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Research Article

THE EFFECT OF SOY ISOFLAVONES AND NONDIGESTIVE OLIGOSCCHARIDES ON BONE TURNOVER MARKERS

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ABSTRACT

Objective: The present study investigated the effects of soy isoflavones alone and combined with nondigestible oligosaccharides on prevention of bone loss in ovariectomized rats.

Methods: Sixty female Albino rats subjected to either; sham operated surgery (Sham, n= 12) or ovariectomized surgery (OVX, n=48), and then assigned to five groups of 12 rats each; {OVX control, OVX supplemented with 5% Raftilose®Synergy1,OVX supplemented orally with a dose of 30.6mg phyto soya extract/rat/day and OVX supplemented with both 5% Raftilose® Synergy1 plus 30.6 mg phyto soya extract/rat/day}.All rats fed a casein based diet (AIN-93M) for12 weeks.At the end of the experiment urine, blood and femur were sampled to investigate; serum calcium, phosphorus and magnesium, bone turnover markers(serum sRANKL, serum osteocalcin, serum total alkaline phosphatase and urinary deoxypyridinoline) and femoral BMD.

Results: Ovariectomy was found to elevate the rate of bone turnover as indicated by the higher levels of bone turnover markers and a reduction of sRANKL level in OVX control group comparing to sham group and as a result femoral BMD was reduced, but in three supplemented OVX groups the elevation in the levels of bone turnover markers were significantly reduced with a significant elevation in sRANKL comparing to OVX control group and this was followed by an improvement in the femoral BMD. The reduction of bone turnover markers, the elevation of sRANKL and the improvement in femoral BMD was found to be higher in the OVX group received a combination of the two supplements than using each supplement alone.

In conclusion: Our data suggests that isoflavones exert their effects on bone by stimulating bone formation and at the same time suppressing bone resorption, and the combined supplementation with NDOs and phyto soya extract rich with isoflavones have a cooperative and additive effect in the prevention of osteoporosis.

Keywords: Osteoporosis, Ovariectomized rats, Nondigestible oligosaccharide, Soy isoflavones.

INTRODUCTION

Osteoporosis is characterized by a reduction in bone density and strength to the extent that fractures occur after minimal trauma. It is well known that estrogen deficiency as in postmenopause and ovariectomy leads to acceleration of bone resorption and rapid bone loss, resulting in the development of osteoporosis [1].

Current therapies recommended for postmenopausal osteoporosis treatment include supplementation with estrogen or hormone replacement therapies (ERTorHRT). However, available evidence appears to suggest that the long-term use of ERT has numerous side effects[2,3].Currently, natural alternatives with estrogen-like activities such as soy isoflavones are being investigated as possible alternatives for HRT[4]. Isoflavones are naturally occurring phytoestrogens particularly soybeans, which are structurally and functionally comparable to 17β-estradiol, exhibiting similar estrogenic action by binding to the estrogen receptors[5]. They have recently received considerable attention for their potential use in the prevention of postmenopausal bone loss[6].

Evidence from clinical trails suggests that an increase in mineral balance –especially calcium- will positively affect bone mass but unfortunately, the solution is not as straight forward as simply, consuming more ca because the percentage absorption is inversely related to intake, so that increasing calcium intake may be partially negated by a corresponding decrease in the efficiency of calcium absorption[7]. An important mechanism to increase fractional calcium absorption may be through the consumption of nondigestible oligosaccharides(NDOs) such as the oligofructose-enriched inulin (Raftilose®Synergyl). The nondigestible oligosaccharides, inulin and its hydrolysate oligofructose, are present in many plants and are extracted commercially from chicory roots[8]. The fermentation of NDOs in the large intestine enhanced mineral absorption so, when NDOs added to a diet they cause increasing in whole body mineral retention and bone mineral accumulation, this in turn is thought to be preferable in preventing osteoporosis[9]. Uehara *et al.,* (2001) reported that NDOs improve the bioavailability of genistein and daidzein in rats given isoflavones conjugates. So the combination of dietary NDOs and soy isoflavones may be more efficient than either alone in the prevention of bone loss in osteoporosis[11].

Our study aimed to evaluate the protective effect of soy isoflavones alone and combined with nondigestible oligosaccharides on prevention of bone loss in ovariectomized rats.

MATERIAL AND METHODS

Animals and diets

Sixty adult female albino rats weight (160g-194g) were purchased from the animal house of the National Research Centre, Giza, Egypt. Rats were housed individually at 21°C in metallic cages with free access to water. All Rats were anesthetized by exposure to diethyl ether and subjected to either bilateral ovariectomized surgery (OVX, n=48), or sham operated surgery (sham, n=12), and the animal assigned to five groups of 12 rats each (sham-operated control, OVX control, OVX supplemeneted with 5% NDO [Raftilose®Synergy l] in the diet, OVX supplemented orally with a dose of 30.6 mg phyto soya extract/rat/day, and OVX supplemented with both 5% Raftilose® Synergy 1 plus 30.6 mg phyto soya extract/rat/day.

Rats were fed a casein based diet prepared according to the (AIN-93M) diet [12] for 12 weeks.

Corn oil was used instead of soybean oil to eliminate any possible interference with isoflavones in soybean oil.

The NDO (Raftilose® synergy 1) used in this study in an oligofructose –enriched inulin. It is a combination of long chains chicory inulin molecules , enriched by a specific fraction of short chains oligofructose produced by partial enzymatic hydrolysis of chicory inulin. It was obtained from Orafti Active Food Ingredients, Tienen. Belgium. The nondigestible oligosaccharide was added at 50g/kg diet by replacing an equal amount of cornstarch in the casein

based diet (AIN-93M)[13,14]. Neutral Phyto soya extract was obtained from Arkopharina, Laboratories pharmaceutiques, France. This neutral phyto soya extract is rich with isoflavones (daidzein and genistein) 41.08 mg/gm soya extract. The chosen dose used in this experiment for each rat was 30.6 mg/day. This dose was calculated according to the human dose recommended.

Table 1: composition of casein based diet (AIN_93M diet)

Ingredients	Casein based diet (g/kg diet)
Corn starch	620.692
Casein (8.85%protein)	140
Sucrose	100
Corn oil	40
Fiber	50
Mineral mixture(AIN-93M-MX)	35
Vitamin mixture(AIN-93M-VX)	10
L-cystine	1.8
Choline chloride	2.5
Tet-bytylhydroquinone	0.008

Analysis

Blood sampling

At necropsy, all rats way anesthetized by exposure to diethyl ether and blood samples were collected from the retro-orbital sinus. Serum was separated by centrifugation at 3000 rpm for 10 minutes and used for the estimation of :

- Serum sRANKL was determined by using a sandwich enzyme immunoassay for quantitative measurement (ELISA) (BioVendor Research and Diagnostic Products).
- Serum calcium, phosphorus and magnesium colorimetrically using commercially available kits.
- Serum osteocalcin (OC), as a marker of bone formation, by Sandwich Enzyme Linked Immunosorbent assay using rat ^{125}I-labelled OC, goat anti –rat OC antibody, and donkey anti- goat second antibody (Biomedical Technologies. Stoughton, MA, USA).
- Serum total alkaline phosphates, as marker of bone formation, colorimetrically using a colorimetric kit (Biomerieux Vitec, Missouri, (USA).

Femur sampling

Directly after the blood collection, the right femurs were excised and cleaned from soft tissues, and then stored in saline water for measuring the bone mineral density (BMD) by dual energy X-ray absorptiometry (DXA) equipped.

Urine sampling

At the end of the 12 weeks of dietary feeding and on the day before necropsy , 24 hours urine sample were collected from each rat and then centrifuged for the determination of :

- Urinary deoxypyridinoline (DPD), as a marker of bone resorption by a competitive enzyme immunoassay in a microtiter stripwell plate utilizing a monoclonal anti-DPD antibody coated on the strip to capture DPD (Metra DPD, Quidel Corporation, San Diego, USA).
- Urinary creatinine which was measured colorimetrically using commercially avilabile kit (Stanbio Creatinine Procedure No. 0400, Stanbio Laboratory, Boerne, TX ,USA) to adjust the DPD values, this was done to eliminate errors due to the difference in renal function of individual rats[15].

Statistical Analysis:

Statistical analyses were performed using the SPSS program, version 9.05 and Microsoft Excel 2003. Data were expressed as mean ± standard deviation (SD). Independent samples T-test was performed to determine the specific differences between means.

RESULTS

Serum calcium, phosphorus and magnesium

Mean concentrations of the serum minerals calcium, phosphorus and magnesium were significantly decreased in the OVX control group compared to sham group. On the other hand, supplementing the diet of the OVX rats with the NDO (Raftilose®Synergy1) alone had a significant positive effect in increasing the mean of serum calcium and magnesium, but not phosphorus, comparing to the OVX control rats. The mean concentrations of serum phosphorus and magnesium, but not calcium, were significantly influenced in OVX rats supplemented with the phyto soya extract alone comparing to the OVX control rats. On the other hand the combination group showed significant increase in the mean concentrations of the three minerals compared to the OVX control group (table 2).

Table 2: Mean serum calcium, phosphorus and magnesium levels in the different studied groups.

	Sham	OVX control	Supplemented OVX groups		
			NDO	Phyto soya extract	{NDO +Phyto soya extract}
Calcium(mg/dl)	9.7 ± 0.54	8.42 ± 0.34$^{\#\#}$	8.92 ± 0.45*	8.59 ± 0.4	9.03 ± 0.25**
Phosphorus(mg/dl)	5.33 ± 0.8	4.39 ± 0.94$^{\#}$	5.01 ± 0.43	6.89 ± 0.74**	5.89 ± 0.89*
Magnesium(mg/dl)	2.11 ± 0.07	1.94 ± 0.03$^{\#\#}$	2.06 ± 0.05**	2.05 ± 0.04**	2.04 ± 0.04**

Values are means ± SD, n = 12.

= Significant at P< 0.05, ## = Significant at P< 0.001 comparing with sham group.

* = Significant at P< 0.05, ** = Significant at P< 0.001 comparing with OVX control group.

Bone turnover markers

In the present study, ovariectomy elevated the rate of bone turnover and this was found in the significant increasing of the bone turnover markers levels (osteocalcin, alkaline phosphatase and deoxypyridinoline) of OVX control group comparing to sham group. This elevation was significantly reduced in the three supplemented OVX groups (NDO group, phyto soya extract group and NDO + phyto soya extract group) compared to the OVX control group and this reduction was noticed clearly in NDO and phyto soya combined group.

Soluble RANKL (sRANKL) elevated significally in both supplemented NDO and phyto soya extract groups when compaired with OVX group, On the other hand the combined group showed significant increase in the mean concentration of sRANKL comparing with OVX group (table 3).

Bone mineral density

Ovariectomy induced a decrease in femoral BMD, and this was obvious in the significant decrease femoral BMD in OVX control group compared to sham group. But this reduction was successfully improved by the supplementations. Since it was found that, the three supplemented OVX groups have significantly higher femoral BMD than the OVX control group, in addition this improvement was exacerbated by the combination of the two supplements (Figure 1).

Table 3: Mean serum osteocalcin , Serum total alkaline phosphatase , urinary deoxypyridinoline and serum sRANKL levels in the different studied groups.

	Sham	OVX control	Supplemented OVX groups		
			NDO	Phyto soya extract	{NDO +Phyto soya extract}
Serum osteocalcin (ng/dl)	14.58 ± 1.24	19.77 ± 2.08##	14.74 ± 0.39**	12.93 ± 1.13**	11.33 ± 1.04**
Serum total alkaline phosphatase (U/l)	118.89 ± 7.12	248.39 ±5.98##	116.65 ±5.64**	106.7 ± 7.83**	98.32 ± 9.85**
Urinary deoxypyridinoline (nmol DPD/ mmol creatinine)	79.34 ± 6.86	313.88 ± 16.4##	142.64±13.3**	148.97 ±5.01**	132.24 ± 6.79**
sRankl (pmol/l)	0.683±0.137	0.299±0.076##	0.991±0.323*	1.106±0.233**	2.035±0.232**

Values are means ± SD, n = 12.

= Significant at P< 0.05, ## = Significant at P< 0.001 comparing with sham group.

* = Significant at P< 0.05, ** = Significant at P< 0.001 comparing with OVX control group.

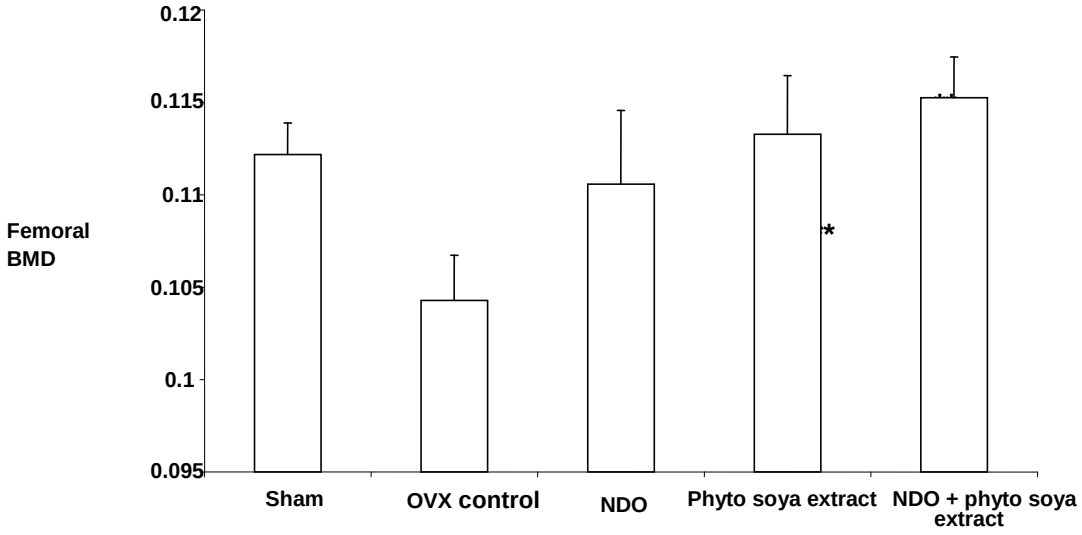

Fig. 1: Comparison of mean values of Femoral BMD between the different studied groups.

= Significant at P< 0.05, ## = Significant at P< 0.001 comparing with sham group.

* = Significant at P< 0.05, ** = Significant at P< 0.001 comparing with OVX control group.

DISCUSSION

Estrogen has wide-ranging effects on the regulation of bone remodeling. Through both direct and indirect effects, it influences calcium bioavailability, the activity of other endocrine factors involved in the regulation of bone remodeling, and the synthesis and activity of various inflammatory cytokines.

In rats, surgical menopause (ovariectomy) leads to a selective reduction in the number of vitamin D receptors (VDR) in jejunal but not renal cells[16]. A reduction in the jejunal VDR number results in reduced responsiveness of intestinal cells to vitamin D signaling and therefore, reduced intestinal calcium absorption[17].The results of the present study revealed significant decreases in mean serum calcium, phosphorus and magnesium in the OVX control rats compared to sham rats. This finding is in agreement with Clara etal.2012 who reported that osteoporotic women have decreased mineral absorption as a result of the reduction in serum 1,25-dihydroxyvitamin D[1,25-(OH)2D] which is known to be a regulator of bone mineral homeostasis. Supplementing the diet of the OVX rats in this experiment with NDO alone had a significant positive effect in increasing the mean concentrations of serum calcium and magnesium when compared to the OVX control rats. These results are in agreement with many studies examined the beneficial effect of NDOs on mineral absorption in animals[14,19] and human subjects[20,21]. Basically it has been speculated that the stimulatory effect of NDO on mineral absorption is mainly due to their prebiotic character. Their colonic fermentation produces SCFA (mainly acetate, propionate, and butyrate) and other organic acids (e.g. lactate) that contribute to a significant reduction in caecal lumen pH. This reduction in caecal pH leads to greater solubilization of calcium and magnesium so that the biologically available concentration of these minerals is increased[22]. In addition both low pH and SCFAs induces caecal development and caecal weight rise at least 2-fold greater than normal, resulting in a greater exchange surface area in the caecum and thus enhanced mineral absorption[23]. In the present work, concerning the effect of phyto soya extract rich with isoflavones on serum mineral concentrations, and the mean concentrations of serum phosphorus and magnesium, but not calcium,there were significantly influenced in OVX rats supplemented with the phyto soya extract compared to OVX control rats. This result is corresponding to (Arjmandi *et al*.,2002),who reported that supplementation with soy protein containing isoflavones prevented the ovariectomy-induced reduction in calcium transport in duodenal and colonic cells in in vitro transport experiments. However, no effect of soy protein supplementation, with or without isoflavones,on urinary calcium excretion or circulating.

In the present study, ovariectomy induced the elevation of bone turnover markers, and this was indicated when comparing the levels of serum osteocalcin, total serum ALP and urinary DPD concentrations in OVX control rats with sham rats. This result was in agreement with several experimental studies[15,25]. OVX-induced osteoporosis belongs to high conversion type where bone formation and bone resorption are all increased but bone resorption is more remarkable[25].

The elevation in the rate of bone turnover after ovariectomy can be attributed to the absence of estrogens. Estrogens have the ability to decrease the differentiation of osteoclast progenitor cells[26], inhibit the bone resorbing activity of terminally differentiated osteoclasts[27]and regulate the life span of mature osteoclasts by inducing apoptosis.

One of the major pathways governing osteoclastogenesis involves a triad of proteins including a receptor (receptor activator of nuclear factor kappa B [RANK]), a ligand (receptor activator of nuclear factor kappa B ligand [RANKL]), and a decoy receptor (osteoprotegerin OPG)[28].

RANKL and OPG are expressed by osteoblasts[29]. However, binding of RANKL to OPG prevents RANKL-RANK binding and therefore indirectly inhibits osteoclastogenesis[30].

The relative levels of RANK, RANKL, and OPG are important for controlling osteoclastogenesis. Daidzein increased secretion of both OPG and sRANKL and increased concentration of membrane-bound RANKL and promote apoptosis of osteoclast progenitors by an ER-mediated mechanism[31]. Genistein modulates expression of both RANKL and OPG. Genistein increased ERβ expression in rat mandibular subchondrial bone[32]. By binding to ERβ in osteoblastic cells *in vitro*, phytoestrogens induce production of OPG. OPG competes with RANKL and prevents maturation of pre-osteoclasts and thus, resorption[18]. The mechanism appears to be linked to genistein's ability to inhibit topoisomerase-II activity[33].

In postmenopausal women supplemented with genistein for 12 months, the ratio of sRANKL: OPG in serum was significantly lower than in non-supplemented controls[34].This may indicate that genistein inhibits RANKL-induced osteoclastogenesis in postmenopausal women[17].Our results showed a significant increase in sRANKL in groups supplemented by isoflavones than OVX group. This elevation is more increased in groups supplemented with both NDO and phyto soy, as it was found that in ovariectomized rats, fructo-oligosaccharide supplementation to a soy-based diet was shown to increase the plasma levels of genistein, daidzein, and improve the protective effect of isoflavones against bone loss secondary to castration[15].

Several studies reported that the rate of bone turnover appears to play an important role as a determinant of bone mass[35]. This could also be noticed in the present experiment where the results revealed that the elevation in the rate of bone turnover,as a result of ovariectomy process,decreased BMD of the right femur in the OVX control rats comparing to the sham rats. The significant reduction in the mean concentrations of bone turnover markers in rats supplemented with NDO in their diet showed that the Nondigestible oligosaccharides have the ability to reduce the elevation in the rate of bone turnover . This results was found in several experimental studies[14,15] and clinical studies[21,36].The reduction effect of NDO on the rate of bone turnover could be attributed to the stimulatory effect of NDO on the enhancement of calcium absorption which is followed by a suppression of PTH and consequently a reduction in the osteoclastic activity thus the rate of bone resorption decreases[37,38]. The suppression of bone resorption would be expected to result in suppression of the bone remodeling rate and a measurable increase in bone mass over time[39] and this was obvious in the present experiment by measuring femoral BMD of the OVX rats supplemented with NDO, where an improvement in femoral BMD was observed in this group comparing to OVX control group.

Phyto soya extract rich with isoflavones also proved to be able to reduce the elevation in the rate of bone turnover induced by ovariectomy, and this was indicated by the significant decrease in bone turnover markers in OVX rats supplemented with phyto soya extract compared to OVX control rats. The mechanisms by which soy isoflavones positively affect bone turnover rate may be directly by interacting with estrogen receptors ER-α and ER-β or indirectly by suppressing the release of proinflammatory cytokines from bone cells which are often elevated after ovariectomy[40].

In addition Hutabarat *et al.,*(2000) explained that soybeans contain not only the phytoestrogen isoflavones but also the phytoestrogens coumestans and lignans and it is possible that these phytoestrogens can also suppress bone resorption under estrogen deficient conditions. The reduction effect of phyto soya extract on the rate of bone turnover was followed by an improvement in the femoral BMD. The femoral BMD was significantly greater in the OVX rats supplemented with phyto soya extract comparing to OVX control rats. Viereck *et al.,*(2002) reported that mechanism by which phyto soya extract can affect bone turnover rate and thus improve BMD may be related to the presence of specific isoflavones, such as genistein, which inhibit osteoclast activity and osteoclast survival in femoral–diaphysial tissues of elderly female rats and ultimately prevent the loss of trabecular bone after ovariectomy. The combination of NDO and phyto soya extract rich with isoflavones have been reported to have an additive effect on the prevention of post-ovariectomy bone loss[15], and this cooperative effect was noticed in the reduction of bone turnover markers and improving femoral BMD higher than using each supplement alone.

In conclusion, adding NDO to a soy-based diet can offer improvements beyond that of soy alone and the results suggest that some of the combinations may affect different areas with varying degrees. Therefore, it is necessary to investigate if these bioactive compounds either together or independently can also restore bone mass in postmenopausal women. NDO combined with soy in the diet provide a valuable alternative to conventional osteoporosis therapies due to their anti-resorptive and anabolic properties.

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