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Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage *in vitro* and *in vivo*

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Summary

Objective: To investigate the direct effect of prolyl-hydroxyproline (Pro-Hyp) on chondrocytes under *in vivo* and *in vitro* conditions in an attempt to identify Pro-Hyp as the bioactive peptide in collagen hydrolysate (CH).

Methods: The *in vivo* effects of CH and Pro-Hyp intake on articular cartilage were studied by microscopic examination of sections of dissected articular cartilage from treated C57BL/6J mice. In this study, mice that were fed diets containing excess phosphorus were used as an *in vivo* model. This mouse line showed loss of chondrocytes and reduced thickness of articular cartilage, with abnormality of the subchondral bone. The *in vitro* effects of CH, Pro-Hyp, amino acids and other peptides on proliferation, differentiation, glycosaminoglycan content and mineralization of chondrocytes were determined by MTT activity and staining with alkaline phosphatase, alcian blue and alizarin red. Expression of chondrogenesis-specific genes in ATDC5 cells was determined by semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR).

Results: *In vivo*, CH and Pro-Hyp inhibited the loss of chondrocytes and thinning of the articular cartilage layer caused by phosphorus-induced degradation. In the *in vitro* study, CH and Pro-Hyp did not affect chondrocyte proliferation but inhibited their differentiation into mineralized chondrocytes. A combination of amino acids such as proline, hydroxyproline and prolyl-hydroxyprolyl–glycine did not affect chondrocyte proliferation or differentiation. Moreover, CH and Pro-Hyp caused two and threefold increases, respectively, in the staining area of glycosaminoglycan in the extracellular matrix of ATDC5 cells. RT-PCR indicated that Pro-Hyp increased the aggrecan mRNA level approximately twofold and decreased the *Runx1* and osteocalcin mRNA levels by two-thirds and one-tenth, respectively.

Conclusion: Pro-Hyp is the first bioactive edible peptide derived from CH to be shown to affect chondrocyte differentiation under pathological conditions.

Key words: Collagen hydrolysate, Prolyl-hydroxyproline, Chondrocyte, Articular cartilage degradation, Extracellular matrix.

Introduction

Collagen hydrolysate (CH), a heterogeneous mixture of oligopeptides and polypeptides similar to gelatin, has been used therapeutically as a dietary supplement to improve conditions of joints. Some animal experiments have suggested that oral ingestion of CH might have beneficial effects on joint conditions such as osteoarthritis (OA)[1,2]. CH is absorbed in its high-molecular-weight form, containing peptides of 2.5–15 kDa[3]. A recent study reported detection of peptides such as prolyl proline (Pro-Pro), alanyl hydroxypropyl glycine (Ala-Hyp-Gly), prolyl-hydroxyproline (Pro-Hyp), prolyl hydroxypropyl glycine (Pro-Hyp-Gly), isoleucyl hydroxyproline (Ile-Hyp), leucyl hydroxyproline (Leu-Hyp) and phenylalanyl hydroxyproline (Phe-Hyp) in human venous blood after ingestion of CH. Pro-Hyp was the most prevalent among those peptides[4,5].

Many reports indicate that various peptides obtained from CH show biological activity. For example, the Asp–Gly–Glu–Ala tetrapeptide regulated the expression of osteoblast-related genes in the bone marrow[6]. Furthermore, the Pro-Hyp dipeptide was suggested to be involved in platelet aggregation[7]. Correspondingly, a hydrogel containing peptides from collagen has been used as a scaffold for a true cartilage-like extracellular matrix in regenerative medicine for effective and lasting repair of articular cartilage[8]. These findings suggest that some peptides that are metabolites act as bioactive peptides and functional molecules in some tissues, and that CH contains bioactive peptides that affect cartilage homeostasis.

This study aimed to examine the protective effect of CH and its specific dipeptide, Pro-Hyp, in relation to primary degenerative articular cartilage of mice.

Materials and methods

CH, AMINO ACIDS, AND PEPTIDES

Enzymatic CH of porcine skin gelatin (01-JP) was a kind gift from Nitta Gelatin (Osaka, Japan). This hydrolysate was of food grade and is commercially available. CH was a mixture of peptides of various molecular weights (average molecular weight of 5000 Da), including dipeptides and tripeptides. Gly, Pro and Hyp were purchased from Sigma Aldrich (Tokyo, Japan). The peptides Pro-Hyp and Gly–Pro-Hyp were purchased from PH Japan (Hiroshima, Japan) and used at 95% purity.

ANIMALS

Ten-week-old male C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). All the diets used in *in vivo* experiments were modifications of the AIN-93G composition, and we referred to the methods of Morishita *et al.*[9]. The diet compositions are shown in Table I. Potassium dihydrogen

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Table I
Compositions of experimental diets

g/kg Diet	High Pi diet			
	N	C	CH	Pro-Hyp
Casein*	200	150	150	150
Lard*	58.3	58.3	58.3	58.3
Corn oil*	11.7	11.7	11.7	11.7
Mineral mixture*	35	35	35	35
Vitamin mixture*	10	10	10	10
Sucrose†	100	100	100	100
Cornstarch*	472.95	472.95	472.95	472.95
Cellulose*	50	50	50	50
KH_2PO_4†	–	59.05	59.05	59.05
L-cystine*	3	3	3	3
Gluten†	–	50	–	47
CH‡	–	–	50	–
Pro-Hyp§	–	–	–	3

*Oriental yeast (Chiba, Japan), AIN-93G was used as a vitamin and mineral mixture composition.
†WAKO (Tokyo, Japan).
‡Nitta gelatin (Osaka, Japan).
§PH JAPAN (Hiroshima, Japan).

phosphate anhydrous (KH_2PO_4) was used as the Pi source and added instead of cornstarch. Gluten, CH or Pro-Hyp was added instead of casein. Gluten hydrolysate from corn (Sigma Aldrich) was used as the negative control. CH contained approximately 6% Pro-Hyp. The mice were randomly assigned to four groups of six mice each: a normal (N) group fed a standard diet containing 0.2 g Pi/100 g; a control (gluten) group fed a diet containing 1.2 g Pi and 5 g gluten/100 g; a CH group fed a diet containing 1.2 g Pi and 5 g porcine skin gelatin/100 g; and a Pro-Hyp group fed a diet containing 1.2 g Pi and 0.3 g Pro-Hyp/100 g. The mice were allowed free access to food and water during the 3-week feeding period. Blood samples were drawn by cardiac puncture of anesthetized mice. The blood samples were stood for 1 h at room temperature and centrifuged at 2000 g for 15 min. The resulting supernatants were employed as serum samples, which were stored at −20°C until use. Serum inorganic P and Ca were measured with Phospher C test WAKO and Calcium E test WAKO, respectively (Wako Pure Chemical, Osaka, Japan). The animals were housed under conditions of constant temperature (20–22°C) and humidity (45–50%), and a standard 12-h light/dark cycle. The study was performed in accordance with the National Institutes of Health (NIH) institutional guidelines for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee of The University of Josai, Saitama, Japan.

HISTOLOGICAL COMPARISON

Dissected articular cartilage was immediately immersion-fixed overnight in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, at 4°C. Articular cartilage sections were cut at a thickness of 10 μm at −22°C with a cryomicrotome (Leica, CM3050S) and stained with Mayer's hematoxylin (H). Each section was compared at the same thickness by measuring instrument of cryomicrotome. Articular cartilages of the same thickness were compared. Each section was observed at ×400 or ×200 magnification, and the shape of chondrocytes, distribution of chondrocytes, thickness of articular cartilage and morphology of subchondral bone were compared. Apoptosis was assessed using an *In Situ* Cell Death Detection Kit (Roche Applied Science) and by observation using the 4′,6-diamino-2-phenylindole (DAPI) fluorescence method.

MICRO-COMPUTED TOMOGRAPHY (CT)

All measurements were performed with a micro-CT desktop system, which is based on a combination of X-ray projection microscopy and a tomographic reconstruction technique (Skyscan 1172, Belgium). In this system, an air-cooled point X-ray source (focal spot size ∼8 μm in diameter, maximum voltage 80 kV) was used to illuminate the object with a divergent beam. All samples and phantoms were scanned under identical conditions. Scanning parameters were as follows: anode voltage, 80 kV; rotation step, 0.5°; and exposure time, 7 s per view. After scanning, virtual cross-sections through the bone were reconstructed with a 32-bit dynamic range and converted into 8-bit map images according to the selected density window using CT-analyzer software.

CELLS AND CULTURE CONDITIONS

ATDC5 cells, a murine chondrocytic cell line, were prepared as described previously[10]. Briefly, the basic medium consisted of dulbecco's modified eagle medium (DMEM)/F12 medium (Invitrogen) containing 1 mM phosphorus, 0.25 mM glycine and 0.15 mM proline. Hydroxyproline was not included in the basic medium. Peptone, a pancreatic digest of casein purchased from Becton−Dickinson, was used as a negative control of CH in the *in vitro* study. In the experiments, the ATDC5 cells were cultured in four different media: the basic medium, and the basic medium supplemented with 1 mg/ml peptone (Pe), 1 mg/ml CH or 2.5 mM Pro-Hyp. Methyl-thiazol-tetrazolium (MTT) assay was performed after 1 day of culture, alkaline phosphatase (ALP) staining was performed after 5 days of culture, and alcian blue (AB) staining and alizarin red (AR) staining were performed after 35 days of culture. In addition, amino acids such as proline, hydroxyproline, and/or glycine, dipeptides or tripeptides were added to the basic culture medium to a final concentration of 2.5 mM before the MTT assay on day 1 of culture and ALP staining on day 5 of culture.

RNA EXTRACTION AND RT-PCR

ATDC5 cells were cultured in 6-cm dishes by seeding at approximately 5×10^5 cells per dish. After culturing for 1 day, the medium was replaced by one of four media: basic medium, 1.25 mM proline and 1.25 mM hydroxyproline (Pro and Hyp), 2.5 mM Pro-Hyp or 2.5 mM Pro-Hyp−Gly. The cells in each of these media were cultured for 3, 24 or 72 h, and then the total RNA was extracted with TRIzol for use in RT-PCR. The method for RNA extraction and the conditions for RT-PCR for type II collagen (Col2), osteocalcin (OCL), *Smad2*, *Smad4* and *Runx2* were as described previously[10,11]. The primer sequences are shown in Table II.

STATISTICAL ANALYSIS

The results are expressed as the mean ± standard deviation (SD). *In vivo* experiments were carried out $n = 6$ animals each groups. Welch's *t* test was used to identify the 'number of cells' and 'thickness of cartilage' differences between control group and Pro-Hyp group. *In vitro* experiments were carried out using five wells for each medium. Each experiment was repeated three times, and representative results are shown. Multiple comparison of mean values was performed by Dunnett's *t* test. The *P* value refers to comparison of a measured parameter in a test medium with that in the basic medium. Statistical significance was set at alpha = 0.05 for one-side test. All statistical computations were performed using SAS version 9.1.3 (SAS Institution, Cary, NC).

Table II
Primers and PCR conditions

Primer	Sequence	Annealing (°C)	Cycles
Runx1 (upstream)	5′-acttcctctgctccgtgcta-3′	58	28
Runx1 (downstream)	5′-ggtagcgagattcaacgacc-3′		
Aggrecan (upstream)	5′-cacgctacaccctggactttg-3′	58	28
Aggrecan (downstream)	5′-ccatctcctcagcgaagcagt-3′		
PEPT1 (upstream)	5′-gcgaggtggtcttctctgtc-3′	58	30
PEPT1 (downstream)	5′-cagaagcaatgaggcaaaca-3′		
PEPT2 (upstream)	5′-tgtcctggttacagcagcag-3′	58	30
PEPT2 (downstream)	5′-caaaacgaattcagcccact-3′		

Results

ADMINISTRATION OF CH AND PRO-HYP AMELIORATES HISTOLOGICAL CHANGES IN PHOSPHORUS-INDUCED CARTILAGE DEGRADATION IN MICE

We examined whether CH and Pro-Hyp affected the articular cartilage of mice *in vivo*. In this study, mice that were fed diets containing excess phosphorus (C) were used as controls (Fig. 1). The final weights and bone lengths of mice were not significantly different among the four groups (data not shown), and neither were the serum inorganic P and Ca levels (Table III). Representative histological sections are shown in Fig. 1(A). Normal integrity of the articular cartilage was observed in the normal (N) group. Microscopic observation revealed that excess phosphorus caused a decrease in small chondrocytes on the articular cartilage surface. Moreover, excess phosphorus induced loss of cells in the middle layer of the articular cartilage. Figure 1(F) shows the number of cell for each 250 μm^2 on a section of articular cartilage in each sample. Figure 1(B) and (E) shows that the thickness of the articular cartilage layer was decreased slightly by excess phosphorus. The thickness of the articular cartilage was reduced in the C group compared to that in the N group. The relative thickness of the articular cartilage of the CH and Pro-Hyp groups increased to approximately 1.3 times that of the C group [Fig. 1(B)]. At the same time, histological observation and micro-CT analysis showed that the subchondral bone was reduced in thickness and bone mass by excess phosphorus [Fig. 1(B)–(D)]. In particular, the edge of subchondral bone was eroded in response to excess phosphorus, and an abnormal shape was observed for the cartilage after it lost its support base [Fig. 1(B) and (D) arrows]. In contrast, CH and Pro-Hyp mitigated the phosphorus-induced effects. Taken together, CH and Pro-Hyp ameliorated the reduction of chondrocytes and subchondral bone [Fig. 1(A)–(D)]. Moreover, with CH and Pro-Hyp, numerous round chondrocytes were observed compared with N [Fig. 1(A)]. In this study, apoptosis was not observed in the sections prepared at 3 weeks, either by TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis or the DAPI method in this high-phosphorus model (data not shown).

CH REGULATES ATDC5 CELL DIFFERENTIATION

Next, we analyzed whether CH and Pro-Hyp affect the proliferation, maturation and differentiation of ATDC5 cells. The proliferation of pre-chondrocytes was quantified by the MTT assay [Fig. 2(B)] and Mayer's hematoxylin (H) staining on day 1 of culture [Fig. 2(A) upper photos]. Metabolic activity of the CH- or Pro-Hyp-supplemented cells was approximately equal to the activity of the negative control cells in the peptone (Pe)-supplemented medium. The staining areas of glycosaminoglycan were compared by AB staining at pH 1.0. After 35 days of culture, ATDC5 cells showed an approximately 1.6 times ($P = 0.03$) greater staining area of glycosaminoglycan in CH and approximately 2.3 times ($P = 0.002$) greater staining area of glycosaminoglycan in Pro-Hyp compared with in Pe medium [Fig. 2(A) middle upper photos and (C)]. Cells that had differentiated into hypertrophic chondrocytes were quantified by measuring the ALP activity by ALP staining on day 5 of culture [Fig. 2(A) middle lower photos, and (D)]. Moreover, mineralization was quantified by measuring calcium deposits by AR staining on day 35 of culture [Fig. 2(A) lower photos and (E)]. The ALP activity and positive areas seen in AR staining of Pe-supplemented cells as a negative control were approximately equal to those in the normal (N) group. The ALP activity and positive areas seen in AR staining of CH- or Pro-Hyp-supplemented cells were less than half of those in Pe-supplemented cells ($P = 0.001$ and $P < 0.0001$). These results indicate that CH contains a bioactive peptide that suppresses the differentiation of ATDC5 cells into hypertrophic chondrocytes and maintain mature chondrocytes that deposit aggrecan in the extracellular matrix.

IDENTIFICATION OF THE BIOACTIVE PEPTIDE THAT AFFECTS ATDC5 CELL DIFFERENTIATION

ATDC5 cells were cultured in the basic medium and also in media prepared by supplementing the basic medium with 2.5 mM of either proline (Pro), hydroxyproline (Hyp), glycine (Gly), proline and hydroxyproline (Pro and Hyp), proline, hydroxyproline and glycine (Pro and Hyp and Gly), Pro-Hyp or prolyl-hydroxyprolyl–glycine (Pro-Hyp–Gly). The cells were stained with Mayer's hematoxylin and examined by MTT assay on day 1 of culture [Fig. 3(A) upper photo, (B)]. As a result of microscopic observation, the morphology of the cells was not changed by Pro, Hyp, Gly, Pro-Hyp or Pro-Hyp–Gly, and none of these affected proliferation of the cells as shown by MTT assay. Next, the cells were stained for ALP on day 5 of culture [Fig. 3(A) lower photo]. None of the amino acid or peptide supplementations with Gly, Pro, Hyp, Pro and Hyp, Pro and Hyp and Gly, or Pro-Hyp–Gly affected the ALP-stained area of the cultured cells compared to the N group. The ALP activity was decreased only by Pro-Hyp, and the ALP-stained area of ATDC5 cells cultured in the Pro-Hyp-containing medium was one-fifth that of the cells cultured in the basic medium [$P < 0.0001$; Fig. 3(C)].

Next, we studied whether the concentration of Pro-Hyp in the medium correlates inversely with the ALP activity of ATDC5 cells. The ATDC5 cells were cultured in media containing 0, 0.1, 0.5, 1, 2.5, 5 or 10 mM Pro-Hyp for 5 days and then subjected to ALP staining [Fig. 4(A) lower photo]. The ALP activity of ATDC5 cells cultured in 1, 2.5 and 10 mM Pro-Hyp was approximately two-thirds, one-fifth, and one-tenth of that of cells cultured in the basic medium (0 mM Pro-Hyp), respectively [Fig. 4(C)]. That is, increasing the concentration of Pro-Hyp in the culture medium reduced the ALP activity of ATDC5 cells in a concentration-dependent manner. These concentrations, however, did not affect ATDC5 cell proliferation as determined by the MTT assay and Mayer's hematoxylin staining [Fig. 4(A) upper photo, (B)].

THE mRNA LEVELS OF CHONDROCYTE-ASSOCIATED GENES AND PEPTIDE TRANSPORTER GENE IN ATDC5 CELLS

We studied whether Pro-Hyp affects chondrogenesis-specific gene expression. The total RNA was extracted 24 h after addition of Pro and Hyp, Pro-Hyp or Pro-Hyp–Gly to the basic medium, and the mRNA levels were measured by RT-PCR [Fig. 5(A)]. Compared to the N group, the cells cultured in the medium containing Pro-Hyp exhibited approximately two-thirds ($P = 0.001$) and one-tenth ($P < 0.0001$) decreases in *Runx1* and OCL mRNA levels, respectively [Fig. 5(B)]. On the other hand, cells cultured in the medium containing Pro-Hyp exhibited an approximately twofold increase in the *aggrecan* mRNA level [$P = 0.004$; Fig. 5(B)]. The *Runx2*, *Smad2* and *Smad4* mRNA levels were not affected by Pro-Hyp. Next, we confirmed whether mRNA of peptide transporter was expressed to ATDC5 cells. PEPT1 mRNA expression was detected by RT-PCR, but it was not affected by Pro + Hyp, Pro-Hyp or Pro-Hyp–Gly. Conversely, PEPT2 mRNA could not be detected in ATDC5 cells by RT-PCR (data not shown).

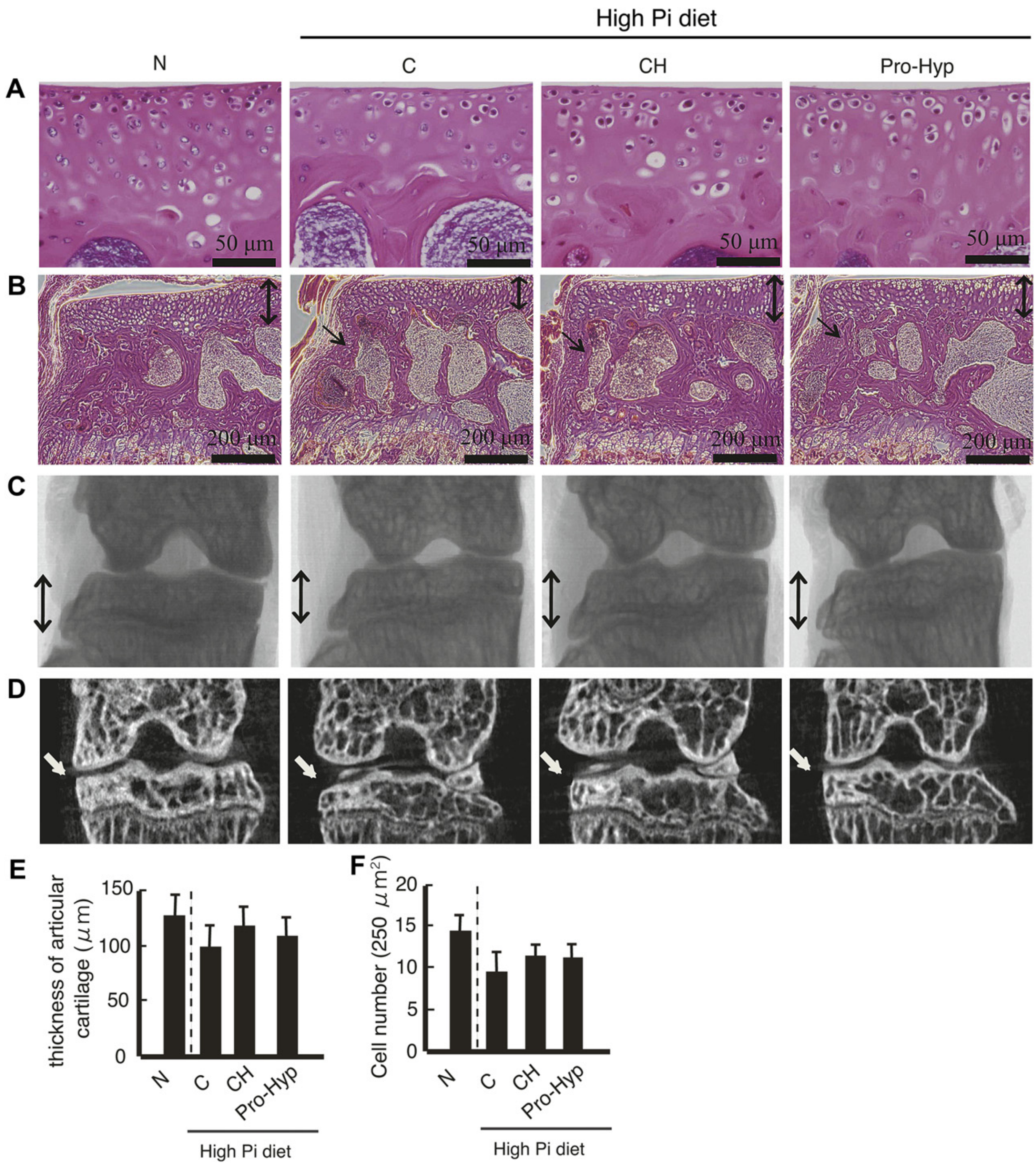

Fig. 1. CH and Pro-Hyp ameliorated the degradation of knee joints in male C57BL/6J mice with phosphorus-induced cartilage degradation. (A) Photograph of high magnification (×400) of histological sections of articular cartilage from the tibial side of the knee. (B) Whole image of histological sections of articular cartilage from the tibial side of the knee (×200). (C) Subchondral bone images prepared by micro-CT analysis. (D) Virtual cross-sections through the bone were reconstructed according to the selected density window using the CT-analyzer software. (E) The thickness of articular cartilage is indicated in the bar graph. The thickness of the articular cartilage was measured at five fixed positions in each mouse. (F) The number of cohndrocyte on sections of articular cartilage is indicated in the bar graph. The number of cohndrocyte was measured at four fixed positions in each mouse. N, C, CH and Pro-Hyp indicate mice that were fed a standard diet, 1.5% phosphorus and 5% gluten as a control diet, 1.5% phosphorus and 5% CH derived from porcine skin, and 1.5% phosphorus and 0.3% Pro-Hyp, respectively.

Discussion

Nutritional factors are important to the maintenance of bone and joint health, and a nutritional imbalance combined with endocrine abnormalities may be involved in the pathogenesis of OA[1,12–14]. High dietary phosphorus intake reduces bone strength and caused soft tissue mineralization[15–17]. A defect in *klotho* gene expression in mice results in articular cartilage degeneration and osteoporosis[18]. Restriction of dietary phosphorus consumption by *klotho* mice

Table III
Serum calcium and phosphorus concentrations

	High Pi diet			
	N	C	CH	Pro-Hyp
Calcium (mg/dl)	10.6 ± 1.7	10.2 ± 1.3	9.6 ± 0.6	11.0 ± 2.0
Phosphorus (mg/dl)	8.5 ± 2.4	8.9 ± 2.7	7.5 ± 1.9	7.7 ± 0.8

Values are mean ± SD; $n = 6$/group.

arrested the cartilage degeneration and osteoporosis[9]. Therefore, it has been suggested that excessive intake of phosphorus accelerates articular cartilage degeneration due to an imbalance in bone and cartilage metabolism. In a preliminary study, we performed observations each week for 4 weeks, and a primary disorder of articular cartilage was noted after 3 weeks on the high-phosphorus diet. Our high-phosphorus diet model is characterized by a primary disorder of articular cartilage due to nutritional imbalance.

There is growing evidence that CH might have beneficial effects on joint conditions[1,2]. However, few studies to date have focused primarily on the effects of treatments on joint conditions[1], and there have been no reports on the mechanism by which CH ameliorates articular cartilage degeneration. Recently, it has been shown that not only amino acids but also oligopeptides are absorbed by the small intestine. Moreover, Pro-Hyp, a dipeptide, was identified as the major constituent of food-derived CH to be detected in human serum and plasma[4]. We hypothesized that Pro-Hyp reaches the articular cartilage and acts as a bioactive peptide, exerting a chondroprotective effect. We studied the effects of CH on articular cartilage *in vivo* from the viewpoint that CH contains Pro-Hyp as a bioactive peptide that ameliorates articular cartilage degeneration.

We predicted that C57BL/6J mice administered CH or Pro-Hyp would show differences in advanced articular cartilage degeneration when excess phosphorus is administered and gluten is used as a negative control. The protein digestibility-corrected amino acid score (PDCAAS) has been adopted by Food and Agriculture Organization/ World Health Organization (FAO/WHO) as the preferred method for the measurement of the protein value in human nutrition. Collagen doesn't contain tryptophan, and PDCAAS of collagen is 0. Gluten shows low PDCAAS value. Therefore, gluten was used for control. Intake of excess phosphorus led to reduced chondrocyte numbers on articular cartilage and decreased density of the cartilage layer and subchondral bone. The serum phosphorus and calcium concentrations were not affected by intake of excess phosphorus. CH and Pro-Hyp suppressed phosphorus-induced degradation of articular cartilage. Moreover, CH and Pro-Hyp caused chondrocytes in articular cartilage to become round.

Next, we used ATDC5 cells for a screening assay to determine the factors that preserve mature chondrocytes. Articular cartilage is thought to preserve mature chondrocytes, keeping them from differentiating into mineralized chondrocytes. In this study, we used the property of ATDC5 cells that they differentiate from prechondrogenic cells into mineralized chondrocytes. We proposed that the factor that prevents differentiation of ATDC5 cells in the mature chondrocyte stage *in vitro* is the same factor that maintains permanent cartilage *in vivo*. Addition of phosphorus to the basic culture medium induced higher mRNA levels for various transcription factors in ATDC5 cells[19,20]. In this study, we experimented with 1 mM phosphorus in the basic medium, since at that concentration it does not induce mRNA levels of transcriptional factors that lead to mineralization.

Our *in vitro* study revealed that CH and Pro-Hyp did not affect ATDC5 cell proliferation, but it remarkably inhibited the ALP activity and showed a larger positive area in AR staining compared with peptone. As for CH, there are a lot of ratios of Pro-Hyp and Pro-Hyp−Gly. Peptone was composed of

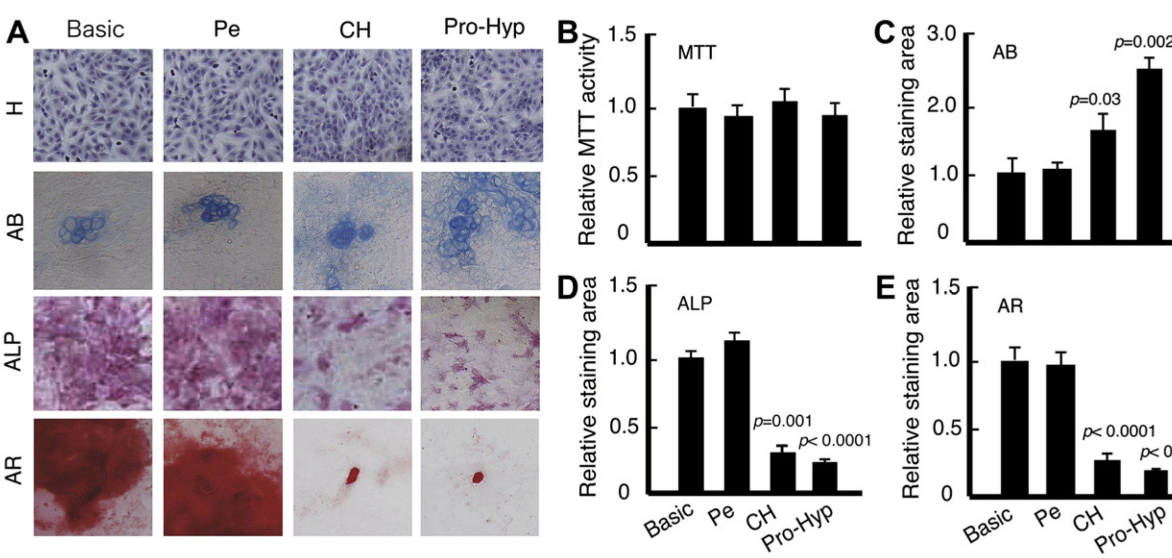

Fig. 2. Effects of CH and Pro-Hyp on the maturation, differentiation and mineralization of chondrocytes (ATDC5 cells). (A) Mayer's hematoxylin (H), AB pH 1.0, ALP and AR stainings were performed to investigate whether CH and Pro-Hyp affect the proliferation, maturation, differentiation and mineralization of ATDC5 cells. (B) The signal intensity of the proliferation of the cells on day 1 was determined by the MTT assay. The signal intensity of the AB-stained cultures on day 35 of culture (C), the ALP-stained cultures on day 5 of culture (D), and the AR-stained cultures on day 35 of culture (E) were determined by densitometry and expressed as bar graphs. (Basic): culture in the basic medium; (Pe): culture in a medium containing 1 mg/ml peptone as the negative control medium; (CH): culture in a medium containing 1 mg/ml CH derived from porcine skin; and (Pro-Hyp): culture in a medium containing 2.5 mM Pro-Hyp. Experiments were carried out in five wells for each medium. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

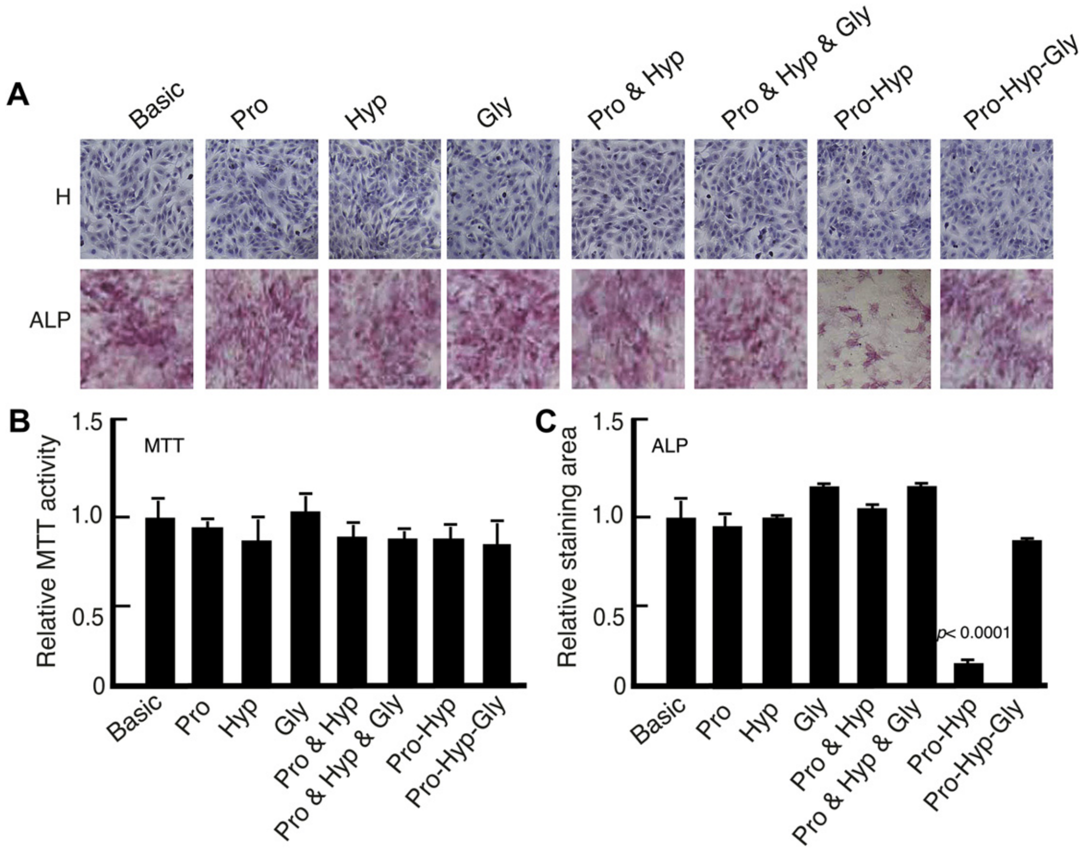

Fig. 3. Effect of Pro-Hyp on ALP activity of ATDC5 cells. (A) The effects of amino acids and peptides on Mayer's hematoxylin-stained area (H) and ALP-stained area. Pro-Hyp and Pro-Hyp−Gly were tested as candidate bioactive peptides. Gly, Pro and Hyp were used as negative controls. Pro and Hyp: addition of both Pro and Hyp to the basic medium; and Pro and Hyp and Gly: addition of Pro, Hyp and Gly to the basic medium. Each peptide and amino acid was added to the basic medium at 2.5 mM. (B) Graph shows the signal intensity of the MTT assay when the cells were cultured with each peptide and amino acid. (C) Graph shows the signal intensity of the ALP-stained area when the cells were cultured with each peptide and amino acid. Experiments were carried out in five wells for each medium. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

various different peptides without specific bioactivity, and thus we used it as a control, avoiding possible effects of specific peptides involved. Moreover, CH and Pro-Hyp induced increased amounts of glycosaminoglycan in the extracellular matrix. Because Pro-Hyp induced increased mRNA levels for aggrecan (Fig. 5), Pro-Hyp might induce chondroitin sulfate. Induction of glycosaminoglycan synthesis in response to Pro-Hyp should be confirmed by biochemical methods such as dithiothreitol (DTT) or 35S incorporation in the future. ALP activity increases at the stage of prehypertrophic chondrocytes. AR staining was used to assess mineralization. Calcification is principally an effect of aging and may contribute to the progression of OA[21−23]. The present data suggest that CH preserves mature chondrocytes and does not allow their differentiation into mineralized chondrocytes.

Many reports have shown that some oligopeptides affect cell function and regulate gene expression *in vivo* and *in vitro*. For example, Pro-Hyp−Gly, Pro-Hyp, etc., stimulate chemotactic activity of fibroblasts, peripheral blood neutrophils and monocytes[24,25]. Asp−Gly−Glu−Ala stimulates osteoblast-related gene expression by bone marrow cells[6]. These reports suggest that some oligopeptides function as bioactive peptides and regulate the function of tissue cells *in vivo*.

The present study found that although such amino acids as glycine, proline, hydroxyproline and Pro-Hyp−Gly did not affect the ALP activity, Pro-Hyp alone caused a reduction in the ALP activity of ATDC5 cells. The basic medium contained about 0.25 mM glycine and proline, but no hydroxyproline. It contained about 1 mM phosphorus, which was not supplemented. In Fig. 3, the final concentrations of Pro, Hyp and Gly were each 2.5 mM. Therefore, amino acids such as glycine, proline and hydroxyproline were not bioactive, but it is surmised that dipeptides such as Pro-Hyp act as bioactive peptides. This suggests that Pro-Hyp might be absorbed in the small intestine after oral administration and that circulating Pro-Hyp might regulate chondrocyte metabolism.

During the process of endochondral ossification, *Runx1* and *Runx2* are coexpressed in limb bud cell condensations that undergo both cartilage and bone differentiation[26−28]. *Runx1* mediates the onset of mesenchymal cell differentiation in chondrogenesis[29]. Another study revealed that the *Runx1* consensus sequence binds to an osteoblast-specific complex and transcriptionally activates the *OCL* gene[27]. In our study, Pro-Hyp regulated the mRNA levels for *Runx1*, *OCL* and *aggrecan* in ATDC5 cells. On the other hand, such free amino acids and tripeptides such as proline, hydroxyproline and Pro-Hyp−Gly did not affect those mRNA levels. The H^+/peptide transporter *PEPT1* and *PEPT2* play a key role in the maintenance of mammalian protein nutrition[30−32]. ATDC5 cells expressed the mRNA of one

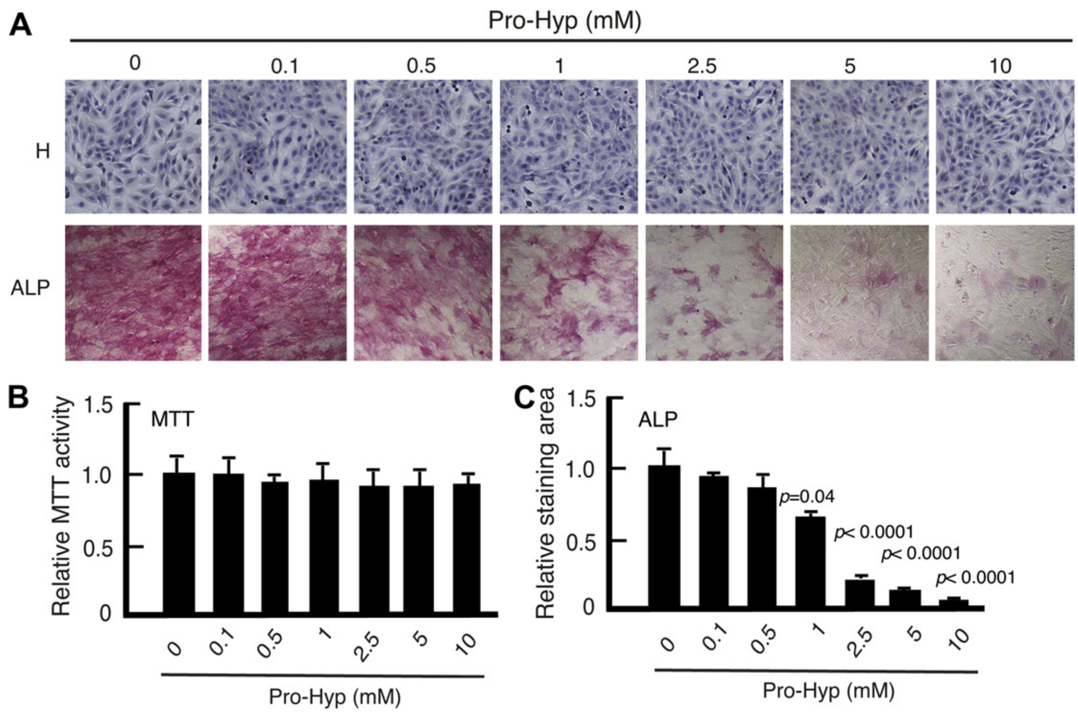

Fig. 4. Concentration-dependent changes in the intensity of the ALP-stained area due to Pro-Hyp. (A) The effects on proliferation and differentiation of ATDC5 cells following the increase in Pro-Hyp concentration were determined by Mayer's hematoxylin staining and ALP staining. (B) Graph shows the relative signal intensity of the MTT assay of Pro-Hyp. (C) Graph shows the relative signal intensity of the ALP-stained area at each concentration of Pro-Hyp. Experiments were carried out in five culture wells. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

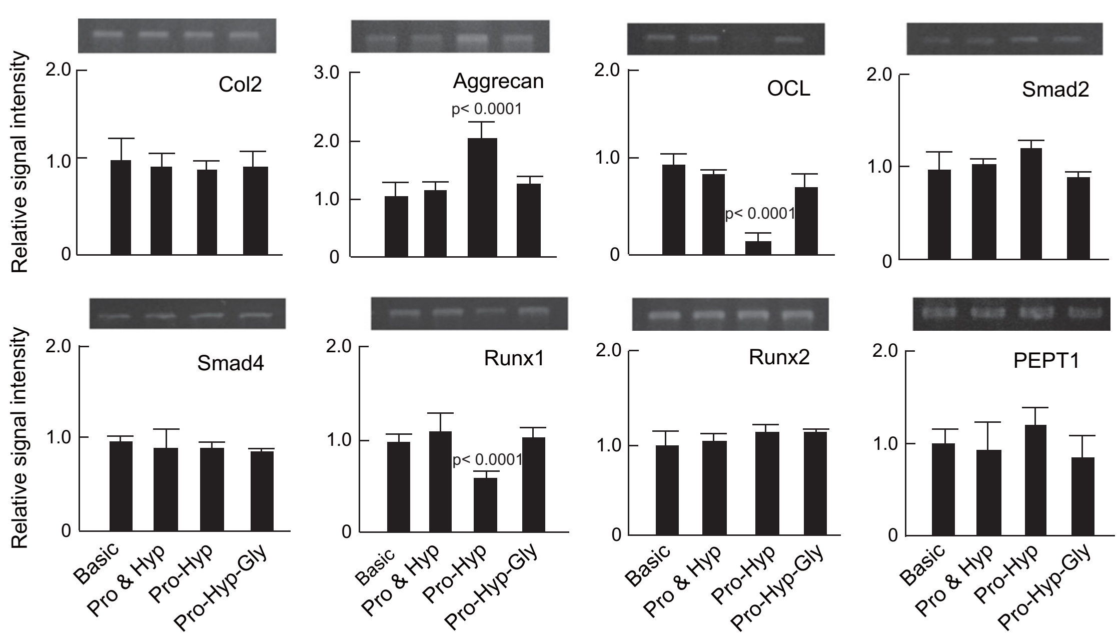

Fig. 5. Effects of Pro-Hyp on mRNA levels for aggrecan, *Runx1*, OCL and PEPT1 in ATDC5 cells. RT-PCR shows that the aggrecan, *Runx1* and OCL mRNA levels were affected by Pro-Hyp in ATDC5 cells. Graphs shows the band intensities determined using ImageJ software, which were obtained from at least three different experiments. The signals were normalized against those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts at 24 h. Experiments were carried out in five culture wells. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

oligopeptide transporter, *PEPT1*, but not *PEPT2*. Therefore, Pro-Hyp is predicted to have a specific binding site for a receptor or intracellular protein, by which it regulates a specific signaling pathway. Consequently, Pro-Hyp might suppress the differentiation to mineralized chondrocytes by repressing the mRNA levels for *Runx1* and *OCL*, and enhancing the mRNA level for *aggrecan*. In the future it will be important to identify the signaling pathway of Pro-Hyp.

Our findings suggest that the Pro-Hyp dipeptide is an important factor that regulates chondrocyte differentiation and plays a role in the maintenance of mature chondrocytes in permanent cartilage. This regulation mechanism by Pro-Hyp suggests that it might be recognized by chondrocytes, leading to reduction in the mRNA level for *OCL via* regulation of the *Runx1* mRNA level and termination of differentiation at the mature chondrocyte stage. Pro-Hyp is present in CH, and this postulated regulation by Pro-Hyp would seem to explain the mechanism of the therapeutic effect of CH in improving joint conditions. It was reported that Pro-Hyp is excreted in human urine[33]. For that reason, Pro-Hyp is formed in the body as a collagen degradation product. This study implies that the bioactive peptide, Pro-Hyp, is derived not only from collagen in living tissues but also from dietary supplements such as gelatin, and that it functions in target tissues.

Further studies are necessary to investigate the effects of CH supplements. Also, understanding the mechanism of action of CH on chondrocyte differentiation would provide a rational basis for the development of chondroprotective therapies for damaged joints.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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Research Article

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A double-blind, placebo-controlled, randomised, clinical study on the effectiveness of collagen peptide on osteoarthritis

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Abstract

BACKGROUND: Recent studies show that enzymatically hydrolysed collagen, the collagen peptide, is absorbed and distributed to joint tissues and has analgesic and anti-inflammatory properties. A double-blind, placebo-controlled, randomised trial with collagen peptides isolated from pork skin (PCP) and bovine bone (BCP) sources was carried out to study the effectiveness of orally supplemented collagen peptide to control the progression of osteoarthritis in patients diagnosed with knee osteoarthritis. Improvement in treatment was assessed with reduction in Western Ontario McMaster Universities (WOMAC), visual analogue scale (VAS) and quality of life (QOL) scores from baseline to 13 weeks (Visit 7). Safety and tolerability were also evaluated.

RESULTS: There was significant reduction from baseline to Visit 7 in the primary end points of WOMAC and VAS scores and in the secondary end point of QOL score in subjects with PCP and BCP groups, while in subjects with placebo group the end point indices remained unaltered. Furthermore, all the score levels of WOMAC, VAS and QOL decreased significantly ($P < 0.01$) in the study group compared to placebo group in Visit 7.

CONCLUSION: The study demonstrated that collagen peptides are potential therapeutic agents as nutritional supplements for the management of osteoarthritis and maintenance of joint health.

Keywords: collagen peptide; osteoarthritis; cartilage; clinical study

INTRODUCTION

Osteoarthritis (OA) is the most common type of arthritis and the major cause of chronic musculo-skeletal pain and mobility disability in the elderly population worldwide. The characteristic features of this chronic, progressive and degenerative disorder of the entire joint include variable inflammation and changes in the structure of bone bordering the joint and in the protective cushion called articular cartilage. Clinical manifestations include joint pain, tenderness, limitation of movement, effusion and varying degrees of inflammation, and finally induce disability in many patients.

The principal components of articular cartilage are the insoluble fibrous protein collagen and the soluble proteoglycans. A complex organisation of collagen, proteoglycans and the fluid environment endows the tissue with the capacity for reversible deformability, a property essential for its physiological function. The integrity of cartilage tissue is dependent on the complex network of type II collagen, proteoglycans and accessory proteins such as fibronectin. These molecules are synthesised and integrated into the residual extracellular matrix (ECM) by chondrocytes. The loss of ECM in cartilage is associated with an increased cleavage of type II collagen by collagenase and an aggrecan cleavage along with the degradation of small proteoglycans.[1] Alterations in the collagen fibril network have been observed including extensive changes in the collagen fibril orientations, especially in the superficial zone and reduction in the collagen content.[2,3] Although the loss of aggrecan in articular cartilage is essential for the progression of OA, the final cartilage damage is inflicted by the loss of the collagen network.[4]

Current pharmacological treatments widely use non-steroidal anti-inflammatory drugs (NSAIDs) as therapeutic agents for OA despite their adverse effects on long-term usage. An alternative treatment with nutritional supplements with higher levels of safety and effectiveness attracts much attention. By nature nutritional supplements are better positioned to provide long-term health benefits.

Collagen-based peptides represent functional peptides that exhibit various physiological activities. Bone mineral density has been shown to be increased by the oral ingestion of gelatin.[5] Folk medicines always mention the positive influence of collagenous preparations as being beneficial to joint health, skin, hair and nails.[6–8]

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Several studies show that enzymatically hydrolysed collagen (known as gelatin hydrolysate or collagen hydrolysate or collagen peptide) is absorbed and distributed to joint tissues and has analgesic and anti-inflammatory properties. The protein has a typical and unique amino acid composition in that it is very rich in glycine, proline and hydroxyproline. Research in mice has demonstrated that after oral administration of radiolabelled gelatin hydrolysate the radioactivity was specifically found in cartilage.[9] Animal experiments have suggested that oral ingestion of collagen peptide might have beneficial effects on joint health such as OA. A recent study in animal models demonstrated that collagen peptide reduced the morphological changes associated with osteoarthritic cartilage destruction in knee joints.[10]

Being a protein with rich source of amino acids specifically found in collagen, it is worthwhile performing a clinical evaluation of the substance to understand the health benefits in the management of OA. Hence the present study was planned with an aim to assess the effectiveness of pork skin collagen peptide (PCP) manufactured from pork skin and bovine bone collagen peptide (BCP) in subjects with clinically diagnosed knee OA.

MATERIALS AND METHODS

Investigational products

PCP was supplied by Nitta Gelatin Inc., Japan, and BCP was sourced from Nitta Gelatin India Ltd, and placebo (maltodextrin) was purchased from Matsutani Chemical Industry Co. Ltd, Itami, Japan. The amino acid profiles in PCP and BCP are illustrated in Table 1.

Table 1. Amino acid composition in pork skin collagen peptide (PCP) and bovine bone collagen peptides (BCP)

Amino acid	Composition ($g\ kg^{-1}$) dry basis PCP	BCP
Valine	0.277	0.277
Tyrosine	0.060	0.023
Threonine	0.219	0.236
Serine	0.413	0.373
Proline	1.620	1.550
Phenylalanine	0.256	0.249
Methionine	0.088	0.063
Lysine	0.414	0.436
Leucine	0.334	0.345
Isoleucine	0.136	0.154
Hydroxylysine	0.104	0.076
4-Hydroxyproline	1.350	1.330
Histidine	0.101	0.070
Glysine	2.640	2.720
Glutamic acid	1.160	1.160
Aspartic acid	0.670	0.670
Arginine	0.910	0.900
Alanine	1.070	1.130

Study design

A double-blind, placebo-controlled, randomised, clinical study in 30 subjects of both sexes between age group 30 and 65 years diagnosed with knee osteoarthritis [visual analogue scale (VAS) score ≥ 40 and Kellgren–Lawrence grade 2 to 4] were enrolled for the study. Subjects were assigned to one of the two treatment groups through computer-generated randomisation code using SAS® software. The study protocol, informed consent document and case report together with all secondary documents used for the study were reviewed and approved by an independent ethics committee. The study was conducted in accordance with the ethical principles as laid out in the current version of the Declaration of Helsinki and ICH-GCP guidelines and was registered (No. CTRI/2009/091/000993) with the Clinical Trial Registry, India. The subjects were advised to consume the investigational product or placebo orally, 5 g dissolved in 250 mL water or milk in the morning and night after food.

Thirty subjects those who fulfilled the study criteria (vital signs, physical examination, pregnancy test, previous medical history, chest X-ray, electrocardiogram, urine examination, serology, haematological and biochemical parameters) and study specific parameters of VAS score, Western Ontario McMaster Universities (WOMAC) score, quality of life (QOL) score and X-ray findings were enrolled and underwent placebo run-in period for 7 days. Subjects took the following baseline therapy during the placebo run-in period:

- Tablet aceclofenac sodium – 100 mg in the morning and at night after food for 7 days
- Tablet pantoprazole – 40 mg oral dosage in the morning before food for 7 days
- Flufenamic acid gel in the morning and at night for 7 days
- Physiotherapy (WAX BATH for the Knee for continuous 3–5 days).
- Placebo 10 g per day (5 mg twice) in the mornings and at night after food for 7 days

After the completion of run-in period the subjects were randomised in a 2:1 ratio to receive either PCP or placebo as illustrated in Fig. 1. As per the randomised ratio, 20 subjects received PCP and 10 subjects received placebo for 13 weeks (91 days). Altogether, 30 subjects took the investigational product daily twice (5 g dissolved in 250 mL of milk or water) in the morning and at night after food for 13 weeks. Static quadriceps exercise was continued throughout the treatment period.

The treatment period was split into seven visits with an interval of 15 days. During the treatment period if extensive knee pain occurred, the subjects were prescribed tablet aceclofenac sodium 100 mg. The efficacy variables were measured using the questionnaire-based assessment of pain, stiffness and physical function were done using the WOMAC score, VAS score and QOL score. The primary efficacy end points are improvement in treatment with a reduction in the WOMAC score by 20 points or more from the baseline to final visit (Visit 7), reduction in VAS score by 40 mm or more from baseline to Visit 7 in 100 mm scale of measurement, and reduction in QOL score by 20 points or more from baseline to Visit 7.

The clinical laboratory evaluation and biochemical evaluation were done by blood and urine analysis to assess the safety of PCP. Vital signs such as physical functions and other observations related to safety were analysed by temperature, pulse rate, respiratory rate, systolic and diastolic blood pressure.

The same study has been applied in second set of 30 subjects who were given BCP (20 subjects) or placebo (10 subjects).

Statistical analysis

The primary efficacy parameter was based on the change in WOMAC and VAS score compared to baseline data over 14 weeks

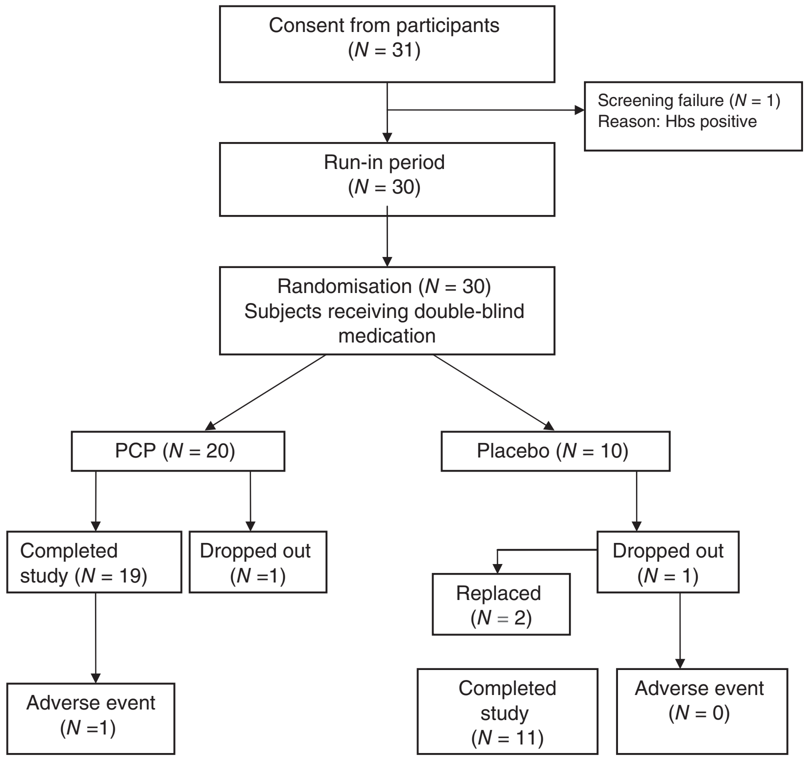

Figure 1. The selection method of subjects for clinical study and study design. The subjects were randomised after a 1 week run-in period with primary therapy into two arms of pork skin collagen peptide (PCP) and placebo in a 2:1 ratio. Nineteen subjects in the PCP group and all 11 subjects in the placebo group completed the study. Hbs: Hepatitis B.

study period. Kolmogorov–Smirnov Z test was performed to assess the normality of WOMAC and VAS score data. The Student paired *t*-test was applied for comparing the baseline and Visit 7 data. For non-normal data, the Mann–Whitney *U*-test was performed for comparison between study groups and the placebo group. The 95% confidence level (CL) around the mean score and the change in WOMAC and VAS score over 14 weeks study period was determined. Assessment of the secondary efficacy parameters was based on the change in QOL score over the 14-week study period compared to baseline data. The Kolmogorov–Smirnov *Z* test was used to assess the normality of QOL score data.

RESULTS

The demographic characteristics of all subjects are recorded in Table 2. All the patients were advised to follow the routine diet whatever they practised at the time of inclusion into the clinical study. None of the patients involved in the clinical study showed any problems with their diet.

The changes of WOMAC score, VAS score and QOL score are given in Table 3. The WOMAC score, VAS score and QOL score have shown a prominent downward trend from baseline to Visit 7 in study group during the treatment period of 13 weeks. As shown in Table 3, there is significant reduction in the score levels of WOMAC ($P < 0.05$ in Visit 4, $P < 0.01$ in Visits 5–7), VAS ($P < 0.01$ in Visits 4–7) and QOL ($P < 0.01$ in Visits 4–7) in subjects with PCP when compared to placebo.

Furthermore, there was a significant reduction ($P < 0.01$) in the score levels of WOMAC, VAS and QOL at Visit 7 compared to the baseline score in subjects who had orally taken PCP, while there was no significant improvement in subjects who had taken placebo. The results further authenticate the role of PCP in improving the status of OA condition.

Table 2. Demographic data of subjects selected for clinical study

	Study I (PCP)		Study II (BCP)	
Variable	Study group (N = 19)	Placebo (N = 11)	Study group (N = 19)	Placebo (N = 11)
Sex				
Male	2	1	8	4
Female	17	10	11	7
Height (cm)				
Mean	158	159	160	159
Std	6.7	7.8	8.7	7.4
Min	145	140	144	146
Max	171	160	175	171
Weight (kg)				
Mean	64.5	58.5	65.6	65.2
Std	7.8	5.4	8.2	9.0
Min	55	49	53	52
Max	82	66	80	78
BMI				
Mean	26.1	23.1	25.9	25.8
Std	3.8	1.9	3.3	3.3
Min	21.1	18.6	21.3	20.3
Max	33.9	26.2	33.3	32.3

Table 3. Changes of primary efficacy parameters for WOMAC and VAS and secondary end point parameter for QOL

	WOMAC score (points)		VAS score (points, in mm scale)		QOL score (points)	
Visit	Placebo (*N* = 11)	PCP (*N* = 19)	Placebo (*N* = 11)	PCP (*N* = 19)	Placebo (*N* = 11)	PCP (*N* = 19)
Baseline	47.3 ± 8.6	47.2 ± 9.8	60.0 ± 6.3	63.2 ± 10.6	53.3 ± 8.8	53.4 ± 10.4
Visit 1	39.9 ± 8.7	35.4 ± 8.1	42.7 ± 13.5	44.2 ± 10.7	44.2 ± 9.8	39.8 ± 8.4
Visit 2	42.6 ± 9.4	39.4 ± 9.4	52.7 ± 12.7	51.1 ± 9.4	47.9 ± 10.4	44.5 ± 9.8
Visit 3	43.8 ± 8.7	37.8 ± 9.5	50.0 ± 12.6	46.8 ± 12.0	48.8 ± 9.8	42.4 ± 10.1
Visit 4	43.6 ± 8.0	36.1 ± 9.6*	50.0 ± 10.0	40.5 ± 11.8**	48.6 ± 8.7	40.1 ± 10.1**
Visit 5	45.6 ± 8.1	34.7 ± 9.2**	50.9 ± 12.2	38.9 ± 11.0**	50.7 ± 9.1	38.6 ± 9.9**
Visit 6	43.6 ± 9.8	32.7 ± 9.5**	54.5 ± 13.7	36.3 ± 13.8**	49.0 ± 11.0	36.3 ± 10.4**
Visit 7	45.5 ± 9.4	31.1 ± 9.8**†	57.3 ± 13.5	31.1 ± 15.2**†	51.2 ± 10.7	34.3 ± 10.8**†

The results are the scores from 30 subjects (19 in PCP group and 11 in placebo group) participated in the study for seven visits once in 15 days during the 13-week study period.
Data are expressed as mean ± standard deviation (SD).
* $P < 0.05$ vs. placebo in WOMAC score, VAS score or QOL score at the same visit.
** $P < 0.01$ vs. placebo in WOMAC score, VAS score or QOL score at the same visit.
† $P < 0.01$ vs. Baseline in WOMAC score, VAS score or QOL score.

Table 4. Response ratio of subjects who have shown the study efficacy improvement in WOMAC, VAS and QOL scores

	WOMAC score		VAS score		QOL score	
Assessment	Placebo (*N* = 11)	PCP (*N* = 19)	Placebo (*N* = 11)	PCP (*N* = 19)	Placebo (*N* = 11)	PCP (*N* = 19)
Percentage of efficacy improvement*	0%	63.2	0%	63.2	9%	63.1

*Efficacy improvement is reduction in WOMAC score by 20 points or more from baseline to final visit (Visit 7), in VAS score by 40 points or more from baseline to Visit 7 and in QOL score by 20 points or more from baseline to Visit 7.

This observation is in correlation with the response ratio of subjects participated in the study. A total of 63% of subjects who have taken PCP have shown efficacy improvement in WOMAC, VAS and QOL score levels while the placebo group could not demonstrate such an efficacy improvement in all the score levels, as shown in Table 4.

The same study with BCP demonstrated similar results (Table 5) as that observed in study with PCP. The results further affirm that the efficacy behaviour of collagen peptide is similar (Fig. 2), whether the source of origin of peptide is from pork skin or bovine bone.

Biochemical evaluations

As part of safety assessment, laboratory analysis was performed for the various biochemical parameters in serum and urine. The results of the baseline and Visit 7 are shown in Table 6. Other than some minor changes none of the biochemical parameters showed any significant variation in the results during the study period. The minor changes observed were not clinically significant. All the data were statistically analysed and found no significant differences from the normal range in PCP and placebo groups. Data are not shown, but BCP also achieved a similar result. These findings demonstrated the safety of PCP and BCP in humans. Moreover the

Table 5. Changes of primary efficacy parameters for WOMAC and VAS and secondary end point parameter for QOL

	WOMAC score (points)		VAS score (points, in mm scale)		QOL score (points)	
Visit	Placebo (*N* = 10)	BCP (*N* = 18)	Placebo (*N* = 10)	BCP (*N* = 18)	Placebo (*N* = 10)	BCP (*N* = 18)
Baseline	50.1 ± 14.7	50.3 ± 9.6	62.0 ± 14.0	66.0 ± 12.3	56.3 ± 15.4	56.9 ± 9.9
Visit 1	35.3 ± 7.4	38.1 ± 7.7	50.0 ± 12.5	40.0 ± 13.4	40.3 ± 7.4	42.1 ± 7.9
Visit 2	45.6 ± 13.9	38.5 ± 8.3	52.0 ± 12.3	44.5 ± 11.5	50.8 ± 14.3	42.9 ± 8.7
Visit 3	47.4 ± 17.9	34.8 ± 8.6	51.0 ± 17.3	38.5 ± 11.8	52.5 ± 18.6	38.7 ± 8.9
Visit 4	46.9 ± 18.3	32.9 ± 9.2**	53.0 ± 14.9	39.5 ± 10.5**	52.2 ± 19.2	36.8 ± 9.3**
Visit 5	45.2 ± 17.5	31.8 ± 9.6**	52.0 ± 13.2	36.5 ± 11.4**	50.4 ± 17.9	35.4 ± 9.8**
Visit 6	45.9 ± 18.6	29.1 ± 10.0**	54.0 ± 19.6	32.0 ± 10.6**	51.3 ± 19.9	32.3 ± 10.2**
Visit 7	47.3 ± 19.4	25.8 ± 11.3**†	55.0 ± 20.1	28.0 ± 10.9**†	52.8 ± 20.9	28.7 ± 11.4**†

The results are the scores from 28 subjects (18 in BCP group and 10 in placebo group) participated in the study for seven visits once in 15 days during the 13-week study.
Data are expressed as mean ± standard deviation (SD).
** $P < 0.01$ vs. placebo in WOMAC score, VAS score or QOL score at the same visit.
† $P < 0.01$ vs. Baseline in WOMAC score, VAS score or QOL score.

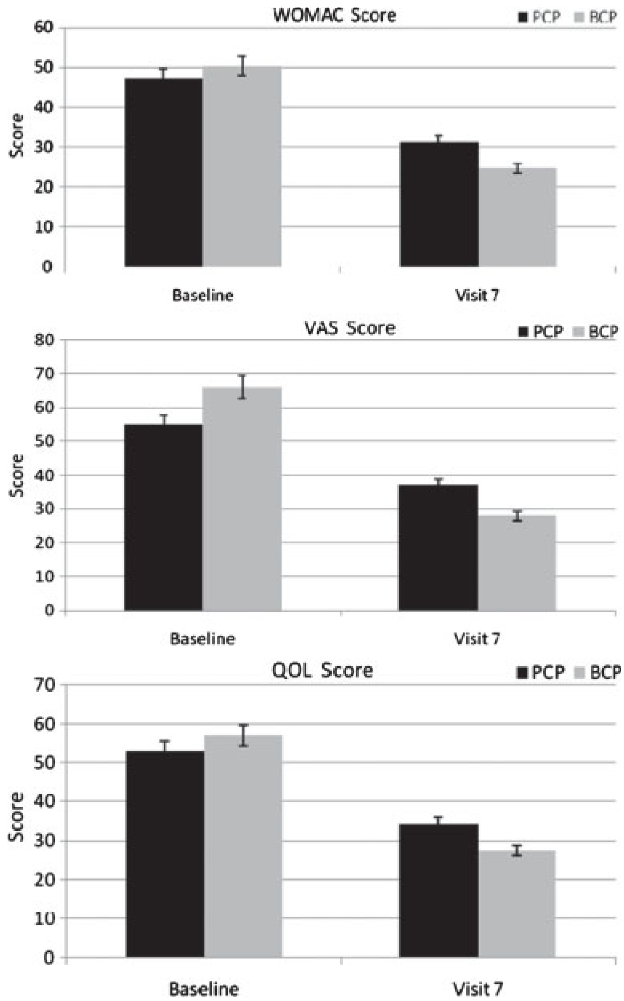

Figure 2. Comparison of Western Ontario McMaster Universities (WOMAC), visual analogue scale (VAS) and quality of life (QOL) scores in the pork skin collagen peptide (PCP) and bovine bone collagen peptide (BCP) groups at baseline and Visit 7. Each bar represents the average score with standard error.

US Food and Drug Administration (US FDA) has classified gelatin and collagen peptide as a Generally Recognised as Safe (GRAS) product.

Adverse events

Among 20 subjects treated with PCP, only one experienced an adverse event of allergic peripheral oedema. The adverse event was reported to be mild in nature and was relieved with concomitant medication. Among 20 subjects treated with BCP, three subjects experienced adverse events such as vomiting, diarrhoea and common cold. Cold and vomiting were reported as mild while diarrhoea was reported as moderate and was cured by medical intervention.

All the adverse events had an unlikely relationship with investigational products and upon re-challenging the investigational products, the events did not re-appear.

DISCUSSION

The present study demonstrated a significant change in the status of OA condition of patients orally administered with PCP or BCP compared to placebo group. Pain stiffness of joints, reduced movement of joints and physical disabilities are the major clinical manifestations in OA. The study carried out in clinically diagnosed subjects having knee OA demonstrated that both PCP and BCP are effective nutritional supplement to improve the overall physical discomforts resulting from the OA. Collagen peptides from two sources have been considered in this study to evaluate whether collagen peptides prepared by the same process from different sources would have the similar efficacy on the management of a human population with OA. This study is the first to compare collagen peptides from different sources. The study clearly demonstrated that, irrespective of the source of collagen peptides, the efficacy level remains the same. A recent study by Trc and Bohmova[11] compared the efficacy and tolerance of hydrolysed collagen and glucosamine sulphate treatment in knee OA. In their multicenter, randomised, double-blind study the subjects were given hydrolysed collagen at a dose of 10 g day^{-1} and glucosamine sulfate 1.5 g day^{-1} for 90 days. The efficacy was measured by WOMAC and VAS score analysis. According to the study results hydrolysed collagen shows superior improvement over glucosamine sulfate. Reduction of the WOMAC and VAS indices has been observed in 80.8% of study population who were orally supplemented with hydrolysed collagen while only 46.6% of study subjects in glucosamine sulfate have shown reduction. Moskowitz,[12] in his review on role of collagen peptide in bone and joint disease concluded that collagen peptide oral consumption in a daily dose of 10 g will have a therapeutic effect on the indications of OA and osteoporosis. In a review of clinical and preclinical studies on collagen peptide Bello and Oesser[13] explained the clinical evidence for the effectiveness of collagen peptide in the treatment of OA. The results of the present study with PCP are in accord with these observations.

The mechanism of action of collagen peptide has been extensively studied.[14–18] Experimental investigations have demonstrated that the degradation products of the collagen are principally able to influence cell metabolism.[19] Studies with labelled collagen peptide have shown that a significant amount of the peptide could be detected in skin and cartilage tissue after one single administration, indicating an accumulation of these peptides within the connective tissue.[9] The study demonstrated the intestinal absorption and cartilage accumulation of collagen peptide. Thus the potential role of collagen peptide in repair of damaged cartilage could be associated with the accumulation of orally administered collagen peptide. Cell culture experiments investigating the efficacy of collagen peptide on the biosynthesis of articular chondrocytes revealed that the treatment of cartilage cells with collagen peptide induced a statistically significant dose dependent increase in type II collagen synthesis of chondrocytes compared to the untreated controls.[19] The major component of collagen peptide that remained in the blood was identified as Pro–Hyp dipeptides.[14] Nakatani *et al.*[20] concluded that Pro–Hyp,a dipeptide in PCP, is an important factor that regulates chondrocyte differentiation and plays a key role in the maintenance of mature chondrocytes in cartilage. They hypothesised that Pro–Hyp in collagen peptide and its regulatory mechanism seem to explain the therapeutic effect of collagen peptide in improving joint conditions. Being one of the most important symptoms, pain reduction indirectly indicates the mark of improvement in joint conditions in patients with OA. Thus the administration of collagen peptide has much relevance with regard to reduction of pain in a patient with OA. As discussed previously, the improvement could be associated with the initiation of the repair process by

Table 6. Biochemical evaluation of subjects participated in the clinical study

Biochemical analysis	Unit	Placebo Baseline	Placebo Visit 7	Pork collagen peptide Baseline	Pork collagen peptide Visit 7
Urea	μmol L^{-1}	10.4 ± 3.3	9.1 ± 2.0	10.4 ± 3.3	8.6 ± 2.1
Uric acid	μmol L^{-1}	179.4 ± 43.8	225.3 ± 46.4	221.1 ± 77.7	247.1 ± 44.5
Creatinine	μmol L^{-1}	79.5 ± 26.0	64.9 ± 16.2	79.7 ± 25.3	73.2 ± 19.1
Total bilirubin	μmol L^{-1}	7.2 ± 2.6	9.9 ± 6.3	6.4 ± 2.6	8.1 ± 4.4
Direct bilirubin	μmol L^{-1}	2.4 ± 1.3	3.3 ± 1.5	3.1 ± 2.1	3.4 ± 1.7
SGOT	U L^{-1}	24.0 ± 6.0	25.9 ± 6.1	23.8 ± 7.5	30.0 ± 13.7
SGPT	U L^{-1}	21.0 ± 7.6	17.5 ± 8.9	21.6 ± 8.9	24.4 ± 17.0
ALP	U L^{-1}	252.8 ± 65.9	186.9 ± 67.3	230.5 ± 82.6	211.4 ± 84.7
Random blood sugar	mmol L^{-1}	6.4 ± 2.1	6.5 ± 3.8	6.3 ± 3.7	5.9 ± 1.3
Total cholesterol	mmol L^{-1}	5.6 ± 0.9	4.6 ± 0.9	5.3 ± 0.8	4.5 ± 1.3
Protein	g L^{-1}	73.6 ± 4.7	74.3 ± 3.8	74.4 ± 3.6	75.4 ± 4.4
Albumin	g L^{-1}	44.3 ± 2.7	44.1 ± 2.9	43.1 ± 2.6	42.7 ± 4.2

Data are expressed as mean ± standard deviation (SD).
ALP, Alkaline Phosphatase; SGOT, Serum Glutamic Oxaloacetic Transaminase; SGPT, Serum Glutamic Pyruvate Transaminase.

accumulation of collagen peptide in cartilage tissue. The accumulated collagen peptide helps to maintain structure and function of cartilage, which in turn results in joint comfort and subsequent improvements in pain. These results clearly indicate that collagen peptide, irrespective of the source – namely pork skin or bovine bone – has a stimulatory effect on important molecules of ECM, namely proteoglycans, and thus might be of therapeutic relevance to slow down or even halt the progression of degradation of articular cartilage tissue in OA.

CONCLUSION

The study clearly demonstrates that both PCP and BCP are effective supplements for the improvement in overall physical problems associated with OA and thereby help to improve the quality of life. It is hypothesised that the supplementation of collagen peptide regulates chondrocyte differentiation and stimulates synthesis of proteoglycans, resulting in the initiation of repair processes in cartilage tissue.

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