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Original

Structure Model Index Changes in the Femoral Epiphyseal Region on Micro-Computed Tomography Caused by a Supplement Diet in Ovariectomized Rats

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Abstract: 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). The present study aimed to clarify whether rats with osteoporosis fed an HMD showed an improved structure model index (SMI) in the femoral epiphyseal region compared with rats fed a normal mineral diet (NMD). The experiment used 20-week-old ovariectomized rats divided into an NMD group (n=6) and an HMD group (n=6). After 24 weeks on the diet, this study examined the changes in SMI and the 3-dimensional pseudocolor map (3D-map) using micro-computed tomography. Compared to the NMD group, the HMD group had significantly greater Trabecular Thickness (Tb. Th) and Trabecular Number, and shorter Trabecular Separation and Trabecular Spacing, indicating highly dense trabeculae. The trabeculae of the NMD group had low Tb. Th and bone volume / tissue volume (BV/TV), indicating a thin, rod-like shape, but the trabeculae of the HMD group had significantly greater Tb. Th and BV/TV, demonstrating a thick, plate-like structure. In the sagittal section image of the 3D-map, the trabecular bone of the NMD group showed low bone mineral density (BMD), represented by light blue and purple colors, on the growth plate side of the inner portion of the cortical bone, indicating near complete resorption of the trabecular bone. The trabecular bone of the HMD group showed moderate to low BMD, represented by light blue and green colors, on the growth plate side, indicating a greater trabecular bone density compared to the NMD group.
The present results showed that the intake of HMD, compared to NMD, maintains the trabecular structure by preventing trabecular bone resorption, indicating the usefulness of HMD intake.

Key words: Micro-computed tomography, Supplement diet, Fructo-oligosaccharide, 3-dimensional pseudocolor map, Structure model index

Introduction

With the increasing aging of society, aging-related diseases have attracted significant social interest. Of these diseases, osteoporosis is known to affect approximately 75 million people in the world[1], and various discussions and research have been proceeding with regards to its treatment. Bisphosphonates (BPs) are the major treatment drugs for osteoporosis[2], and they are used widely for the treatment of other bone disorders such as Paget's disease of bone, multiple myeloma, and osteogenesis imperfecta. However, since BP drugs may induce side effects of osteonecrosis of the jaw after invasive dental treatments such as teeth extractions[3], research and development of treatment methods for osteoporosis, as well as novel preventive drugs, are currently ongoing.

The epiphyseal region of the trabecular bone can be categorized into primary and secondary trabecular bones. The trabeculae of the primary trabecular bone are modified through bone resorption and undercuts to eventually become secondary trabecular bone. Changes in the trabecular structure occur due to this bone remodeling process, and osteoclast-induced bone resorption and osteoblast-induced bone formation are constantly repeated to maintain balance. Due to decreased estrogen secretion in osteoporosis, there is an imbalance in remodeling, causing bone

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Table 1. Compositions of the normal mineral diet (NMD) and the high mineral diet (HMD).

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

resorption to exceed bone formation. This subsequently leads to bone fragility. Since the presence or absence of this trabecular bone greatly affects the mechanical strength of the bone, the formation and maintenance of the trabeculae are crucial.

Traditionally, trabecular structure analysis has been conducted two-dimensionally with morphometry, but this method has disadvantages of being labor-intensive and time-consuming, because it requires the preparation of decalcified and undecalcified histological sections of the bone samples prior to analysis. With the popularization of micro-computed tomography (micro-CT), the current trabecular structure analysis allows for the assessment of three-dimensional spatial information of the trabeculae, measured as the "structure model index" (SMI), and various studies using this technique have been reported[4,5]. The advantages of SMI measurement using micro-CT include: information about the interior of the bone sample can be ascertained non-invasively with a short duration of imaging; observations can be evaluated with analytical software; and repeated measurements can be made over time *in vivo* in small animals. Furthermore, with the appearance of high resolution micro-CT on the market, measurements and analysis can be carried out easily.

In conventional micro-CT imaging, black and white images are evaluated because the color intensity representing X-ray penetration and non-penetration through materials is judged. Moreover, it is difficult to observe changes in bone quality with regard to the bone mineral density (BMD) presented in a 3-dimensional pseudocolor map (3D-map) of conventional X-ray or pathological images, suggesting the usefulness of this analytical system including micro-CT. Thus, in previous studies, we analyzed bone samples with micro-CT imaging and subsequently performed bending tests and sectioning, thereby permitting multiple analyses on the same sample[6-10].

We have previously conducted research involving the development of a high mineral diet (HMD) that contains fructo-oligosaccharide (FOS), isoflavone (ISO), and calcium citrate (CC) with calcium phosphate as an osteoporosis prevention supplement[10]. An earlier analysis of some of the results showed that HMD is beneficial for bone quality improvement, as demonstrated by the elevation in BMD and bone mineral content (BMC) of the femoral diaphysis in ovariectomized (OVX) rats after 24 weeks of the diet[10]. Therefore, in this study, the aim was to elucidate the usefulness of changes in SMI at the femoral epiphyseal region in HMD-fed OVX rats using micro-CT imaging. This report is a follow-up of our previous findings on the bone quality changes of the diaphysis[10].

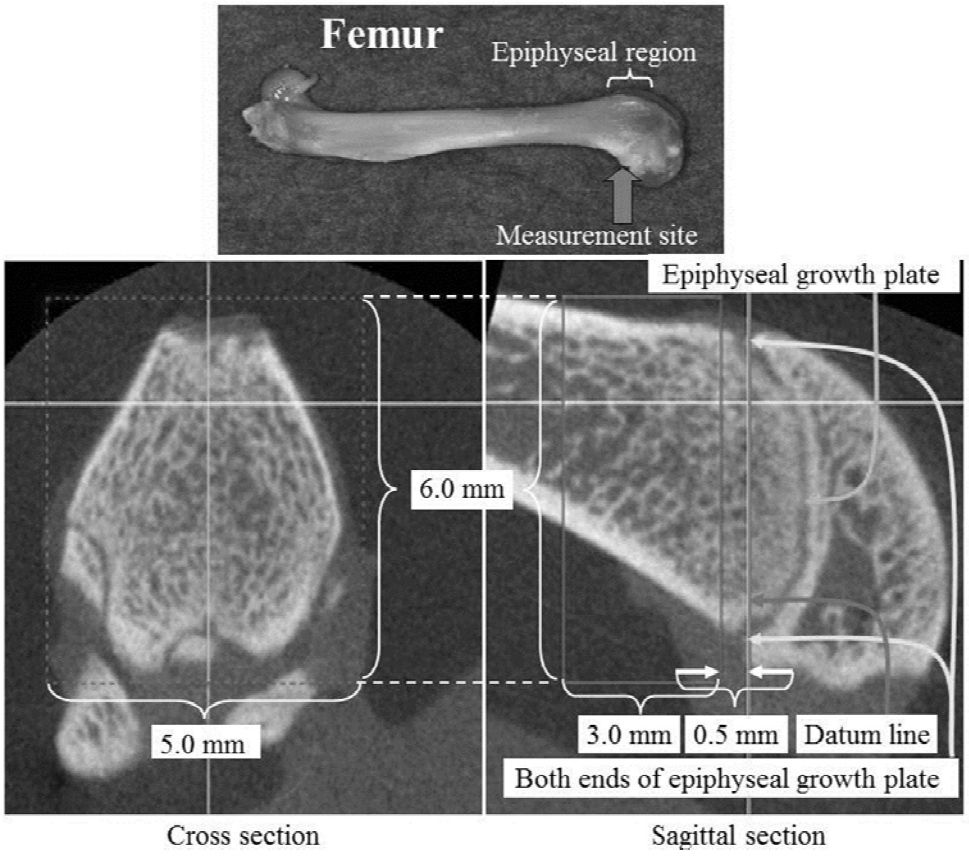

Figure 1. Measurement sites in the femur (top). The measurement range (6.0 mm × 5.0 mm × 3.0 mm) connecting the two ends of the growth plate in the femoral epiphyseal region from the micro-CT image (bottom).

Materials and Methods

All experiments were conducted according to the Guidelines for the Treatment of Animals, Nihon University, Tokyo, Japan.

Animals

Twelve, 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).

Preparation of rats

The 12 rats were randomly allocated to 2 groups. Group 1 (n = 6) consisted of OVX rats on a normal mineral diet (NMD, Oriental Yeast Co., Tokyo, Japan), and Group 2 consisted of OVX rats on an HMD (Oriental Yeast Co.). The compositions of the NMD and HMD are listed in Table 1. Both groups of rats were on the prescribed diet at 20 weeks of age. The compositions of the NMD and HMD were conducted according to the method reported by Nakada *et al*.[10)]

Table 2. Body weight gain in each group.

	After intervention (20 weeks old)	24 weeks (44 weeks old) (g)
OVX + NMD	196.5 ± 1.7	289.9 ± 13.0
OVX + HMD	196.0 ± 4.7	266.4 ± 10.2

Measurement of body weight

The body weights of both groups were measured at the following times: after intervention (20 weeks old) and 24 weeks (44 weeks old) after intervention. Animals were then euthanized with carbon dioxide, and bilateral femora were removed at 24 weeks after intervention.

Micro-CT

Micro-CT settings

The micro-CT (R_mCT; Rigaku Co., Tokyo, Japan) imaging conditions 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 femoral epiphyseal region (Fig. 1) and phantoms for CT value proofreading after 24 weeks.

Measurement of SMI

Table 3. BMD and BMC of the cortical bone and BMD and BMC of the trabecular bone for the 2 groups at 24 weeks after the intervention.

	Cortical bone		Trabecular bone	
	BMD (mg / cm^{-1})	BMC (mg)	BMD (mg / cm^{-1})	BMC (mg)
OVX + NMD	1078.1±14.5	15.1±0.7 **	705.3±12.3	0.2±0.1 **
OVX + HMD	1076.7±9.9	16.9±0.4	722.5±11.6	0.5±0.1
p value	0.75	0.004	0.065	0.004

Mann-Whitney's U test, n = 6, **: p < 0.01

The significance of differences between the 2 groups at any given time is shown by Mann-Whitney's U test (*P < 0.05, **P < 0.01).

Table 4. Results of the structure model index for the 2 groups at 24 weeks following the intervention.

	Trabecular bone					
Volume	TV (mm^3)	BV (mm^3)	BS (mm^2)	BS / BV (1 / mm)	BV / TV (%)	BMC / TV (mg / cm^3)
OVX + NMD	20.3±1.9 *	0.29±0.1 *	7.7±3.6 **	44.5±2.7	1.4±0.6 *	9.2±3.7 *
OVX + HMD	25.2±1.1	0.73±0.2	18.4±5.2	47.4±5.2	2.9±0.7	18.3±4.2
p value	0.04	0.04	0.006	1.09	0.04	0.01

Parallel Plate Model	Tb. Th (μm)	Tb. N (1 / mm)	Tb. Sp (μm)	Tb. Spac (μm)
OVX + NMD	41.3±1.9 *	0.27±0.1 *	4096.2±1423.3 *	3414.5±1510.7 **
OVX + HMD	50.2±4.5	0.53±0.2	1210.1±442.2	1216.7±440.5
p value	0.04	0.016	0.04	0.006

Mann-Whitney's U test, n = 6, * : p < 0.05, **: p < 0.01

The significance of differences between the 2 groups at any given time is shown by Mann-Whitney's U test (*P < 0.05, **P < 0.01).

Digital images were converted to a 16-bit gray-scale TIFF format using the Atlas TIFF Convertor® (Rigaku Co.), and they were observed using TRI/3D-Bon BMD (TRI/3D-Bon; Ratoc System Engineering, Tokyo, Japan). CT-value corrections and BMD measurements were conducted according to the method reported by Nakada *et al.* [10]. BMD and BMC of both cortical and trabecular bones, and tissue volume (TV), bone volume (BV), bone surface (BS), BS/BV, BV/TV, BMC/TV, trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), and trabecular spacing (Tb. Spac) of the trabecular bone were the measurement variables for the SMI.

Observation of 3D-map

Bone status (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). 3D-map observation was conducted according to the method reported by Nakada *et al.*[10]

Statistical analysis

All values in the tables and figures are shown as means ± standard deviation. Mann-Whitney's U test was used for statistical analyses of SMI with the null hypothesis that no difference would exist between the NMD and the HMD in the OVX. Values of P < 0.05 were considered significant.

Results

Body weight

Table 2 shows the body weight measurements for the 2 groups at each observation time. The body weight for the NMD group increased more than that of the HMD group.

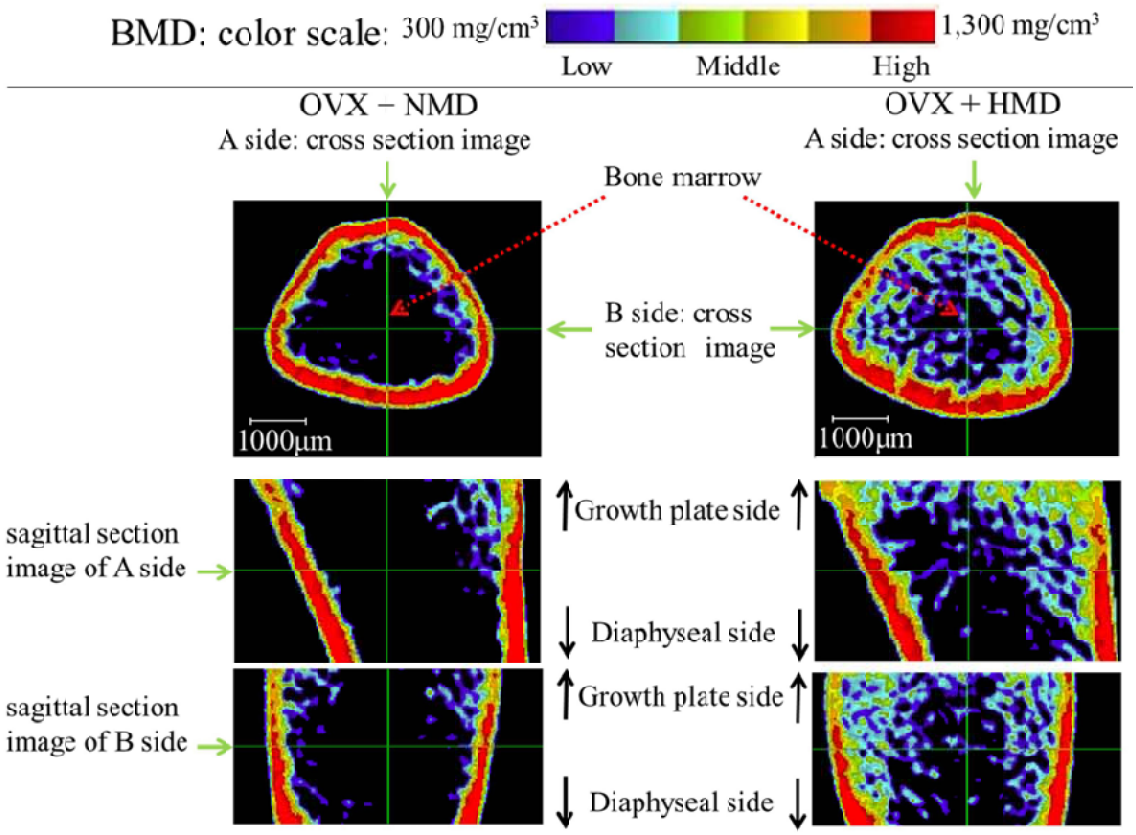

Figure 2. 3D-map of cross-sectional and sagittal section images of the femoral epiphyseal region for the 2 groups (OVX+NMD and OVX+HMD) 24 weeks after intervention. BMD color scale: Red and orange indicate high BMD, yellow and green indicate medium BMD, and light blue and purple indicate low BMD.

Measurement of SMI

The SMI results are shown in Tables 3 and 4. BMD of the cortical bone was the same in the two groups of animals, and BMC was significantly greater in the HMD group than in the NMD group. The BMD of the trabecular bone had a tendency to be higher in the HMD group than in the NMD group, and BMC was significantly higher in the HMD group than in the NMD group. In terms of the trabecular bone volume, the HMD group showed significantly greater TV, BV, BS, BV/TV, and BMC/TV than the NMD group. BS/BV had a slightly higher tendency in the HMD group than in the NMD group. In the Parallel Plate Model of the trabecular bone, the HMD group showed significantly greater Tb. Th, Tb. N, Tb. Sp, and Tb. Spac values than the NMD group.

Observation of 3D-map

Fig. 2 shows the 3D-map for the 2 groups after 24 weeks. High, medium, and low BMD are indicated, respectively, by red and orange, yellow and green, and light blue and purple.

In the cross-section of NMD bone, the outer periphery of the cortical bone was depicted in red, which represents high BMD. The inner portion of the cortical bone showed a layer of yellow, which represents moderate BMD. In the sagittal section, the growth plate side of the inner portion of the cortical bone showed low-BMD trabecular bone toward the bone marrow side, represented by light blue and purple colors. A small amount of trabecular bone was observed on the growth plate side, but this amount decreased further closer to the diaphyseal side. Due to both growth plate and diaphysis resorption, trabeculae were not observed in the trabecular bone at the center of the bone marrow cavity.

In the cross-section of the HMD bone, as in the NMD group, the outer periphery of the cortical bone was shown in red, which represents high BMD. The inner portion of the cortical bone showed a layer of yellow, which represents moderate BMD. In the sagittal section, the growth plate side of the inner portion of the cortical bone showed moderate to low-BMD trabecular bone toward the bone marrow side, represented by light blue and green colors. A large amount of trabecular bone was detected on the growth plate side, but this amount decreased towards the diaphyseal side. The trabeculae at the center of the bone marrow showed low BMD on the growth plate side represented by light blue and purple colors, but only a small amount was observed on the diaphyseal side.

Discussion

Rats in the NMD group were heavier than rats in the HMD group at 24 weeks following initiation of the diet intervention. This may have occurred because the 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 consider that this caused greater body weight loss in the HMD group than in the NMD group.

Observation of a 3D-map is an analytical system that uses both micro-CT and TRI/3D-Bon, allowing qualitative analysis of the trabecular bone structure with a 3D representation, and it has been used in many studies to date.[8-10)]

Osteoporosis is a systemic disease that results in reduced bone mass and poor trabecular bone structure.[11,12)] It is defined as "a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture".[13)] Bone strength is associated with 2 factors: BMD and bone quality, contributing about 70 % and 30 %, respectively.[13)] BMD is related to the microstructure, metabolic turnover, micro-damage, calcification, and collagen crosslinking[13)]. BMD is an important parameter in predicting fractures. However, the fracture risk increases with age even if the BMD remains constant.[14)] Therefore, it is necessary to improve not only BMD but also bone quality, including bone strength.

Numerous treatments, including calcium intake,[15)] estrogen supplementation[16)] (hormone replacement therapy), and active vitamin D_3,[4)] have been reported in studies related to osteoporosis prevention. Studies of oligosaccharides and soy beans have also been reported[17-24)]. Therefore, we developed a new osteoporosis-prevention supplement diet (HMD) that contains calcium phosphate dibasic along with FOS, ISO, and CC as additives. Thus, using micro-CT imaging, the changes in SMI, which is an important factor for the bone strength at the femoral epiphyseal region, were elucidated in this study. Some of the trabecular characteristics of osteoporosis include: decreased BV/TV, decreased connectivity, decreased SMI, increased distance between trabeculae, decreased number of trabeculae, and loss of homogeneity. When osteoporosis develops, the trabecular bone applies stress on the narrowed trabeculae, potentially causing the bone to bend easily and develop trabecular fractures or microcracks. Furthermore, local development of minute bone resorption lacunae can cause a series of trabecular fractures, resulting in bone collapse. For these reasons, trabecular microstructure is as important as bone mass as a factor that determines bone strength.

As shown in the 3D-map, the trabecular bone of the bone marrow is almost completely absorbed in the NMD group, leading to lower SMI values than in the HMD group. The HMD trabeculae, which are seen as having higher BMD compared to the NMD group on the 3D-map, were shown to advance from the inner wall of the cortical bone in the direction of the bone marrow. These trabeculae increased Tb. Th and Tb. N and decreased Tb. Sp and Tb. Spac, thereby maintaining highly dense trabeculae.

OVX groups reportedly show greater bone resorption than bone formation due to reduced estrogen secretion, leading to decreased BMD and bone strength[9)]. However, although the HMD group was also exposed to an environment that reduces estrogen secretion, SMIs were higher in the HMD group than in the NMD group. Therefore, it was thought that the NMD group showed a thin rod-like trabecular structure due to the low Tb. Th and BV/TV, but the HMD group showed a thick plate-like structure due to the high Tb. Th and BV/TV of the trabeculae. Moreover, the trabecular bone of the HMD group had higher Tb. Th and Tb. N, and shorter Tb.Sp and Tb. Spac, leading to trabeculae with higher density compared to the NMD group, resulting in stronger bones. These findings demonstrated that the intake of HMD, compared to that of NMD, maintains a healthy trabecular structure by preventing trabecular bone resorption, showing the usefulness of HMD intake.

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