Chicken Collagen Type II Reduces Articular Cartilage Destruction in a Model of Osteoarthritis in Rats

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ABSTRACT

Aim: To evaluate the therapeutic effects of domestic chicken collagen type II (CCII) on rat osteoarthritis (OA) and analyze concomitant changes in the level of Matrix metalloproteinase (MMP)-13, MMP-9, Cathepsin K and their mRNA as well as the tissue inhibitor of matrix metalloproteinase (TIMP)-1 mRNA in articular cartilage of osteoarthritic rats.

Methods: Osteoarthritis models were surgically induced. Morphology of articular cartilage was done by haematoxylin and eosin staining and Mankin score was calculated, immunohistochemistry of MMP-13, MMP-9 and Cathepsin K was done by ABC method while the mRNA level for MMP-13, MMP-9, cathepsin K as well as TIMP-1 was evaluated by RT-PCR method.

Results: Oral administration of CCII reduced the morphological changes of osteoarthritic cartilage (shown by Mankin score), decreased levels of MMP-13, MMP-9, cathepsin K as well as their mRNA in articular cartilage from osteoarthritic rats while it exhibited no effect on TIMP-1 mRNA.

Conclusion: Oral CCII reduced articular cartilage degradation of osteoarthritic rats and may probably be a potent drug candidate for OA treatment.

Colágeno de Pollo Tipo II Reduce la Destrucción del Cartílago Articular en un Modelo de Osteoartritis en Ratas

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RESUMEN

Objetivo: Evaluar los efectos terapéuticos del colágeno de pollo doméstico de tipo II (CPII) en la osteoartritis de ratas (OA) y analizar los cambios concomitantes en el nivel de metaloproteinasa de la matriz (MMP-13). MMP-9, catepsina K y su mRNA, así como el inhibidor tisular de la metaloproteinasa de la matriz (TIMP)-1 mRNA en el cartílago articular de ratas osteoartríticas.

Métodos: Modelos osteoartríticos fueron obtenidos mediante inducción quirúrgica. La morfología del cartílago articular se realizó mediante tinción H-E, y se calculó la puntuación de Mankin. Se realizó la inmunohistoquímica de MMP-13, MMP-9 y catepsina K mediante el método ABC, en tanto que el nivel de mRNA para MMP-13, MMP-9, y catepsina K así como el TIMP-1, fue evaluado mediante el método RT-PCR.

Resultados: La administración oral de CPII redujo los cambios morfológicos del cartílago osteo-artrítico (mostrado por la puntuación Mankin), disminuyó los niveles de MMP-13, MMP-9, catepsina K así como su mRNA en cartílago articular de las ratas osteoartríticas mientras que no mostró efecto sobre TIMP-1 mRNA.

Conclusión: El CP oral redujo la degradación del cartílago articular de las ratas osteoartríticas y puede ser probablemente candidato a un medicamento potente para el tratamiento de la OA.

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INTRODUCTION

Osteoarthritis (OA) is by far the most common joint disorder in ageing. The underlying cause and precise mechanism of OA remains unknown, and there is no effective and safe drug for OA treatment. Currently, OA treatment starts with simple analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) which display various side effects including gastric and cardiovascular complications (1, 2). Among them, selective COX-II inhibitors exhibit less complications than other NSAIDs but still have severe side effects such as heart attacks and stroke (2). The safer drug for OA treatment seems to be diacerein which acts against IL-1 (3). But it should be mentioned that even diacerein has side effects of diarrhoea and none of the available OA drugs are specially designed to block the progressive degradation of the cartilage (2, 3). So to find safe and effective drugs for OA treatment is significant. Chicken collagen type II (CCII), a kind of collagen protein, has been used recently in RA patients for phase II clinical trial in China and yield promising results without any side effects. This drug yields its therapeutic action by inducing oral tolerance. Oral tolerance means a state of immunological unresponsiveness induced by oral administration of antigen (4, 5). The gastrointestinal tract is the major site of antigenic contact in the body. The gut-associated-lymphoid tissue (GALT) provides for absorption of nutrients, avoidance of hypersensitivity to food antigens and exclusion of pathogens. Both Th1 and Th2 cells are present in the GALT. Oral administration of a number of CCII lead to upregulation of Th2/Th3 cells which secrete anti-inflammatory cytokines and as a result inhibits inflammatory events that lead to autoimmunity (4, 5). Part of the mechanism of OA resembles that of RA so we guess that oral administration of CCII may have promising results for OA treatment. Therefore this study was done to explore therapeutic effects of oral CCII on rat OA and analyze concomitant changes in the level of MMP-13, MMP-9, cathepsin K and their mRNA level as well as tissue inhibitor of matrix metalloproteinase (TIMP)1 mRNA level in articular cartilage of osteoarthritic rats.

MATERIALS AND METHODS

Rat OA model

One hundred and thirty-two Wistar rats (Shanghai experimental animal centre attached to the Chinese Academy of Science, Shanghai, China) were used in this study. Osteoarthritis was surgically induced according to the method of Hayami et al (6).

Compounds and Machines

The dry powder of soluble dispersal mixture of domestic CCII was provided by Professor REN Geng-fu (product of Shanghai institute of herbal biology, Shanghai, China), patent number: ZL 02 1 45192.3, plot number: 00031004, its excipient: mannitol.; chondroitinase ABC: 0.25 unit/mL (FLUKA, USA), primary polyclonal antibody against MMP-13: 15 µg/mL (CHEMICON International Inc, USA); primary mon-

oclonal antibody against MMP-9: 10 µmg/mL (CHEMICON International Inc, USA), primary antibody against cathepsin K: µ1 mg/mL (Santa Cruz Biotechnology, USA) ABC kit (Sino-American Biotechnology Co China) biotin-conjugated secondary antibody (KPL Co Europe) Access RT-PCR system (Promega) TRIzol (Gibco) light microscope (Leica DMLS, Leica) was connected with a personal computer; Biophotometer RS232C: Eppendorf.

One hundred and thirty-two rats were divided into five groups at random: group 1 (n = 36), OA rats that received placebo (sterile saline solution, 1 mL/day). Groups 2 and 3 (n = 24), OA rats treated with CCII (20 µg/day and 80 µg/day, namely 0.002% CCII solution 1 ml and 0.008% CCII solution 1 ml orally, respectively); group 4 (n = 24), OA rats given excipient (0.25% mannitol 1 ml/day, orally). Group 5 (n = 24), composed of normal rats, was used as the control group and received placebo similar to group 1. The domestic CCII dosages were selected based on the dosages of the drug given to patients for the treatment of symptomatic rheumatoid arthritis (RA). All treatments began on the day of operation and lasted for eight weeks. All rats were killed by neck disjoint one week after treatment finished. Half of these rats were used for morphological and immunohistochemical study, the rest for biomolecular study.

Specimen preparation

For morphometric and immunohistochemic study, specimens consisted of the whole right knee joints removed without opening the joint capsule. Each specimen was divided into two equal sections and fixed in 10% (v/v) formalin, decalcified with 10% (v/v) formic acid in formalin for 12 hours and embedded in paraffin. The anterior half was subjected to immunohistochemical observation. The posterior half was used for the bone histomorphometric analysis.

For RNA analyses, the articular cartilage tissue (including full depth of articular cartilage, synovium and some bone, size about 1.5 H 1cm) was harvested from each group after treatment finished. The samples were put in a tube filled with 0.7 ml TRIzol (Gibco) immediately and frozen in liquid nitrogen.

The sections (5 µm) of each specimen were subjected to haematoxylin and eosin staining. The bone morphology was performed in three nonconsecutive sections from each specimen. From each section, 3 representative fields (original magnification H 400) were identified by two pathologists. The average value of mankin score was calculated on each section.

Specimens were processed for immunohistochemical analysis thus: sections (5 µm) of paraffin-embedded specimens were placed on anti-shed slides, deparaffinized in xylene, rehydrated in a reversed graded series of ethanol and preincubated with chondroitinase ABC in phosphate-buffered saline (PBS) for 60 minutes at 37°C followed by further incubation in triton X-100 (0.3%) for 30 minutes. The specimens were then stained by ABC method according

to the manufacturer's instructions. To determine the specificity of staining, negative control was done by omitting primary antibody.

Each section was examined with a light microscope (Leica DMLS, Leica) and photographed with a HPIAS-1000 high clear pathology picture analysis system (9.0 version). The different antigen levels were quantified by determining the percentage of cells staining positive for the specific antigen. The microscopic fields examined were similar to those previously described for the morphometric analysis and the results from the three fields were averaged for each section. The data obtained from each specimen were considered as independent variables for the purpose of statistical analysis.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction was performed with Trizol according to the manufacturer's instructions. The concentration of RNA was measured with a spectrophotometer. RT-PCR was performed with equal amounts of RNA using access RT-PCR system (promega) according to the manufacturer's instructions. The PCR consisted of 40 cycles. PCR products were visualized on 2% agarose gel (containing 1% ethidium bromide). The analysis of PCR products was performed by determining the photo-density of electrophoresis strap and β-actin was used as internal control. The sequence for the primers and the conditions for their use are summarized in Table 1.

Table 1: Details of PCR primers used in this study

Gene	Temperature $^{\circ}$C	Primer sequences	bp
β-actin	54.5	5-cacccgcgagtacaaccttc-3	207
	54.4	5-cccatacccaccatcacacc-3	
MMP-13	62.36	5-ccctggagccctgatgtttt-3	142
	62.41	5-ctctggtgtttgggggtgct-3	
MMP-9	60.0	5-ggagcatggcaacggagacg-3	285
	60.8	5-gccccaacccacccacagac-3	
Cathepsin K 258	59	5-gccaccttcgcgttccttcagtaa-3	
	59	5-tagccgcctccacagccatagttc-3	
TIMP-1	60.8	5-gccccaacccacccacagac-3	256
	60.7	5-aacggcccgcgatgagaaac-3	

The Statistical packages for the Social Sciences (SPSS) software was used. The comparison among the different treatment groups was done using the analysis of variance (ANOVA) and unpaired student t test. *P* values < 0.05 were considered significant.

RESULTS

Therapeutic effect of CCII on the morphological changes of rat OA

Table 2: Effect of CCII on Mankin score (X ± s)

Group	n	Mankine score
Nor	12	1.555 ± 0.483
OA	18	6.723 ± 1.499**
CCII (20 µg.d^{-1})	12	5.188 ± 1.042*$^{\Delta\Delta}$
CCII (80 µg.d^{-1})	12	5.249 ± 1.583**$^{\Delta}$
Exipient (2.5 mg.d^{-1})	12	6.388 ± 0.621**

**p < 0.01 *vs* normal group; $^{\Delta}$p < 0.05 *vs* OA group; $^{\Delta\Delta}$p < 0.01 *vs* OA group

Fig. 1: Morphology of CCIIon articular cartilage of OA rats (HE staining, x 100)
A: Normal cartilage; B: Cartilage from OA rats without active treatment; C: Cartilage from OA rats treated with CCII80µg.d^{-1}

The articular cartilage in normal rats exhibited smooth surfaces with intact layers of flattened cells in the superficial zone and hypertrophic-like chondrocytes in the deep zone. The articular cartilage zone was found to be thick. The mankin score of normal rats was the lowest (Table 2, Fig. 1A). In OA rats without effective treatment, the articular cartilage exhibited very rough and uneven surfaces. Its superficial fibrils were exposed. Frequently, deep cracks were observed in the cartilage. The cartilage of OA rats was significantly eroded. In some sections, obvious cartilage loss and articular surface distortion were observed. In these rats, the mankin score was the highest (Table 2, Fig. 1B).

Table 3: Effect of CCII on the production of MMP-9, MMP13 and Cathepsin K (X ± s)

Group	n	MMP-9 (%)	MMP13 (%)	Cathepsin K (%)
Nor	12	4.533 ± 1.628	11.042 ± 1.675	5.683 ± 1.317
OA	18	24.400 ± 6.444**	26.522 ± 4.162**	25.556 ± 4.756**
CCII(20 µg.d^{-1})	12	17.317 ± 5.650**$^{\Delta}$	19.233 ± 3.978**$^{\Delta}$ $^{\Delta\#}$	20.592 ± 2.657**$^{\Delta\Delta}$
CCII (80 µg.d^{-1})	12	16.667 ± 6.556**$^{\Delta}$	23.750 ± 3.800**	22.042 ± 3.025**$^{\Delta}$
Exipient (2.5mg.d^{-1})	12	20.583 ± 8.186**	26.342 ± 4.325**	24.008 ± 3.875**

**p < 0.01 *vs* normal group; $^{\Delta}$p < 0.05 *vs* OA group; $^{\Delta\Delta}$p < 0.01 vs OA group; $^{\#}$P < 0.05 *vs* CCII (20 µg.d^{-1})
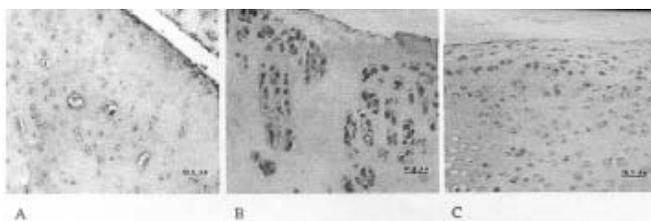
Fig. 2: Immunohistochemical staining of MMP-13 (ABC method, x 100)
A: Normal cartilage; B: Cartilage from OA rats without active treatment; C: Cartilage from OA rats treated with CCII80 μg.d⁻¹

cial fibrils of cartilage was also exposed (Table 2, Fig. 1E). In some sections, obvious distortion of cartilage surface was observed. The mankin score of this group exhibited no significant difference as compared to OA rats without effective treatment ($p > 0.05$).

Therapeutic effects of CCII on the immunohistochemical changes of OA. In normal articular cartilage, rare MMP-13 or Cathepsin K positive cells were observed. However, there was a marked increase in the positive chondrocytes in OA

Table 4: Effect of CCII on the special mRNA levels of articular cartilage (X ± s)

Group	n	MMP-9	MMP13	CathK	TIMP
Nor	12	51.17 ± 5.10	55.97 ± 16.57	46.61 ± 13.05	51.08 ± 9.48
OA	18	81.42 ± 6.05**	117.90 ± 12.27**	122.39 ± 13.07**	98.70 ± 8.58**
CCII (20 μg.d⁻¹)	12	70.10 ± 8.72**ΔΔ	67.44 ± 14.83ΔΔ	83.76 ± 9.09**ΔΔ	92.71 ± 3.54**
CCII (80 μg.d⁻¹)	12	61.04 ± 6.98**ΔΔ	66.30 ± 17.01ΔΔ	77.30±13.18**ΔΔ	93.32 ± 6.71**
Exipient	12	80.34 ± 7.79**	111.86 ± 11.68**	121.40 ± 18.79**	98.18 ± 6.33**

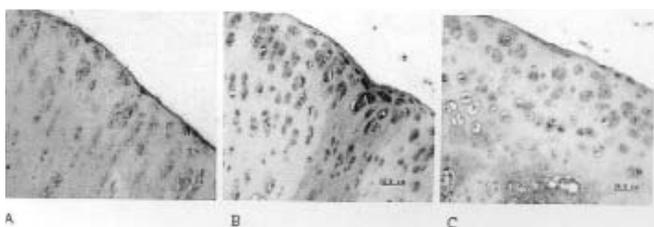**p < 0.01 *vs* normal group; ΔΔp < 0.01 *vs* OA group; #P < 0.05 *vs* CCII (20 μg.d⁻¹)

Fig. 3: MMP-9 immunohistochemical staining of MMP-9 (ABC method, x 400)
A: Normal cartilage; B: Cartilage from OA rats without active treatment; C: Cartilage from OA rats treated with CCII80 μg.d⁻¹

Administration of CCII (20 μg/d or 80 μg/d) beginning the day of operation made, the mankin score of OA cartilage decreased but it was still comparable to normal cartilage. Some roughness of cartilage surface could be observed in specimens from these rats (Table 2, Fig. 1C and D).
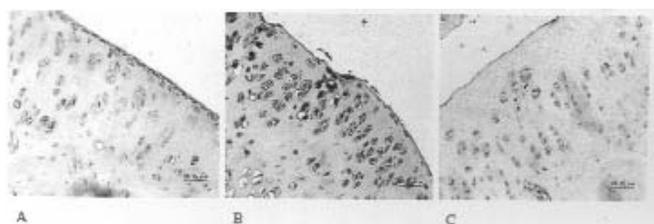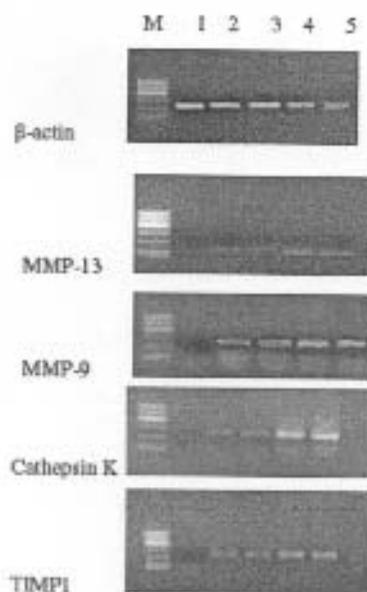

Fig. 4: Immunohistochemical staining of Cathepsine K (ABC method, x 400)
A: Normal cartilage; B: Cartilage from OA rats without active treatment; C: Cartilage from OA rats treated with CCII80 μg.d⁻¹

Occasionally, small cracks were observed in the cartilage surface. Excipient (1ml/d) exhibited no protective effect on delaying cartilage destruction. The articular cartilage of this group exhibited very rough and uneven surfaces, the superfi-

Fig. 5: CCII inhibits special mRNA level. Lane 1: normal rats; Lane 2: CCII80 μg.d⁻¹ Lane 4: Excipient 2.5 mg.d⁻¹; Lane 5: OA group; β-actin was used as control.

rats. The increase was particularly pronounced in the field near leisional areas of cartilage. The cells staining positive for MMP-13 were mainly chondrocytes of superficial layers while cathepsin K positive cells were mainly present in the superficial and deep zone of osteoarthritic cartilage. Some osteocytes were also staining positive for MMP-13 and Cathepsin K (data not shown). In specimens treated with CCII (20 μg.d⁻¹ or 80 μg.d⁻¹), the percentage of MMP-13 or Cathepsin K positive chondrocytes was significantly smaller

than that of OA rats without effective treatments ($p < 0.01$) but was comparable to the normal rats ($p < 0.01$). There was no significant difference in the percentage of MMP-13 or Cathepsin K positive chondrocytes between two CCII groups (CCII20 μg/d *vs* CCII80 μg/d, $p > 0.05$) while excipient showed no significant effect on these enzymes (Table 2, Fig 2, 3 & 4).

Special mRNA levels
Transcript levels for MMP-13, cathepsin K and tissue inhibitor of matrix metalloprotease 1(TIMP-1) from articular cartilage of rat osteoarthritic knee, CCII groups and non-operated control rats were analysed by semi-quantitative RT-PCR method. Fig 5 illustrates changes in mRNA levels of different groups. The mRNA level for MMP-13 or cathepsin K were significantly elevated in the articular cartilage of osteoarthritic rats (OA group *vs* control group, $p < 0.05$) while oral administration of CCII significantly reduced these changes, however the difference between two CCII groups was not significant. TIMP1 mRNA level also elevated significantly in OA cartilage, neither CCII nor excipient exhibited any effects on it.

DISCUSSION
It is generally accepted that in animal experiments, OA induced by surgical operation which lead to joint instability provide pathological conditions similar to human OA. As a result, operation-induced OA has been widely used for anti-osteoarthritic drug screening. Hulth's method of operation is most frequently used for OA study around the world. In Hulth's operation, the anterior and posterior cruciate ligaments (PCL) as well as the medial collateral ligament were severed, and part of the meniscus was resected. In our study, operation procedure was simplified by omitting PCL severing, so injury was lightened greatly because big vessels near PCL would escape being resected by mistake. Eight weeks after operation, OA was successfully induced in all operated rats similar to previous work (5) and the joint changes resembled that of human osteoarthritic joints.

The hallmark of OA is the imbalance of articular cartilage degradation and regeneration led by the imbalance of cartilage degrading proteases and their inhibitors which are regulated by various cytokines including pro-inflammatory and anti-inflammatory cytokines (7–9). The mechanism and pathological changes of OA resembles RA such as autoimmune response, excessive production of cartilage degradation, inflammation of synovial membrane, imbalance of cartilage destruction and regeneration. Inflammation in OA is not thought to be part of disease initiation but to be secondary and thereby, to contribute to disease progression and cartilage degradation (7–9). Cartilage degradation and bone destruction were thought to contribute to more autoantigen release and exposure which lead to further autoimmunity in OA, and autoimmunoreaction result in aggravating inflammation, so a vicious cycle forms. Different studies have

described some of the features of autoimmunity in OA patients and animals: raised levels of synovial fluid rheumatoid factors, immune complexes, antibodies to collagen type I and II (9–10). We postulate that CCII may have protective effect on OA. Domestic CCII was extracted from chicken sternal cartilage and purified by high pressure liquid chromotography. Its purity was determined using SDS-PAGE. Its immunogenicity titer, which was determined by ELISA in rabbit anti-serum that had been immunized by CCII previously, was 16 000. One feature of soluble dispersal mixture of domestic CCII is that it can be dissolved in saline solution or water because it was manufactured and prepared by special technique. Another feature of domestic CCII dispersal mixture is that its immunogenicity or antigenicity, which was assayed with ELISA, is very stable and can be stored at room temperature. So it would be an ideal drug for OA treatment that can be stored and administered expediently if it had protective effect on OA cartilage. The study of CCII for RA treatment has been well described. However, rarely have studies been seen on the effect of domestic CCII for OA treatment **in** animals or patients. So the morphologic study was done to explore the possible effect of domestic CCII on OA. Our data showed that oral administration of CCII delayed the degradation of articular cartilage in osteoarthritic rats. CCII in the dosage of 20 μg/d showed the same effect as 80 μg/d in delaying the cartilage degradation (CCII20 μg/d *vs* CCII80 μg/d, $p > 0.05$) but the mechanism by which CCII exhibited its protective effect remains unknown.

From previous study, matrix degrading proteases play a key role during OA. Among these proteases, cathepsin K and MMP-13 posess a strong scavenging effect for type I and II collagen as well as other cartilage matrix components (11–14) while MMP-9 has strong scavenging effect to type IV and V collagen (7, 19–23). The amount of active MMP-13 is directly related to the degree of cartilage degradation in OA (11–13). Cathepsin K is known to work in conjuction with MMP-13 in the induction of bone resorption (14–15). MMP-9 was secreted by macrophagocytes as zymogen and activated in chondrocytes by MMP-13 and (or) cytokines (7, 21). So the immunohistochemistry of MMP-13, MMP-9 and Cathepsin K was done to explore the mechanism by which OA was downregulated following gastric administration of CCII.

This study showed that there were few MMP-9, MMP-13 or cathepsin K positive chondrocytes in normal cartilage, but the percentige of MMP-13, MMP-9 or cathepsin K positive chondrocytes increased significantly in OA cartilage, and it was about 9, 5 and 6 fold higher than that of normal cartilage respectively. Chondrocytes of superficial cartilage were the main source of MMP-13 supply. The distribution of MMP-9 positive chondrocytes was well in line with that of MMP-13. Cathepsin K is mainly present in the superficial and deep zone of OA cartilage. The percentage of MMP-13, MMP-9 or Cathepsin K positive chondrocytes was closely correlated to the severity of OA that was evaluated by mankin

score (r = 0.94, 0.93 and 0.96 respectively). This was well in line with Hellio and Konttinen YT's study (4–5, 9). The results of biomolecular study was consistent with that of immunohistochemistry, which illustrated that MMP-13, MMP-9 or Cathepsin K positive chondrocytes elevated in osteoarthritic cartilage were due to their mRNA level increasing. In addition, mRNA level for the tissue inhibitor of matrix matelloproteinases (TIMP)-1 was examined. Data from this study showed that the mRNA level for TIMP-1 was elevated also in OA rat cartilage. This may probably in part counteract the effect of MMP-13 and Cathepsin K, and as a result restrain articular cartilage degradation. But the increasing of TIMP-1 mRNA was not so predominant as MMP-13, MMP-9 and cathepsin K, so it was not enough to contrast the effect of the protease on cartilage degradation during OA. Administration of CCII decreased the mRNA level for these three proteinase but not for TIMP-1, so CCII contributed to the correction of the imbalance between collagen degradation enzymes and their inhibitor. This would be a reasonable explanation for delaying the degradation of articular cartilage from OA rats. The flaw of this study is that the immunohistochemistry of TIMP-1 had not been done so the protein products of TIMP-1 mRNA remain unknown. The data suggests that CCII exhibits protective effect on osteoarthritic cartilage at least in part by reducing the increased synthesis of MMP-13, MMP-9 and Cathepsin K (three major proteases involved in the degradation of articular cartilage during OA) and confirmed the notion that MMP-13, MMP-9 and cathepsin K are present in increased amounts in osteoarthritic cartilage.

In summary, the results presented in this study indicate that oral administration of CCII delayed but did not completely block the degradation of articular cartilage in OA rats. MMP-13, MMP-9 or cathepsin K positive chondrocytes increased in articular cartilage of OA rats and the chondrocytes of superficial cartilage were the main source of MMP-13 supply while cathepsin K was mainly present in the superficial and deep zone of osteoarthritic cartilage. The distribution of MMP-9 positive chondrocytes was the same as MMP-13. Oral administration of CCII reduced MMP-13, MMP-9 and cathepsin K level significantly by inhibiting their transcription but had no significant effect on TIMP-1 mRNA, which contributed to correcting the imbalance between collagen degradation enzymes and their inhibitors. As a result, oral CCII contributed to delaying the degradation of osteoarthritic cartilage. This study shows the important anatomical, biochemical and biomolecular changes that take place in OA joints and the effect of CCII on these changes. The exact mechanism by which CCII reduced the OA cartilage damage remains uncertain and cannot be answered within the scope of this study. However, it is interesting to note that treatment with domestic CCII could effectively reduce cartilage damage. The data obtained in this study suggested that CCII may be a potent drug candidate for OA treatment.

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