

ORIGINAL ARTICLE

**COMPARISON BETWEEN THE EFFECT OF COLLAGEN TRIPEPTIDE AND SODIUM HYALURONAN TO PREVENT KNEE OSTEOARTHRITIS: A PRELIMINARY IN VIVO STUDY**

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**Abstract Objective** Collagen peptides have recently been shown to have several biological activities, and have been used as preservatives and immunotherapeutic agents. The purpose of this study was to investigate the ability of weekly intra-articular injections of collagen tripeptide (Ctp) to prevent knee osteoarthritis (OA) compared with sodium hyaluronan (HA).

**Methods** Thirty rabbits with anterior cruciate ligament (ACL) transection were randomly divided into three groups: Ctp, HA, and saline (control). All animals were administered the same amount of each reagent once weekly. Articular cartilages of the medial condyle of femur were examined by gross morphological and histopathological examination conducted at 5 (n=6) and 10 (n=4) weeks.

**Results** The Ctp and HA injection groups tended to have improved gross morphological scores and exhibited preventive effects compared with control groups at 10 weeks after ACL transection. But the scores were not significantly different among the 3 groups. The overall means of histological grading scores were not significantly different among the 3 groups however, the score for the Ctp group had improved from 5 weeks to 10 weeks.

**Conclusions** Intra-articular injections of Ctp may inhibit progression of knee OA, and further examination is needed to clarify the physical characteristics and regenerative functions of Ctp, and to determine the optimal dose and duration of Ctp injection therapy for the knee OA.

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**Key words:** Osteoarthritis; collagen peptide; hyaluronic acid; cartilage; injection.

原 著

**コラーゲン・トリペプチドおよびヒアルロン酸関節内投与における変形性膝関節症予防効果の比較**

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**抄録 目的** コラーゲントリペプチド(Ctp)関節内投与による変形性膝関節症(膝OA)予防効果を、現在臨床応用されているヒアルロン酸(HA)と比較した。

**方法** 日本白色家兎30匹を用い右膝関節前十字靭帯切離によるOA発症モデルを作製。それらを生食、Ctp、HA投与群に分類し、週1回関節内投与を施行した。6匹を5週投与後、4匹を10週投与後屠殺し、大腿骨内側顆を用いてOA進行度を肉眼的、組織学的に評価した。

**結果** 肉眼的評価では10週投与時でCtp及びHA投与群で生食投与群に比し膝OAの進行は軽度であったが、統計学的有意差は認めなかった。組織学的評価でも3群間に有意差は認めなかったが、Ctp投与群では5週から10週にかけてスコアの改善を認めた。

**考察** Ctp関節内投与によるOA予防効果が示唆された。生理活性の詳細な検討と、治療に向けた至適な投与濃度及び投与期間の更なる検討が必要である。

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**キーワード:** 変形性膝関節症; コラーゲンペプチド; ヒアルロン酸; 軟骨; 注射。

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## Introduction

Osteoarthritis (OA) of the knee is a major cause of disability worldwide<sup>1</sup>. Knee OA is induced by complex mechanisms such as proteoglycan (PG) degradation and disruption of the collagen network, all of which lead to progressive destruction of joints and functional loss<sup>2</sup>. Although several symptomatic therapies have been attempted for Knee OA, no radical treatment methods have been established, with the exception of total knee arthroplasty. Much research has been performed to intervene in the disease process and retard or even prevent progression of joint damage. Intraarticular injection of sodium hyaluronan (HA), which is one of these strategies, has been used for the treatment of pain associated with knee OA, and has been shown to have an anti-inflammatory effect and reduces pain clinically<sup>3,4</sup>.

Collagen peptides, which are enzymatic degradation products of collagen, have recently been shown to have several biological activities and have been used as preservatives and immunotherapeutic agents<sup>5</sup>. Collagen tripeptide (Ctp) prepared from porcine type I collagen, using a bacterial collagenase that degrades the peptide bonds of collagen at the amino-terminal end of Gly, is a highly purified, non-antigenic, and low-allergenic tripeptide fraction containing Gly-Xaa-Yaa sequences. A previous study showed that chondrocyte proliferation and synthetic ability were stimulated by supplementation of culture medium with tripeptide<sup>6</sup>.

We hypothesized that Ctp administration within the joint would suppress the loss of cartilage matrix and consequently prevent knee OA progression. The purpose of this study was to examine the preventive effect of knee OA development by intra-articular injection of Ctp compared with HA used for the treatment of knee OA. This study was conducted using an

experimental model in which knee OA develops as a result of anterior cruciate ligament (ACL) transection<sup>7-9</sup> in Japanese white rabbits.

## Materials and Methods

### Experimental materials

Ctp was provided by Central Research Institute, Jellice Corp. (Sendai, Japan). The tripeptide content consisting of Gly-Xaa-Yaa sequences was more than 90%. Solutions of 3.0 µg/ml Ctp and HA (Supartz, Seikagaku Corp., Tokyo, Japan) were used.

### Experimental animals and ACL transection (ACLT) surgery for induction of osteoarthritis

Thirty mature female Japanese white rabbits (body weight  $3.3 \pm 0.8$  kg) were utilized in the study. Unilateral ACL transection was performed under anesthesia induced by intravenous injection of 30 mg/kg sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan). The ACL was exposed through a medial parapatellar incision and transected at the midsubstance with a sharp blade. Complete transection of the ACL was confirmed by a positive anterior drawer sign. The capsule was sutured to render it watertight, followed by skin closure. All animals were allowed normal cage activity.

All animal experiments in this study followed the Guidelines for Animal Experimentation of Hirosaki University.

### Experimental protocol for treatment

After ACL transection, all the rabbits were divided into three groups of 10 rabbits each: Group 1 was injected with sterile physiological normal saline as a control, group 2 with Ctp, group 3 with HA. Using a 27-gauge needle inserted through the lateral infrapatellar area toward the intercondylar space of the femur in each animal in a deep knee-flexed position, 0.3 ml of each reagent was administrated

intra-articularly into the right knee with ACL transection. The first injection was given immediately after ACL transection; six animals of each group were administered once weekly for 5 weeks and four animals of each group for 10 weeks, and all animals were sacrificed one week after the final injection was administered, by an overdose of sodium pentobarbital. The knee joints were then harvested and evaluated. The dose of 900 µg of Ctp was chosen based on the local effective concentration of the previous *in vitro* preliminary study that fibroblasts' gene expressions were stimulated by supplementation of culture medium with Ctp of this concentration.

#### **Gross morphological examination**

Gross morphological changes of the medial condyles of femur were assessed and graded as previously described<sup>10</sup> after application of india ink (grade 1, 2, 3, 4a, 4b, 4c = score 0, 1, 2, 3, 4, 5) —grade 1 (intact surface): surface appears normal and does not retain any ink; grade 2 (minimal fibrillation): site appears normal before staining, but retains the india ink as elongated specks or light gray patches; grade 3 (overt fibrillation): the cartilage is velvety in appearance and retains ink as intense black patches; grade 4 (erosion): loss of cartilage exposing the underlying bone; grade 4a: erosion of 0 to 2 mm; grade 4b: erosion of 2 to 5 mm; and grade 4c: erosion of >5mm. In a blinded manner, the assessment was conducted by two independent examiners, who were blinded to each other's findings and to the treatment group assignment of the animals. Finally, the two scores from the examiners were averaged to obtain an overall score.

#### **Histopathological examination**

Dissected medial condyles of femur were fixed in 10% neutral buffered formalin after gross morphological examination. Specimens

were decalcified in 4% EDTA solution, dehydrated with a gradient ethanol series, and embedded in paraffin blocks. Based on macroscopic observation, 4 µm sections including the most severely degenerated area were stained with hematoxylin and eosin (H&E) and with Alcian blue for light microscopic examination. Histological sections were visualized using an Olympus BX41 microscope (Olympus, Tokyo, Japan) and Olympus DP2-BSW software (Olympus). Histological sections were assessed in a blinded manner by a pathologist who was unaware of the treatment group assignment of the animals, and quantified using the histological grading method proposed by Mankin and coworkers<sup>11</sup> (Table. 1).

#### **Statistical analysis**

All data are expressed as mean ± standard deviation. A Tukey's honestly significant difference test was used to evaluate the statistical significance of difference in the macroscopic and histologic results. P values less than 0.05 were considered statistically significant.

## **Results**

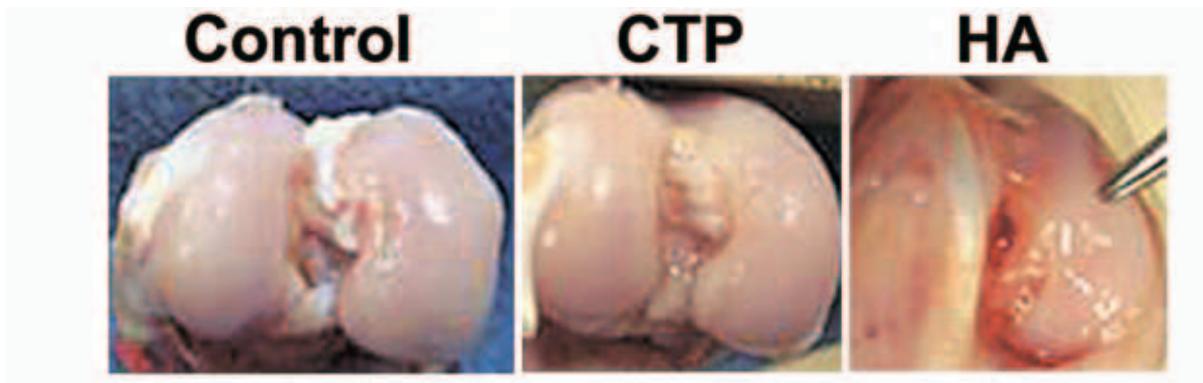
There were no adverse effects due to injections in any rabbit, and no evidence of postoperative infection was noted. At sacrifice, all ACLT knees showed complete transection of the ACL, with only a stump remaining.

#### **Gross morphological assessment**

Specimens injected for 5 weeks of all groups showed no morphological change (Fig. 1), however specimens injected for 10 weeks exhibited changes consistent with the development of knee OA and showed mild to severe degradation of the condyle cartilage (Fig. 2). Both treatment groups injected for 10 weeks after ACL transection exhibited slightly

**Table 1** Mankin scale

<b>1. Cartilage Anatomy</b>
0: Normal
1: Surface roughness, fissures extending into the radial layer
2: Pannus
3: Superficial layer of the cartilage is lost
4: Mild disorganization, loss of columnar alignment of cells, scarcity of cellular clusters
5: Fissures extending to the calcified cartilage layer
6: Complete loss of cellular organization, clusters of cells, osteoclastic activity
<b>2. Cellular abnormalities</b>
0: Normal
1: Hypercellularity, including small clusters of superficial cells
2: Clusters of cells
3: Hypercellularity
<b>3. Matrix Staining</b>
0: Normal/slightly decreased staining
1: Decreased stained of radial layer
2: Decreased stained of interterritorial matrix
3: Only pericellular matrix is stained
4: Unstained
<b>4. Tidemark integrity</b>
0: Intact
1: Destruction

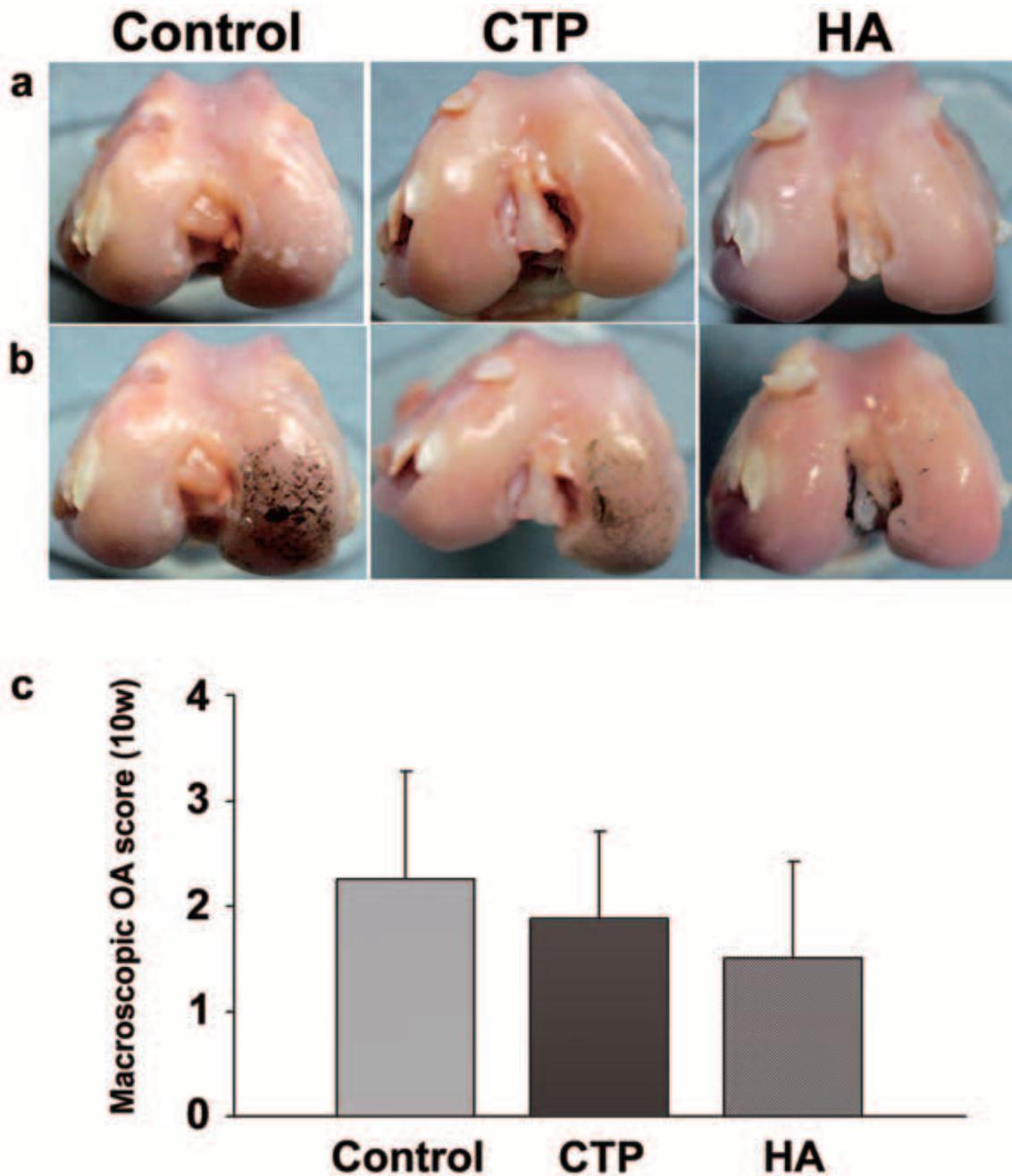
**Figure 1** Representative macroscopic appearances at 5 weeks after anterior cruciate ligament transection. Ctp=collagen tripeptide; HA=sodium hyaluronan.

resistance to the changes of knee OA than saline group (saline group  $2.3 \pm 1.0$ , Ctp group  $1.9 \pm 0.8$  and HA group  $1.5 \pm 0.9$  and saline group) ( $p > 0.05$ ).

#### Histopathological assessment

The cartilage on the medial condyle of femur in the ACL transection knees of all groups exhibited pathological changes of some degree of knee OA. In specimens injected for 5

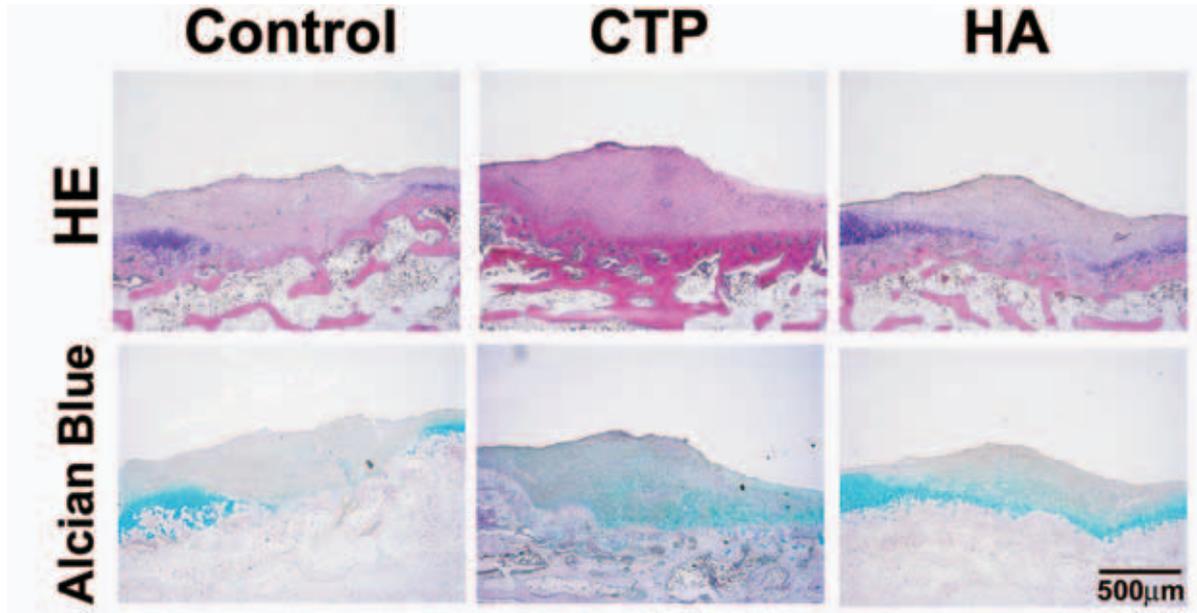
weeks of all groups, fibrous tissue was formed over the degenerated area (Fig. 3), in which fibroblast-like cells were increased (Fig. 4). The degenerative area of saline group was extended to more deeper zone compared with that of Ctp group and HA group. In case of 10 weeks injection, the fibrous tissue was decreased and the structure and matrix staining of deep layer were improved in Ctp group (Fig. 5). The overall means of Mankin grading scores



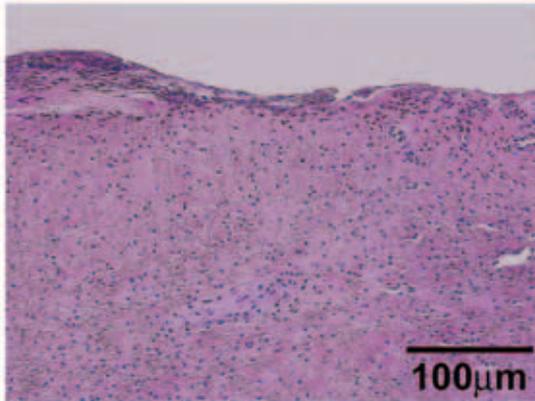
**Figure 2** Evaluation of knee OA grades by gross morphological assessment of the articular cartilage from the medial condyle of femur. (a) Representative macroscopic appearances at 10 weeks after anterior cruciate ligament transection. (b) Surface of the cartilage was stained with India ink to identify any fibrillation and erosion. (c) Quantification of macroscopic analysis (grade 1, 2, 3, 4a, 4b, 4c = score 0, 1, 2, 3, 4, 5). The scores are displayed as means  $\pm$  standard deviation (10 weeks; n=4). There were no statistically significant differences in any scores among the three groups. Ctp=collagen tripeptide; HA=sodium hyaluronan.

were not significantly different among groups, but HA group injected for 5 weeks tended to decrease in comparison with other 5 injected groups (saline group  $2.8 \pm 0.8$ , Ctp group  $2.3 \pm 1.4$  and HA group  $1.3 \pm 1.0$ ) (Fig. 6). Furthermore,

in case of 10 weeks injection, the score for the Ctp in addition to HA groups were lower than that for saline group (saline group  $2.8 \pm 1.5$ , Ctp group  $1.8 \pm 0.5$  and HA group  $2.0 \pm 0.8$ ) (Fig. 6). While the overall mean score of HA group



**Figure 3** Representative histologic appearance of cartilage of medial condyle of femur (H-E and Alcian blue staining) of control, Ctp and HA group at 5 weeks. Ctp=collagen tripeptide; HA=sodium hyaluronan.



**Figure 4** High magnification of fibrous tissue in Ctp group injected for 5 weeks.

was increased from 5 weeks to 10 weeks, that of Ctp group was decreased. The mean score for criteria related to matrix staining and tidemark integrity of Ctp group injected for 10 weeks were lower than that of saline group and HA group (Table. 2). On the contrary, the mean score for cellular abnormalities of Ctp group was higher than that of saline group and HA group in both 5 and 10 injected groups.

## Discussion

The Ctp used in this study was a highly purified tripeptide fraction containing Gly-Xaa-Yaa sequences<sup>12</sup>. Articular cartilage has extremely small pores (estimated at 50 Å) in the superficial zone, and so only low-molecular-weight compounds (<20 kDa) in synovial fluid may diffuse into the tissue<sup>13</sup>. Our experimental material, Ctp, has a possibility to move freely through the tissue because they are not heavy molecular compounds. Transporter systems for amino acids in cartilage chondrocytes have not yet been identified, but glycine, proline, glutamine and glutamate transporters in chondrocytes have recently been investigated<sup>14, 15</sup>. While the Ctp-treated group tended to have a higher score in gross morphological assessment than the HA-treated group, the Mankin score had decreased from 5 weeks to 10 weeks after ACLT, and the score was lower than in the HA-treated group at 10 weeks. Concerning the mean scores for criteria related to cellular abnormalities, Ctp groups had higher cell numbers than the

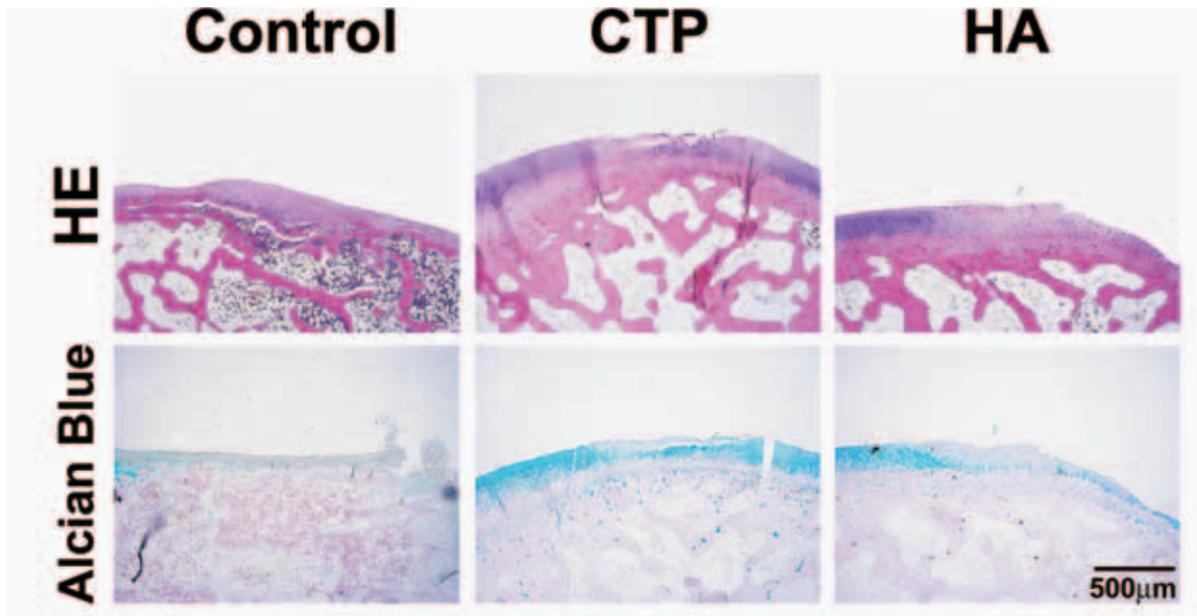


Figure 5 Representative histologic appearance of cartilage of medial condyle of femur (H-E and Alcian blue staining) of control, Ctp and HA group at 10 weeks. Ctp=collagen tripeptide; HA=sodium hyaluronan.

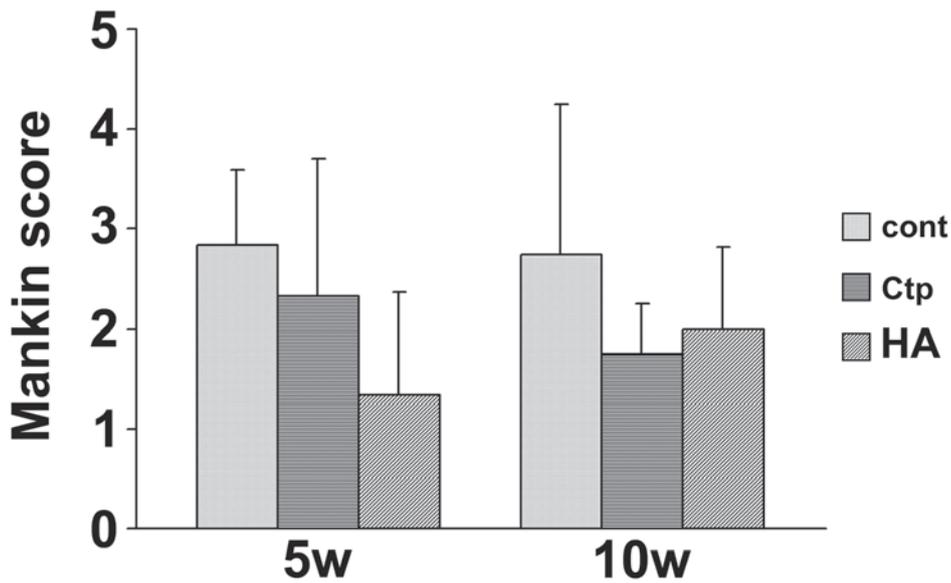


Figure 6 Quantification of histological analysis using the Mankin grading method at 5 and 10 weeks after anterior cruciate ligament transection. The scores are displayed as overall means  $\pm$  standard deviation (5 weeks: n=6, 10 weeks: n=4). There were no statistically significant differences in any scores among the three groups. Ctp=collagen tripeptide; HA=sodium hyaluronan.

non-Ctp groups. Furthermore, the Ctp group treated for 10 weeks had lower scores for criteria related to matrix staining and tidemark integrity. We speculated that Ctp had a regenerative effect by stimulating proliferation

of cells containing chondrocytes and fibroblast-like cells and repairing cartilage matrix. Previous study have shown that amino acids are expected not only to control chondrocyte gene expression<sup>16)</sup> but also to control synthesis

**Table 2** Histological evaluation by the Mankin grading method at 5 and 10 weeks after anterior cruciate ligament transection. There were no statistically significant differences in any scores among the three groups. Data represent the means  $\pm$  standard deviation for each group: Ctp=collagen tripeptide; HA=sodium hyaluronan.

Group weeks(n)	Control		CTP		HA	
	5(n=6)	10(n=4)	5(n=6)	10(n=4)	5(n=6)	10(n=4)
Structure	0.8 $\pm$ 0.4	0.8 $\pm$ 1.0	0.3 $\pm$ 0.5	0.3 $\pm$ 0.5	0.2 $\pm$ 0.4	0.3 $\pm$ 0.5
Cell abnormalities	0.7 $\pm$ 0.5	1.0 $\pm$ 0.8	1.2 $\pm$ 0.4	1.3 $\pm$ 0.5	0.8 $\pm$ 0.4	0.8 $\pm$ 0.5
Matrix staining	0.5 $\pm$ 0.5	0.5 $\pm$ 0.6	0.5 $\pm$ 0.5	0.3 $\pm$ 0.5	0.0 $\pm$ 0.0	0.5 $\pm$ 0.6
Tidemark integrity	0.8 $\pm$ 0.4	0.5 $\pm$ 0.6	0.3 $\pm$ 0.5	0.0 $\pm$ 0.0	0.3 $\pm$ 0.5	0.5 $\pm$ 0.6
Total	2.8 $\pm$ 0.8	2.8 $\pm$ 1.5	2.3 $\pm$ 1.4	1.8 $\pm$ 0.5	1.3 $\pm$ 1.0	2.0 $\pm$ 0.8

of collagen by chondrocytes<sup>17</sup>). And, amino acids stimulate fibroblasts proliferation<sup>18</sup>). Therefore, amino acids in Ctp might be selected to provide substrates for fibril forming collagen and proteoglycan, based on their prevalence in the triple-helical structure of collagen, and depending on their specific biochemical and physiologic characteristics<sup>19, 20</sup>).

The total Mankin score of the HA-treated group was lowest among the 3 groups at 5 weeks after ACLT, and maintained the low level until 10 weeks. HA covers cartilage surfaces as an amorphous layer, and partially protects cartilage against cartilaginolytic enzymes and penetration of inflammatory cells<sup>21, 22</sup>). Furthermore, a decrease in HA level in the synovial fluid of OA may result in damage to the cartilage surface and increase the permeability of cartilage, allowing proteins and other high molecular weight substances to penetrate into the cartilage matrix<sup>23</sup>). Intraarticularly injected HA binds free proteoglycan in the cartilage matrix, and inhibits further proteoglycan release, thus protecting the cartilage matrix and cells<sup>24, 25</sup>). Exogenous HA is known to stimulate endogenous HA production by positive feedback<sup>26</sup>). From these findings, the lower score of the HA-treated group in this study might also be brought about by its cartilage protective effect.

Further examination is needed to clarify the physical characteristics and regenerative

functions of Ctp, and to determine the optimal dose and duration of Ctp injection therapy for the OA knee, based on in vitro studies and several additional in vivo studies.

Experimental osteoarthritis induced by ACL transection is a useful tool in the investigation of knee OA development<sup>7-9</sup>). The biochemical and pathological changes that PG degradation and disruption of the collagen network lead to loss of hyaline cartilage, are identical to those seen in human knee OA and the lesion site, as in humans, is mainly on the medial condyle of femur, and eventually extends over the lateral condyle of femur<sup>10</sup>). Therefore, medial condyles of femur were used to examine the knee OA development in this study.

The Mankin grading scores of the HA and saline groups were lower than those of previous study<sup>27-29</sup>). The possible reasons for the difference might be due to: (1) duration after ACLT for histology examination: 5 and 10 weeks were used in our study versus more than 20 weeks in other studies; (2) examined site: unilateral knees were used in our study, and as the knee OA progressed, rabbits avoided weight-bearing on the affected legs. However, the improved Mankin scores in the HA-treated ACLT knees in our study are in agreement with previous reports<sup>27-29</sup>).

In conclusion, our results show the regenerative effects of intra-articular injections of Ctp on degenerative changes in the cartilage,

in the ACL transection joint with no obvious adverse effects. These results suggest that Ctp injection may be able to modify the progression of OA of the knee and may potentially be applied as an effective treatment.

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