression. Meanwhile, ISO is known to bind to the estrogen receptors in OVX animal models[23,24] and directly inhibit osteoclastic activity in cells, thereby preventing bone loss.[25] Accordingly, we suggest that the combined use of ISO and MBP results in enhanced effects on unloading-related bone loss through different regulatory mechanisms for bone metabolism. Further studies are needed to

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elucidate the precise mechanisms through which ISO and/or MBP attenuate unloading-induced bone loss.

In this study, the final body weights and the body weight gains in the UL-C, UL-ISO, UL-MBP and UL-I + M groups were lower than those in the Normal and Loading groups (Table 3). One of the reasons for the weight loss in the UL-C, UL-ISO, UL-MBP and UL-I + M groups may be lower food intake. Some studies involving hind-limb unloading mice using the tail-suspension model showed persistently lower body weights than those of controls. These reports concluded that the reduction in body weight was due to persistent endocrine stress.[26] In addition, it is possible that tail suspension induces hypokinesia and alters some factors that regulate energy metabolism or body weight in mice. However, the unloading mice in the present study appeared to be normal condition during the experiment, because the plasma albumin concentrations, which reflect general health condition to some degree, in the UL-C, UL-ISO, UL-MBP and UL-I + M groups were not lower than those in the Normal and Loading groups.

The diet containing ISO significantly increased plasma genistein, daidzein and equol concentrations (Table 3). Equol is a metabolic product of daidzein that exhibits stronger estrogenic activity than daidzein. Therefore, some part of the ISO effects on BMD may be due to equol. The plasma ISO levels in the present study were almost the same as those in a previous report that revealed the bone-sparing effects of ISO in rodents.[5]

In conclusion, we found that the combination of ISO and MBP had cooperative effects on preventing unloading-induced bone loss and MBP inhibited the increased expression of osteogenic genes in unloading mice. As a result, we suggest that MBP prevents unloading-induced bone loss by normalizing the bone remodeling balance. These results suggest that the combination of soy ISO and MBP may be useful for bone health in subjects with disabling conditions as well as astronauts.

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Abbreviations

BMD	bone mineral density
DXA	dual-energy X-ray absorptiometry
ISO	isoflavone
MBP	milk basic protein

Conflict of Interest

No potential conflicts of interest were disclosed.

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