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Effects of Nanocalcium Supplemented Milk on Bone Calcium Metabolism in Ovariectomized Rats

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ABSTRACT : This study examined effects of calcium supplemented milk on bone loss in ovariectomized rats. Twenty four Sprague-Dawley female rats, 7 weeks-old, were divided into 4 groups, ovariectomized and fed diets containing: 1) control, no Ca supplemented milk, 2) ovx 1, Ca carbonate supplemented milk, 3) ovx 2, ionized Ca supplemented milk, and 4) ovx 3, nano Ca supplemented milk. All rats were fed 1 ml of milk containing 20 mg supplemented Ca. After 18 wk feeding, body weight gain and food efficiency ratio were significantly different between ovx 1 and ovx 3. Serum concentration of calcium and phosphorus were not different among groups. However, there was a significant difference in calcium content of dry femoral weight in ovx 3 compared with the control and ovx 2. In addition, femoral bone mineral density (g/cm^2) was significantly greater in ovx 3 than in other groups ($p<0.05$). The ovx 3 group showed the highest stiffness (N/mm), maximum energy (N) in femur and trabecular bone area (%). The present study indicated that nano Ca supplementation in milk may be an effective way to enhance bone calcium metabolism for ovariectomized rats. (**Key Words :** Nanocalcium, Bone Ca Metabolism, Milk, Ovariectomized Rat)

INTRODUCTION

Postmenopausal osteoporosis is a serious problem in elderly women and is characterized by a decrease in bone mass, leading to fracture and imbalanced turnover of the bone. Ovariectomized rats exhibit a decrease in mineral density, volume and strength of bone, and an increase in bone turnover that was seen in women suffering from osteoporosis (Tamaki et al., 1998).

It is well known that dietary calcium intake, moderate physical exercise and estrogen replacement are necessary for the prevention of osteoporosis. Whereas the beneficial effects of estrogen replacement have been clearly established in the treatment of estrogen-deficiency-induced bone loss, the beneficial effects of calcium supplementation as a method of preventing bone loss are less obvious. Thus, it is important to understand the effects on bone health of dietary calcium deficiency as well as calcium supplementation (Shen et al., 1995).

Dietary calcium and estrogen deficiencies both can lead to osteoporosis in humans and animals (Dawson-Hughes, 1991; Thomas et al., 1991; Christiansen, 1992; Gallagher, 1993). Variable results from different dietary calcium supplementation studies may be due to different dietary calcium intake of the patients and lack of investigative control over intake among different studies (Arnaud and Sanchez, 1990; Nordin et al., 1991). Thus, it is important to understand the effects on bone health of dietary calcium deficiency as well as calcium supplementation.

Milk is an excellent source for calcium, considering the absorption of calcium and its bioavailability (Wong and Lacroix, 1980; Kansal and Chaudhary, 1982; Poneros-Schneider and Erdman, 1982). Calcium from calcium carbonate has also been reported as a good calcium source compared with calcium from milk (Mortenson and Charles, 1996), but other studies have reported that calcium from milk and whey is better than calcium from calcium carbonate (Wong and Lacroix, 1980; Tsuchiya et al., 1993).

In order to increase daily calcium intake, increased consumption of milk is recommended. Recently, there are commercially available calcium-fortified milk products. As mentioned above, it is considered that calcium bioavailability in various types of calcium is equal if not less than that in milk. However, the calcium bioavailability of those is not well understood when those are added into milk.

Since the intake of calcium has never been sufficient, many studies have been taken to determine as excellent

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Table 1. Composition of AIN-76A purified diet

Ingredient	grams/kg
Casein	200
Corn starch	150
Sucrose	502.996
Corn oil	50
Cellulose	50
Mineral mix[1]	35
Vitamin mix[2]	10
Biotin	0.004
Choline bitartrate	2

[1] AIN-76 Mineral mix (g/kg): $CaHPO_4$ 500, NaCl 74, K citrate monohydrate 220, K_2SO_4 52, MgO, Mn carbohydrate 3.5, Fe citrate 6.0, Zn carbonate 1.6, Cu carbonate 0.3, KIO_3 0.01, $Na_2SeO_4 \cdot H_2O$ 0.01, $CrK(SO_4) \cdot 12H_2O$ 0.55, Sucrose 118.

[2] AIN-76 Vitamin mix (g/kg): thiamin·HCl 0.6, riboflavin 0.6, phydoxine·HCl 0.7, nicotinic acid 3, D-calcium pantothenate 1.6, folic acid 0.2, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 0.8, DL-α-tocopheryl acetate 20, cholecalciferol 0.00025, menaquinone 0.005.

calcium source for bone metabolism (Hirasawa et al., 2001). Milk is an excellent source for calcium, considering the absorption of calcium and its bioavailability (Toba et al., 1999). Among various sources of dietary calcium, little information is available about nano-sized calcium in present. Therefore, for the purpose of finding another type of calcium supplements, we have compared nanocalcium with other kinds on bone metabolism when supplemented to milk in ovariectomized rats.

MATERIALS AND METHODS

Animals and diets

Twenty four 7-week-old female Sprague-Dawley rats were purchased from Jung-Ang Lab. Animal, Inc. (Seoul, Korea). They were allowed to adapt to our environs and were provided with a chow diet and distilled water *ad libitum*. A week later, the animals were ovariectomized and divided into 4 groups as follows: 1) control, no Ca supplemented milk fed, 2) ovx 1, Ca carbonate supplemented milk fed, 3) ovx 2, ionized Ca supplemented milk fed, and 4) ovx 3, nano Ca supplemented milk fed. Diet was formulated by the recommendations of the American Institute of Nutrition (Table 1). In the main diet, 0.35 g Ca was contained in 20 g diet, which is about daily consumption of rats. In addition, rats fed Ca supplementation consumed 20 mg of Ca from other sources in 1 ml milk daily by gastric intubation and allowed access to diets and distilled water *ad libitum*. Rats were housed in individual cages at 22.2±2°C, 50±5% humidity and lightened from 19:00 to 07:00 h. All Ca supplements were obtained from NanoTechWorld (Pohang, Korea). Calcium carbonate and ionized Ca were commercial products, and nano Ca was produced by NanoTechWorld and size was in the range of 30-900 nm.

Sampling

At the end of the experimental period, all rats were fasted for 24 h and blood was collected in EDTA-coated tubes from tail and then immediately centrifuged at 3,000 rpm for 15 min at 4°C for collecting serum for further chemical analysis (Li et al., 2007). The excised tibias were fixed in 10% buffered formalin for 24 h and processed for bone histomorphometry (Shen et al., 1993). Femurs were preserved in 70% ethanol for subsequent bone mineral density. The liver and kidneys were removed after exsanguination, blotted with absorbent paper and weighed.

Biochemical assays

Serum and tissue Ca and P were measured by atomic blood chemical analyzer with Roche testing kits (Roche COBAS MIRA PLUS, Switzerland) (Chen et al., 2006).

Bone measurement

Bone Ca and P in dry left femur were determined by Inductively Coupled Plasma (ICP, Shimadzu, Japan). BMD of the right femur was measured by X-ray bone densitometer (Lunar Co., USA), and stiffness and maximum energy of the right femur were measured by Bone strength meter (Iwoo, Korea). Trabecular bone area was determined manually using a grid-point counting technique, and was expressed as a percentage (points on bone/points on tissue inside the measurement area). The micrographs used for the measurement of trabecular bone area were taken at a magnification of ×400. Trabecular bone measurements were performed at a 6-mm^2 area. The femora were subsequently removed and immersed in the same fixative for approximately 12 h at 4°C. The femoral specimens were then demineralized with 10% EDTA solution and dehydrated with increasing concentrations of ethanol before being embedded in paraffin. The specimens for transmission electron microscope (TEM) were post-fixative with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 4 h at 4°C. They were then dehydrated with ascending concentrations of acetone and embedded in epoxy resin (Epok, Polysciences, Inc., Warrington, PA, USA). For immunoelectron microscopy, the demineralized specimens were dehydrated with *N,N*-dimethylformamide before embedding into glycomethacrylate (GMA) as described (Asawa et al., 2004). Ultrathin sections were made by microtome (Leica, Wesin, Austria) and stained with tannic acid, ulanyl acetate, and lead citrate for TEM observation (JEM-100CX II, JEOL Ltd., Tokyo, Japan) at 80 kV.

Statistical analysis

The ANOVA and Duncan's multiple tests were used to analyze the differences between groups and all data were presented as mean±SD and the level of significance was

Table 2. Body weight gain, food intake and food efficiency ratio (FER) in ovariectomized rats[1]

Group	Initial body weight (g)	Final body weight (g)	Total weight gain (g)	Daily food intake (g/d)	FER (%)
Control[2]	164.7±4.3[a]	305.2±11.2[ab]	140.5±12.0[ab]	12.1±0.8[ab]	9.3±0.7[a]
ovx 1[3]	169.5±13.5[a]	316.8±15.5[a]	147.3±20.8[a]	12.4±0.9[a]	9.5±1.6[a]
ovx 2[4]	165.6±12.3[a]	294.0±20.8[b]	128.4±28.1[ab]	11.2±0.5[b]	9.1±1.9[ab]
ovx 3[5]	169.7±5.1[a]	287.3±6.5[b]	117.7±4.5[b]	12.3±0.6[a]	7.6±0.5[b]

[1] Values within the same column with different superscripts are significantly different at p<0.05.
[2] Control: ovariectomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3: ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

Table 3. Contents of Ca and P in serum during the experimental period in ovariectomized rats[1]

Group	Serum Ca (mg/dl)	Serum P (mg/dl)
Control[2]	10.2±1.3[a]	4.4±0.4[a]
ovx 1[3]	10.2±1.5[a]	4.5±0.9[a]
ovx 2[4]	9.9±0.7[a]	4.5±0.9[a]
ovx 3[5]	9.8±0.4[a]	4.5±0.1[a]

[1] Values within the same column with different superscripts are significantly different at p<0.05.
[2] Control: ovariectomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3: ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

determined at p<0.05 (SAS, 1985).

RESULTS

Body weight and food intake

After the experimental diets had been administrated for 18 weeks, body weight gain in ovx 3 which was fed the nano Ca supplemented milk, was slightly but not significantly lower than those in control and ovx 2 (Table 2). However, a significant difference was found between ovx 1 and ovx 3 groups. Among Ca supplemented groups, ionized Ca supplement inhibited the food intake, but food efficiency (FER) in the nano Ca supplemented group was significantly lower than those in control and ovx 1 group, and was slightly but not significantly lower than that in ovx 2 group. It is not certain why FER was lower in nano Ca supplemented group than no Ca and Ca carbonate supplemented groups, however, we may speculate the nano Ca impaired the intestinal food absorption in rats.

Table 5. Length of femur and tibia in the ovariectomized rats[1]

Group	Femur length (mm)	Tibia length (mm)
Control[2]	35.8±0.8[b]	40.7±0.5[ab]
ovx 1[3]	36.8±0.6[a]	41.3±0.4[a]
ovx 2[4]	35.8±0.6[b]	40.2±0.8[b]
ovx 3[5]	35.5±0.5[b]	40.3±0.4[b]

[1] Values within the same column with different superscripts are significantly.
different at p<0.05.
[2] Control : overictomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3: ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

Calcium and phosphorus contents

There was not a significant difference among groups with regard to serum Ca, which was a nearly normal level (Table 3). Also, serum P was not affected by Ca supplemented diet. There was no increase in serum Ca and P in the Ca supplemented diet groups.

Calcium and phosphorus contents per g tissue were shown in Table 4. Even though wet weight of liver and kidney were similar, calcium concentrations per g liver and kidney were significantly higher in nano Ca supplemented group (ovx 3) among other groups (p<0.05). However, there was no difference in P concentrations per g tissue among

Table 4. Contents of Ca and P in liver and kidney in ovariectomized rats[1]

Group	Liver			Kidney		
	Wet weight (g)	Ca (μg/g)	P (mg/g)	Wet weight (g)	Ca (μg/g)	P (mg/g)
Control[2]	9.07±0.84[a]	12.2±8.4[b]	2.0±0.3[a]	1.78±0.09[a]	38.7±7.1[b]	1.7±0.3[a]
ovx 1[3]	9.11±0.79[a]	12.6±6.5[b]	2.1±0.3[a]	1.90±0.10[a]	44.1±15.5[b]	2.0±0.4[a]
ovx 2[4]	8.76±0.56[a]	9.6±5.6[b]	1.7±0.3[a]	1.78±0.09[a]	42.0±25.3[b]	1.8±0.3[a]
ovx 3[5]	8.95±0.62[a]	25.9±15.5[a]	1.9±0.3[a]	1.85±0.11[a]	77.8±23.4[a]	1.8±0.3[a]

[1] Values within the same column with different superscripts are significantly different at p<0.05.
[2] Control: ovariectomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3 : ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

Table 6. Ash weight, Ca and P contents of left femur in the ovariectomized rats[1]

Group	Ash weight (g)	Ca/ash (%)	P/ash (%)
Control[2]	0.309±0.012[b]	24.42±2.49[c]	12.67±1.83[b]
Ovx 1[3]	0.332±0.020[ab]	28.22±1.67[ab]	15.14±0.88[a]
Ovx 2[4]	0.314±0.027[ab]	26.13±0.93[bc]	13.63±1.05[ab]
Ovx 3[5]	0.336±0.017[a]	29.75±4.13[a]	15.58±2.08[a]

[1] Values within the same column with different superscripts are significantly different at p<0.05.
[2] Control: ovariectomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3: ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

Table 7. Stiffness and maximum energy in the ovariectomized rats[1]

Group	Stiffness (N/mm)	Maximum energy (N)
Control[2]	115.0±17.8[b]	83.5±7.7[b]
ovx 1[3]	120.8±7.2[b]	88.2±9.4[b]
ovx 2[4]	119.5±16.1[b]	88.4±11.6[b]
ovx 3[5]	143.7±12.4[a]	101.5±4.8[a]

[1] Values within the same column with different superscripts are significantly different at p<0.05.
[2] Control: ovariectomized+no Ca supplemented milk.
[3] ovx 1: ovariectomized+Ca carbonate supplemented milk (20 mg/ml milk).
[4] ovx 2: ovariectomized+ionized Ca supplemented milk (20 mg/ml milk).
[5] ovx 3: ovariectomized+nano Ca supplemented milk (20 mg/ml milk).

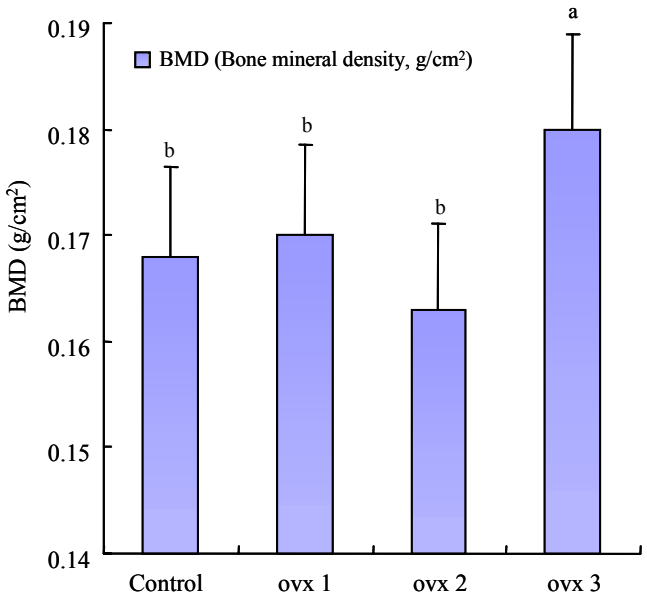

Figure 1. Bone mineral density in ovariectomized rats. Control: no Ca supplemented milk fed, ovx 1: Ca carbonate supplemented milk fed, ovx 2: ionized Ca supplemented milk fed, and ovx 3: nano Ca supplemented milk fed.

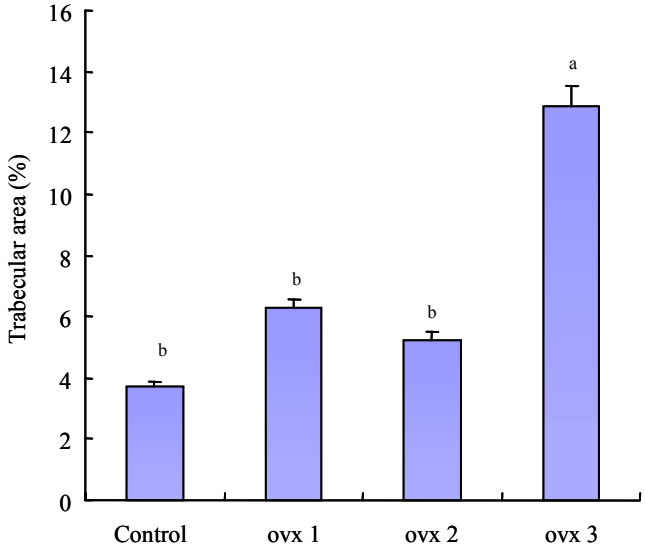

Figure 2. The ratio of trabecular area in ovariectomized rats. Control: no Ca supplemented milk fed, ovx 1: Ca carbonate supplemented milk fed, ovx 2: ionized Ca supplemented milk fed, and ovx 3: nano Ca supplemented milk fed.

treatments.

Femoral length, ash weight and calcium and phosphorus content

Calcium carbonate supplemented group (ovx 1) showed the greatest femur length compared with other groups (p<0.05) (Table 5). Also, tibia length in ovx 1 was significantly higher. Nano Ca supplemented animals has a significantly higher femur ash weight than the other groups (Table 6). The nano Ca and Ca carbonate supplemented groups also had a significantly greater femur Ca and P content than the no Ca supplemented group.

The present study was designed to examine the effects of 3 different types of Ca supplementation in preventing bone loss due to ovariectomy. Our data on bone density and bone Ca confirm the observations of other investigators that bone loss due to ovarian hormone deficiency is prevented by effective nano Ca supplementation. In accordance with other findings (Ishida et al., 1998), the femurs of rats in the ovx group, fed the Ca-deficient diet, had lower density and strength, lower ash weight, and lower calcium and phosphorus content.

Bone mineral density, stiffness and maximum energy

The influence of the difference sources of Ca on BMD in the right femur are shown in Figure 1. The nano Ca intake in ovx 3 significantly enhanced the BMD in femur (p<0.05), whereas no difference was found among other 3 groups. The femur showed a significant loss of bone mineral density in the ovx group when compared with the Ca supplemented groups. In addition, animals in the nano Ca supplemented group had significantly higher stiffness and maximum energy of the femur compared with other groups (p<0.05) (Table 7). The present study indicated that

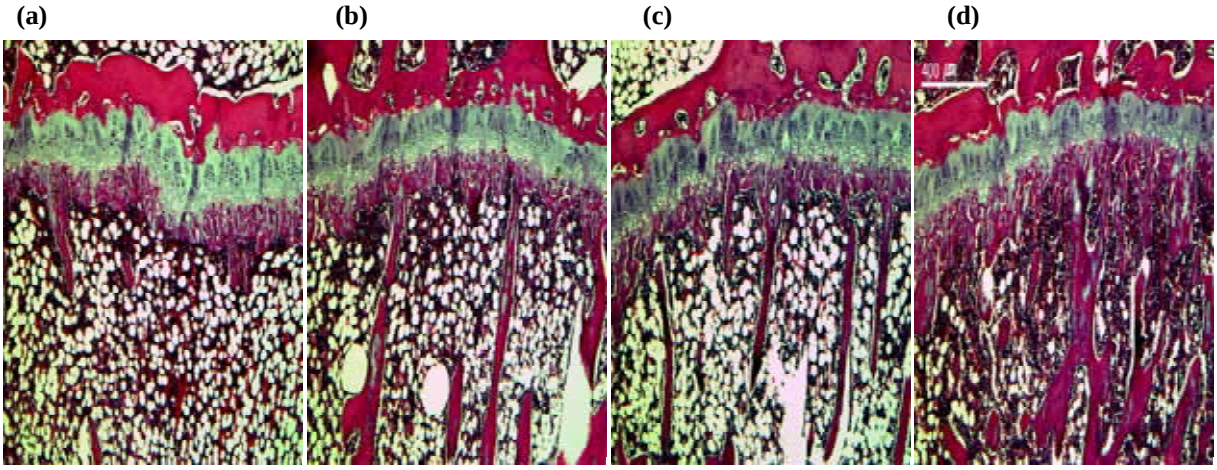

Figure 3. Histological trabecular bone in tibia in ovariectomized rats. (a) Control: no Ca supplemented milk fed, (b) ovx 1: Ca carbonate supplemented milk fed, (c) ovx 2: ionized Ca supplemented milk fed, and (d) ovx 3: nano Ca supplemented milk fed.

nano Ca may have a preventive effect on ovariectomized-induced bone loss in femur.

Since it is well known that a highly absorptive Ca source in the intestine will be an effective Ca supplement for bone metabolism (Hirasawa et al., 2001), nano Ca could be considered as an effectively absorptive source for dietary Ca.

Trabecular bone area

The trabecular bone area (%) was measured and compared among the four experimental groups (Figure 2). As expected, the trabecular bone area was significantly lower in control, which was ovariectomized and fed the no Ca supplemented commercial milk, than in other groups ($p<0.05$). The nano Ca supplementation (ovx 3) resulted in the greatest level of trabecular bone area of all of the groups.

The different histological areas of the proximal tibia sections from all group rats are depicted in Figure 3. The nano Ca supplemented group (ovx 3) displayed markedly elongated trabeculae while the trabeculae of no Ca supplemented group (control) has fragmented metaphyseal trabeculae. Control rats showed extremely resorbed metaphyseal bone but few trabeculae when compared with other groups which were supplemented Ca. The ovx 1 and ovx 2 displayed relatively shortened trabeculae compared with that in ovx 3.

DISCUSSION

Dietary calcium requirements for immature rats to achieve normal skeletal status have been estimated at approximately 0.3 to 0.4% (Bell et al., 1941). However, the exact calcium requirement for maintenance of skeletal health in mature animals is not well known. Using 0.01 to 0.1% of calcium in the diet, previous studies have shown decrease in bone calcium content (Wong et al., 1980) and bone mineral density (Sissons et al., 1984), circulating parathyroid hormone (Rader et al., 1979), and 1,25-$(OH)_2D_3$ levels (Thomas et al., 1991). Although it is difficult in practice to obtain accurate dietary calcium intake information in humans, a very controlled dietary regimen can be planned and maintained easily in rats. Such a rat model of dietary calcium deficiency could offer useful information regarding the effect of dietary calcium deficiencies on calcium homeostasis and skeletal health.

It is well known that the development of a highly absorptive Ca source in the intestine will be an effective Ca supplement for bone metabolism. In the present study, we compared various kinds of Ca supplements on bone metabolism in ovariectomized rats. The results showed that nano Ca supplementation increased significantly BMD and bone strength more effective than other Ca supplements, which may suggest the high intestinal absorption of nano Ca in ovariectomized rats. Taking a nutrient substance suitable to one's age, lifestyle and dietary habit is important. Therefore, it is important for aged people, who tend to have decreased intestinal Ca absorption, to take Ca supplements. Our results suggest that nano-sized Ca would be one effective supplement for bone and calcium metabolism in older people.

In the bone observation, this study allowed us to define the effects of nano Ca in lessening the decrease in bone mineral density and increase in bone resorption induced by ovariectomy. The difference in metaphyseal trabeculae was clearly found between nano Ca supplemented and ovariectomized rats. The nano Ca supplemented femur has elongated trabeculae whereas no Ca-supplemented group displayed fragmented metaphyseal trabeculae. Thus, our observation on the effects of nano Ca supplementation indicated that nano Ca may induce in high intestinal

absorption and inhibit bone loss by ovaiectomy.

The present study may suggest that the effect of nano Ca supplementation was significant on preventing bone loss. This is the first evidence, to our knowledge, that nano Ca supplementation provides a partial explanation for the beneficial effect on bone health in ovariectomized rats. Further studies are needed to clarify the mechanism of action of dietary nano Ca in preventing bone loss after ovariectomy.

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