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Original

A New Osteoporosis Prevention Supplements-diet Improve Bone Mineral Density in Ovariectomized Rats on Micro-CT

Hiroshi Nakada[1,6], Toshiro Sakae[2,6], Takehiro Watanabe[3], Takahiro Takahashi[3], Kanami Fujita[3], Yasuhiro Tanimoto[4,6], Mari Teranishi[5], Takao Kato[5,6] and Yasuhiko Kawai[1,6]

[1]Department of Removable Prosthodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
[2]Department of Histology, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
[3]Nihon University Graduate School of Dentistry at Matsudo, Removable Prosthodontics, Matsudo, Japan
[4]Department of Dental Biomaterials, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
[5]Department of Oral Implantology, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
[6]Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
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Abstract: Osteoporosis is a skeletal disorder characterized by reduced bone strength and an increased risk of fractures. Bone strength is exacerbated by various factors that influence bone mineral density (BMD) and bone quality with increasing age. We have developed a novel supplement diet for osteoporosis prevention that contains fructo-oligosaccharide, isoflavone, and calcium citrate in addition to calcium phosphate (high mineral diet: HMD), thereby increasing the Ca and P content. The present study aimed to clarify whether rats with osteoporosis fed a HMD showed improved BMD compared with rats fed a normal mineral diet (NMD). The experiment used 20-week-old ovariectomized rats divided into an NMD group (Group 2, n=8) and a HMD group (Group 3, n=8). This study also used 20-week-old sham-ovariectomized rats fed NMD as controls (Group 1, n=8). After 8 weeks and 24 weeks on the diet, this study examined the changes in BMD, bone mineral content (BMC), and 3-dimensional (3D)-map using micro-computed tomography (CT) imaging of the shaft of the femur. In the 3D-map, both Groups 1 and 3 showed high BMD on the inner portion of the cortical bone, while Group 2 showed slightly lower BMD at the same location. Decreased estrogen secretion in Group 2 significantly affected bone metabolism, lowering both BMD and BMC compared to Group 1. However, while Group 3 also showed decreased estrogen secretion, BMD and BMC were higher than in Group 2. These findings indicate that HMD increases both BMD and BMC, and is therefore more effective than NMD for improving BMD and bone quality.

Key words: Micro-computed tomography, Bone mineral density, Bone mineral content, Fructo-oligosaccharide, Ovariectomized rat

Introduction

Osteoporosis affects approximately 75 million people in Europe, the United States, and Japan[1], and this number is expected to rise with the aging of the “baby boomers”. This disease, characterized by bone fragility and increased susceptibility to fractures, represents a serious medical problem with extensive social and economic implications. Osteoporosis is a systemic disease that results in reduced bone mass and poor trabecular structure[2,3]. Bone strength is associated with two factors: bone mineral density (BMD) and bone quality, contributing about 70% and 30%, respectively[4]. Bone quality is related to bone microstructure, metabolic turnover, micro-damage, calcification, and collagen crosslinking[4]. BMD is thus an important parameter for predicting fracture.

Numerous internationally recognized drugs are used to treat osteoporosis in the clinical setting, including calcium, bisphosphonate, estrogen, calcitonin, and active vitamin D_3 formulations. However, current treatment drugs are known to delay the rate of bone resorption, but do not regenerate bone that has already been lost. Inadequate intake of Ca and protein is the nutritional cause of osteoporosis in the elderly and postmenopausal women[5]. Therefore, to improve bone mass and bone quality, consumption of minerals or supplements that can be easily taken can effectively counterbalance those nutrients lacking in daily life.

We have previously used ovariectomized (OVX) rats in preliminary studies where rats were given a low mineral diet. We subsequently analyzed the femoral shaft and metaphyseal area in

Corespondence to: Department of Removable Prosthodontics, Nihon University School of Dentistry at Matsudo, Chiba , 271-8587 Japan; Phone: +81-47-360-9379; Fax:+81-47-360-9376; E-mail address: nakada.hiroshi@nihon-u.ac.jp

Table 1. Compositions of the Diets Used.

Composition	Normal mineral diet: NMD	High mineral diet: HMD (%)
α-cornstarch	40.00	40.00
β-cornstarch	22.07	2.45
Casein	14.00	14.00
L-cystine	0.18	0.18
Sucrose	10.00	7.00
Soybean oil	4.00	4.00
Cellulose powder	5.00	5.00
Mineral mixture	3.50	3.50
Vitamin mixture	1.00	1.00
Choline bitartrate	0.25	0.25
Tert-butylhydroquinone	0.00	0.00
Additives		
Fructo-oligosaccharides		10.00
Isoflavone		0.50
Calcium citrate tetrahydrate		4.00
Calcium phosphate, dibasic		8.10
Ca/P content %		
Ca	0.51	3.00
P	0.30	1.76

Table 2. Body Weight Gain in Each Group.

	After intervention (20 weeks old)	8 weeks (28 weeks old)	24 weeks (44 weeks old) (g)
Group 1: SHAM + NMD	195.4 ± 3.1	225.7 ± 3.1	276.6 ± 13.8
Group 2: OVX + NMD	196.5 ± 1.7	222.6 ± 8.8	289.9 ± 13.0
Group 3: OVX + HMD	196.0 ± 4.7	222.7 ± 7.7	266.4 ± 10.2

rats. Through our findings regarding the correlations between BMD, bone volume, and bone breaking strength, we concluded that mineral intake is essential for bone maintenance[6,7]. OVX rats are frequently used as an animal model of osteoporosis[8-10]. OVX rats develop bone mutations associated with reduced secretion of estrogen[11] and exhibit subsequent changes in bone metabolism and systemic conditions[12,13] and bone loss[9,11].

Previous investigations assessing methods to improve osteoporosis have included various approaches, such as supplementation with fructo-oligosaccharide (FOS)[14], isoflavone (ISO)[15], and calcium citrate (CC)[16]. FOS has been reported to possess the ability to promote the intestinal absorption of Ca, magnesium, and iron[17,18]. ISO is known to be effective for suppressing loss of bone mass since it reduces cholesterol, can act like estrogen, and has antioxidant properties[19-22]. CC has been reported to lower pH within the large intestine, while Ca is known to be transported to the large intestine by chelating with citric acid, thus promoting absorption[16]. However, no reports to date have evaluated the synergistic drug efficacy in bone of increasing Ca and P content in a mixture that contains the three compounds mentioned above in addition to calcium phosphate (dibasic: CP). We therefore developed a new osteoporosis-prevention supplement diet (high mineral diet: HMD) that contains CP along with FOS, ISO, and CC as additives. The current study demonstrated the effects of HMD and normal mineral diet (NMD) in OVX rats by assessing changes in bone quality using micro-computed tomography (CT) to determine BMD.

Materials and Methods

Animals

Twenty-four 20-week-old female Wistar rats (Sankyo Labo Service, Tokyo, Japan) were housed in individual metal cages at room temperature (23 ± 1 °C) and 50 ± 1% humidity, with ad libitum access to food and water. The experimental protocol was approved by an animal experiment ethics committee (Nihon University Animal Care and Use Committee: Approval no. Ap11-MD023). All experiments were conducted according to the Guidelines for the Treatment of Animals, Nihon University, Tokyo, Japan.

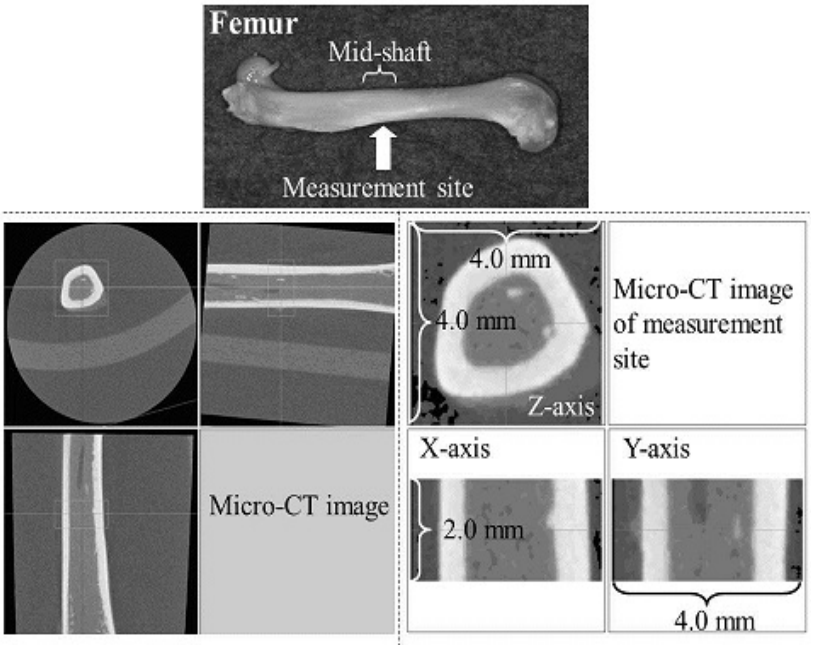

Figure 1. Measurement sites in the femur (top). The measurement range (4.0 mm × 4.0 mm × 2.0 mm) for BMD and BMC is at the femoral mid-shaft.

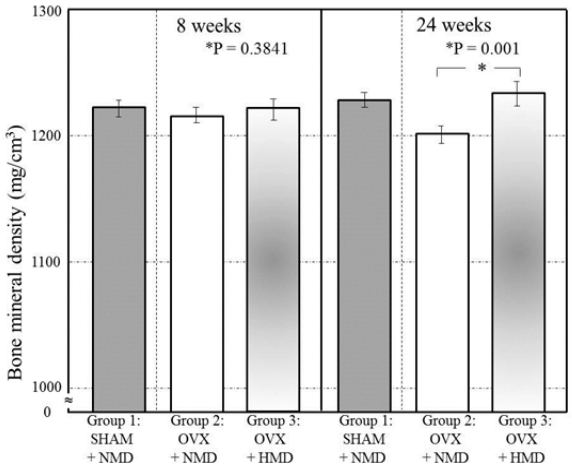

Figure 2. Bone mineral density of cortical bone for the 3 groups at 8 and 24 weeks after starting the intervention. Differences between OVX groups at any given time were analyzed using Student's t-tests (*P < 0.05).

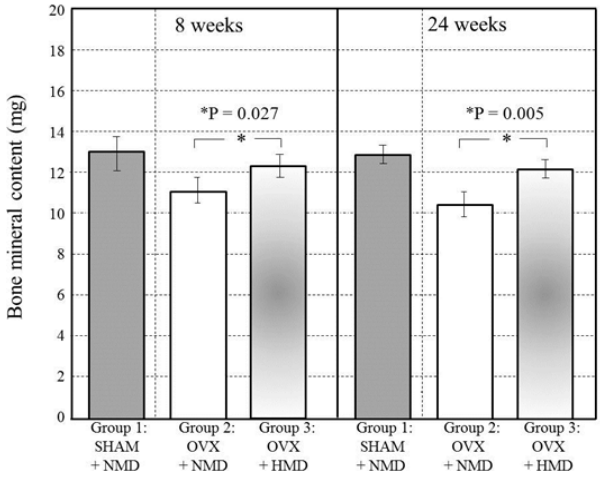

Figure 3. Bone mineral content of the cortical bone for the 3 groups at 8 and 24 weeks after starting the intervention. Differences between OVX groups at any given time were analyzed using Student's t-tests (*P < 0.05).

Preparation of rats

The 24 rats were randomly allocated to 3 groups. Group 1 (n = 8) comprised sham-operated rats who were provided with a normal mineral diet (NMD; Oriental Yeast Co., Tokyo, Japan). Group 2 (n = 8) consisted of OVX rats on NMD, and Group 3 consisted of OVX rats on a HMD (Oriental Yeast Co., Tokyo, Japan). As shown in Table 1, the Ca and P contents of HMD were increased from 0.51 % to 3.00 % and 0.30 % to 1.76 %, respectively.

In the sham operation group, rats were anesthetized with isoflurane, and bilateral ovaries were raised up and then returned to their original position at 20 weeks old. In the OVX groups, rats were anesthetized with isoflurane. The abdominal area was sterilized with 75 % ethanol and was surgically opened. Bilateral ovaries were raised up and completely excised, thus completing the ovariectomy. The uterus and adipose tissue were then placed back into the abdomen, and the incision was sutured. All 3 groups of rats were on the prescribed diet at 20 weeks old.

Measurement of body weight

Body weights of all rats were measured at 8 weeks (28 weeks) and 24 weeks (44 weeks) after intervention. Animals were then euthanized with carbon dioxide, and both femora were removed

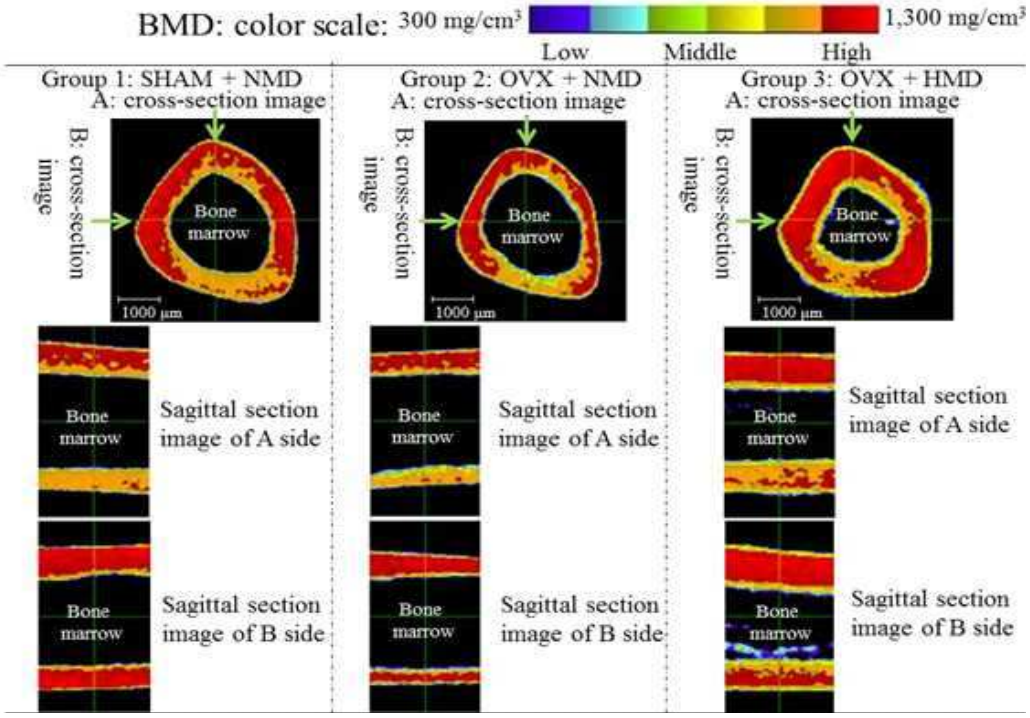

Figure 4. Three-dimensional pseudocolor map of cross-section and sagittal section images of the femoral mid-shift region for the 3 groups (Group 1: SHAM+NMD; Group 2: OVX+NMD; Group 3: OVX+HMD) at 24 weeks after starting intervention. BMD color scale: red and orange, high BMD; yellow and green, medium BMD; and light blue and purple, low BMD.

(n = 4 per period and group).

Micro-CT

Micro-CT settings

The full length of the femur was measured with slide calipers, and the midpoint of the full length was used as the measurement site (4.0 mm × 4.0 mm × 2.0 mm) (Fig. 1). Imaging conditions for micro-CT (R_mCT; Rigaku, Tokyo, Japan) were as follows: tube voltage, 90 kV; tube current, 88 μA; magnification, ×6.7; measurement time, 17 s; resolution, 30 μm; slice thickness, 240 μm; and slice spacing, 240 μm. Micro-CT images were taken of the femur and phantoms for proofreading of CT values after 8 and 24 weeks.

Measurement of BMD and BMC

Digital images were converted to 16-bit gray-scale TIFF format using the Atlas TIFF Convertor® (Rigaku), and were observed using TRI/3D-Bon BMD (TRI/3D-Bon; Ratoc System Engineering, Tokyo, Japan). For BMD and BMC measurements, a hydroxyapatite calibration curve was prepared from images of phantoms (hydroxyapatite content: 200 mg/cm^3, 300 mg/cm^3, 400 mg/cm^3, 500 mg/cm^3, 600 mg/cm^3, 700 mg/cm^3, 800 mg/cm^3, and 1550 mg/cm^3), and femora were measured with the TRI/3D-Bon trabecular structure analysis routine (auto-detection mode) using the obtained CT values.

Observation of 3D-map

Bone situation (inferred from BMD values) was determined from a 3D-map showing BMD distributions obtained by micro-CT, represented in pseudocolors (high BMD: red and orange; middle BMD: yellow and green; low BMD: light blue and purple). The 3D image analysis was conducted using TRI/3D image analysis software from bone density values. The 3D-map conditions were: 1 pixel, 30 μm; range, 300-1300 mg/cm^3.

Statistical analysis

All values in the tables and figures are shown as mean ± standard deviation. Student's t-test was used for statistical analyses of body weight, BMD and BMC with the null hypothesis that no difference would exist between NMD and HMD in the OVX groups. Values of $P < 0.05$ were considered significant.

Results

The results from sham rats (Group 1) were used as control data for comparison with the changes in OVX rats on either NMD (Group 2) or HMD (Group 3).

Body weight

Table 2 shows body weight measurements for the 3 groups at each observation time. From 8 to 24 weeks after the intervention,

body weight had increased more in Group 2 than in Group 3.

BMD measurement

Changes in BMD with time for each group are shown in Fig. 2. Group 2 decreased from 8 weeks to 24 weeks. Group 3 slightly increased from 8 weeks to 24 weeks. The BMD was significantly higher for Group 3 than for Group 2 at 24 weeks. Group 1 showed no change with observation time. BMD values for the 3 groups at 8 weeks were as follows: Group 1, 1221.4 ± 6.8 mg/cm^3; Group 2, 1215.8 ± 6.4 mg/cm^3; and Group 3, 1220.6 ± 8.2 mg/cm^3. BMD values for the 3 groups at 24 weeks were as follows: Group 1, 1228.4 ± 6.0 mg/cm^3; Group 2, 1200.5 ± 7.1 mg/cm^3; and Group 3, 1233.7 ± 9.5 mg/cm^3.

BMC measurement

Changes in BMC with time for each group are shown in Fig. 3. Group 2 decreased from 8 weeks to 24 weeks. Groups 1 and 3 showed no change from 8 weeks to 24 weeks. BMC was significantly higher for Group 3 than for Group 2 at 8 and 24 weeks. BMC values for the 3 groups at 8 weeks were as follows: Group 1, 12.9 ± 0.8 mg/cm^3; Group 2, 11.1 ± 0.6 mg/cm^3; and Group 3, 12.3 ± 0.5 mg/cm^3. BMC values for the 3 groups at 24 weeks were as follows: Group 1, 12.9 ± 0.4 mg/cm^3; Group 2, 10.4 ± 0.6 mg/cm^3; and Group 3, 12.2 ± 0.5 mg/cm^3.

Observation of 3D-map

Fig. 4 shows the 3D-map for the 3 groups after 24 weeks. Red and orange, yellow and green, and light blue and purple in the 3D-map indicate high, medium, and low BMD, respectively.

The outer portion of the cortical bone in Group 1 had high BMD depicted primarily in red and partially in orange on cross-sectional imaging, and the inner portion of the cortical bone had slightly lower BMD indicated in orange. The sagittal section on the A-axis of the cortical bone showed both red and orange in the upper part of the screen, but primarily orange in the lower part of the screen. In Group 2, the outer portion of cortical bone was shown in red and orange on cross-sectional imaging, while the inner portion was shown in orange and yellow, indicating lower BMD. In the sagittal section image on the A-axis of the cortical bone, cortical bone was shown in red and the bone marrow side was shown in orange in the upper part of the screen, while cortical bone was primarily shown in orange and the bone marrow side was shown in yellow in the lower part of the screen. The cross-sectional image of the cortical bone in Group 3 indicated that the outer portion of the cortical bone was primarily red and partially orange, while the inner portion was orange and yellow. The sagittal section image on the A-axis was shown in red and orange in the upper part of the screen while the lower part was shown primarily in orange and partially in red. Sagittal section images on the B-axis showed similar BMD trends to those seen with the A-axis in all three groups. Images of Group 2 showed thinning of cortical bone around the entire circumference compared to Groups 1 and 3. Furthermore, Group 3 showed the formation of cancellous bone indicated by the light blue and purple colors in the bone marrow area.

Discussion

Rats in Group 2 were heavier than rats in the other groups at 24 weeks following initiation of the diet intervention. This finding corroborates previous reports claiming that OVX groups (Groups 2 and 3) experience greater body weight gain compared to sham groups (Group 1)[6,7,23,24]. Compared to the sham group, OVX groups secrete less estrogen, which in turn affects hormone metabolism[25]. We therefore considered that body weight gain occurred due to the accumulation of fat. HMD contained lower amounts of β-cornstarch to compensate for the additions of FOS, ISO, and CC as well as CP, causing a reduction in fuel for the body. We believe that this caused greater body weight loss in the HMD group (Group 3) than in the NMD groups (Groups 1 and 2). However, body weight was only slightly lower in Group 3 than in Group 1, signifying virtually no difference in results.

Observation of a 3D-map is an analytical system that uses both micro-CT and TRI/3D-Bon, allowing qualitative analysis of the trabecular structure with a 3D representation, and has been utilized in many studies to date[6,7,26]. In all three groups, the BMD of cortical bone shown in the lower part of the 3D-map cross-section was considered to be affected by external and gravitational forces as well as exercise stimulation depending on the site. In the 3D-map, the outer portion of the femur shows high BMD in Group 1. However, the inner portion of the femur, or the bone marrow side, showed lower BMD, as indicated by the orange color. In contrast, the femur of Group 3 showed high BMD all the way to the inner portion, indicating a clear difference in changes in BMD.

Numerous treatments, including calcium intake[27], estrogen supplementation[28] (hormone replacement therapy), and active vitamin D_3[29] have been reported in studies related to osteoporosis prevention. Studies that utilize oligosaccharides and soy beans have also been reported[21,22,30-33]. FOS is distributed to the bone throughout the body via intestinal mineral absorption and subsequent delivery by blood, and is reported to elevate BMD and BMC, as well as bone quality[33]. FOS intake is postulated to play an important role in the calcification of bone, because facilitates mineral absorption from the intestinal tract. ISO is reportedly found in bean products and possesses a similar structure to estrogen, and so can act as a selective estrogen receptor modulator (SERM) to promote the differentiation of osteoblast and inhibition of osteoclast formation[15,23,34,35]. However, intake in large quantities or long-term intake can extend the menstrual cycle and affect the hormonal balance[36], so the level of ISO intake must

be taken into consideration.

CC was included in the supplement diet, since it is known to prevent ureteral calculi caused by Ca intake in high concentrations. In a previous report that fed mice a diet containing 5% FOS and 0.5% ISO, significant elevations in BMD were observed compared to mice fed a diet supplemented with ISO alone[37]. Ohta et al. reported that absorption rates of Ca and Mg increased when e”3% FOS was added, and that absorption rate of P increased when e”5% was added[14]. In addition, animals fed 15% FOS experienced transient diarrhea or loose stools[14]. Ohta et al. also reported that absorption of Ca and P from the intestinal tract are sustained due to FOS in the diet, and that this effect is dose-dependent[14]. For these reasons, the present study created a supplement diet containing 10% FOS and 0.5% ISO. With regard to Ca and P ratio, a 2.4% Ca content in the diet does not harm the body[27]. In addition, a diet containing 6% Ca and 5.2% P has been reported to lower BMD and bone strength compared to other experimental groups, and bone quality did not improve even if large amounts of Ca and P were consumed[29]. The present study therefore set the Ca content to 3%, which is known to promote greater mineral absorption. P content was calculated using the Ca/P ratio in bone of 1.67 as a reference value, and the amounts of CC and CP were adjusted accordingly.

Treatment of osteoporosis patients and those affected by osteoporosis requires the consumption of greater amounts of Ca and P than normally required due to the inadequate intake of these minerals, and patients must also engage in moderate physical activity. However, consuming large amounts of specific minerals is not considered an effective method to supply the body, and may cause nutritional imbalances. It is therefore necessary to promote prevention of osteoporosis and rapid aging by activating the metabolism through the addition of FOS, ISO, and CC as well as increased Ca and P content in the diet, thereby inducing the body to efficiently absorb minerals. OVX groups reportedly show greater bone resorption than bone formation due to reduced estrogen secretion, leading to decreased BMD and bone strength[7]. It was supposed that Group 2 experienced lower BMD and BMC compared to Group 1 due to a large effect of reduced estrogen secretion on bone metabolism. However, although Group 3 on HMD was also exposed to an environment that reduces estrogen secretion, BMD and BMC were higher than those in Group 2. This revealed that HMD is a more effective supplement than NMD because it elevates BMD and BMC. In OVX groups, HMD was more effective than NMD in improving BMD and bone quality. Maturation of bone calcification depends on ingested mineral components, and the properties of bone matrix are determined by the crystal structure, metabolic turnover and microstructure of collagen crosslinking. Changing of bone quality was reported to be caused by nano-level abnormalities in collagen crosslinking and minerals[38], and bone strength was reported to influence collagen crosslinking markedly[39]. The HMD intake effect on bone strength, metabolic turnover and collagen crosslinking, remains to be investigated.

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