Journal of Hard Tissue Biology 17[1] (2008) p17-22

CODEN-JHTBFF, ISSN 1341-7649

Original

Effect of Oral Administration of High Advanced-Collagen Tripeptide (HACP) on Bone Healing Process in Rat

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(Accepted for publication, February 8, 2008)

Abstract: The influence of oral administration of High Advanced-Collagen Tripeptide (HACP) developed by Jellice Co., Ltd (Sendai, Japan) for bone repairing process was investigated in rat. Cortical bone defects (1mm diameter) of the tibia were created through the medial cortex and medulla. After 1 day of operation, HACP/physiological saline solution (80mg/2ml/Kg) was orally administrated as experimental group. Only physiological saline solution was orally administrated as control group. There were no significant differences in body weight, serum levels of total proteins, calcium concentration and alkaline phosphatase activity between HACP and control groups during administration periods. After 3 weeks of administration, the tibia bone was excised. Micro high-resolution microfocus x-ray computed tomography showed the formation of primary woven bone in HACP group. Histological sections stained with hematoxylin and eosin were observed under light microscope. In control group, blood clot inside the bone defect with a thin connective tissue surrounding the defect was observed. On the contrary, bone defect area was filled with granulation tissue, blood clot and a great number of osteoblasts in the HACP group.

The present results suggested that oral administration of HACP may provide a beneficial effect on bone healing process.

Key words: Collagen, Oral administration, Bone repairing process

Introduction

Collagen is the major protein of connective tissue in living animals with a 20-30% of structural protein throughout the whole body. Collagen especially existed in the skin, bone, cartilage and tendon supporting their structure and function. It is well known that the decrease of collagen synthesis, which is related with aging, cause the functional decline of the internal organs[1)].

Recently, collagen and collagen peptide have been marketed as a functional food for the maintenance of normal bone integrity, as well as for the treatment of joint problems[2)]. It was reported that orally administrated collagen hydrolysate was adsorbed from the intestine in the high molecule weight form of the peptide[3)]. Orally administrated collagen or collagen peptide provide beneficial effects on bone metabolism in vivo. Moreover, oral intake of collagen hydrolysate with calcitonin has a greater effect in inhibiting bone resorption than calcitonin alone for osteoporosis[4)]. Collagen has also been used as preservatives for lyophilization of biological substances such thrombin[5)]. It has been reported the usefulness of oral administration of collagen for immunotherapeutic agents such as human rheumatoid arthritis treatment [6,7)]. However, marketed collagen has a wide variation of composition or molecular weight due to the difference of collagen source or preparation methods, and caused a variation of beneficial effect after administration[8)].

Collagen has an amino acid unit of Gly-X-Y-, and glycine in amino acid unit is regularly repeated. X and Y are often occupied by proline hydroxyproline and alanine. Collagen tripeptide, High Advanced-Collagen Tripeptide (HACP) developed by Jellice Co., Ltd (Sendai, Japan), has 20 % of basic unit of Gly-X-Y in HACP. HACP was prepared from hog skin by collagenase digestion

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technique[9,10].

It was reported that oral administrated HACP distributed around the connective tissue such as skin, bone or tendon, and that the fracture healing of femur of rat was progressed by oral administration of HACP[11,12]. However, to our knowledge, there are few studies related the effectiveness of HACP oral administration and there is no evidence to confirm this issue in detail.

In the present study, we aimed to investigate the effectiveness of oral administration of HACP on bone repairing process in rat.

Materials and Methods

Animals & surgical procedure

The animal study was conducted in accordance with the animal experimental ethical guidelines of Nihon University School of Dentistry at Matsudo (Certificate Number: CA-06-29). Eight 11 week-old female Wister/ST rats (Sankyo Labo Service, Tokyo, Japan) weighing about 221.5 g were used. They were randomly divided into 2 groups, HACP-supplemented group (HACP group) and non-HACP supplemented group (control group). All rats were housed in metal cages in a temperature controlled (23± 1°C) and maintained under a 12-hour light-dark cycle. The rats were fed with a commercially diet (MF, Pellet f=12mm, Oriental Yeast Co., Ltd. Tokyo, Japan) and had free access to tap water throughout the experiment.

Surgery was performed under general anesthesia with a intramuscular injection of ketamin hydrochloride (Ketalar® Intramuscular, 80mg/kg, Daiichi Sankyo Propharma Co., Ltd. Tokyo, Japan), and local anesthesia was performed by injection of lidocaine (1% Xylocain®). To reduce the perioperative infection risk, a prophylactic antibiotic (Shiomalin® equivalent to latamoxef sodium), was administrated postoperatively by subcutaneous injection.

Surgery was performed under sterile condition. The hind legs of the rat were shaved, washed and disinfected with iodine tincture. A longitudinal incision was made on the medial surface of the tibia, and the bone was exposed by blunt dissection. Cortical bone defects (1mm diameter) were created by a straight fissure bur (Beldenta MG Burs, MGST36, ISO008, J. Morita Tokyo Mfg. Corp, Tokyo, Japan) through the medial cortex and medulla. These defects were prepared with a very gentle surgical technique using a low rotational drilling speed (500rpm) and continuous internal cooling. Afterwards, soft tissues were closed in a separate layer using restorable Vicryl® 3-0 sutures. Postoperatively, the animals were placed in a standard cage. They were fed with water and rat diet *ad libitum*, and were allowed to move unrestricted at all times.

After 1 day of operation, HACP/physiological saline solution (80mg/2ml/Kg) was orally administrated using a stomach radio sonde in the HACP group. Only physiological saline solution was orally administrated in the control group.

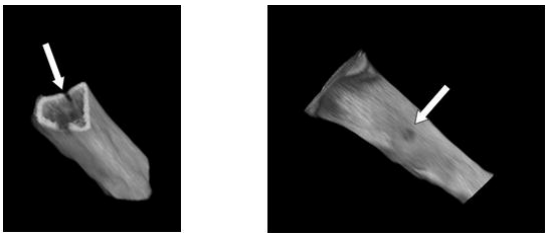


Figure 1. Preparation of bone defect in rat tibia (arrow)

Body weight and serum assay

Body weight measurement and serum assay were preformed every one week after the administration of HACP. Blood was collected from the pulmonary vein of rats. Serum levels of total protein (TP), calcium concentration (Ca) and alkaline phosphatase activity (ALP) were measured using Fuji Dri-Chem (Fujifilm Medical Co. Ltd.,Tokyo, Japan).

Micro CT and histological observation

After 3 weeks of administration of HACP, rats were sacrificed with CO_2 anesthesia. The tibia bone was excised immediately after sacrifice, and was fixed in 10% buffered formalin solution.

Before preparing histological sections, healing condition of holes in tibia was observed using micro high-resolution microfocus x-ray computed tomography (micro-CT, TOSCANER-30000*i*hd, Toshiba IT & Control Systems Corporation, Tokyo, Japan) with a voltage of 74 kV, a tube current of 110*i*A, a slice thickness of 0.160mm, and a slice pitch of 0.8mm.

Fixed bone specimens were decalcified with 5% formic acid for 5 days, and then embedded in paraffin blocks in a routine manner. Afterwards, histological sections of 4*i*m thick were prepared and stained with hematoxylin and eosin. The stained sections were observed using a light microscope (Eclipse E800M, Nikon, magnification x 100).

Statistical analysis

All measurements were statistically evaluated using one-way ANOVA and Fisher's test for multiple comparison among the means at p=0.05.

Results

During the test periods, the experimental animals remained in good health. After sacrifice, no clinical signs of inflammation or adverse tissue reactions were seen. The bone defects before HACP administration are shown in Fig.1. The defects were almost in the center of the cortical side of tibia.

The change of body weight during 3 weeks is shown in Fig.2. No significant differences were detected between HACP and control group ($p>0.05$). The changes of TP, Ca concentration and ALP activity are shown in Figs.3-5. The mean value of Ca

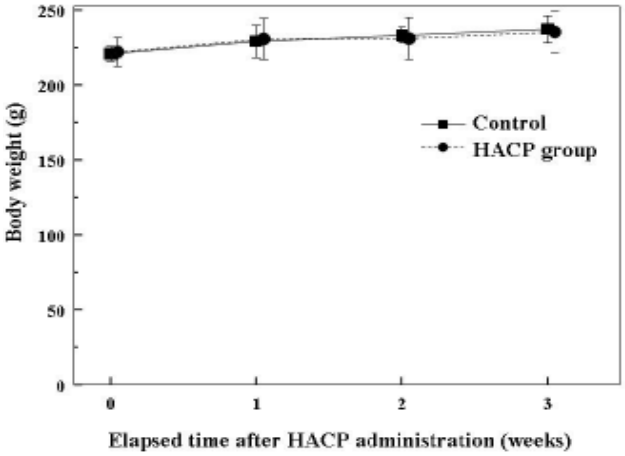


Figure 2. Changes of body weight during test periods

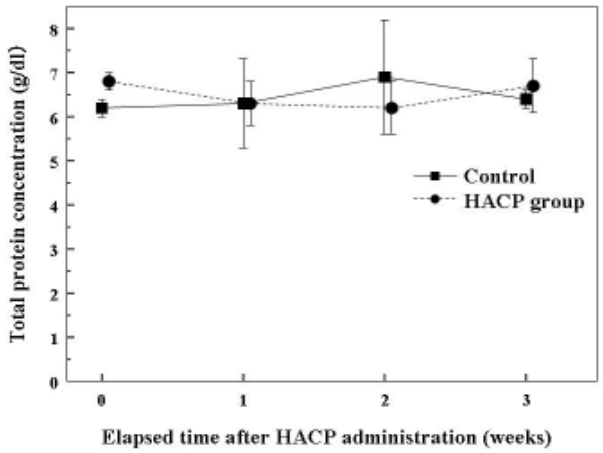


Figure 3. Changes of total protein concentration during test periods

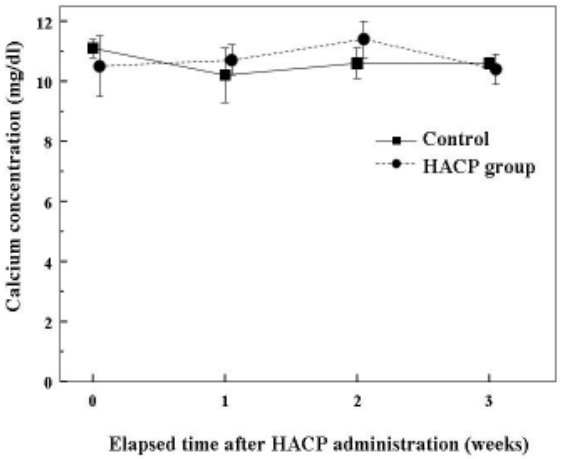


Figure 4. Changes of calcium concentration during test periods

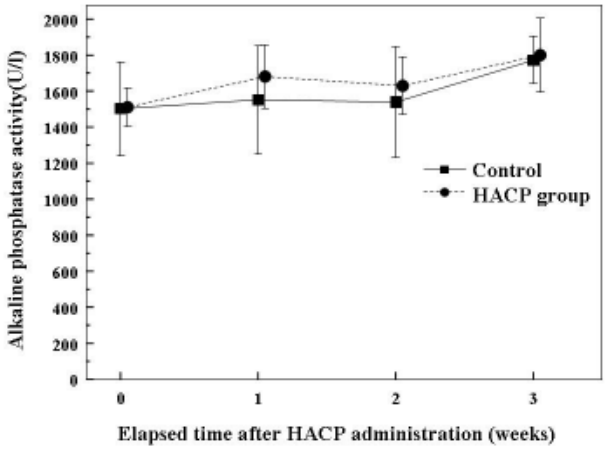
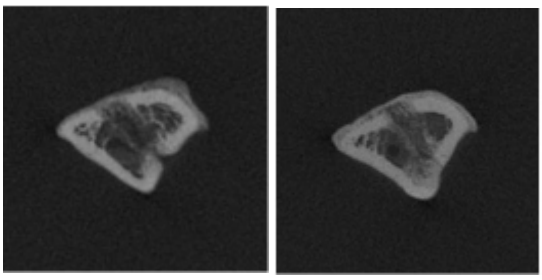


Figure 5. Changes of alkaline phosphatase activity during test periods



Control HACP Group

Figure 6. Micro-CT appearances of rat tibiae after 3weeks

concentration and APL activity tended to be slightly higher in the HACP group than in the control group, although there were no significant differences among TP, Ca concentration and APL activity in the HACP group compared with the control group ($p>0.05$). According to the administration periods, also did not show any significant differences among TP, Ca concentration and APL activity ($p>0.05$).

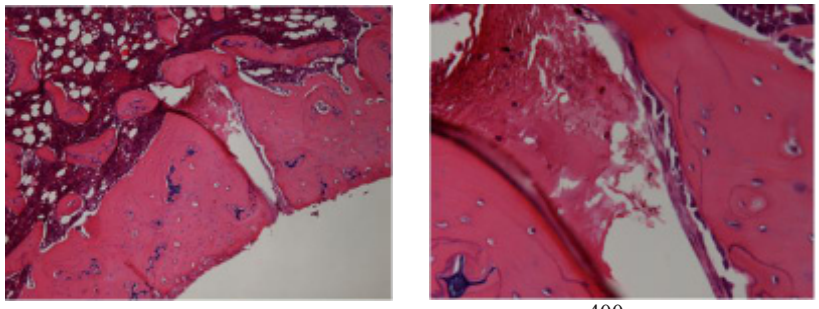
Fig. 6 shows the micro-CT appearances of rat tibiae after 3 weeks of HACP administration. In the control group, the presence of original bone defect was clearly identified. On the contrary, formation of primary woven bone was only observed in the HACP group.

Histological appearances of bone defects after 3 weeks of administration are shown in Fig. 7 (control) and Fig. 8 (HACP group). In the control group, blood clot was observed inside the defects with thin connective tissue surrounding the bone defects. Few osteoblasts was also identified. On the contrary, the bone defect areas were filled with granulation tissue with blood clot and a great number of osteoblast were observed in the HACP group.

Discussion

In Japan, collagen peptide is one of the most common ingredients of functional foods. The aim of the present study was to evaluate the effect of oral administration of HACP: High Advanced-Collagen Tripeptide for bone repairing process in rat.

Previous studies reported the effect of oral administration of collagen peptide, dolomite and fluoride[13-15]. Wu et al[13] demonstrated that oral administration of collagen peptide recovered the bone mineral density of femur and lumbar spine in calcium-deficient rats without adverse effects, and that collagen



x100 x 400

Figure 7. Histological appearances of repaired bone in control group

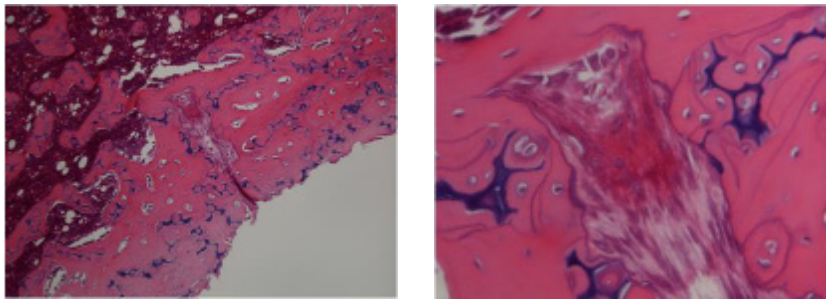


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Figure 8. Histological appearances of repaired bone in HACP group

peptide intake did not affect bone metabolism in growing rats. They also reported that 100-fold administration of collagen increased the femoral bone mineral density in growing rats.

In the present study was used HACP, which is a collagen peptide and soluble powder. It is supposed that tripeptide unit of HACP, Gly-X-Y, is easily absorbed from the intestine. The present study revealed that HACP administration enhances bone healing process in tibiae defects of rats, although calcium concentration or APL activity in serum was not influenced by HACP administration.

The mechanism of the effect of collagen intake on bone healing process was not clear. However, several predictable mechanisms were postulated. One possibility is that the intake of HACP supplies bone matrix protein for calcification. Oesser et al[3] reported that gelatin hydrolysate could be absorbed in the intestine and adsorbed gelatin hydrolysate was accumulated in cartilage tissue. It is speculated that adsorbed HACP supply the bone matrix surface providing a better effect in mineralization. It is also likely that the absorbed HACP stimulate osteoblast differentiation. Mizuno et al[16,17] reported that type I collagen interacted with integrin receptor on the cell membrane and caused the differentiation of bone-marrow cells into osteoblasts.

Tsuji[8] reported that collagen hydrolysate, which was digested by collagenase and contained tripeptide, Gly-X-Y, showed greater collagen synthesis ability in dermis than non-hydrolysate collagen or pepsin digested collagen by oral administration in rat. Moreover, this collagen hydrolysate enhanced the expression of m-RNA in the fibroblast culture. Sakai et al [9] also found that HACP enhanced the collagen synthesis of dermal fibroblasts. Yamato and Sakai[11] investigated the distribution of HACP into the living tissue after the oral administration to rat using tritium labeling to Gly-Pro-Hyp. HACP peptide was more rapidly adsorbed and moved into blood system than proline, which was control amino acid, and was selectively adsorbed into skin, bone and connective tissue after 24 hours of oral administration by autoradiogram measurement. It is also reported that collagen type I expression was enhanced in human osteoblastic cell cultures by the addition of HACP through upregulation of the transcription factor, Osterix[12]. It is speculated in the present study that HACP, which was absorbed through peptide transporter on intestine, provided inductive effect on osteoblats and was used as a component for collagen synthesis in vivo. Moreover, some biological activity could be improved the adsorbed HACP hydrolysate. Assay for other factors, such as osteocalcin concentration, urinary excretion of deoxypyridinoline should be needed to elucidate the in vivo mechanism of HACP administration. The next concern is HACP administration will have a benefit for bone disease such as osteopososis. The effect of oral administration of HACP to ovariectomized rats should be further investigated.

In conclusion, oral administration of HACP may provide beneficial effects on bone healing process.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C)(19592260) from the Japan Society for the Promotion of Science. We thank to JELLICE Co., Ltd., for the supply of HACP material.

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