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Influence of the Anti-Osteoporosis Effect of Fish Collagen Supplement in Mice

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Abstract

Background: *Osteoporosis (OA) is the second most serious epidemic worldwide in chronic progressive disease. Complex fish collagen powder contains astaxanthin extract, vitamin C, vitamin K_1, vitamin D_3, vitamin E, vitamin A, and whole milk powder to protect bone health. This novel combination for improving bone loss has not yet been studied or discussed.*

Objective: *This trial investigated whether complex fish collagen (FC) powder containing astaxanthin extract, vitamin C, vitamin K_1, vitamin D_3, vitamin E, vitamin A, and whole milk powder had an improved effect on osteoporosis.*

Methods: *The experiment has forty male Sprague-Dawley (SD) rats ages five weeks. The mice were divided into five groups (control, LCa+H_2O, complex fish collagen doses 0.5-flod, 1-flod, and 2-flod) for twelve weeks. The complex fish collagen powder uses blood serum biochemical value, bone tissue, and biomechanics were analyzed.*

Results: *Twelve weeks of low calcium feeding resulted in a significant reduction in the N-terminal telopeptides of type I collagen, the N-telopeptide (NTX) concentration, and the osteocalcin (OCN) concentration in the serum compared to the LCa+H_2O group, the LCa+FC-L, LCa+FC-M, and LCa+FC-H group. According to the micro-computed tomography analysis, in comparison to the LCa+H_2O group, the femoral bone mineral density, trabecular area percentage, and trabecular thickness of the LCa+FC-L, LCa+FC-M, and LCa+FC-H groups increased significantly, and the trabecular separation decreased significantly.*

Conclusion: *By translating the experimental data into a daily supplement for adults, it is recommended that a daily intake of FC can help bone remodeling and bone strength, prevention effect on osteoporosis disease.*

Keywords: *Fish Collagen; Osteoporosis; Bone Health.*

INTRODUCTION

Based on the World Health Organization (WHO), osteoporosis is the second most serious epidemic worldwide in chronic progressive disease. In of individuals aged 50 years or more at high risk of osteoporotic fracture worldwide, 2010 was estimated at 158 million people. (Oden, McCloskey et al. 2015, Sheik Ali 2023, Gong, Azad et al. 2024)

Calcium is one of the most abundant elements in the human body and is a major component of the mineralized tissues, where more than 99% of the total body calcium is contained. It plays a key role in skeleton mineralization and is required for normal growth, development, and bone strength. Calcium is an essential element and is, therefore, essential to introduce the recommended quantity through the diet. (Nozue 2018, Vannucci, Fossi et al. 2018)

Fish collagen is mainly composed of type I collage. after hydrolysis, the product loses its gelling ability and becomes soluble in water. Collagen peptide differs from other proteins, as it contains amino acids such as glycine, proline, and hydroxyproline in an accessible form at around 10-20 times higher (Delmas 1993, Tian, Li et al. 2021, Yamada, Yamamoto et al. 2021, Gong, Azad et al. 2024). Collagen is stabilized by the formation of covalent cross-links between the end of one collagen molecule and the helical portion of the adjacent collagen molecule. There are two major cross-link molecules, pyridinoline and deoxypyridinoline. These residues are the most important means of stabilizing collagen molecules by intramolecular cross-links and are released from bone only during bone resorption or collagen breakdown (Delmas 1993, Seibel 2005).

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Hydrolyzed collagen from both fish and shrimp was described to contain both, a biologically related calcitonin and/or calcitonin-gene-related peptide isolated fish-bone peptides with a high affinity to calcium and a high content of phosphopeptides (Delmas 1993, Tian, Li et al. 2021, Yamada, Yamamoto et al. 2021, Davan, Fakurazi et al. 2023, Gong, Azad et al. 2024).

Nutritional factors play a critical role in the prevalence rate of osteoporosis with Ca^{2+} and vitamin D considered as important for bone health. The role of vitamin D deficiency in osteoporosis is mediated by the role of its active form, 1,25-dihydroxy vitamin D, in the regulation of mineral metabolism and bone remodeling through its influence on osteoblast formation, new bone, and osteoclast activity. However, evidence demonstrates that Ca^{2+} and vitamin D, including minerals and trace elements, vitamins, and polyphenols, can modify the risk of developing osteoporosis. It has been demonstrated that several vitamin groups, including vitamins A, E, K, C and B, are involved in regulation of bone translation, and that their insufficiency may be considered as a dietary risk factor for osteoporosis (Lips and van Schoor 2011, Sadat-Ali, Al Elq et al. 2011, Burt, Billington et al. 2019, De Martinis, Allegra et al. 2021, Skalny, Aschner et al. 2024).

Although everyone knows that supplements with more calcium can help with bone health, the above factors for bone health required by the human body are still insufficient. Therefore, the purpose of animal experiments was designed based on the concept of fish collagen supplements to prove whether the health care of animal bones can promote achieved bone health.

MATERIAL & METHODS

Test Substances

The FC powder was provided by HealthTake Corporation (16g per powder). The formula contained fish collagen extract, astaxanthin extract, vitamin C, vitamin K_1, vitamin D_3, vitamin E, vitamin A, whole milk powder, magnesium sulfate, and sucralose.

Experimental Dose

The recommended dosage of FC for adult humans is one package per day (16g per powder). The oral dose for rats was calculated according to the metabolic conversion ratio of 6.2 between humans and rats. The rats were dosed with 0.83, 1.65, and 3.3 g/kg of FC per day, which are 0.5, 1, and 2 folds (indicate the low dose, medium dose, high dose) of the human dose, respectively.

Study Design

The animal experiment was approved by the China Medical University Institutional Animal Care and Use Committee (No: CMUIACUC-2021-22) and was performed according to the codes of ethics for institutional animals of China Medical University. 40 four-week-old male SD rats were purchased from BioLASCO Taiwan and reared in the animal center of China Medical University. The rearing condition was controlled in an environment alternating between light and dark at a constant temperature of 22±4 °C and constant humidity for 12 hours.

The rats were divided into the control diet group (D10012G, Ca 0.5% Research Diet Inc., New Brunswick, NJ) and three low calcium (LCa) diet groups (D20122201, Ca 0.126% Research Diet Inc., New Brunswick, NJ). During this period, the body length and body length plus tail length of the rats were measured using a tape measure once a week. The body length is the length from the tip of the rat's nose to the anus (the position of the anus can be observed by holding the rat with both hands and lying on its side). The length of body length plus tail length is the length from the tip of the nose to the end of the tail when the rat is in the prone position.

After 12 weeks of administration of the test materials, the rats were narcotized, blood was drawn from the abdominal aorta, and two femurs and two tibias were taken out for analysis. Weigh the feed and body weight once a week. The body weight is the basis for the dosage of collagen powder for that week.

Body Height, Weight, and Food Intake

The body length and body length plus a tail length of the rats were measured weekly during the experiment, and the rats were weighed at the same time. Two to three rats were reared in each rearing box, the feed was weighed, and the daily average food intake per rat was estimated and recorded.

N-terminal Telopeptides of the Type I Collagen and Osteocalcin Concentrations in the Serum

The N-terminal telopeptides of the type I collagen (NTX) in the rats were determined by using the commercially available ELISA kit for cross-linked N-telopeptide of type I collagen (Rat) reagent (Cloud- Clone Corp, TX, USA) on the principle of enzyme immunoassay, according to the operation instruction. The procedure is briefly described below. An amount consisting of 50μL of the standard sample and the serum sample was put in the 96-well plate of the kit, and 50μL of detection reagent A was put in each well. The well plate reacted at 37°C for one hour, and then the well plate was washed with a buffer solution. An amount consisting of 100μL of detection reagent B was then put in the well plate, and the well plate reacted at 37°C for 30 minutes. The well plate was washed with a buffer solution, filled with 90μL of a substrate solution, and then reacted at 37°C for 20 minutes. Finally, each well was filled with 50μL of a stop solution to stop the reaction. The absorbance value of the well plate at 450 nm was tested by a spectrophotometer, the standard regression curve of the absorbance value corresponding to the standard concentration was made, and the curvilinear equation was obtained. The sample absorbance value was substituted into the curvilinear equation, the dilution factor was restored, and the NTX concentration in the serum of each rat was calculated.

The rats' osteocalcin (OCN) levels were determined using the commercially available Rat Osteocalcin ELISA Kit (Cat. No. BT-490, Biomedical Technologies Inc., MA, USA) on the principle of enzyme immunoassay, according to the operation instruction. The procedure is briefly described below. An amount consisting of 100μL of the standard samples at different concentrations (0.25-20 ng/mL) and the rat serum diluent (five times dilution using a sample buffer solution) was put in the appended 96-well plate of the kit and reacted at 2-8°C for 24 hours. The well plate was washed with a phosphate buffer, after which 100μL of osteocalcin rat antiserum was put in the well plate and reacted at 37°C for one hour. Afterward, the well plate was washed with the phosphate buffer according to the cleaning step. Each well was then filled with 100μL of donkey anti-goat immunoglobulin G (IgG) peroxidase and reacted at room temperature for one hour. Then, each well is filled with 100μL of a tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) mixed solution and reacted at room temperature in the dark for 30 minutes. Finally, 100 μL of a stop solution was applied to stop the reaction. The absorbance value of the well plate at 450 nm was tested with a spectrophotometer, the standard regression curve of the absorbance value corresponding to the standard concentration was made, and the curvilinear equation was obtained. The sample absorbance value was substituted into the curvilinear equation, the dilution factor was restored, and the OCN concentration in the serum of each rat was calculated.

Bone Tissue Analysis

The right femur was taken out after each rat was sacrificed, and a computed tomography (CT) image of the femur was captured using micro-computed tomography at a resolution of 18μm. The trabecular area percentage, bone and tissue volume, trabecular thickness, trabecular number, and trabecular separation were analyzed by analytical software. The analytic position of distal diaphysis on the right femur was the region from the growth plate to 2,700μm below the growth plate (towards the proximal end), excluding the cortical bone. The femoral bone mineral density was analyzed using the same region but included the cortical bone.

Biology Analysis

The three-point bending was determined by a bone stress determinant (RT1-TST, Royalty Tec. Ins. LTD, Taiwan). The fixed points at both ends were 5 mm, respectively, and the speed was 0.5 mm/min. The force and distance were recorded, and the cross- sectional area was worked out by image analysis software after tomography. The femur was analyzed by the test system, and the obtained parameters of the bone mechanics analysis included the maximum load (i.e., the force converted from the recorded maxi mum weight), with Newton (N) as the unit. The absorbed energy was the integral of the area under the curve, and the unit was mJ (i.e., N multiplied by distance), as shown in Figure 1A. The stiffness was represented by the slope, and the N of the maximum load was divided by the distance (N/mm). Young's modulus, or the elastic modulus (E), was calculated using the following equation(Turner and Burr 1993):

pressure $\sigma = FLc/4I$

torsion $E = F/d \times L^3/48I$

E is elastic modulus

F is the applied load (N)

c is the distance from the center of mass (equal to ½b)

d is the displacement (mm)

L is the span between the two support points of the bending fixture (mm) (as shown in Figure 1B)

I is the cross-sectional moment of inertia

$I = \pi[ab^3-(a-2t)(b-2t)^3]/64$

a: mediolateral direction (as shown in Figure 1C, D)

b: anteroposterior direction (as shown in Figure1 C, D)

t: the average of the cortical thickness

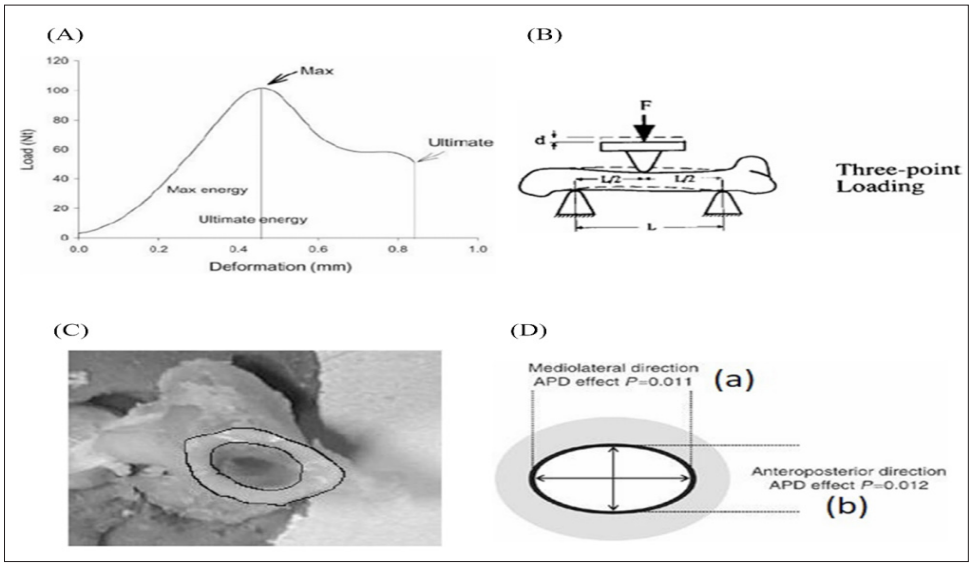

Figure 1. Femoral three-point bending measurement using bone stress tester.

Statistical Analysis

The data derived from this test were analyzed by one-way analysis of variance and Duncan's multiple range test was performed. The statistical result was represented using English letters, with the same letter indicating no statistical difference between groups.

RESULTS

Influence of NTX and OCN Concentrations in Serum

Figure 2 shows the changes in serum NTX and OCN concentrations in each group of animals after 12 weeks of the experiment. The LCa+H_2O group NTX and OCN concentrations in serum were enhanced than the control group. On the other hand, in the LCa+FC group, different doses were reduced in serum NTX concentrations. In particular, the LCa+FC medium and higher doses were reduced in serum OCN concentrations, but the lower dose was no different.

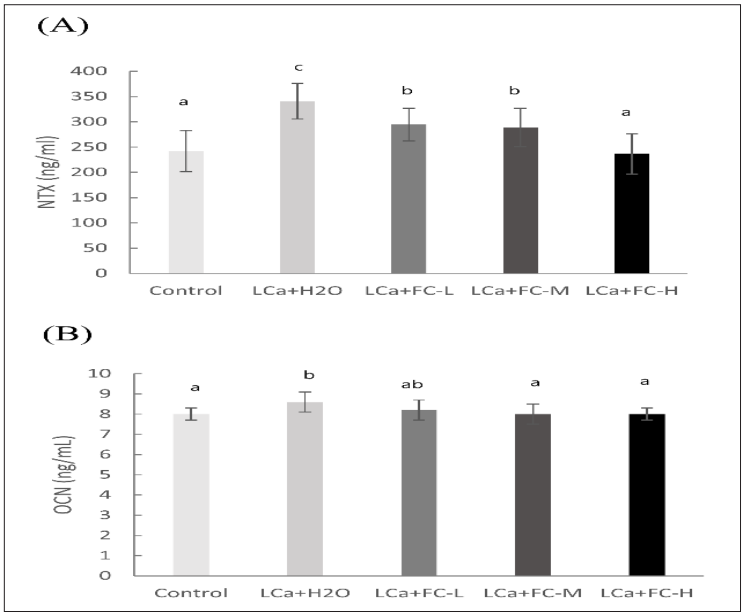

Figure 2. Effect of FC supplement on NTX and OCN concentrations in serum (A) NTX (B) OCN.

The reported values are the mean ± SD (n=10). Different superscript letters (a, b and c) indicate significant differences between groups ($p < 0.05$).

Influence on Right Femur Length and Bone Mineral Density

The bone mineral density of the right femur was analyzed by micro-computed tomography. As shown in Table 1, the bone mineral density of the LCa+H_2O group was significantly lower than the control group. The LCa+FC group's different doses significantly enhanced the bone mineral density.

Table 1. Effect of FC supplement on bone mineral density of the Ovariectomized Rats

Treatments	Dose (g/kg)	Bone mineral density (g/cm^3)
Control	-	0.636 ±0.017^c
LCa+H_2O	-	0.600 ±0.012^a
LCa+FC	0.83	0.619 ±0.010^b
	1.65	0.624 ±0.020^{bc}
	3.30	0.624 ±0.022^{bc}

The reported values are the mean ± SD (n=10). Different superscript letters (a, b, and c) indicate significant differences between groups ($p < 0.05$).

The right femur morphology was analyzed by micro-computed tomography. Influence on trabecular area percentage, thickness, number, and separation of the right femur. As shown in Figure 3, the trabecular area percentage, thickness, and number of the LCa+H_2O group were significantly lower than the control group. Compared to the LCa+FC group, different doses were elevated than the LCa+H_2O group. The trabecular separation analysis showed that the trabecular separation of the LCa+H_2O group was promoted by the control group. Therefore, the LCa+FC group's different doses significantly declined the trabecular separation than the LCa+H_2O group.

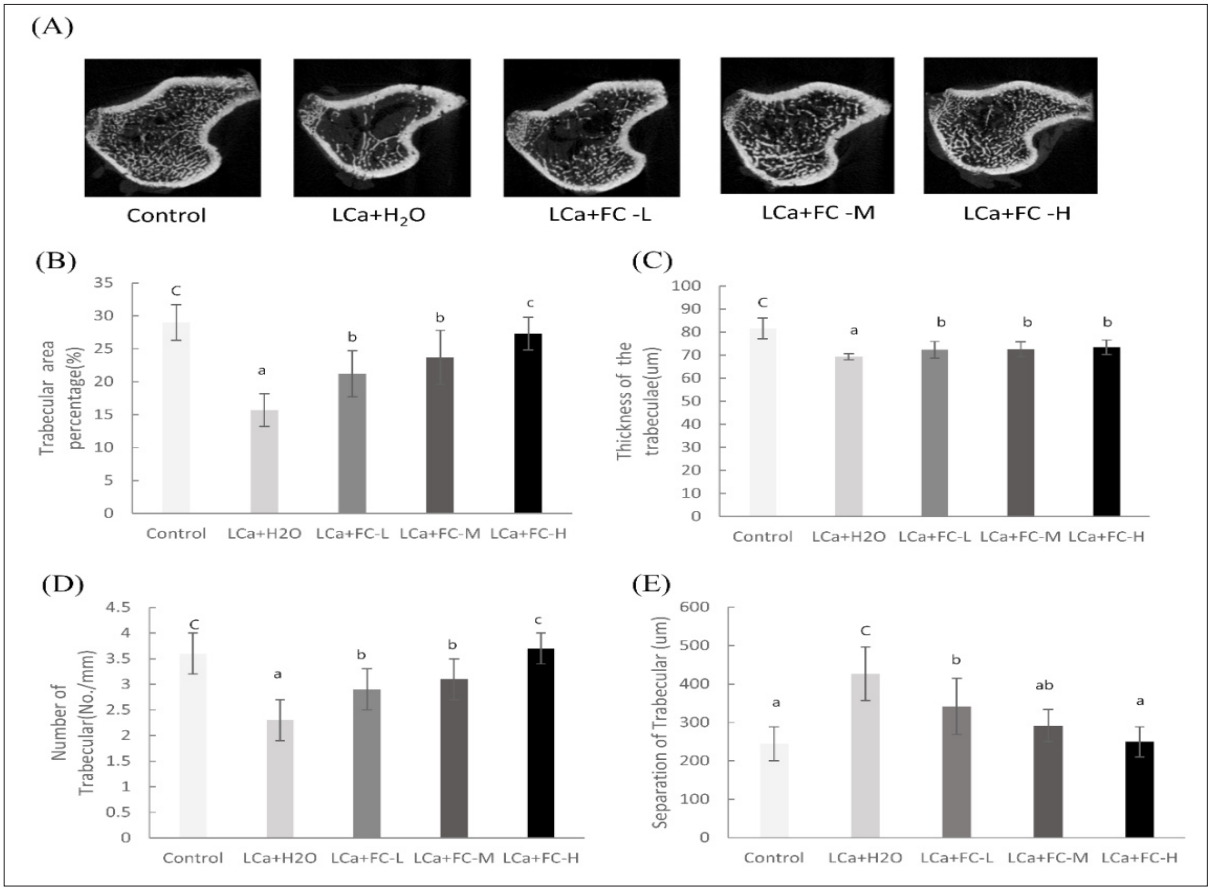

Figure 3. Effect of FC supplement on Right Femur Length and Bone Mineral Density (A) Micro-computed tomography analysis of the femora (B) Trabecular area percentage (C) Thickness of the trabeculae (D) Number of Trabecular (E) Separation of Trabecular.

The reported values are the mean ± SD (n=10). Different superscript letters (a, b and c) indicate significant differences between groups ($p < 0.05$).

Influence on Right Femur Length Cortical Bone

The right femur cortical bone morphology was analyzed by micro-computed tomography. As shown in Figure 4. The LCa+H_2O group that cortical bone aera and thickness were significantly lower than the control group. However, the LCa+FC group's different doses significantly promoted the trabecular separation than the LCa+H_2O group.

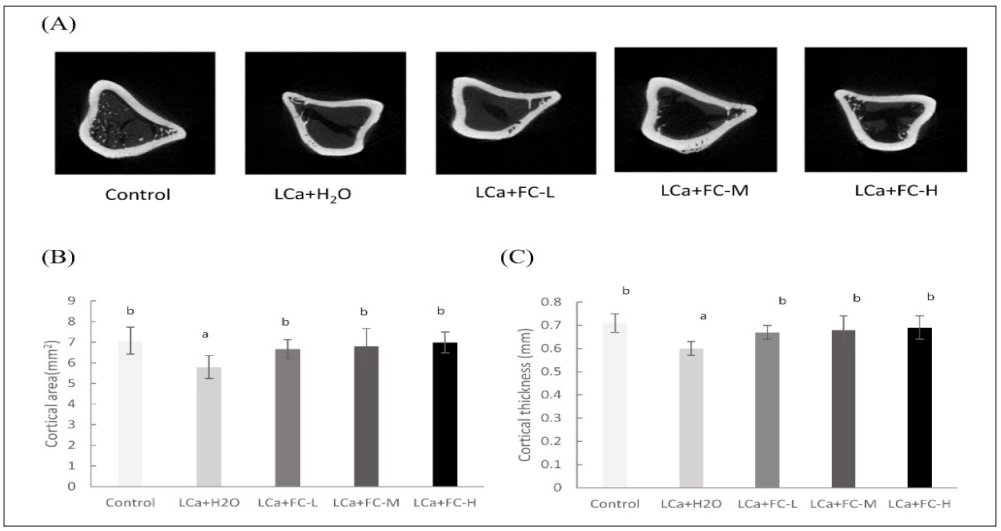

Figure 4. Effect of FC Supplement on Right Femur Length cortical bone (A) Micro-computed tomography analysis of the femora (B) Cortical aera (C) Cortical thickness.

The reported values are the mean ± SD (n=10). Different superscript letters (a and b) indicate significant differences between groups ($p < 0.05$).

Influence on Maximum Load, Stiffness, and Young's Modulus of the Right Femur Length

The maximum load, Stiffness, and Young's modulus of the right femur length are shown in Figure 5. The LCa+H_2O group that the maximum load, Stiffness, and Young's modulus of the right femur length were significantly lower than the control group. Therefore, the LCa+FC group had different doses of the maximum load. The stiffness and Young's modulus of the right femur length were significantly higher than the LCa+H_2O group.

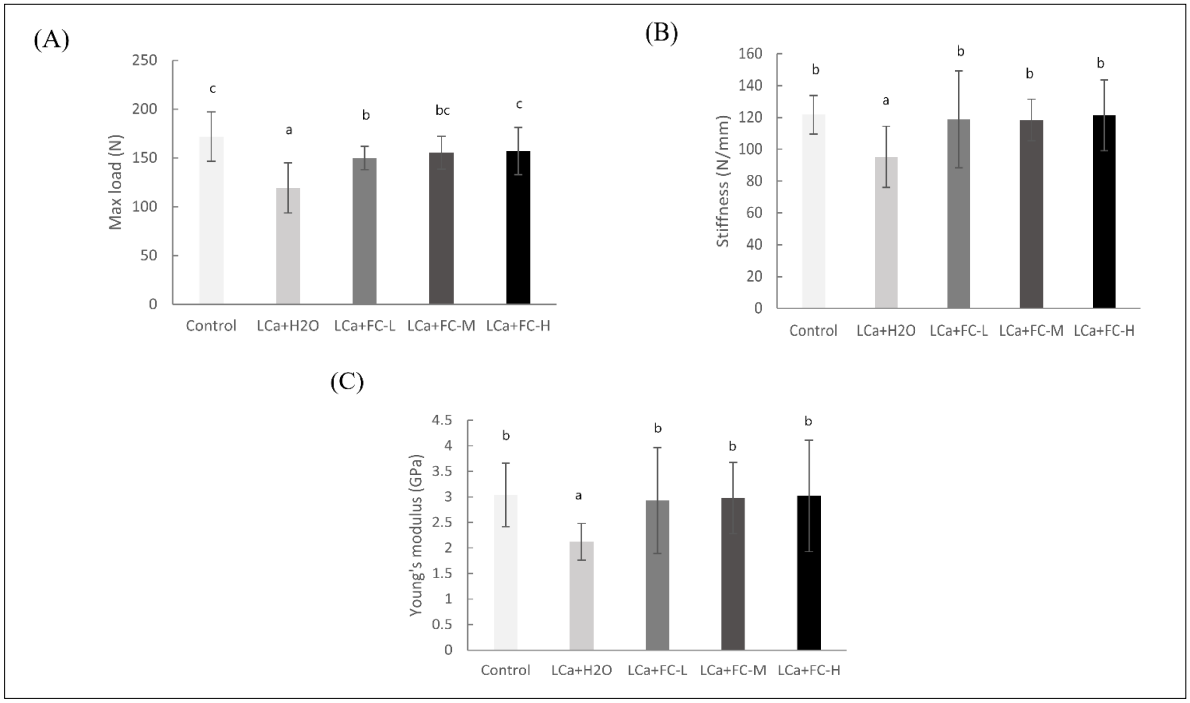

Figure 5. Effect of FC Supplement on maximum load, stiffness and Young's modulus of the Right Femur Length (A) Maximum load (B) Stiffness (C) Young's modulus.

The reported values are the mean ± SD (n=10). Different superscript letters (a, b and c) indicate significant differences between groups ($p < 0.05$).

DISCUSSION

Osteoporosis is a major problem for many older people and is caused by rising and lying down habit. Moreover, osteoporosis influences personal health and mobility and, in turn, greatly increases national healthcare expenses. In this study, an FC supplement can protect bone health and bone growth. Ultimate by micro-computed tomography, finds bone remodeling and bone strength.

Fish collagen, belonging to collagen type I, could provide bone resorption; only about 40% of the cross-links are released as free pyridinium cross-links. The remaining 60% are in the form of peptide-attached cross-links. To measure the N-terminal telopeptide (NTX) and the C-terminal telopeptide (CTX), Cross-laps in serum have been described. Recent studies of these assays have shown that they are better than other markers to assess bone resorption. The NTX assay, which is based on a cross-linked peptide from the N-terminal telopeptide of collagen I, appears to measure all collagen type I degradation products (Hwang, Kim et al. 2018;Seibel 2005). In this study NTX assay, the LCa+FC group's different doses were significantly reduced in serum NTX concentrations. This demonstrates that fish collagen can improve bone resorption and bone health.

Calcium accounts for 1±2% of adult human body weight. Over 99% of the total body calcium is found in the bones. Therefore, in addition to the obvious structural role of the skeleton, it also serves as a reservoir for calcium. Dietary calcium intake has an important impact on bone metabolism and bone health (Cashman 2002, Yao, Hu et al. 2021, Bakirhan and Karabudak 2023, Skalny, Aschner et al. 2024). Calcium is required for normal growth and development of the skeleton. During skeletal growth and maturation, i.e., until the age of the early twenties in humans, calcium accumulates in the skeleton at an average rate of 150mg per day (Cashman 2002, Bakirhan and Karabudak 2023). Human clinical trial calcium supplement intake can change bone mineral density in older adults (Bristow, Bolland et al. 2022).

Accordingly, it has been demonstrated that supplementation with astaxanthin in a model of periodontitis reduced osteoclast activity and increased osteoblast number in the mandible. In particular, a previous study observed reduced levels of bone resorption biomarkers, such as serum calcium, that bone mineral density and microarchitecture were recovered after 6 weeks of astaxanthin supplementation (Hwang, Kim et al. 2018). Previously published works have suggested the positive effects of astaxanthin in OA experimental models. OA occurs due to chondrocyte dysfunction following an inflammatory state, with an overproduction of MMPs inducing the degeneration of articular cartilage. In this study

by micro-computed tomography LCa+FC group's different doses can improve bone area, thickness, and density. This corroborates that LCa+FC supplements can enhance bone remodeling and bone strength to prevent osteoporosis.

CONCLUSION

The animal experiment results of this study show that for the osteoporosis of the ovariectomized rats, that NTX assay LCa+FC different doses group significantly reduce in serum NTX concentrations. That demonstrates that FC supplements can improve bone resorption and bone health. By micro-computed tomography, the LCa+FC group's different doses can improve bone area, thickness, and density. However, FC supplements can enhance bone remodeling, strength, and growth to prevent osteoporosis and improve bone health.

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