Oral Radiol
DOI 10.1007/s11282-013-0160-2

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ORIGINAL ARTICLE

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Received: 29 September 2013 / Accepted: 16 October 2013

Abstract
Objectives To characterize the osteoporosis-preventive effects of a novel material consisting of a complex of type 1 collagen and hydroxyapatite (CCH) in osteoporosis model mice.
Methods Ten-week-old ovariectomized (OVX) ICR mice were used. The five study groups comprised a sham group, OVX control group, calcium carbonate ($CaCO_3$) group, vitamin K_2 [menaquinone 7 (MK7)] group, and CHH group ($n = 10$ each). The $CaCO_3$ and CHH groups were fed a special diet containing 2 % of the respective substance, whereas the MK7 group received a special diet containing 0.5 % MK7. After maintenance of the animals under these conditions for 8 weeks, their femurs were removed and blood samples were collected. The bone mineral densities (BMDs) of the distal femoral epiphysis were analyzed by peripheral quantitative computed tomography. Three-dimensional images were rendered with micro-focus computed tomography, and the trabecular structures were determined.
Results The significantly lower BMDs in the epiphyseal and diaphyseal regions of the OVX control group femurs compared with the sham group femurs were clear osteoporotic signs. The trabecular structure analyses revealed 12 parameters that were significantly lower in the OVX control group compared with the sham group. No distinct changes in the BMD or trabecular structure were observed in the $CaCO_3$ group. Significant improvements in the BMD in the MK7 group were limited to three parameters, whereas 11 trabecular structure parameters were significantly improved. The CHH group showed significant improvements in seven BMD parameters and seven trabecular structure parameters.
Conclusions CHH improves trabecular structure and BMD in a well-balanced manner.

Keywords Osteoporosis · Bone quality · Bone density · Collagen · Mineral

Introduction

Patients with osteoporosis are prone to fractures of the vertebral bodies, femoral neck, distal wrist, proximal humerus, and other sites, and are consequently at risk of experiencing a substantially reduced quality of life (QOL). Femoral neck fractures often leave patients bedridden, and dramatically lower their QOL and overall prognosis [1]. Bisphosphonates, a class of therapeutic drugs widely used for osteoporosis, increase bone mineral density (BMD), but also increase the risk of osteonecrosis of the jaw. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is becoming common, and more than 1,500 articles associated with BRONJ have been published worldwide [2]. Reduced QOL through such adverse reactions to bisphosphonates is a concern [3]. Regarding the oral and maxillofacial region, this background information illustrates the importance of preventing osteoporosis before the disease strikes and reduces patient QOL. Dietary supplements in the field of complementary and alternative medicine have gained interest as a means of preventing osteoporosis. Regimens consisting of a variety of dietary supplements have been proposed for this purpose, many of which involve supplemented minerals that suppress decreases in

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Published online: 28 November 2013

BMD. However, bone quality has recently gained attention as a factor other than BMD that affects the bone mechanical properties, because the incidence of femoral neck fractures in osteoporosis is not necessarily correlated with BMD [4], the frequency of new fractures remains high in patients with steroid-induced osteoporosis but high BMD [5], and sodium fluoride increases the incidence of fractures despite leading to increased BMD [6].

The Consensus Development Panel of the American National Institutes of Health states, "Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality," and continues, "bone quality refers to architecture, turnover, damage accumulation (e.g., microfractures), and mineralization" [7].

Typical dietary supplements used for osteoporosis are calcium and vitamin K_2. Calcium preparations are often used to compensate for or prevent decreases in BMD [8–10]. Although calcium supplementation has been shown to increase BMD [7], the results of an animal study demonstrated that calcium supplementation has limited effects on bone quality [11]. Conversely, vitamin K_2 prevents fracture, but minimally increases BMD [12].

Dietary supplements should ideally contain substances that improve both BMD and bone quality to maximally improve patient QOL. A calcium–collagen matrix may have an effect on bone quality. Fujita et al. [13] reported that calcium–collagen matrix supplementation decreased urinary excretion of crosslinked N-terminal telopeptides of type 1 collagen. On the basis of this finding, we noted CHH (RBS Co. Ltd., Shimane, Japan) as a dietary supplement for preventing osteoporosis that improves both BMD and bone quality. CHH is a novel material made by depositing hydroxyapatite onto type 1 collagen extracted from fish scales. CHH contains type 1 collagen, calcium, magnesium, phosphorus, sodium, and other minerals present as a complex. A product patent for this material has been approved in both Japan and the USA.

We conducted the present study in a mouse model of osteoporosis to investigate the inhibitory effects of CHH on BMD and trabecular deterioration associated with osteoporosis. We also compared CHH with two types of dietary supplement (calcium carbonate and vitamin K_2) with different actions.

Materials and methods

Experimental design

Ten-week-old female ICR mice (mean body weight, 20 g) were used in the study and subjected to ovariectomy (OVX group) or sham surgery (sham group). The mice were given a regular diet for 2 months, and then divided into five groups of 10 animals each. In the OVX control and sham groups, the regular diet contained 1.5–1.8 % calcium carbonate. In the $CaCO_3$ group, the mice were given a diet containing 2 % calcium carbonate. In the vitamin K_2 [menaquinone 7 (MK7)] group, the mice were given a diet containing MK7 (J-OIL MILS Inc., Tokyo, Japan) at 500 μg/100 g. In the CHH group, the mice were given a diet containing CHH at 2 g/100 g. Feeding under these conditions was continued for 2 months, and the mice were then euthanized by decapitation. Blood was collected and the separated serum was used for measurements of calcium (Ca), phosphorus (P), magnesium (Mg), and alkaline phosphatase (ALP). The femurs were resected and bone parameters were analyzed using peripheral quantitative computed tomography (pQCT). Microcomputed tomography (μCT) of the distal femoral epiphysis was also performed for three-dimensional (3D) analysis of the BMD and trabecular structure. Figure 1 shows the experimental protocol. This study was carried out using the same conditions and same period as our previously reported experiment [14]. Therefore, the results of the control and OVX groups were the same as in the previous report. The results for the experimental groups are new and interesting. The mice were housed in our approved animal holding facility and treated according to the guidelines of Kanagawa Dental College on Animal Care.

Measurements of plasma components

To separate plasma, blood samples were centrifuged (1,000 rpm, 10 min, 4 °C) with heparin sodium. We measured Ca levels using a chelate color development method, P levels with a direct molybdenum blue method, Mg levels with a xylidyl blue method, and ALP activity using a phenyl phosphate substrate method. A clinical biochemical test kit (TEST WAKO, Wako Pure Chemical Industries, Osaka, Japan) was used for these measurements.

Measurements of BMD and cross-sectional morphology

The BMD and cross-sectional morphology were measured at the femoral epiphysis of mice in the OVX control and experimental groups using a pQCT bone densitometer (XCT Research SA^+; Stratec, Pforzheim, Germany). First, a scout view of the mandibular bone was obtained, and a reference line was set at the epiphyseal region. A position approximately 2.0 mm distal to the reference line was selected as the analysis point. One slice was scanned using a voxel size of 80 × 80 × 480 μm. On the basis of the volume data for this slice, the BMD of trabecular bone and the BMD, sectional area, thickness, periosteal perimeter,

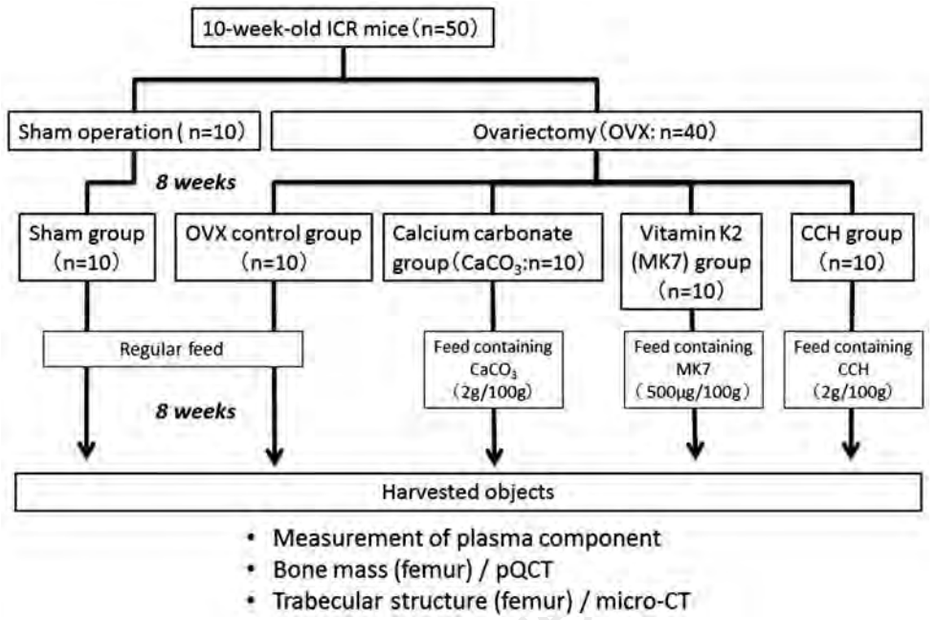

Fig. 1 Experimental protocol

and stress/strength index (SSI) of cortical bone were determined. The inner threshold for trabecular bone analysis was set at 395 mg/cm^3, and indicated by P-Mode 20, which can automatically detect the inner threshold [15]. The threshold for cortical bone analysis was set at 690 mg/cm^3 according to the Cort mode.

Measurements of 3D structural parameters

For trabecular structural analysis, 3D image data of the femur were collected using μCT (MCT-CB130 MF; Hitachi Medico, Tokyo, Japan). The volume data for 100 slices were obtained from each sample under the following exposure conditions: tube voltage, 40 kV; tube current, 100 μA; and voxel size, 43 × 43 × 43 μm. TRI 3D-BONE software (Rotoc System Engineering, Tokyo, Japan) was used for measurements of trabecular structure parameters [16].

Bone histometric analysis

3D reconstructed images were used for analysis of trabecular structure parameters, such as bone volume (BV) per tissue volume (TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular spacing (Tb.Spac), fractal dimension (FD), trabecular bone pattern factor (TBPf), and structure morphological index (SMI) [17–20].

Star volume analysis

The continuity of trabecular bone structures was evaluated by star volume analysis [21, 22]. In this type of analysis, a trabecular bone structure is defined according to two types of measurement: trabecular star volume (V*tr) and marrow space star volume (V*m.space). V*tr represents the mean volume of a trabecula from a designated internal point in all directions to the margin of the trabecula. V*m.space represents the mean volume of the marrow cavity surveyed from a designated point in all directions without obstruction by the trabecula. With increasing trabecular number and continuity, V*tr becomes larger and V*m.space becomes smaller, and vice versa.

Node-strut analysis

3D skeletal images with a thickness of 1 pixel were produced from the 3D trabecular images by thinning processing. The structural parameters of the 3D skeletal line network connectivity were measured by node-strut analysis [23, 24].

In the present study, the point at which three or more trabecular skeletal elements intersected was defined as a node (Nd), and a terminus (Tm) was defined as a terminating end point, one that was not contiguous with other skeletal elements. A strut was defined as a linear skeletal element connected to Nd or Tm or cortical bone (Ct). The numbers of Nd per TV (N.Nd/TV), Tm per TV (N.Tm/TV), and Ct per TV (N.Ct/TV) were determined by numerical parameters. The proportion of the length of each strut element to the TV was determined as the total strut length (TSL)/TV. The strut length between Ct and Nd per TV and strut length between Tm and Tm per TSL were also determined. According to these definitions, higher values of the Nd, Ct, and TSL parameters indicated greater

Table 1 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for body weight and resected femoral bone weight (wet)

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
Body weight (g)	45.8 ± 6.9	45.7 ± 4.3	50.7 ± 11.4	55.4 ± 7.1*	51.8 ± 9.3
Femoral bone weight (g)	0.125 ± 0.012	0.127 ± 0.011	0.133 ± 0.015	0.134 ± 0.012*	0.135 ± 0.007*

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

* $P < 0.05$

Table 2 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for serum levels of calcium (Ca), phosphorus (P), magnesium (Mg), and alkaline phosphatase (ALP)

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
Ca (mg/dl)	9.42 ± 1.32	9.82 ± 1.08	10.2 ± 1.56	9.34 ± 1.50	9.80 ± 1.64
P (mg/dl)	7.01 ± 0.78	7.02 ± 0.72	6.65 ± 0.96	7.12 ± 0.64	7.06 ± 0.76
Mg (mg/dl)	2.72 ± 0.72	3.10 ± 1.05	2.77 ± 0.95	2.63 ± 0.63	2.71 ± 0.92
ALP (BLunit)	4.26 ± 1.98	3.88 ± 1.54	3.74 ± 1.67	3.77 ± 1.76	3.44 ± 1.59

No significant differences were observed between the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

skeletal connectivity, whereas higher values of the Tm parameter indicated lower skeletal connectivity.

Evaluation methods

Differences in mean values between the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group) were calculated for each parameter to determine the mean percentages. Significant differences between groups were evaluated by analysis of variance (ANOVA) and a post hoc test (Fisher's protected least significant difference (PLSD)). StatView version 5.0 software (Abacus Concepts, Berkeley, CA, USA) was used for all analyses. Differences were considered significant for values of $P < 0.05$.

Results

Table 1 compares the body weight and femoral bone weight (wet) between the OVX control group and each experimental group. The lowest body weight was observed in the sham group. The femoral bone weight tended to be higher in all groups compared with that in the OVX control group, but the differences were not significant. In the MK7 group, the body weight and femoral bone weight were significantly increased compared with the OVX control group. In the CHH group, the femoral bone weight was significantly increased compared with the OVX control group.

Table 2 compares the serum mineral (Ca, P, Mg) and ALP concentrations between the OVX control group and each experimental group. The serum Ca, P, and Mg levels did not differ significantly among the groups. The levels of ALP (for which high values are associated with bone metabolic abnormalities) tended to be lower in the experimental groups compared with the OVX control group, but the between-group differences were not significant.

Table 3 compares the total bone mineral content, BMD of trabecular bone, and BMD, sectional area, thickness, periosteal perimeter, and SSI of cortical bone in the femoral epiphysis between the OVX control group and each experimental group. The sham group had a significantly higher total bone mineral content, cortical bone BMD, cortical bone cross-sectional area, cortical bone thickness, and SSI than the OVX control group, and the values were significantly lower at the endosteal perimeter. These findings show that ovariectomy caused osteoporotic bone loss in the OVX control group. In the CHH group, the values were similar to or lower than those in the sham group, whereas the total bone mineral content, BMD of cortical bone, cross-sectional area, thickness of cortical bone, and SSI were significantly greater than those in the OVX control group. In the MK7 group, only the cross-sectional area and endosteal perimeter were significantly greater than those in the OVX control group, whereas the other parameters showed no significant differences. In the $CaCO_3$ group, no significant differences were seen in any of the parameters compared with the OVX control group. Among the experimental groups, the CHH group showed the best improvement in the BMD of the femoral epiphysis.

Table 4 compares the total bone mineral content, BMD of trabecular bone, and BMD, cross-sectional area, thickness, periosteal perimeter, and SSI of cortical bone in the femoral diaphysis between the OVX control group and each experimental group. The sham group had a significantly

Table 3 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for bone mineral density (BMD) and cross-sectional morphometric indices of cortical bone in the femoral epiphysis

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
Total bone mineral content (mg/mm)	442.5 ± 32.8	547.8 ± 69.9**	457.9 ± 36.7	473.2 ± 58.3	500.0 ± 71.4*
Trabecular bone					
BMD (mg/cm^3)	173.1 ± 24.5	206.1 ± 37.1	180.9 ± 14.7	168.0 ± 64.9	189.8 ± 39.0
Cortical bone					
BMD (mg/cm^3)	853.6 ± 28.1	921.9 ± 41.8**	874.4 ± 20.5	872.0 ± 36.3	892.8 ± 25.6*
Sectional area (mm^2)	1.40 ± 0.18	1.99 ± 0.30**	1.59 ± 0.30	1.73 ± 0.33*	1.88 ± 0.43**
Thickness (mm)	0.20 ± 0.03	0.29 ± 0.04**	0.22 ± 0.04	0.24 ± 0.05	0.26 ± 0.06**
Periosteal perimeter (mm)	7.80 ± 0.14	7.85 ± 0.46	8.07 ± 0.35	8.13 ± 0.28	8.06 ± 0.40
Endosteal perimeter (mm)	6.57 ± 0.19	6.04 ± 0.55**	6.72 ± 0.36	6.66 ± 0.30*	6.42 ± 0.47
SSI (mm^3)	1.18 ± 0.10	1.45 ± 0.26**	1.36 ± 0.22	1.39 ± 0.23	1.49 ± 0.28**

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

SSI stress/strength index

* $P < 0.05$, ** $P < 0.01$

Table 4 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for bone mineral density (BMD) and cross-sectional morphometric indices of the cortical bone in the femoral diaphysis

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
Total bone mineral content (mg/mm)	710.6 ± 105.8	848.6 ± 52.1**	742.3 ± 60.7	787.8 ± 106.3	792.4 ± 71.7*
Cortical bone					
BMD (mg/cm^3)	1,160.1 ± 58.1	1,233.2 ± 23.6**	1,192.7 ± 35.3	1,205.4 ± 65.2*	1,207.3 ± 33.1*
Sectional area (mm^2)	1.42 ± 0.83	1.62 ± 0.17*	1.54 ± 0.19	1.59 ± 0.23	1.53 ± 0.18
Thickness (mm)	0.32 ± 0.02	0.38 ± 0.03**	0.32 ± 0.03	0.34 ± 0.04	0.35 ± 0.04
Periosteal perimeter (mm)	5.51 ± 0.35	5.46 ± 0.36	5.78 ± 0.37	5.72 ± 0.40	5.51 ± 0.38
Endosteal perimeter (mm)	3.52 ± 0.47	3.09 ± 0.40*	3.74 ± 0.35	3.57 ± 0.43	3.32 ± 0.49
SSI (mm^3)	0.82 ± 0.09	0.95 ± 0.16	0.98 ± 0.16*	0.96 ± 0.20	0.91 ± 0.14

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

SSI stress/strength index

* $P < 0.05$, ** $P < 0.01$

higher total bone mineral content, cortical bone BMD, cortical bone sectional area, and cortical bone thickness than the OVX control group, and values were significantly lower at the endosteal perimeter. In the CHH group, the values were similar to or lower than those in the sham group, whereas the total bone mineral content and BMD of cortical bone were significantly greater than those in the OVX control group. In the MK7 group, only the cortical bone BMD was significantly greater than that in the OVX control group, and the other parameters showed no significant differences. In the $CaCO_3$ group, only the SSI was significantly greater than that in the OVX control group, and the other parameters showed no significant differences. Among the experimental groups, the CHH group showed the most improvement in the BMD of the femoral diaphysis.

Table 5 compares the morphometric indices at the femoral epiphysis between the OVX control group and each experimental group. Most trabecular structural parameters were worse in the OVX control group than in the sham group, indicating an osteoporotic condition. In the CHH group, compared with the OVX control group, Tb.N was significantly higher, whereas Tb.Sp, Tb.Spac, and TBPf were significantly lower. The trabecular structure improved to the same extent as in the sham group. In the MK7 group, the trabecular structure improved with significant increases in BV/TV, Tb.N, and FD, but significant decreases in Tb.Sp, Tb.Spac, and TBPf, compared with the OVX control group. In the $CaCO_3$ group, only Tb.Sp and Tb.Spac were significantly decreased compared with the OVX control group.

Table 5 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for morphometric indices in the femoral epiphysis

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
BV/TV (%)	4.40 ± 2.41	10.9 ± 5.33**	5.22 ± 1.65	10.73 ± 5.66**	8.89 ± 5.17
Tb.Th (μm)	42.8 ± 7.8	47.8 ± 3.8	40.7 ± 5.7	48.2 ± 8.2	47.2 ± 6.0
Tb.N (1/mm)	0.98 ± 0.41	2.24 ± 0.95**	1.27 ± 0.35	2.13 ± 0.82**	1.80 ± 0.86*
Tb.Sp (μm)	1,188 ± 642	475 ± 210**	806 ± 266*	479 ± 190**	640 ± 335**
Tb.Spac (μm)	1,230 ± 636	523 ± 207**	846 ± 264*	527 ± 183**	687 ± 331**
FD	1.57 ± 0.21	1.74 ± 0.13*	1.57 ± 0.10	1.76 ± 0.16*	1.72 ± 0.16
TBPf	20.5 ± 4.5	16.9 ± 2.6*	19.3 ± 3.4	16.5 ± 3.5*	16.5 ± 1.9*
SMI	3.20 ± 0.13	3.04 ± 0.16	3.12 ± 0.27	3.06 ± 0.19	3.16 ± 0.16

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

BV/TV bone volume per tissue volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation, *Tb.Spac* trabecular spacing, *FD* fractal dimension, *TBPf* trabecular bone pattern factor, *SMI* structure morphometric index

* $P < 0.05$, ** $P < 0.01$

Table 6 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for parameters obtained by star volume analysis in the femoral epiphysis

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
V*m.space	0.28 ± 0.04	0.16 ± 0.07**	0.29 ± 0.04	0.24 ± 0.08	0.23 ± 0.06
V*tr	0.007 ± 0.004	0.011 ± 0.003	0.008 ± 0.004	0.010 ± 0.005	0.012 ± 0.005*

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

*V*m.space* marrow space star volume, *V*tr* trabecular star volume

* $P < 0.05$, ** $P < 0.01$

Table 7 Comparisons between the OVX control group and the sham, $CaCO_3$, MK7, and CCH groups for parameters obtained by node-strut analysis in the femoral epiphysis

Parameters	OVX control	Sham	$CaCO_3$	MK7	CCH
N.Nd/TV (1/mm^3)	2.67 ± 3.58	7.82 ± 5.70*	3.74 ± 1.96	8.45 ± 7.03*	6.22 ± 4.76
N.Tm/TV (1/mm^3)	3.56 ± 2.56	3.55 ± 3.04	2.36 ± 1.43	2.38 ± 2.29	2.40 ± 2.17
N.Ct/TV (1/mm^3)	5.04 ± 2.28	15.12 ± 11.80**	4.67 ± 2.78	12.85 ± 5.64*	10.37 ± 8.56
TmTm/TSL	8.31 ± 6.232	3.58 ± 3.27*	4.92 ± 4.66	2.79 ± 3.67**	1.31 ± 1.35**
CtNd/TV (1/mm^2)	0.45 ± 0.48	2.26 ± 1.49**	0.73 ± 0.57	2.09 ± 1.14**	1.83 ± 1.25*
TSL/TV (1/mm^2)	1.60 ± 1.49	5.20 ± 2.94**	2.19 ± 1.19	5.60 ± 3.65**	4.18 ± 2.81

ANOVA and a post hoc test (Fisher's PLSD) were conducted to compare the OVX control group ($n = 10$) and the experimental groups ($n = 10$/group). Values are shown as the mean ± standard deviation

Nd node, *Tm* terminus, *Ct* cortical, *N.Nd/TV* number of Nd per tissue volume, *N.Tm/TV* number of Tm per tissue volume, *N.Ct/TV* number of Ct per tissue volume, *TmTm/TSL* strut length between Tm and Tm per total strut length, *CtNd/TV* strut length between Ct and Nd per tissue volume, *TSL/TV* total strut length per tissue volume

* $P < 0.05$, ** $P < 0.01$

Table 6 compares the star volume analysis of the femoral epiphysis between the OVX control group and each experimental group. Compared to the OVX control group, V*tr was significantly increased in the CHH group, indicating an improvement in trabecular continuity.

Table 7 shows the results for the node-strut analysis of the femoral epiphysis. In the MK7 group, compared with the OVX control group, N.Nd/TV, N.Ct/TV, CtNd/TV, and TSL/TV were significantly increased, and TmTm/TSL was significantly decreased, indicating an improvement in the trabecular connectivity. In the CHH group, CtNd/TV was

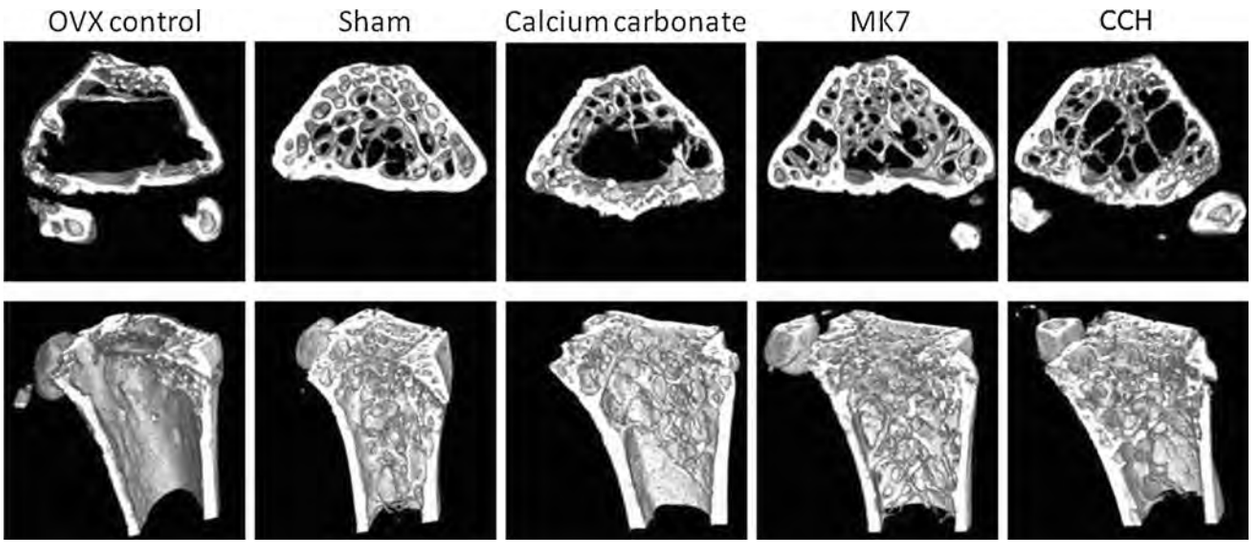

Fig. 2 3D reconstructed images of representative axial and sagittal sections from the OVX control, sham, $CaCO_3$, MK7, and CCH groups

significantly increased, and TmTm/TSL was significantly decreased, compared with the OVX control group. Trabecular connectivity was improved in the MK7 and CHH groups.

Figure 2 shows representative axial and sagittal sections with 3D imaging for the structural analysis in the OVX control group and each experimental group. The axial and sagittal section images demonstrate improved trabecular structure in the $CaCO_3$, MK7, and CHH groups.

Discussion

CHH is a novel material made by depositing hydroxyapatite onto type 1 collagen extracted from fish scales. Having identified CHH as a dietary supplement that purportedly enhances BMD and bone quality, we compared this product with two types of dietary supplement with different actions. The first supplement was MK7, which minimally improves BMD, but lowers fracture frequency by improving the skeletal architecture through enhancement of bone matrix protein [25]. The other supplement, $CaCO_3$, is the most commonly used dietary supplement for preventing osteoporosis. $CaCO_3$ is often taken to supplement calcium deficiencies. Previous clinical studies have reported that calcium supplementation suppresses postmenopausal decreases in BMD when taken therapeutically [26, 27].

In the present study, no distinct increases in the BMD of the epiphyseal or diaphyseal regions were noted in the $CaCO_3$ and MK7 groups. In contrast, increases in the total bone mineral content and cortical bone BMD as well as morphological changes in cortical bone were observed in the CHH group. The significant increase in the femoral bone weight in the CHH group relative to the OVX control group also substantiated this increase in BMD. Calcium consumption was expected to increase the BMD, but did not do so in this study. $CaCO_3$ is used to address insufficient calcium consumption and absorption in the elderly. However, the amount of calcium absorbed through the intestines is dependent not only on the amount of dietary calcium consumed, but also on the form of the calcium ingested and the amount of phosphate bound to the calcium in the intestines. On the other hand, calcium absorption is affected by vitamin D_3 and citric acid intake. An increase in BMD was achieved in a previous study in which postmenopausal women took vitamin D_3 together with calcium [28]. In addition, an in vivo study reported that citric acid supplementation improved the trabecular structure in OVX mice [29]. However, a study on osteoporosis model mice found no substantial effects from supplementation with $CaCO_3$ alone [11]. The effect achieved by adding $CaCO_3$ alone to the regular diet given to young ovariectomized mice in the present study was likely to be small, because the regular diet already contained sufficient calcium (1.5–1.8 %). This suggests that the improved BMD in the CHH group represents a result that would not be achievable with calcium supplementation alone.

Significant increases in the trabecular structural parameters of BV/TV and FD were observed in the MK7 group. The node-strut analysis further revealed significant increases in N.Nd/TV and N.Ct/TV. This compound provided the best improvement in the trabecular structure. Fujikawa et al. [11] found that the trabecular structure was significantly improved following coadministration of calcium phosphate and MK7, compared with administration

of calcium phosphate alone [11]. Vitamin K_2 is noted for its ability to promote bone formation, suppress bone resorption, and improve the bone trabecular structure [30–33]. Substantial improvements in the trabecular structure were also seen in the CHH group in the form of significant decreases in Tb.Sp, Tb.Spac, and TBPf and significant star volume and node-strut increases. The only trabecular structure effects observed in the $CaCO_3$ group were decreased Tb.Sp and Tb.Spac. These findings suggest that CHH has a trabecular mechanism of action that differs from that of calcium monotherapy. By acting on bone matrix protein without substantially improving the BMD, CHH appears to promote mineralization to a similar extent to MK7.

$CaCO_3$ produced no distinct changes in the BMD or trabecular structure. MK7 substantially improved the trabecular structure, but had limited effects on the BMD. The effects of CHH on the BMD and trabecular structure were well balanced. These effects of CHH are attributable to the compound actions of the minerals and type 1 collagen derived from fish scales. The non-denatured type 1 collagen found in fish scales is a natural marine type 1 collagen that consists of many amino acids, including glycine, proline, hydroxyproline, and aniline. In an investigation of collagen peptides, Wu et al. [34] found that orally administered collagen peptides have the ability to increase bone mass. Another study reported that supplementation with 900 mg of calcium and 3.5 g of collagen to patients with osteoarthritis experiencing backache and knee-joint pain resulted in marked alleviation of subjective pain [13]. These reports suggested that collagen supplementation may protect collagen against degradation through inhibition of cytokine-induced release of metalloproteinases, including collagenase. Hata et al. [35] reported that orally administered collagen tripeptide promotes fracture healing. Collagen intake is also expected to have beneficial effects on osteoporosis. CHH contains calcium, magnesium, phosphate, and other minerals bound to collagen peptides. Magnesium is particularly strongly correlated to bone quality. Okada et al. [36] showed that magnesium deficiencies degrade the trabecular structure without decreasing the BMD, indicating that magnesium consumption has a stronger effect on bone quality than on BMD. These compound effects appear to be the reason why CHH improved both the BMD and bone quality.

Regarding the oral and maxillofacial region, the present results suggest that intake of CCH will prevent osteoporosis and reduce the risk of BRONJ. In addition, the improvements in BMD and bone mass should also appear in the mandible. A previous study described that osteoporosis influences the mandible [37]. On the other hand, it is known that dietary supplement administration to OVX rats may change the trabecular bone pattern in the femur, while also causing slight changes in the mandibular bone [38]. Previously, several dietary supplements for osteoporosis in consideration of application to the oral and maxillofacial region have been reported [14, 29, 39]. It is supposed that the improvements in bone mass and BMD induced by CCH will influence the mandible. The effects of this dietary supplement on the mandible should be further considered in future studies.

Our findings show that CHH improves both the BMD and trabecular structure. Taken as a dietary supplement, CHH could be a beneficial form of complementary and alternative medicine for preventing osteoporosis.

Acknowledgments This work was supported by a research fund (No. 2008025) from RBS Co. Ltd.

Conflict of interest Yusuke Kozai, Mikiko Iino, Hisashi Innami, Ryota Kawamata, Hiromi Wakao, and Takashi Sakurai declare that they have no conflict of interest.

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