PE9.19 Effect of intake of proteins in porcine cardiac muscle on in vivo bone formation of ovariectomized rat model 120.00

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Abstract—this research was carried out to examine anti-osteoporosis effect of porcine cardiac muscle proteins (PCMP) using ovariectomized rat model. The intake of proteins (PCMP) extracted from porcine cardiac muscle improved in vivo bone formation of ovariectomized rat model. The weight of femurs and tibiae in rats group fed PCMP was larger than those in control rats group fed casein instead of PCMP. The bone density of femurs in PCMP-fed rats was higher than that in control ones. These improvements were thought to be caused by the enhancement of absorption of calcium ions in small intestine of rats. The solubility of calcium ions in the phosphate buffer was examined to clarify the mechanism for enhancement of calcium ions absorption (Ca absorption). The addition of PCMP to 20mM phosphate buffer (pH 7) containing calcium ions increased the solubility of calcium ion. The hydrolysate of PCMP by pepsine, trypsin and chymotrypsin also enhanced the calcium ion solubility in phosphate buffer, suggesting that this enhancement of calcium solubility lead to the improvement of in vivo bone formation of ovariectomized rat model by the enhancement of calcium absorption.

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I. INTRODUCTION

CALCIUM is an essential nutrient for human health as bone component and regulation of physiological functions. The recommended intake of Ca from dietary is about 700 mg/day for adult. However, the intake of Ca from dietary is not enough compared to recommended level. Since the calcium intake from dietary and bone resorption can maintain serum Ca level in the normal range, lack of Ca intake from dietary leads to a metabolic bone disease such as osteoporosis. Especially, persons advanced in years tend to have this disease because of lack of Ca by metabolic problem. The prevention of them from osteoporosis is very important problem to be resolved. Some functional materials are developed from food staffs. Casein phosphopeptides derived from milk1-3), the complex of maleic acid and citric acid, poly-glutamic acids derived from Natto and phosvitin peptides derived from hen egg yolk4, 5) have been shown to possess the activity of enhancement of Ca absorption by increase of soluble Ca ions in phosphate. In Japan, it is well known that a mountain of porcine cardiac muscle rises with increasing consumption of pork. The utilization of porcine cardiac muscle is also important problem. Cardiac muscle contains calcium binding proteins such as calsequestrin, troponin C and so on. In this study, we investigated the effect of intake of proteins (PCMP) in porcine cardiac muscle on in vivo bone formation of ovariectomized rat model.

II. MATERIALS AND METHODS

A. Preparation of porcine cardiac muscle proteins (PCMP)

Porcine cardiac muscle was washed with distilled water and minced with meat chopper. Two hundreds and fifty grams of minced cardiac muscle were homogenized with 1 L of 35% saturated ammonium sulfate in 0.1 M potassium phosphate buffer (pH7.2) containing 0.5mM PMSF, 1mM monoiodo acetate for 1min. This homogenate was centrifuged at 10,000 x g for 20 min, and then the supernatant was filtered with gauze. After the filtrate was dialyzed with distilled water in order to remove ammonium sulfate, it was freeze-dried. This powder was used in following experiments as porcine cardiac muscle protein (PCMP).

B. Amino acid composition analysis

PCMP and casein were hydrolyzed in 6M HCl at 110 degrees Celsius for 24h. The analysis of amino acids in

hydrolysate was performed with an amino acid analyzer (JLC-500/V2, JEOL Co., Tokyo).

C. Animals and diets

Six-week-old ovariectomized female SD rats were housed in mesh-bottom cages in a controlled environment (at 24 degrees Celsius) and acclimatized for1 week. Then, rats were randomly divided into two groups of 6 rats each. Rat in control group (control group) was fed 20% casein, and another one was fed 7.5% PCMP and 12.5% casein (PCMP group) as protein in diet (Table 1). Fifteen grams of Diet was given to each rat every day for 4weeks, while distilled water was offered ad libitum for 30 days.

D. Bone density and bone calcium content

Femurs and tibiae of rats in control and PCMP groups were removed after slaughter, and cleaned by removing muscle and connective tissues completely. The right femur and tibia were smashed up by grinder and burnt to ash at 580 degrees Celsius for 2h. Ash powder was resolved with 0.1 % nitric acid solution and its calcium content was measured with ICP. Bone density of the left femurs was determined by pQCT (peripheral Quantitative Computed Tomography) method.

E. Measurement of ratio of Ca absorbed from diet

Feces were collected for 3days at each period (before feeding, at 13-15 day and 28-30 day after feeding). Collected feces for 3 days were burnt to ash at 580 degrees Celsius for 2h. Ash powder was resolved with 0.1 % nitric acid solution and its calcium content was measured with ICP. The ratio of Ca absorbed from diet was calculated as follow; The ratio of absorbed Ca (%) = (Ca content in diet – Ca content in feces) / (Ca content in diet) x 100

F. Hydrolysis of PCMP by digestive proteases

PCMP was incubated with 1/200 part of pepsin in distilled water adjusted to pH 2.0 at 37 degrees Celsius for 12h. After pH adjustment of the reaction mixture to 8.0, the solution was incubated with 1/200 part of trypsin and chymotrypsin for 12h. After incubation, 3 times volumes of ethanol were added to reaction mixture to halt the enzyme reaction. This solution was centrifuged and the supernatant was obtained. Furthermore, salt in the supernatant was removed by microacylizer (Asahi Kasei Co., Tokyo). The desalted solution was used as PCMP hydrolysate.

G. SDS-PAGE

SDS-PAGE was carried out on 7.5% polyacrylamide slab gels with a 3% stacking gel, according to the method of Laemmli6). The gels were stained with Coomassie brilliant blue R-250 for proteins.

H. Measurement of activity in Ca solubility

Calcium-solubilizing activity was determined according to the method described in Choi's report5). The activity was evaluated by addition to20mM of phosphate buffer (pH 7.0) containing Ca ions. After addition of PCMP or PCMP hydrolysate to phosphate buffer containing Ca, this mixture was incubated at 37 degrees Celsius for 1h and centrifuged at 10,000 x g for 15min. The contents of Ca soluble in supernatant were determined by the titration of 0.01 M EDTA and indicator of o-cresolphthalein complexone.

I. Statistical analysis The statistical analysis was carried out using Microsoft excel 2003 software. All analyses were determined by t-test at $p<0.05$. The results shown were expressed in means ± SD.

III. RESULTS AND DISCUSSION

1. Effect of PCMP intake on bone information (1) Amino acid composition of PCMP Amino acid analysis showed that a major amino acid of PCMP and casein was glutamic acid. Glutamic acid ratio in composition of PCMP was larger than that of casein. (2) Changes in body weight of ovariectomized rat model Body weights of rats in control and PCMP groups gradually increase for 30 days (Fig. 1).

There is no significant difference in body weight between both groups. (3) Effect on bone length of femur and tibia The bone length of femur and tibia of rats in both groups was measured after slaughter. The average bone length of femurs and tibiae of rats was 3.5 and 3.9 cm. respectively. There is no difference in bone length of femurs and tibiae between both groups. (4) Effect on bone weight and Ca content of femur and tibia The bone weight of femur and tibia in rats of PCMP group was significantly larger than that in rats of control group. The bone weights of femurs in rats of PCMP and control groups were 503 and 483 mg,

respectively, while those of tibiae were 394 and 375 mg, respectively (Fig. 2).

The Ca content of femurs and tibiae in rats of PCMP group was higher than that in rats of control group. (5) Effect on bone density of femur The bone density of femur of ovariectomized rat model was measured after slaughter by pQCT method. The bone density of femur in rats of PCMP group was higher than that in rats of control group. Especially, the bone density of spongy parts in femurs of the former rats was significantly higher than that of the latter rats (Fig. 3). (6) Effect on Ca absorption in small intestine Apparent ratio of Ca absorption from diet in small intestine was estimated by measuring Ca content in feces and diets. The ratio of Ca absorption for 3days at 13-15 day after feeding was lower than that before feeding in ovariectomized rat model of both groups (Fig. 4). There is no difference in Ca absorption ratio at this period between both groups. At 28-30 day after feeding, the ratio of Ca absorption in rats of control group was further lower than that at 13-15 day. This seems to be caused by ovariectomy in female rats. On the other hand, the ratio of Ca absorption in rats of PCMP group was higher than those at 13-15 day and before feeding. This increase in Ca absorption in rats of PCMP group was thought to lead to the improvement in bone information of ovariectomized rat model. Therefore, PCMP was suggested to possess anti-osteoporpsis effect.

2. Anti-osteoporosis effect of PCMP The Ca-solubilizing activity of PCMP was examined to clarify the mechanism for enhancement of Ca absorption. (1) Ca-solubilizing activity of PCMP Ca-solubilizing activity of porcine cardiac muscle proteins (PCMP) was measured in phosphate buffer (pH 7.0 or 8.0) containing Ca ions at different concentrations (0-500 ppm). The addition of PCMP to the phosphate buffer (pH 7.0) containing Ca ions increased Ca content soluble in the phosphate buffer (Fig. 5). This solubility by addition of PCMP was higher with increasing amount of added PCMP.

The Ca solubility in the mixture containing 30 mg/ml PCMP was three times higher than that without PCMP. PCMP also possessed Ca-solubilizing activity at pH 8. (2) Ca-solubilizing activity of PCMP hydrolysate When PCMP are, in general, taken by oral administration, they are hydrolyzed by digestive proteases such as pepsin, trypsin and chymotrypsin. So, the Ca-solubilizing activity of PCMP hydrolysate by proteases was also examined. Hydrolysis of PCMP by proteases was checked by SDS-PAGE. PCMP were completely hydrolyzed to generate oligopeptides with low molecular weights. The addition of PCMP hydrolysate (30 mg/ml or 60 mg/ml) as well as PCMP showed Ca-solubilizing activity in the phosphate buffer (pH 7.0) (data not shown). The Ca solubility in the mixture containing 60 mg/ml PCMP hydrolysate was three times higher than that without PCMP hydrolysate. These results indicated that peptides in PCMP hydrolysate resulted in increase of Ca solubility (Ca-solubilizing activity). These peptides seem to enhance Ca absorption in small intestine and improve the in vivobone formation in ovariectomized rat model.

IV. CONCLUSION

The intake of porcine cardiac muscle proteins (PCMP) showed the improvement of in vivo bone formation such as bone weight, Ca content and bone density of femurs and tibiae in ovariectomized rat model. This improvement was caused by enhancement of Ca absorption in small intestine of rats. Furthermore, increase of Ca solubility (Ca-solubilizing activity) by PCMP and PCMP hydrolysate seems to contribute to enhancement of Ca absorption in small intestine. Next problem is to identify the peptide possessing Ca-solubilizing activity. V.

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