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EFFECT OF SOYMILK AND FERMENTED SOYMILK POWDERS ON OSTEOPOROSIS IN OVARIECTOMIZED RATS

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SUMMARY

The present study aimed to evaluate the effect of fortified biscuits (manufactured using wheat flour, 72% extraction and fortified with 15% soymilk (SM) or fermented soymilk (FSM) powders) on osteoporosis status of ovariectomized rats. Serum calcium, phosphorus concentrations and alkaline phosphates activity, radiographic evaluation, physical properties (length, breaking force and bone density) and bone mineral density were measured as indicators for the efficiency of the tested fortification processes. It was concluded that biscuits contained soymilk and fermented soymilk powders caused no significant differences in serum ca and p concentrations in both of ovariectomized and normal rats. On contrary, serum alkaline phosphates activity was higher in ovariectomized rats fed on diets containing SM and FSM powders compared with those found in rats fed on a basal diet or control biscuit. The femoral bone lengths were also taller in ovariectomized rats fed on diets containing SM and FSM powders than normal rats taking the same diets. Breaking forces of rats femurs were slightly higher in normal rats and ovariectomized rats fed diets containing soymilk and fermented soymilk compared with femurs of those fed on the diet control biscuit. On the other hand, bone density and bone mineral density values were elevated in ovariectomized rats fed on the tested diets(SM and FSM powders) compared with those fed on a basal diet or control biscuit.

INTRODUCTION

Soybeans are rich source for protein, fat, and a good source for energy, vitamins and minerals (Nwokolo and Smartt, 1996).Soymilk is a water extract of cooked whole soybean, resembling dairy milk in appearance and composition (Liu, 2000). Fermentation of soymilk with lactic acid bacteria preserving soymilk and modifying the flavor and texture characteristics to make it more acceptability and also improve the digestibility (Mital and Steinkraus, 1979). During fermentation the β-glycosyl bond of genistin is cleaved to produce genistein (Slavin et al., 1998).

Osteoporosis could be primary related to the age and sex or secondary due to an identifiable drug or disease that cause loss of bone tissue (Dodd, 1996). In most cases, bones weaken where the rate of bone degeneration is greater than the rate of bone formation, resulting in bone mass loss, that makes the bone weak and brittle and can easily to fracture (Faraj and Vasanthan, 2004). In human beings postmenopausal women are at risk for osteoporosis, and the loss is associated with reduction in estrogen level (Marcus, 1996).A sharp decrease in ovarian estrogen production is the predominant cause of rapid hormone - related bone loss during the first decade after menopause (Gruber et al., 1984). Hormone replacement therapy (HRT) has been used to prevent osteoporosis in such cases. HRT may increase the incidence of some types of cancer besides other side effects (Blum et al., 2003). On the other hand, it was found that consumption of soy products containing isoflavones could minimize risk factors for cardiovascular disease and osteoporosis (Potter et al., 1998). The high concentration of isoflavones subclass of the familiar flavonoids (found in soybean and soybean products) are pharmacologically and structurally similar to the synthetic phytoestrogens as tamoxifen and ipriflavone that prevent or reduce bone loss (Arjmandi et al., 1996). Hou and Chang, (2002) stated that the main isoflavones found in soybean are genistein, daidzein and glycitein. Genistein binds weakly with estrogen receptor ER alpha but binds strongly with ER beta as estrogen does (Kuiper et al., 1998).This may explain genistein's ability in preventing bone loss in ovariectomized rats (Blair et al., 1996).The scientific community has firmly established a connection between diet and risk of chronic diseases such as cancer and heart disease. Soy foods are gaining popularity because of their specific health benefits (Faraj and Vasanthan, 2004). Isoflavones, when provided either purified or in soy protein, attenuate the loss of bone mass that occurs during and after menopause (Alekel et al., 2000).

MATERIALS AND METHODS

Soymilk and fermented soymilk were obtained from soy processing unit, Food Technology Research Institute (FTRI), Agriculture research Center, Giza, Egypt. Soymilk and fermented soymilk dehydration by lyophilized with a freeze drier (WH-FreeZone 4.51 Benchtop freeze dry system, Labconco Co.) according to Wang et al., (2004). Biscuits samples were prepared with replacement wheat flour (72%extraction) by 15% soymilk or fermented soymilk powders according to the method described by Wade (1988).

Chemical analysis: moisture, protein, ether extract, crude fiber, and ash, contents of tested samples were determined according to A.O.A.C (2000).

Experimental animals:

Seventy adult Sprague-Dawely female rats were used in this study, and were three months old with average weight was 180 ± 5.0 gm. The rats were housed in the Department of animal behavior - Faculty of Veterinary Medicine-Cairo University

, well aerated cages supplied with drinking wa- er bottles at room temperature (25±2°C). Rats were kept for the first week to be adapted to the new environment and were fed on a basal diet according to (NRC, 1995) to supply 15% protein (using casein 85% protein), 12% oil, 4% salt mixture and 1% vitamins mixture. Minerals and vitamins mixtures in the basal diet were prepared according to Reeves et al., (1993). After adaptation period, rats were divided into two equal main groups (GA and GB). Those in GA were fed on basal diet for thirty five days then they were sub divided into 5 equal groups (GA_1, GA_2, GA_3, GA_4 and GA_5). Ovariectomy was performed in rats of group B according to Waynforth, (1980). The rats were anaesthetized using Thiopental sodium 1.25 % (0.4 ml /rat) interaperitoneal according to Lumley et al., (1990).The rats were casted on its ventral surface and the back of each rat till the tail was prepared for aseptic surgery (Fig. 1). A midline dorsal skin incision of 1-2 cm was inflected half way between the middle of the back (the hump) and the base of the tail (Fig. 2). Pointed scissors were inserted subcutaneously through the incision and pushed down on either side of the rat for a short distance to spread and tear the connective tissue to allow access to the muscles. Few millimeters below the spinal muscles, a small cut was made with pointed scissors into the muscles on both sides. The entrance of the peritoneal cavity is gained by making (Fig. 3). The ovaries were easily reached through the muscle opening and were found surrounded by a variable amount of fat (Fig.4). The ovarian blood vessels were legated at the junction between the fallopian tube and the uterine horn using chromic cat gut 2/0 to avoid bleeding. The ovaries were then removed and the horn was replaced into the abdominal cavity (Fig.5). The abdominal wound was closed using chromic cat gut 2/0 and simple continuous suture pattern (Fig.6). Lastly the skin incision was closed using No.0 silk and simple interrupted suture pattern (Fig. 7). The skin sutures were removed after one week (Fig.8). The ovariectomized rats (GB) were fed for thirty five days on a basal diet and sub divided into 5 equal groups (GB_1, GB_2, GB_3, GB_4, and GB_5). After 35 days rats of groups GA_5 and GB_5 were sacrificed to verify bone loss. The other four sub groups in both group A and group B were fed for 2 months on a diet formulated using biscuits containing 15% SM or FSM powders table (1). Table (2) illustrates the chemical analysis of biscuits samples. Calcium and phosphorus levels were adjusted in all diets to obtain the same levels of both ingredients using calcium phosphate and calcium carbonate. After two months blood samples were collected from the orbital plexus venous to obtain serum and then the rats were sacrificed. Serum stored at-20°C. Estimation of serum 17β-estradiol was estimated according to Ismail et al. (1986).

Table (1): Experimental groups and diets.

Groups	Diets
GA_1,GB_1	Basel diet
GA_2, GB_2	Control biscuit
GA_3, GB_3	Biscuit with 15% SM
GA_4, GB_4	Biscuit with 15% FSM

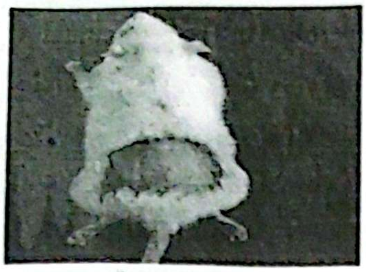


Fig (1)

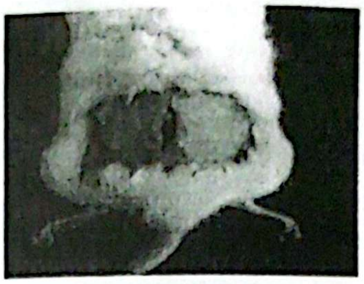
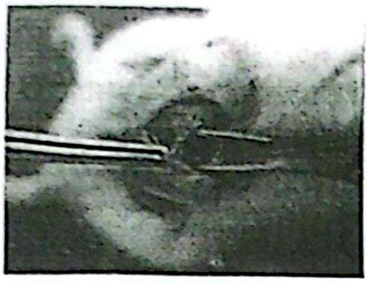


Fig (2)



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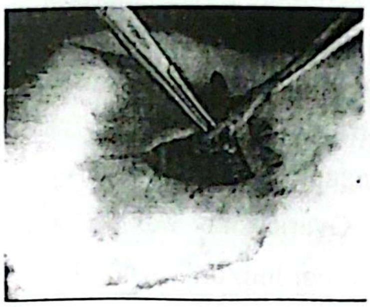

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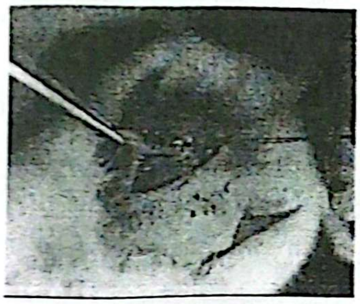

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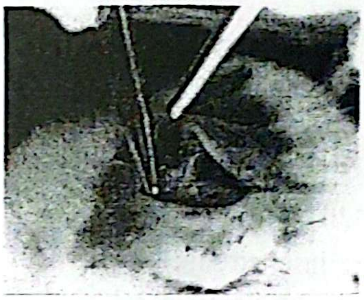


Fig (6)

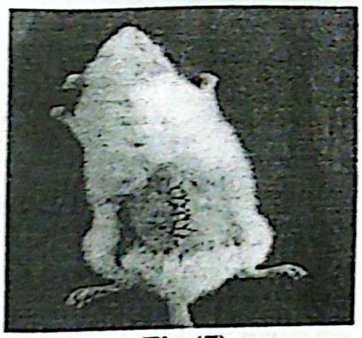


Fig (7)

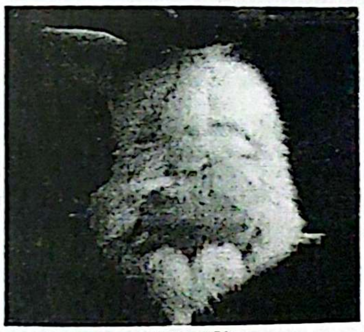


Fig (8)

Ovariectomy procedure of rats

Estimation of serum calcium and phosphorus levels was carried out according to Gindler and King (1972) and El-Merzabani et al. (1977) respectively. Alkaline phosphates activity was measured according to Belfied and Goldberg (1971). Both femurs were harvested and cleaned from the surrounding soft tissue. Contact radiography was performed using x-ray (Fischer Imaging) at 40Kv and 10MAs according to Goseki et al. (1996). The length of femurs was measured using a Varnier caliper using the method of Arjmandi et al. (1996). Bone density was measured by Archimedes's principle (Kalu et al., 1991). Breaking force was measured according to the protocol of Gomez-Aldapa et al. (1999). Bone mineral density (BMD) was determined using Dual energy X-ray absorption-meter as described by Blum et al. (2003). Statistical analysis of data was outlined by Gomez and Gomez (1984). The least significant difference test (LSD) at 5% level was carried out as mentioned by Waller and Duncan (1969).

RESULTS AND DISCUSSIONS

The chemical composition of biscuits contained 15% soymilk or fermented soymilk powders are presented in Table (2). The results showed that biscuits made using wheat flour (72% extraction) which substituted by15% SM or FSM powders contained high protein, ether extract and ash and low total carbohydrates contents compared to control biscuit sample. This results agreement with Hafez (2004). Table (3) shows the estimated values of serum 17 β-estradiol in rats feed on different diets for three months. These data indicated that its concentration was lower in ovariectomized rats (GB_5) than in normal rats (GA_5). Also, soy feeding did not alter serum 17β-estradiol concentrations either normal or ovariectomized rats. A result indicating that the isoflavones in soy unlike estrogen are not uterotrophic. Therefore, the mechanism of action for soy isoflavones is different from that of estrogen in uterine tissue and blood (Ettlinger, et al., 1988). Serum calcium and phosphorus concentrations are presented in Tables (4and 5). No significant difference between ovariectomized and normal groups using the different dietary treatments. These results were in agreement with Arjmandi et al. (1996). This is probably because ca-regulating hormones remained functioning to maintain calcium and phosphorus even in absence of estrogen (Goseki et al., 1996). Table (6) shows that there is increase in serum alkaline phosphates activity (ALP) as an index of bone formation in ovariectomized rats feeding on basal diet for 35 days compared with normal rats. Similar to what happen in cases of rickets or osteomalacia, this increase could be attributed to partial degrading and releasing of ALP in a large quantity from the osteoblast cell membrane into serum under disadvantageous circumstances in the ovariectomized animals. (Goseki et al., 1996). At the end of the experiment (3months), serum ALP activity was higher in ovariectomized groups fed on diets containing SM or FSM compared with those fed on a basal diet or diet containing control biscuit. These results were in agreement with Fanti et al. (1998). They reported that genistein in soy slowed the rate of bone loss and promoted bone formation in rats. Contact X-ray films (Fig. 9) of rat femurs fed on different diets are less radio-opaque in GB_5 compared with GA_5 after one month. These results might indicate that bone insulin- like growth factor in the ovariectomized group was depleted. In the meantime, reduction in the bone morphogenetic protein (BMP) activity may be one of the critical factors in the pathogenesis of post- menopausal osteoporosis (Goseki et al., 1996). On the other hand, normal groups (GA_3, GA_4) showed more radio- opaque bones than other groups. Moreover, ovariectomized groups (GB_3, GB_4) showed more radio- opaque femurs than those fed on basal diet. It was found that isoflavones present in soybean has the ability to increase bone insulin- like growth factor.1mRNA transcripts that correlates with both bone mineral density and the rate of bone formation (Kalu et al., 1994).Physical characteristics of the rat femurs (length, breaking force and bone density) fed with different diets are illustrated in Tables (7), (8), and (9). Table (7) shows that the femur length of ovariectomized rats (GB_5) fed on basal diet for 35days was increased compared with normal rats (GA_5). A result that might a sequel to ovariectomy that enhances synthesis of Insulin-growth factor-1 (IGF-I) that consequently increases bone size (Daniel, 1991). Also, the femoral bone lengths were greater in ovariectomized rats fed on diets containing soymilk and fermented soymilk than normal rats taking the same diets. Such explanations agree with that of Arjmandi et al. (1998). Breaking force for the rat femurs is presented in Table (8). Data show that breaking force in normal group (GA_5) shows significant increase compared with ovariectomized rats (GB_5) fed on basal diet for 35 days. Results show also that breaking force of rat femurs tended to increase in normal rats and ovariectomized rats fed diets containing soymilk and fermented soymilk compared with femurs of those fed on diet containing control biscuit. These results are in agreement with Omi et al. (1994). They reported that dietary soy proteins improve the bone strength due to increase the efficiency of calcium absorption. Ash calcium content values of the femurs fed on different diets are presented in Table (9). Bone density (BD) is directly related to these values. The data show that ovariectomized rats

(GB_5) had less bone density than the normal rats (GA_5) as a sequel to deficiency of ovarian hormone in ovariectomized rats (Arjmandi et al., 1996). This hormone inhibits bone loss by stimulating osteoblast function or attenuating osteoclast activity, or both (Wronski et al., 1988). Rats fed on soymilk and fermented soymilk showed elevated BD values in ovariectomized rats compared with ovariectomized rats fed on basal diet or diet containing control biscuit. On the other hand, the decrement of BD values was noticed in ovariectomized rats fed on basal diet and control biscuit. Similar results were obtained by Omi et al. (1994). They reported that the cause of the positive effect on BD of ovariectomized rats fed soymilk compared to casein basal diet may be due to enhancing calcium absorption. Bone mineral density (BMD) of the femur is shown in Table (10) and Fig (10). Results revealed that BMD was significantly increased in normal group (GA_5) compared with ovariectomized group (GB_5). After rats were fed on soy diets for sixty days, the BMD was significantly greater at bones in ovariectomized groups (GB_3, GB_4) compared with those fed on a casein diet (GB_1) and diet containing control biscuit (GB_2), It is believed that genistein inhibit osteoclast activity and osteoclast survival in elderly female rat's femoral diaphysial tissues and prevent the loss of trabecular bone after ovariectomy (Gao and Yamaguchi 1999).In the meantime, no significant variance was recorded in the normal rats fed on soy (GA_3, GA_4) compared with the casein-fed ones (GA_1). This could be attributed to isoflavones-containing soy protein diets would delay the decrease in BMD compared with diet control biscuit (potter et al., 1998).

Table (2): Chemical analysis of biscuits and casein (g/100g).

Sample	Protein	Fat	Fiber	ash
Control biscuit	10.42	15.87	2.14	1.99
Biscuit with 15% SM	13.03	19.96	1.57	2.19
Biscuit 15% FSM	12.71	20.35	1.89	2.65
Casein	85	---	----	2.66

SM= Soymilk powder FSM= Fermented soymilk powder

Table (3): Serum 17 β- estradiol concentration (pg/ml) in normal and ovariectomized rats fed on different diets for three months.

Experimental period	Diet	Normal Group (A)	Ovariectomized Group(B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	57.00 ± 2[ab]	17.00 ± 5[c]
3 months	Basal diet	55.13 ± 1[b]	15.33 ± 2[cd]
	Control biscuit	54.9 ± 0.9[b]	14.33 ± 5[d]
	Biscuit with 15% SM	58.47 ± 2[a]	15.53 ± 1[cd]
	Biscuit with 15% FSM	59.00 ± 2[a]	17.00 ± 4[c]
L.S.D at 5% Treatments	1.632		
Time	1.032		

SE=standard error.

SM = Soymilk powder FSM = Fermented soymilk powder

Means with the same letter(s) are not significantly different ($p < 0.05$).

Table (4): Serum calcium concentration (mg/dl) in normal and Ovariectomized rats fed on different diets for three months.

Experimental period	Diet	Normal Group (A)	Ovariectomized group(B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	6.91 ± 0.29[abc]	6.94± 0.28[ab]
3 months	Basal diet	6.92 ± 0.35[abc]	6.86 ± 0.30[bc]
	Control biscuit	6.82 ± 0.32[c]	6.97± 0.21[ab]
	Biscuit with 15% SM	6.87 ± 0.35[bc]	7.02± 0.37[a]
	Biscuit with 15% FSM	6.90± 0.34[abc]	6.98± 0.35[ab]
L.S.D at 5% Treatments	0.076		
Time	0.048		

SE=standard error.

SM = Soymilk powder FSM = Fermented soymilk powder

Means with the same letter(s) are not significantly different ($p < 0.05$).

Table (5): Serum phosphorus concentration (mg/dl) in normal and Ovariectomized rats fed on different diets for three months.

Experimental period	Diet	Normal group (A)	Ovariectomized Group (B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	3.96 ± 0.44 [ab]	3.97 ± 0.58 [ab]
3 months	Basal diet	3.90 ± 0.44[bc]	3.96 ± 0.56[ab]
	Control biscuit	3.84± 0.39[c]	3.85 ± 0.59[c]
	Biscuit with 15% SM	3.87 ± 0.45[c]	3.92 ± 0.55[abc]
	Biscuit with 15% FSM	3.98 ± 0.46 [ab]	4.04 ± 0.57[a]
L.S.D at 5% Treatments	0.054		
Time	0.034		

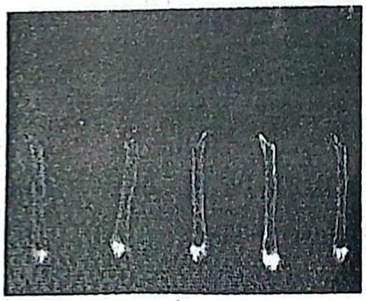
SE=standard error.

SM = Soymilk powder FSM = Fermented soymilk powder

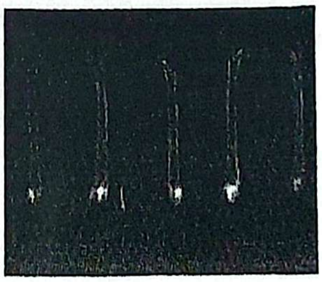
Means with the same letter(s) are not significantly different ($p < 0.05$).

After one month

After one month



GA5



GB5

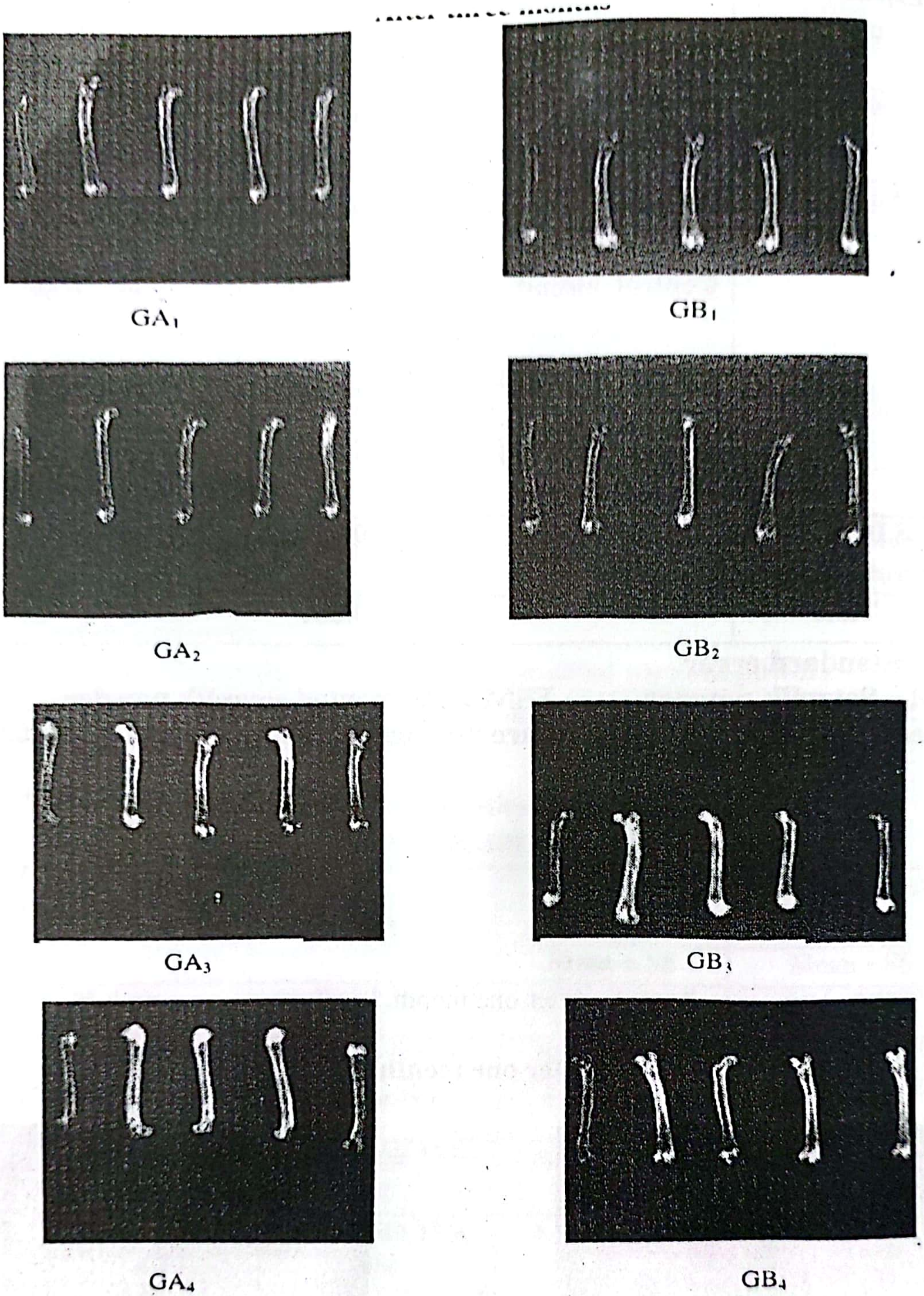


Fig. (9): X- ray contact films of different rats groups

Table (6): Serum alkaline phosphates activity (U/L) in normal and ovariectomized rats fed on different diets for three months.

Experimental period	Diet	Normal group (A)	Ovariectomized Group(B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	95.85 ± 1.16^{ef}	111.27 ± 0.95^{a}
3 months	Basal diet	99.76 ± 1.01^{cd}	97.60 ± 0.611^{de}
	Control biscuit	94.66 ± 1.20^{f}	99.40 ± 0.61^{cd}
	Biscuit with 15% SM	95.33 ± 0.88^{ef}	101.39 ± 0.52^{c}
	Biscuit with 15% FSM	95.97 ± 1.15^{ef}	104.77 ± 0.66^{b}
L.S.D at 5% Treatments	2.315		
Time	5.370		

SE=standard error.

SM = Soymilk powder   FSM = Fermented soymilk powder

Means with the same letter(s) are not significantly different ($p < 0.05$).

Table (7): length (m m) of the femur in normal and ovariectomized rats fed on different diets for three months

Experimental period	Diet	Normal Group (A)	Ovariectomized Group(B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	29.80 ± 0.36^{g}	31.09 ± 0.17^{def}
3 months	Basal diet	30.41 ± 0.06^{fg}	31.43 ± 0.09^{cd}
	Control biscuit	30.7 ± 0.29^{ef}	31.90 ± 0.17^{bc}
	Biscuit with15%SM	30.97 ± 0.35^{def}	32.30 ± 0.15^{ab}
	Biscuit with15%FSM	31.23 ± 0.09^{cde}	32.63 ± 0.18^{a}
L.S.D at 5% Treatments	0.451		
Time	0.285		

SE=standard error.

SM = Soymilk powder   FSM = Fermented soymilk powder

Means with the same letter(s) are not significantly different ($p < 0.05$)

Table (8): breaking force (Neiton*) of the femur in normal and ovariectomized rats fed on different diets for three months

Experimental period	Diets	Normal group (A)	Ovariectomized Group(B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	87.28 ± 0.39^{bc}	84.40 ± 0. 37^{cd}
3 months	Basal diet	88.53 ± 1.18^{b}	87.69 ± 0.87^{bc}
	Control biscuit	85.40 ± 1.68^{bc}	81.80 ± 1.15^{d}
	Biscuit with15%SM	95.53 ± 1.25^{a}	84.42 ± 1.35^{cd}
	Biscuit with15%FSM	96.10 ± 0.92^{a}	86.07 ± 0.78^{bc}
L.S.D at 5% Treatments		2.236	
Time		1.414	

Neiton*=Kg x 9.18 SE=standard error.
SM = Soymilk powder FSM = Fermented soymilk powder
Means with the same letter(s) are not significantly different ($p < 0.05$).

Table (9): bone density of the femur (g/cm³ bone vol.) in normal and ovariectomized rats fed on different diets for three months

Experimental period	Diet	Normal Group (A)	Ovariectomized Group (B)
		Mean ± SE	Mean ± SE
35 days	Basal diet	1.44 ± 0.012^{ab}	1.31 ± 0.033^{b}
3 months	Basal diet	1.46 ± 0.046^{a}	1.37 ± 0.042^{ab}
	Control biscuit	1.42 ± 0.25^{ab}	1.36 ± 0.013^{ab}
	Biscuit with 15%SM	1.48 ± 0.075^{a}	1.42 ± 0.08^{ab}
	Biscuit with15% FSM	1.49±0.043^{a}	1.45±0.015^{ab}
L.S.D at 5% Treatments		0.093	
Time		0.059	

SE=standard error.
SM = Soymilk powder FSM = Fermented soymilk powder
Means with the same letter(s) are not significantly different ($p < 0.05$).

Table (10): bone mineral density of the femur (g/cm²) in normal and ovariectomized rats fed on different diets for three months.

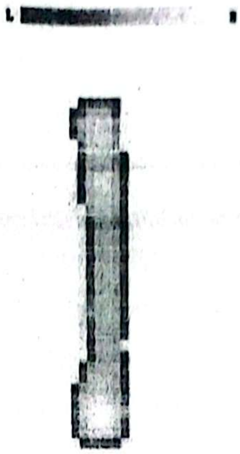
Experimental period	Diet	Normal Group (A)	Ovariectomized Group (B)
		Mean ±SE	Mean ±SE
35 days	Basal diet	0.102± 0.005^{cd}	0.097±0.004^{d}
3 months	Basal diet	0.115± 0.008^{abc}	0.105±0.008^{bcd}
	Control biscuit	0.113± 0.004^{abc}	0.103±0.003^{cd}
	Biscuit with15%SM	0.117± 0.005^{ab}	0.108± 0.001^{abcd}
	Biscuit with15%FSM	0.121±0.004^{a}	0.110±0.001^{abc}
L.S.D at 5% Treatments		0.008	
Time		0.005	

SE=standard error.
SM= Soymilk powder FSM = Fermented soymilk powder
Means with the same letter(s) are not significantly different ($p < 0.05$).

After one month

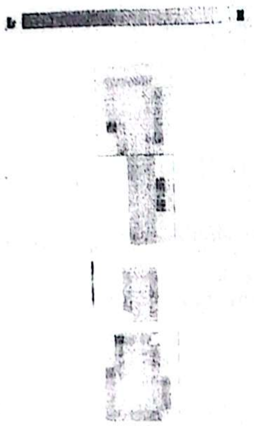
After one month

GA$_5$

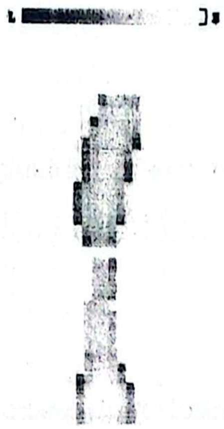


GB$_5$

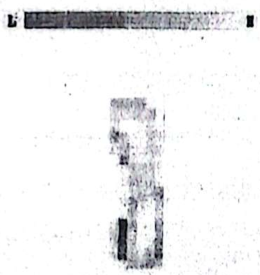
After three months

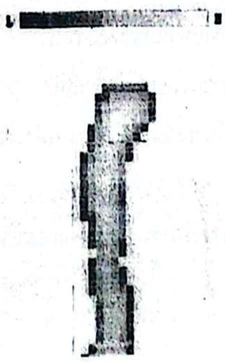


GA$_1$



GB$_1$





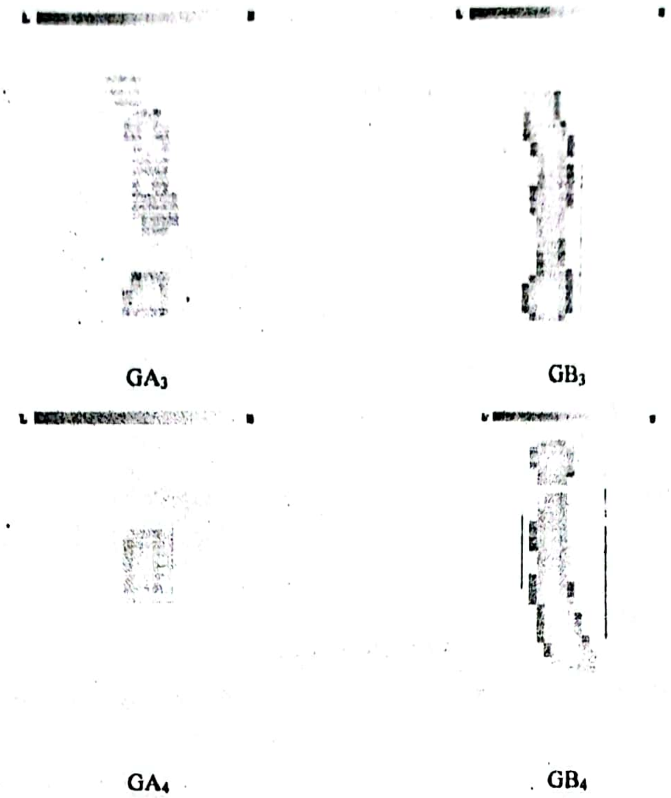


Fig. (10): Bone mineral density of femurs using Dual X-ray Absorpitometry.

CONCLUSION

1-Soymilk and fermented soymilk powders attenuate the loss of bone mass.

2- Feeding of soymilk cause a positive effect on bone density of ovariectomized rats.

3-Soymilk and fermented soymilk powders would delay the increase in bone mineral density.

4- Dietary soymilk improves the bone strength.

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