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Isoflavone Aglycone from Fermented Soy Pulp Prevents Osteoporosis in Ovariectomized Rats

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Abstract: This study was done to investigate the effects of fermented soy pulp on the osteoporosis in ovariectomized rats. Sprague-Dawley female rats were randomly assigued to four groups as Sham Control (SC), Ovariectomized Control (OC), Ovariectomized and Soy Pulp (OSP) fed and Ovariectomized and Fermented Soy Pulp (OFSP) fed. All rats were fed on purified diets, supplemented with non-fermented and fermented soy pulp on basic diet for 7 weeks. It was observed that isoflavone aglycone was very high in soy pulp fermented for 12 h in comparison to non-fermented soy pulp. Body weight of the rats increased significantly ($p<0.05$) in comparison to other groups. Atrophy of uterus in OFSP group was significantly ($p<0.05$) prevented in comparison to OC group. The concentration of estradiol in OFSP group was higher than those of OC and OSP groups. The bone density in OFSP group was significantly ($p<0.05$) higher than those of OC and OSP groups. The histopathology indicated that OFSP group has better retarded the progress of osteoporosis than other groups. The results showed that isoflavone from the fermented soy pulp has prevented the osteoporosis in ovariectomized rats must be due to its estradiol like function. It is expected that the fermented soy pulp might serve as a functional food in osteoporosis of postmenopausal women.

Key words: Fermented soy pulp, isoflavone aglycone, ovariectomy, osteoporosis, bone density

INTRODUCTION

Osteoporosis associated with post menopausal ovarian hormone deficiency is the most common cause of age-related bone loss. It is generally recognized that estrogen deficiency following menopause plays a major role in osteoporosis (Nagata *et al*., 2002). Estrogen deficiency increases bone turnover, which leads to a decrease in bone mass and increases the risk of osteoporotic fractures in postmenopausal women (Lindsay *et al*., 1976). Although, hormone replacement therapy has been most

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commouly used for postmenopausal syndrome, it has some side effects such as increased risks of cardiovascular disease and breast cancer (Recker, 1993). Ipriflavone a synthetic flavonoid derivative have been reported to prevent bone loss in earlier studies (Benvenuti *et al*., 1991). Soy foods are known to contain isoflavones genistein and daidzein as aglycone forms with structures similar to 17-β-estradiol, which may be a potential alternative to hormone replacement therapy (Knight and Eden, 1996).

Asian populations with their high intake of soy foods are known to have low incidence of osteoporosis (Nagata *et al*., 1998). Lower incidence of osteoporosis in Japanese women is attributed to high consumption of soy foods (Horiuchi *et al*., 2000). Some animal studies demonstrated that dietary soy or genistein prevented bone loss (Arjmandi *et al*., 1996; Ishida *et al*., 1998; Ishimi *et al*., 2002; Tsuang *et al*., 2008). However, some studies reported significantly positive correlation between soy food intake and bone mineral density (Tsuchida *et al*., 1999; Somekawa *et al*., 2001).

Isoflvones are generally found in soy as glycosides (King and Bignell, 2000) and less as aglycone unless they have been fermented (Ribeiro *et al*., 2007). Generally processing of soybeans increases the concentration of aglycones through the hydrolysis of glucosides (Hutchins *et al*., 1995). Some lactobacilli and bifidibacteria are known to hydrolyze beta glucosides and enhance the bioavailability of isoflavones by fermentation (Hutchins *et al*., 1995; Chun *et al*., 2007). The aglycone form is more readily absorbed and bioavailable than the glucoside form (Izumi *et al*., 2000). These knowledge has led to the development of aglycone enriched products by fermentation using bifidobacteria (Otieno *et al*., 2006).

In the process of soymilk or tofu production, soy pulp (okara meal) is generated as a by-product and is mostly treated as industrial waste. Due to its water-holding capacity, almost the same amount of fresh soy pulp is produced from every kilogram of soybeans processed into such soybean products (Khare *et al*., 1995). Recently, fermented soy pulp has been developed as a new food source, since it is considered a suitable replacement for digestible food and has several beneficial health effects (O'Toole, 1999). Similarly, some researchers reported that *Lactobacillus* increases the biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk (Otieno *et al*., 2006; Pham and Shah, 2008). The endogenous estrogen level may indicate the relationship between soy intake and osteoporosis and the soy intake may affect the relationship between endogenous estrogen and osteoporosis. Hence, in this study we investigated the effect of soy pulp fermented by *Lactobacillus* on osteoporosis in ovariectomized rats.

MATERIALS AND METHODS

Experimental Plan

This research project was conducted from 01-05-2006 to 31-3-2008 in the Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul.

Twenty four female young adult Spragne-Dawley rats of 4 weeks age were purchased from Central Lab Animal, Inc. (Seoul, Korea) and housed in an environmentally controlled laboratory upon arrival. The rats were maintained in stainless steel cages and kept on a 12/12 h, light/dark cycle. Temperature and humidity were kept at 25±2°C and 60±5%, respectively. One week after acclimatization, six rats were randomly assigned for bilateral laparotomy as Sham Operated Contol (SOC) and remaining for bilateral ovariectomy. One week after recovering from surgery, the ovariectomized rats were divided into three groups (Table 1): overiectomized with normal diet (OC); with normal diet and soy pulp supplement (OSP) and with normal diet and fermented soy pulp supplement (OFSP). All animals were fed a Ca and P-deficient purified rodent diet (Dyets; Bethlehem, PA, USA) with adlibitum water. The composition of the experimental control diet is shown in Table 2. The SC and OC animals were fed a purified control diet, OSP and OFSP animals were fed a

Table 1: Summery of experimental groups of rat and treatment received by them

	SC	OC	OSP	OFSP
Ovariectomy	-	+	+	+
Normal diet	+	+	+	+
SP	-	-	+	-
FSP	-	-	-	+

SC: Sham control, OC: Ovariectomized control, OSP: Ovariectomized and soy pulp fed, OFSP: Ovariectomized and fermented soy pulp fed, SP: Say pulp, FSP:Fermented say pulp, +: Present, -: Absent

Table 2: Composition of normal diet used for feeding rats in the experiment

Ingredients	g kg^{-1}	kcal kg^{-1}
Corn starch	458.742	1651.471
Sucrose	100	400
Dextrose	155	589
Soybean oil	40	360
Cellulose, microcrystalline	50	0
Mineral mix No. 213263	35	114.8
Vitamin mix No. 310025	10	38.7
Potassium citrate	6.95	0
L-cystine	1.8	7.2
Choline bitartrate	2.5	0
Total	1000	3662.371

AIN-93M purified rodent diet (Dyets Inc.; pennsylvania, USA)

Table 3: Composition (%) of non-fermented (SP) and fermented (FSP) soy pulp used as supplement in this experiment

Ingredients	SP	FSP
Carbohydrate	66.70	63.90
Lipids	5.80	7.10
Ash	5.00	5.00
Moisture	1.00	3.00
Ca	0.49	0.47
P	0.23	0.23
Mg	1.27	1.26

similar control diet with the addition of lyophilized SP and FSP at 54 and 27 g kg^{-1} of feed, respectively. The total isoflavone aglycone content in SP and FSP was 3.4 and 17.9 mg kg^{-1}, respectively, which makes the supplement for rats as 1.8 and 4.8 mg kg^{-1} feed for OSP and OFSP group, respectively. Food intake was recorded every 3 days and body weight was measured weekly. All animal procedures conformed to the principles in the guide for the care and use of laboratory animals (National Research Council, 1996). At the end of the 7 week treatment period, blood, uterus and bone specimens were collected after killing the rats under appropriate anaesthesia.

Preparation and Composition of Fermented Soy Pulp

Fermentation of Soy Pulp (FSP) was done according to a previous report (Tsangalis *et al.*, 2002). Briefly, sterile soy pulp was inoculated with or without a 5% active culture of *Lactobacillus acidophilus* strain ATCC 4356 and incubated at 37°C for 0, 12, 24, 36 and 48 h with mild shaking. Then FSP and non-fermented Soy Pulp (SP) were lyophilized and finally ground to powder. Calcium (Ca), phosphorus (P) and magnesium (Mg) contents in FSP and SP were determined using inductively coupled plasma (ICP-AES, Jobin Yvon JY 38 Plus, France). The proximate compositions of FSP and SP viz. carbohydrate protein, lipids, moisture and ash were determined as per AOAC (1990) and presented in Table 3.

Isoflavone Concentration, β-glucosidase Activity

Isoflavone concentrations in FSP and SP were measured using the method of Tsangalis *et al.* (2002). We found that the isoflavone aglycone; daidzein and genistein content in soy pulp fermented

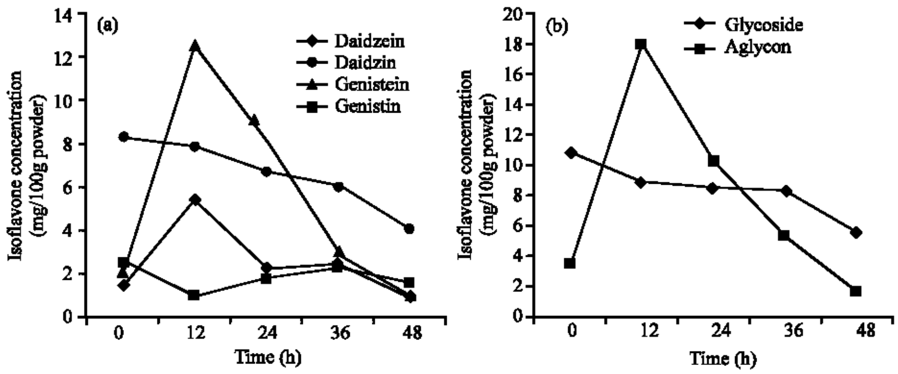

Fig. 1: Isoflavone glycoside (a) and aglycone (b) concentrations (mg 100 g^{-1} powder) in soy pulp fermented at 37°C for different time period with *Lactobacillus acidophilus* (strain ATCC 4356)

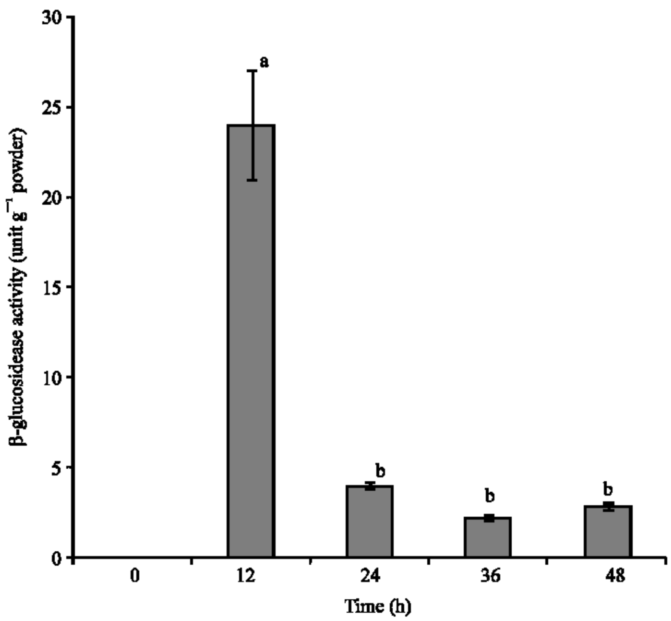

Fig. 2: β-Glucosidase activity in soy pulp fermented at 37°C for different time period with *Lactobacillus acidophilus* (strain ATCC 4356). Enzyme activity was defined as the amount of β-glucosidases that released 1 μmol of p-nitrophenol from the substrate p-nitrophenyl-D-glucopyranoside per minute

for 12 h was higher than that fermented for 0, 24, 36 and 48 h (Fig. 1). We measured beta-glucosidase activity in FSP to confirm whether this enzyme activity was involved in the biotransformation of isoflavone glycosides to isoflavone aglycones in FSP. The β-glucosidase activity was determined by measuring the rate of hydrolysis of p-nitrophenyl β-D-glucopyranoside using the method of Matsuura *et al*. (1995) with some modifications. The amount of p-nitrophenol released was measured using a spectrophotometer at 420 nm. β-Glucosidase activity decreased in the order of 12, 24, 48 and 36 h (Fig. 2). Thus, the β-glucosidase activity was highest at 12 h, which corresponded to the highest isoflavone aglycone concentration at 12 h in FSP. Therefore, we used the powder of soy pulp fermented for 12 h as an animal dietary supplement in this experiment.

Experimental Analysis and Histology

The serum estradiol concentration was measured by radioimmunoassay according to the method of Batzer (1980) and Erikson (1978). The bone density of the right femur was measured after treatment by dual X-ray absorptiometry (Gilsanz, 1999) and expressed as real density (g cm^{-2}). To study whether osteoporosis or bone regeneration occurred in the rats, we measured the formation of trabecular bone and fatty accumulation and the numbers of osteoclasts by comparing each femur, including cartilaginous tissue. The photomicrographic findings for trabecular formation, fatty accumulation and numbers of osteoclasts were graded into three categories, marked (3+), moderate (2+) and mild (+), to indicate the degree of osteoporosis and regeneration for the evaluation of different treatments.

Statistical Analysis

All the analysis were done in triplicate and data obtained were analyzed by ANOVA (Snedecor and Cochran, 1989) after estimation of the means and standard errors. Significant differences among treatment groups were determined by Duncan's multiple-range test at $p<0.05$ (Snedecor and Cochran, 1989).

RESULTS

The purpose of this study was to investigate whether fermented soy pulp containing higher isoflavone aglycone concentrations and whether it can prevent osteoporosis in overiectomized rats. We induced bone loss in ovariectomized rats and tried to see the effect of isoflavone supplement through fermented and unfermented soy pulp feeding. Soy pulp fermented for 12 h was found to have highest level of isoflavone aglycone (Fig. 1) and it was also supported by the highest level of aglycone due to glucosidase activity at 12 h (Fig. 2). Therefore, we used soy pulp fermented for 12 h as the supplement in rat feeding trial in this study. The total isoflavone aglycone (daidzein and genistein) concentrations (mg/100 g powder) in FSP and SP were 17.90 and 3.45, respectively (Fig. 1).

Food Intake, Growth and Uterine Weight

There was no significant difference in food intake among the groups, however, body weight gains were significantly ($p<0.05$) different among groups (Table 4). The highest body weight gain was observed in the OSP group followed by the OC, OFSP and SC groups. Ovariectomy resulted in extensive atrophy of the uterus in rats due to estrogen deficiency except in SC group. Overy weight

Table 4: Body weight, food intake, uterus weight, serum estradiol concentration and bone density in different experimental groups

	Dietary group			
Parameters	SC	OC	OSP	OFSP
Initial body weight (g)	238±4	222±7	233±4	223±5
Final body weight (g)	273 ± 7^{c}	320 ± 3^{ab}	333 ± 6^{a}	310 ± 10^{b}
Change in body weight (%)	12.8	30.6	30	28
Food intake (g day^{-1})	19.8±0.6	20.7±0.9	20.2±0.7	19.9±1.1
Uterus weight (g)	0.463 ± 0.025^{a}	0.050 ± 0.003^{c}	0.053 ± 0.003^{bc}	0.072 ± 0.010^{b}
Serum estradiol (pg mL^{-1})	72.3 ± 8.8^{a}	46.6 ± 7.3^{b}	49.0 ± 1.5^{b}	58.0 ± 4.0^{ab}
Bone density (g cm^{-2})	0.129 ± 0.002^{a}	0.107 ± 0.003^{c}	0.109 ± 0.002^{c}	0.117 ± 0.002^{b}

Each value is expressed as the Mean±SEM for six rats. ^{a,b}Means within row bearing different superscript are significantly different ($p<0.05$). SC: Sham control, OC: Ovariectomized control, OSP: Ovariectomized and soy pulp fed, OFSP: Ovariectomized and fermented soy pulp fed

Table 5: Histopathological observations in femur after 7 weeks of feeding trial in rats

	Dietary group (n = 2)			
Histopathological features	SC	OC	OSP	OFSP
Formation of trabecular bone	+++	+	+	+++
Fatty accumulation	+	+++	++	+
Number of osteoclasts	++	++	+++	+

SC: Sham control, OC: Ovariectomized control, OSP: Ovariectomized and soy pulp fed, OFSP: Ovariectomized and fermented soy pulp fed. +: Mild, ++: Moderate, +++: Marked

was significantly ($p<0.05$) higher in OFSP group than the SC group, however there was no significant difference between OFSP and OSP groups.

Serum Estradiol and Bone-Density

The serum estradiol level reduced significantly ($p<0.05$) in all overiectomized rats (46-58 pg mL^{-1}) compared to that of sham control (72.3 pg mL^{-1}). Though there was no significant difference in serum estradiol levels between OC and OSP and OFSP but there was significant ($p<0.05$) difference between estradiol level of SC and OC and OSP (Table 4).

The bone density reduced significantly ($p<0.050$) in overiectomized groups and a bone-sparing effect was shown on bone density (Table 4). The decrease of bone density in the OFSP group was significantly ($p<0.05$) lower than in the OC and OSP groups. On the other hand, bone density in all overiectomized groups was significantly ($p<0.05$) reduced compared to the Sham group although the contents of Ca, P and Mg in both FSP and SP were similar (Table 3).

Histopathological Observation

Formation of trabeculae in the OFSP group was graded as marked (3+) and similar to that in the Sham group (Table 5), but it was mild (+) in the other ovariectomized groups (OC and OSP). Similarly, accumulation of fat and the number of osteoclasts were mild (+) for OFSP as in the Sham group but it was more in other groups (OC and OSP).

DISCUSSION

The results suggest that body weight gain after ovariectomy is increased even without any feed supplement due to the estrogen deficiency. Uterine weight in all ovariectomized groups was dramatically lower than the Sham group, but in the OFSP group it was significantly ($p<0.05$) higher than the other OC groups. The atrophy in uterus due to estrogen deficiency is well established in previous research (Arjmandi *et al*., 1996). Supplementary FSP significantly ($p<0.05$) improved the atrophy, whereas, no similar effect was found in the OSP group which justify the estradiol like function of isoflavone aglycone in fermented soy pulp. This difference might be explained by the higher isoflavone aglycone concentration in FSP than in SP, because the isoflavone aglycones are metabolized into equol or p-ethylphenol by intestinal microflora, which improve their absorption (Uehara *et al*., 2001; Rafii *et al*., 2003). Therefore, easier access of intestinal microflora to higher isoflavone aglycones might have a modulatory effect in OFSP rats.

Daidzein and genistein have been reported to have an estradiol-like function (Knight and Eden, 1996; Bingham *et al*., 1998) and can improve osteoporosis. Ishida *et al*. (1998), Ishimi *et al*. (2002) have demonstrated that ingestion of soy isoflavones has preventive effects on ovariectomy-induced osteopenia in rats. In this study, isoflavone aglycones were orally consumed by rats at approximately 0.37 and 0.11 mg kg^{-1} body weight day^{-1}. The dose of isoflavone received by rats has already been earlierly reported to be efficient in preventing osteoporosis (Picherit *et al*., 2000; Dai *et al*., 2008). The decrease in bone density was also observed for the effects of soybean products in rats (Tsuchida *et al*., 1999; Omoni and Aluko, 2005). The reduction of bone density in overiectomized groups was possibly related to estradiol reduction induced by the ovariectomy (Nagata *et al*., 2002). However, the prevention of bone loss in OFSP might not be related to the serum estradiol level because we could not observe a significant difference in serum estradiol levels between OC and OSP or OC and OFSP. The beneficial effect on bone loss is also not due to the contaminated minerals because the prevention of bone density effect was ouly found in OFSP group although the contents of Ca, P and Mg in both FSP and SP were similar. Hence, this effect might be due to the estradiol like effect of higher content of soy isoflavone aglycone in fermented soy pulp.

The marked formation of trabeculae and mild accumulation of fat and the number of osteoclasts in the OFSP group indicated the efficient prevention of osteoporosis, which was not observed in the OC and OSP groups. These results also indicated that the dietary supplement with FSP containing a high level of isoflavone aglycones might played an important role in preventing the effect of ovariectomy by reducing the osteoporosis via their estradiol-like function. The results of this study suggest that the higher concentrations of isoflavone aglycone in fermented soy pulp might have prevented osteoporosis in overiectomized rats.

CONCLUSION

In conclusion, present results show that feeding fermented soy pulp to ovariectomized rat prevents bone loss, which might be related to the increase in isoflavone aglycone concentrations in the fermented soy-pulp. However, further study is necessary to understand the mechanism of action of fermented soy pulp isoflavones in ovariectomized rats.

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