The Effects of Undenatured Type II Collagen on Inflammatory Mediators and Oxidative Stress in an Osteoarthritis Rat Model

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Abstract. The present study aimed to investigate the effects of undenatured type II collagen on monosodium iodoacetate-induced osteoarthritis (OA) rats, and elucidate its underlying anti-inflammatory and antioxidant mechanisms. Several parameters such as body mass, pain threshold of knee joint, supporting force of the left and right feet, serum levels of inflammation mediators and cartilage metabolic marker, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity and knee joint histopathology were measured. The results demonstrated that undenatured type II collagen enhanced the pain threshold of knee joint and reduced the difference in supporting force between the left and right feet. Moreover, undenatured type II collagen increased serum TGF-β concentration and decreased the serum levels of TNF-α, MMP-13 and CTX-II in OA rats. In addition, decreased serum MDA content and increased serum SOD activity were found in undenatured type II collagen treatment group when compared to those in model group. Histopathological data indicated that undenatured type II collagen exhibited protective effects on both synovial and cartilaginous tissues in OA rats. Taken altogether, the findings of this study reveal that undenatured type II collagen can relieve and prevent OA symptoms by regulating inflammatory mediators and oxidative stress. Additionally, this collagen helps to reduce knee OA pain and maintain normal joint function.

Keywords: Undenatured Type II Collagen, Osteoarthritis, Inflammatory Mediators, Oxidative Stress

1. Introduction
Osteoarthritis (OA) is a chronic joint disease characterized by the degeneration, destruction and hyperosteoogenesis of articular cartilage. At present, dietary supplements such as glucosamine, chondroitin sulfate and frankincense as well as turmeric and fish oil are commonly used to reduce OA symptoms. However, these dietary supplements possess several limitations [1].
Since the 1990s, undenatured type II collagen has been used for the treatment of rheumatoid arthritis [2], and its role in reducing OA symptoms is studied subsequently [3, 4]. Several clinical trials have reported that undenatured type II collagen exerts greater efficacy compared to glucosamine and chondroitin [5, 6], significantly improves knee extension after 4 months [7] and reduces WOMAC in OA patients [8]. A more recent study has demonstrated that undenatured native chicken type II collagen can diminish the deterioration of articular cartilage in a rat OA model induced by partial medial meniscectomy [9]. Undenatured type II collagen has the advantages of being convenient, safe and non-toxic, as well as high antigenic specificity. It therefore has the potential to be a revolutionary functional ingredient for the prevention and treatment of OA.

2. Materials and Methods

2.1. Materials
Undenatured type II collagen supplied by Beijing Semnl Biotechnology Co., Ltd. (China) was extracted from chicken thoracic cartilage at a low temperature using Semnl patent technology (lot number: 20171120). Glucosamine and chondroitin sulfate were purchased from Zhejiang AOXING Biotechnology Co., Ltd. (China). Monosodium iodoacetate (CAS number: 305-53-3; lot number: C10111711) was obtained from Shanghai Macklin Biochemical Co., Ltd. (China). The ELISA kits for TNF-α, IL-1β, IL-6, MMP-13, CTX-II, TGF-β, IL-4 and IL-10 (lot number: Mar2018) were supplied by Shanghai Enzyme-linked Biotechnology Co., Ltd. (China). Antibodies were obtained from Abcam (USA). Malondialdehyde (MDA) kit (batch number: 20180414) and total superoxide dismutase (SOD) kit (batch number: 20180412) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.2. Experimental Animals and Feeding Conditions
Male SPF SD rats weighing 200-230 g were purchased from Changsheng Bio-technology Co., Ltd. (China). The animal certification number is 211002300027659, while the laboratory animal license number is SCXK (Liao) 2015-0001. The rats were kept in the animal room (barrier system) of Jilin Academy of Traditional Chinese Medicine. The license number for these experimental animals is SYXK (Ji) 2015-0009. The animal room was maintained at 19-23°C, 40-65% relative humidity and 12:12 h light/dark cycle. After sterilization by autoclaving, all cages and covers were replaced 2-3 times a week.

The feed was purchased from Beijing Keao Xieli Feed Co., Ltd. (China) with a production license of Jing (2014) 06054 (lot numbers: 18013221 and 18033211). Pure water was prepared by the KFL-400 water filter system of Kflow Environmental Technology Co., Ltd. (China), and then placed into sterilized water bottles prior to rat consumption. The rats adapted to the environment for three days were used in the subsequent experiments.

This study was approved by the Experimental Animal Ethics Committee of Jilin Academy of Traditional Chinese Medicine, and was conducted in accordance with the regulations of the same committee. The approval number obtained from the Animal Ethics Committee is JLSZKYDWLL2018-004.

2.3. Joint Pain Screening and Animal Grouping
To ensure the objectivity of this experiment, rats with the same pain tolerance (400-650 g) were selected using the joint stress test. The right joint of each rat was fixed on the YLS-3E electronic tenderness-meter. Specifically, top-down pressure from the flat head was applied on the right knee joint, and the values of pain threshold were determined by animal hoarseness after pain. The measurements of tenderness were recorded when the animals reached the pain threshold. Rats with an extremely high or low pain threshold value were excluded, while those with a threshold value of 400-650 g were selected for further analyses.
All rats were grouped according to their joint pressure-pain thresholds. Subsequently, the rats were divided into five groups: control group (n=12), model group (n=12), glucosamine + chondroitin sulfate group (n=12), high-dose (n=12) and low-dose (n=13) undenatured type II groups.

2.4. Model Establishment and Dose Selection
Apart from the 12 rats in control group, the remaining rats were intraperitoneally anesthetized with 2% isoflurane. Before injection, the hair on the right knee joint was removed, the operation theater was sterilized; a 0.5 cm incision was made to expose the musculoskeletal tissue. A microsyringe needle was then injected into the knee joint cavity through the medial patellar tendon, and 25 uL of sterile normal saline containing 40 mg/mL monosodium iodoacetate was injected. Finally, suturing of the skin incision was carried out.

The conversion of animal doses to human equivalent doses was performed based on body surface area, and the resultant dose level was adopted as an integer value. The dose of glucosamine + chondroitin sulfate was 309 mg/kg BW + 103 mg/kg (two times a day), which is equivalent to glucosamine (1500 mg/day) + chondroitin sulfate (500 mg/day) for a 60 kg human. The doses of undenatured type II collagen were 4 and 8 mg/kg in low- and high-dose treatment groups, respectively, which are equivalent to 40 mg/day and 80 mg/day for a 60 kg human. The volume of gastric perfusion was 10 mL/kg for all rats. Before gavage administration to the rats, all treatment solutions were freshly prepared with 0.5% sodium carboxymethyl cellulose and mixed well.

Undenatured type II collagen was administered daily to the rats on the day of model establishment for five weeks. All rats were weighed once a week, and the dosage was adjusted according to the changes in body weight.

2.5. Observation Index Measurement
Behavioral experiments were carried out after four weeks of treatment. Following euthanasia, blood samples were collected from the abdominal aorta of rats. Serum was then separated from the whole blood by centrifuging at 3000 r/min for 10 minutes, and the serum indices were then measured. The right hind legs of rats were amputated by femur and middle tibia, followed by the dissection of residual gastrocnemius muscle, quadriceps femoris muscle and posterior femoris muscle. The intact structure of knee joint was exposed and fixed with 4% paraformaldehyde for further pathological analysis.

2.5.1. Determination of Bipedal Support Balancing In Rats
The upper body of the rat was fixed in a small fixator, while the two hind feet were placed on the left and right pedals of the supporting force measurement device. The instrument readings were recorded when the rat was standing up naturally, and the force difference between the left and right pedals was calculated.

2.5.2. Tenderness Test for the Knee Joint of Rats
The right joint of the rat was placed on the YLS-3E electronic tenderness instrument. Specially, top-down pressure from the flat head was applied on the right knee joint of the rat. The values of pain threshold were determined according to the animal hoarseness after pain. The readings on tenderness meter were recorded when the animals reached their pain thresholds.

2.5.3. Determination of Relative Indices
The serum levels of TNF-α, IL-1β, IL-6, MMP-13, CTX-II, TGF-β, IL-4 and IL-10 in rats were determined using the commercial ELISA kits. The serum content of MDA in rats was assessed by the thiobarbituric acid method, while the serum activity of SOD in rats was detected using the hydroxylamine method.

2.6. Morphological and Histopathological Examination of Rat Cartilage
All sections were examined after systematical evaluation and analysis of the articular cartilaginous and synovial tissues (Table 1). Both histopathological analysis and scoring of cartilaginous tissues were performed according to the Mankin scoring system (Table 2).

**Table 1. Pathological scoring criteria for synovial tissues**

| Synovial tissue structure          | 0               | 1                  | 2                  | 3                  |
|-----------------------------------|-----------------|--------------------|--------------------|--------------------|
| Inflammatory cells                | None            | Sparse and scattered | Quite dense | Massive            |
| Fibrous tissue proliferation      | None            | Few proliferation  | Medium            | Massive            |
| Synovial tissue hyperplasia       | None            | Monolayer          | 2 layers          | 3 layers           |
| Macrophage proliferation          | None            | Sparse             | Quite dense       | Massive            |

### 2.7. Data Analysis

The results were presented as mean ± standard deviation (SD). One-way (single-factor) analysis of variance (ANOVA) was used to compare the differences among groups by using SPSS software (SPSS, version 19.0, IBM Inc., USA). Least significant difference (LSD) test was used for comparison between groups. P values of less than 0.05 were considered statistically significant.

**Table 2. Mankin scoring standards for articular cartilage**

| Cartilaginous structure                     | Smooth as usual | Surface irregular fissures | Fractures reach the migration layer | Fractures reach the radiation layer | Fractures reach the calcified layer | Exfoliation of cartilage layer |
|---------------------------------------------|-----------------|---------------------------|-------------------------------------|-------------------------------------|------------------------------------|-------------------------------|
| Score                                       | 0               | 1                         | 2                                   | 3                                   | 4                                  | 5                             |
| Chondrocytes                                | Amount as usual | Diffuse increase in quantity | Existence of a large number of cluster-like cell aggregates | Significant reduction in quantity |                                    |                               |
| Score                                       | 0               | 1                         | 2                                   | 3                                   |                                    |                               |
| Tidal line integrity                        | Intact          | Multiple tidal line       | Tidal line of subchondral vascular invasion |                                    |                                    |                               |
| Score                                       | 0               | 1                         | 2                                   |                                    |                                    |                               |

### 3. Results

#### 3.1. Effects on the Body Weight of Rats

**Table 3. Effects on the body weight of rats**

| Group                  | Body weight (g) | Rat body weight at different time point after treatment (g) |
|------------------------|-----------------|-----------------------------------------------------------|
|                        |                 | First week       | Second week      | Third week        | Forth week      | Fifth week      |
| Control                | 268.92 ± 12.96**| 324.30 ± 14.63** | 366.38 ± 19.22* | 397.85 ± 27.41*  | 419.52 ± 34.16 | 446.00 ± 34.16 |
| Model                  | 246.65 ± 11.10  | 302.58 ± 24.81   | 351.33 ± 26.45   | 389.87 ± 24.01   | 420.52 ± 24.18 | 435.29 ± 24.18 |
| Glucosamine + chondroitin sulfate | 243.65 ± 10.55 | 298.75 ± 24.81   | 342.08 ± 26.45   | 380.87 ± 24.01   | 403.92 ± 24.18 | 418.00 ± 24.18 |
| High-dose              | 240.33 ± 9.77   | 302.58 ± 14.34   | 351.33 ± 19.24   | 389.08 ± 23.78   | 420.15 ± 32.33 | 434.24 ± 30.90 |
| Low-dose               | 243.29 ± 12.91  | 294.85 ± 22.32   | 339.73 ± 29.25   | 374.55 ± 28.66   | 400.05 ± 28.46 | 417.35 ± 28.39 |

Note: * P<0.05, ** P<0.01; compared with the model group.
At the end of the fifth week, the body weight of rats was not significantly different between control and model groups. Notably, an intragastric administration of 4 or 8 mg/kg undenatured type II collagen once daily for five weeks did not markedly affect the body weight of OA rats. Likewise, glucosamine + chondroitin sulfate demonstrated no significant effect on the body weight of OA rats. These results can be seen in Table 3.

3.2. Effects on the Pressure Pain Threshold and Foot Supporting Force of Rat Knee Joints
In comparison with model group, the pressure pain thresholds of rat knee joints were increased in low- and high-dose undenatured type II collagen treatment groups ($p<0.05$). When compared with the model group, the supporting forces between left and right feet were markedly reduced in high-dose undenatured type II collagen treatment group and glucosamine + chondroitin sulfate group, and the differences were statistically highly significant ($p<0.01$) and statistically significant ($p<0.05$), respectively. These results can be seen in Table 4.

| Group                               | Initial pain threshold (g) | Pain threshold at the fifth week (g) | Supporting force difference between the left and right feet (g) |
|-------------------------------------|---------------------------|-------------------------------------|---------------------------------------------------------------|
| Control                             | 460.6±74.70               | 800.0±161.61**                      | 6.97±7.15**                                                  |
| Model                               | 458.9±59.42               | 465.4±123.51                        | 69.39±19.92                                                  |
| Glucosamine + chondroitin sulfate   | 460.6±54.40               | 484.5±139.91                        | 52.40±11.59*                                                |
| High-dose                           | 462.0±81.48               | 552.5±129.21*                       | 46.04±18.13**                                               |
| Low-dose                            | 460.3±67.04               | 585.5±145.41*                       | 56.58±15.62*                                                |

Note: * $p<0.05$, ** $p<0.01$; compared with the model group.

3.3. Effects on the Serum Levels of IL-1β, IL-6, TNF-α and IL-4
The serum levels of IL-1β, IL-6 and IL-4 were not significantly affected ($p>0.05$) by high- and low-dose undenatured type II collagen as well as glucosamine + chondroitin sulfate. As shown in Table 5, the serum levels of TNF-α were significantly decreased in high- and low-dose undenatured type II collagen treatment groups ($p<0.01$ and $p<0.05$, respectively) compared to model group. Nonetheless, the serum level of TNF-α did not differ significantly between glucosamine + chondroitin sulfate group and model group ($p>0.05$).

| Group                               | IL-1β (Pg/mL) | IL-6 (Pg/mL) | TNF-α (Pg/mL) | IL-4 (Pg/mL) |
|-------------------------------------|---------------|--------------|---------------|--------------|
| Control                             | 7.62±2.33     | 31.69±7.87   | 37.47±10.82*  | 24.77±6.69   |
| Model                               | 9.15±2.58     | 31.13±10.06  | 46.50±8.76    | 22.47±6.78   |
| Glucosamine + chondroitin sulfate   | 8.44±1.56     | 30.41±6.64   | 40.15±17.13*  | 22.90±4.37   |
| High-dose                           | 9.72±3.32     | 32.75±9.53   | 34.22±13.86   | 23.46±3.60   |
| Low-dose                            | 10.17±3.80    | 31.66±7.84   | 36.68±13.86*  | 23.44±5.85   |

Note: * $p<0.05$, ** $p<0.01$; compared with the model group.

3.4. Effects on the Serum Levels of IL-10, TGF-β, MMP-13 and CTX-II
The serum levels of IL-10 were not significantly affected ($p>0.05$) by high- and low-dose undenatured type II collagen as well as glucosamine + chondroitin sulfate. Notably, the serum levels of TGF-β in high- and low-dose undenatured type II collagen treatment groups were significantly higher than those in model group ($p<0.01$ and $p<0.05$, respectively), while glucosamine + chondroitin sulfate administration did not affect the serum level of TGF-β ($p>0.05$). Moreover, low-dose undenatured type II collagen significantly reduced the serum levels of MMP-13 as compared to model group ($p<0.01$), while both high-dose undenatured type II collagen and glucosamine + chondroitin sulfate
exhibited no significant effect on the serum levels of MMP-13 in OA rats. Furthermore, high-dose undenatured type II collagen significantly reduced the serum level of CTX-II compared to model group ($P<0.05$), while the serum levels of CTX-II were not significantly affected by glucosamine + chondroitin sulfate or low-dose undenatured type II collagen treatment in OA rats. All these results are demonstrated in Table 6.

| Group                        | IL-10 (Pg/mL) | TGF-β (Pg/mL) | MMP-13 (ng/mL) | CTX-II (ng/mL) |
|------------------------------|---------------|---------------|----------------|----------------|
| Control                      | 15.23±3.09    | 62.22±32.65   | 43.76±10.49    | 4.23±1.74      |
| Model                        | 13.58±3.71    | 76.57±33.66   | 53.52±9.48     | 5.27±1.32      |
| Glucosamine + chondroitin sulfate | 13.52±2.16    | 89.29±33.57   | 48.76±10.04    | 4.76±1.10      |
| High-dose                    | 15.38±1.92    | 103.51±26.19* | 48.64±7.83     | 4.02±1.24*     |
| Low-dose                     | 15.18±2.76    | 119.09±35.41**| 43.12±8.96**   | 4.49±1.40      |

Note: * $P<0.05$, ** $P<0.01$; compared with the model group.

3.5. Effects on the Histopathological Alterations of Knee Joint Synovial and Articular Cartilage Tissues

The synovial cells in control group were translucent and arranged in a single flat shape, with no vascular hyperplasia, fibrosis or inflammatory cell infiltration. The HE-stained cartilage matrix was pinkish, while chondrocytes were blue. Specifically, the surface of articular cartilage tissue was smooth and regular, its structure was normal with no fracture, and the cells were arranged neatly. Besides, the chondrocytes were divided into four distinct layers: the surface, transitional, radiation and calcification layers. These layers were clear and the tidial lines were obvious.

In model group, synovial cells were multilayered, irregularly arranged and proliferated actively. The synovial capillary was proliferated and expanded, while the fibrous tissue was proliferated with a large number of infiltrating inflammatory cells and fibroblasts. In addition, the chondrocytes were arranged irregularly, while the cartilage surface was rough with fractures and reached the calcified layer. Moreover, the pyknotic and necrotic chondrocytes could be observed around the fissures. The layers of cartilage were not in order, while the chondrocytes were clustered into small aggregates and their shape was round and polygonal. In comparison with control group, the model group displayed obvious knee joint inflammation, which was mainly manifested by the destruction and collapse of articular cartilage tissue as well as synovial tissue proliferation and inflammatory response.

Fig 1. Pathological images for the synovial tissue of knee joint in each group.

(a) Synovial tissue in control group (x100) were smooth and continuous, without inflammatory reactions. (b) In the model group (x100), the synovial tissue was expanded and protruded, with the presence of inflammatory cell infiltration and edema. (c) In glucosamine + chondroitin sulfate group (x100), synovial tissue proliferation and tissue edema were observed, with a reduced inflammatory cell infiltration. (d) In high-dose undenatured type II collagen group (x100), proliferative synovial cells were found, while the inflammatory cells and tissue were infiltrated and expanded, respectively. (e) In low-dose undenatured type II collagen group (x100), tissue edema was subsided, inflammatory cell infiltration was decreased and histiocytic proliferation was noted in synovial tissue.
In glucosamine + chondroitin sulfate group, the degree of articular cartilage lesion, joint bone destruction, cell proliferation, and synovium swelling, hyperemia and edema were all alleviated when compared to model group (Fig. 1 and 2). Additionally, the numbers of infiltrating inflammatory cells and proliferative capillaries in glucosamine + chondroitin sulfate group were lower than those in model group. Furthermore, the pathological analysis revealed that glucosamine + chondroitin sulfate was effective for relieving synovial tissue inflammation in OA rats (P<0.05), but not articular cartilage tissue (Table 7).

Table 7. Pathological scores of knee joint synovial and articular cartilage tissues in each group

| Group                        | Pathological score for synovial tissue | P   | Pathological score for articular cartilage tissue | P   |
|------------------------------|----------------------------------------|-----|--------------------------------------------------|-----|
| Control                      | 0.12±0.35                              | 0.000 | 0.00±0.00                                       | 0.000 |
| Model                        | 9.25±2.92                              | 5.12±0.83 | 4.25±1.83                                       | 0.512 |
| Glucosamine + chondroitin sulfate | 5.75±1.83*                  | 0.012 | 3.50±0.81*                                       | 0.035 |
| High-dose                    | 5.62±1.56*                             | 0.019 | 3.62±0.92*                                       | 0.040 |
| Low-dose                     | 6.12±1.90*                             | 0.043 |                                                  |      |

In high-dose undenatured type II collagen group, the degree of synovial hyperplasia was markedly reduced, including the decreased fibrous tissue proliferation, inflammatory cell infiltration and synovial tissue telangiectasia and hyperemia (Fig. 1). Macrophage proliferation and aggregation could be observed in synovial tissue. When compared with the synovial tissue score of model group, high-dose undenatured type II collagen treatment significantly attenuated the inflammatory response of synovial tissue (P<0.05; Table 7). As illustrated in Fig. 2, the surface of cartilage tissue in high-dose undenatured type II collagen group was rough. Moreover, the dehydrated and pyknotic chondrocytes could be observed in this treatment group. The cartilage layer was not in order, and the chondrocytes were clustered into small aggregates with circular and polygonal shapes. Moreover, some fractures could be observed on the cartilage surface. The pathological analysis indicated that a high dose of undenatured type II collagen could effectively improve the degenerative change and injury of articular cartilage tissue (P<0.05).

Fig 2. Pathological images for the articular cartilage tissue of knee joint in each group. 
(a) The surface of articular cartilage tissue in control group (x100) was smooth and continuous, with no fracture, abnormality or massive cell proliferation. (b) In model group (x100), articular cartilage surface was damaged and partially missing, its structure was collapsed, with a decrease in chondrocyte proportion. (c) In glucosamine + chondroitin sulfate group (x100), chondrocyte proliferation with superficial fraction was found on the surface of articular cartilage. (d) In high-dose undenatured type II collagen group (x100), the continuity of cartilage surface was excellent with no obvious collapse, while clustered chondrocyte aggregation was observed in the cartilage tissues. (e) In low-dose undenatured type II collagen group (x100), the amount of articular chondrocytes was reduced, while the articular surface was continuous and smooth.

In low-dose undenatured type II collagen group, the degree of synovial hyperplasia was slightly alleviated, including the reduced inflammatory cell infiltration and slight fibrous tissue hyperplasia, as well as synovial tissue hyperemia and capillary dilatation (Fig. 1). Macrophage proliferation and
aggregation could be found in synovial tissue. In comparison with the synovial tissue score of model group, low-dose undenatured type II collagen could, to some extent, improve the inflammation of synovial tissue. As presented in Fig. 2, the cartilage surface was rough, and the dehydrated and pyknotic chondrocytes were observed. Additionally, the cartilage layers were not in order, and the chondrocytes tended to be clustered in small aggregates with circular and polygonal shapes. Fractures could be seen on the cartilage surface, and the depth of cartilage destruction was reduced. The pathological analysis indicated that a low dose of undenatured type II collagen could effectively improve the degenerative change and injury of articular cartilage tissue ($P<0.05$).

4. Discussion

Age is the most important risk factor for the onset of OA. As a consequence of aging population growth, there is an urgent need to discover potential raw materials for the treatment of OA symptoms. Undenatured type II collagen has been approved as a general food product in China [10]. In Japan, it has been used as a functional food for joint support [11]. Undenatured type II collagen can be used to enhance joint health, relieve joint pain, promote joint flexibility, prevent OA symptoms [12], and improve the quality of life, physical and mental health of OA patients. Therefore, it has the potential to become a revolutionary functional raw material for reducing the symptoms of arthritis, with broad market development prospects. The possible mechanism of undenatured type II collagen for OA improvement may be through oral immune tolerance [13, 14]. Notably, undenatured type II collagen can be processed by antigen-presenting cells and presented by major histocompatibility complex to activate regulatory T cells in Peyer’s patches (a group of gut-associated lymphoid tissues). However, thus far, no systematic study has focused on the effects of different doses of undenatured type II collagen on inflammatory mediators and oxidative stress as well as the pathophysiology of synovium and cartilage tissues in OA rats.

In this study, undenatured type II collagen and glucosamine + chondroitin sulfate appeared to decrease the supporting force difference between the left and right feet of OA rats, suggesting that these two treatments can alleviate OA knee pain and help maintain body balance. Additionally, undenatured type II collagen could increase SOD activity and decrease MDA content, however, glucosamine + chondroitin sulfate could only decrease MDA content. This indicates that undenatured type II collagen is able to enhance the body's antioxidant capacity. The findings of this study are consistent with those of previous studies [4, 6]. Gupta et al. [6] have studied dogs with moderate OA, and their results demonstrate that chicken undenatured type II collagen, glucosamine + chondroitin sulfate, and chicken undenatured type II collagen + glucosamine + chondroitin sulfate can all reduce OA pain. However, the pressure plate test used in this study indicated that only chicken undenatured type II collagen could reduce OA-induced pain, indicating that its mechanisms may be different from those of glucosamine and chondroitin sulfate. It was also found that undenatured type II collagen and glucosamine + chondroitin sulfate could decrease MDA activity. David et al. [4] have evaluated the roles of orally administered undenatured type II collagen and glucosamine + chondroitin sulfate in reducing OA symptoms, by applying the Western Ontario and McMaster Universities (WOMAC), visual analogue scale (VAS) and Lequesne methods [4]. They found that the effectiveness rates of undenatured type II collagen, glucosamine and chondroitin sulfate for reducing OA symptoms were 33-14%, 40-15.4% and 20-6%, respectively. In addition, Lugo et al. [8] have compared the effects of orally administered undenatured type II collagen and glucosamine + chondroitin sulfate on OA symptoms in a randomized, double-blinded, multi-centered study using the WOMAC, VAS and Lequesne methods, and achieved similar results.

OA is characterized by degeneration of articular cartilage and degradation and the mechanism is not very clear. Many studies have been proved that cytokines and inflammatory mediators play an important role in OA [13, 15]. It is worth noting that TNF-α can promote inflammation, while TGF-β can inhibit inflammation. For the first time, the results of this study demonstrated that undenatured type II collagen significantly reduced TNF-α level and increased TGF-β level in animal model, suggesting that it can alleviate inflammation by regulating inflammatory cytokine levels. In addition,
we found that undenatured type II collagen could protect synovial and cartilage tissues in rats with OA, which are also consistent with the results of Bagi et al. [9]. There were few reports about the changes in other inflammatory cytokines after oral undenatured type II collagen treatment. For instance, Ausar et al. have reported that the level of TNF-α is decreased after 12 weeks of oral undenatured type II collagen administration in OA patients. Carina et al. have shown that undenatured type II collagen and chitosan-fed rats exhibit a reduced secretion of IL-2 and an increased production of IL-10 in Peyer’s patches and spleen, respectively, as well as increased mRNA levels of TGF-β, IL-4 and IL-10 in Peyer’s patches. In our study, there were no significant alterations of IL-1β, IL-6, IL-4 and IL-10 levels in treatment groups compared to control group, probably due to the following three reasons: (1) Serum inflammatory markers may reflect the whole-body condition of OA, and local small-scale injury has little effect on body serum. (2) It took four weeks to detect these immune factors and the rats may have entered the recovery stage during experimentation, hence these indicator levels may be elevated initially but declined to normal after the acute stage. (3) Monosodium iodoacetate, collagenase or other induction methods of OA animal models may be relatively different from human OA. Osteoarthritis is characterized by the degenerative changes in joints among middle-aged and elderly population, which tends to deteriorate over time. However, the OA rats are caused by external stimulation, and their states will get better by time.

This study systematically examined the therapeutic roles of undenatured type II collagen in OA rat model, and found for the first time that it could reduce TNF concentration and elevate the levels of TGF, MMP-13 and CTX-II, which have not been reported in previous studies. TNF-α can not only induce the production of reactive oxygen species (ROS) and lead to cellular injury, but also inhibit the expression of SOD1 and SOD3 and reduce the concentration of SOD [3]. Excessive ROS accumulation will lead to the decrease of type II collagen and proteoglycan in the extracellular matrix, destroy the structure of collagen network, and make the extracellular matrix lose the ability to support and protect chondrocytes as well as the stress-carrying function. After taking undenatured type II collagen, the concentration of TNF-α in the body decreased significantly. The problem of insufficient SOD expression caused by arthritis was so alleviated. At the same time, due to the significant increase of TGF-β concentration, the abnormal apoptosis of chondrocytes caused by oxidative stress was reduces, and the mitosis of osteoblasts was promoted. This may be another reason why the SOD content significantly increased in vivo. MDA is a kind of aldehydes produced in the process of lipid peroxidation caused by free radicals. Its content is closely related to the degree of lipid peroxidation. The decrease of MDA content reflects the lipid peroxidation caused by adverse stimulation was relieved. These findings strongly support that the mechanism underlying the effects of undenatured type II collagen is bystander suppression, which represents one type of oral immune tolerance. In addition, undenatured type II collagen could lower the pain difference between the left and right joints, and repair the damaged synovial membrane and articular cartilage tissue. However, the dose-dependent effects of undenatured type II collagen seem to be not significant. Thus, further experiments are needed to analyze its dose effects on OA patients, in order to provide a reference value for human.

5. Conclusions
This study investigated the effects of undenatured type II collagen on monosodium iodoacetate-induced OA rats by assessing their body weight, knee joint pain, left and right foot supporting force, serum inflammation and cartilage metabolic markers, MDA content, SOD activity and knee joint histopathological changes. The results indicated that undenatured type II collagen exerted no significant effect on rat body weight, but tended to increase the pain threshold of OA knee joints and reduce the supporting force difference between the left and right feet of OA rats. In addition, undenatured type II collagen increased TGF-β concentration and deceased the serum levels of TNF-α, MMP-13 and CTX-II in OA rats. Moreover, it decreased the serum content of MDA and increased the serum activity of SOD in OA rats. Histopathological data revealed that undenatured type II collagen exhibited protective effects on synovial and cartilage tissues in the knee joints of OA rats. These
findings suggest that undenatured type II collagen can relieve and prevent OA symptoms by regulating inflammatory mediators and oxidative stress, and may help to reduce knee OA pain and maintain normal joint function.

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