Original Article

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Hyaluronic acid in combination with chondroitin sulfate and hyaluronic acid improved the degeneration of synovium and cartilage equally in rabbits with osteoarthritis

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ABSTRACT: The purpose of this study was to compare the chondroprotective effects of chondroitin sulfate (CS)-hyaluronic acid (HA) (CS-HA) injection and HA injection in an experimental model of osteoarthritis. After induction of osteoarthritis in rabbits, 28 rabbits were randomized into four groups: control group, 'HA' group, 'CS' group, and 'CS-HA' group. After 7 days, rabbits in the control group, 'HA' group, 'CS' group and 'CS-HA' group were respectively treated with normal saline, HA, CS, or CS-HA injection in the knees. All animals were treated once weekly. The animals were treated continuously for 5 weeks. Histological and biochemical evaluations were performed. As shown by histological observation, CS-HA injection treatment showed a chondroprotective effect on osteoarthritis. However, the histological scores of 'HA' group and 'CS-HA' group were not significantly different ($p > 0.05$). The results of biochemical evaluation showed that the expression levels of IL-1β, TNF-α, TIMP-1 and NO in synovial fluid of treated groups were all different from the control group ($p < 0.05$). However, the expression levels of these biochemical molecules in three treated groups were not significantly different ($p > 0.05$). In conclusion, CS-HA injection showed no obvious advantage over HA injection in osteoarthritis treatment.

Keywords: Osteoarthritis, histological evaluation, Mankin score, biochemical evaluation

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1. Introduction

Osteoarthritis is among the most frequent and symptomatic medical problems for the middle-aged and elderly. The main features of osteoarthritis include slow-developing joint pain, stiffness, and hypertrophy accompanied by limitation of motion (*1,2*). Osteoarthritis is also referred to as osteoarthrosis, degenerative arthropathy, hypertrophic arthritis or senile arthritis (*3*). In clinical practice, osteoarthritis of the knee is most common. The exact etiology, pathogenesis, and progression of this disease have yet to be determined (*4*). Studies have indicated that inflammation of the synovium might play an important role in its pathogenesis (*5,6*).

Chondroitin sulfate (CS) is a natural complex polysaccharide belonging to glycosaminoglycans (GAGs) composed of alternate disaccharide sequences of differently sulfated residues of D-glucuronic acid (GlcA) and of D-*N*-acetyl-galactosamine (GalNAc) linked by (1→3) bonds (*7*). CS is currently recommended by the European League Against Rheumatism (EULAR) as a symptomatic slow acting drug for osteoarthritis (SYSADOA) in Europe in the treatment of knee and hand osteoarthritis based on research evidence and meta-analysis of numerous clinical studies (*7-10*). Furthermore, recent clinical trials demonstrated its possible structure-modifying effects (*10,11*). CS prevents joint space narrowing and reduces joint swelling and effusion. To produce these effects, CS elicits an anti-inflammatory effect at the chondral and synovial levels (*12*).

Hyaluronic acid (HA) is a glycosaminoglycan composed of D-glucuronic acid and D-*N*-acetyl-glucosamine, with versatile biological activities. High molecular weight HA has been used in the treatment of human and animal osteoarthritis. Intra-articular HA treatment of the knee of patients with osteoarthritis has been verified to reduce painful symptoms and improve joint mobility. The purpose of intra-articular HA therapy is to make up for the loss of viscoelasticity of synovial fluid induced by inflammation and to protect against the

degradation of cartilage (*13,14*).

The aim of this study was to evaluate the effect of intra-articular injection of the combination of CS and HA on osteoarthritis to probe its feasibility in treating osteoarthritis. Meanwhile, the levels of biochemical molecules such as IL-1β, TNF-α, TIMP-1, iNOS and NO were also monitored to evaluate the effect of the combination of CS and HA on inflammation.

2. Materials and Methods

2.1. *Preparation of injections*

HA (Injection Grade) with M_r of 1.5~2.0 × 10^6 was obtained from a bacterial strain of *Streptococcus zooepidemicus* and was provided by Shandong Freda Biopharm Co., Ltd. (Ji'nan, Shandong, China). CS purified from porcine cartilage (M_w 35~50 kDa, Injection Grade) was purchased from DongCheng Biochemicals Co., Ltd. (Yantai, Shandong, China). The ratio of CS-A to CS-C was 5.6:1. An injection of CS and HA (CS-HA injection) was prepared according to the following steps: 2.0 g of CS was dissolved in 100 mL of phosphate buffer (pH 7.4); the solution was adjusted to pH 7.30 with 0.05% NaOH; then the solution was heated and kept at 100°C for 30 min; after cooling, the solution was filtered using a 0.45 μm filtration membrane; then 1.0 g of sodium hyaluronate was added to the solution and dissolved sufficiently; the compound solution was sterilized twice with flowing steam, for 30 min each; CS-HA injections were filled under aseptic environments and each injection contained 0.3 mL of compound solution.

An injection of CS (CS injection) was prepared: 2.0 g of CS was dissolved in 100 mL of phosphate buffer (pH 7.4); the solution was adjusted to pH 7.30 and was heated and kept at 100°C for 30 min; after cooling, the solution was filtered using a 0.45 μm filtration membrane; then the solution was sterilized with flowing steam and each injection contained 0.3 mL of solution. An injection of HA was also prepared. Briefly, 1.0 g of HA was added into 100 mL of phosphate buffer (pH 7.4) and dissolved sufficiently. Then the sterilization and aseptic filling were accomplished. Each injection contained 0.3 mL of solution.

2.2. *Induction and treatment of osteoarthritis in rabbits (animal experimentation)*

Papain was from Sigma-Aldrich (St Louis, MO, USA). Adult skeletally mature New Zealand White rabbits (body weight 2.5~3.0 kg) provided by Centre for Drug Safety Evaluation of Shandong Province (Ji'nan, Shandong, China) were housed individually in cages. Osteoarthritis was induced according to the method described in our previous report (*14*): 0.3 mL of sterile papain solution was injected into the both knees of the rabbits (1 mL of the solution containing 4.0 mg of papain and 50 mg of cysteine hydrochloride) under general anesthesia. After osteoarthritis induction, 28 rabbits were randomized into four groups: control group, 'HA' group, 'CS' group and 'CS-HA' group. After 7 days, rabbits in the control group (n = 7) were injected with 0.3 mL of normal saline in the knees. Rabbits in the 'HA' group (n = 7), 'CS' group (n = 7), and 'CS-HA' group (n = 7) were treated with HA, CS, and CS-HA injection in the knees, respectively. All animals were treated once weekly for 5 weeks.

2.3. *Histological evaluation*

On day 7 after the last treatment, the animals were sacrificed and the articular cartilage and synovium were collected. Synovial fluid was also collected. Routine histological methods, involving fixation in 10% formaldehyde, were followed by decalcification in 10% nitric acid. Standard hematoxylin-eosin (HE) staining was performed, and the specimens were assessed by an independent pathologist who was experienced in the examination of osteoarthritis specimens. The articular cartilage injuries found in the rabbits' knees were evaluated and recorded using the Mankin score (*15*).

2.4. *Biochemical evaluation*

0.3 mL of the collected synovial fluid was centrifuged at 5,000 rpm for 30 min. The levels of IL-1β, TNF-α, and TIMP-1 were determined using enzyme linked immunosorbent assay kits (Xuanhao Science and Technology Development Co., Ltd., Shanghai, China). The levels of iNOS and NO in synovial fluid were tested using detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.5. *Statistical analysis*

A t-test was used to analyze data. $p < 0.05$ was considered significant.

3. Results

Histological analysis using the Mankin score is shown in Table 1. The results showed different levels of degenerative changes in controls and the three treated groups. The histological scores of 'HA' group and 'CS-HA' group were not significantly different ($p >$

Table 1. Evaluation by the Mankin score

Groups	Mankin score
Control	8.25 ± 2.22
HA	5.50 ± 0.49*
CS	6.33 ± 1.52
CS-HA	5.17 ± 0.24*

* Compared with control group, $p < 0.05$.

0.05), but were both higher than the control group ($p < 0.01$). Histological score of the 'CS' group was not significantly higher than control group ($p > 0.05$).

As shown in Figure 1A, the structure of normal synovial membrane was intact and epithelial cells were regular and flat. As shown in Figure 1B, lamination of the synovial membrane of animals in the control group disappeared. Some of the epithelial cells swelled and displayed hyaline-like degeneration or shedding. There was mild capillary proliferation in the synovial membrane and focal ischemic necrosis in the synovial cavity. The fibrous tissue swelled and large amounts of capillaries expanded, and inflammation was obvious. As shown in Figure 1C, proliferation of synovial cells of animals in the 'CS' group was obvious. The thickening of synovial membrane was alleviated compared to the control group. There was local hyperemia, edema, infiltration of large amounts of plasmocytes and small amounts of lymphocytes. As shown in Figure 1D, the proliferation of synovial cells and synovial thickening in the 'HA' group was obvious. The proliferation level of the 'HA' group is higher than that of the 'CS' group. Furthermore, local edema and congestion were lighter than those of the 'CS' group. The blood vessels showed mild hyperplasia and the blood circulation was better recovered than that of the 'CS' group. As shown in Figure 1E, in the 'CS-HA' group, epithelial cells proliferated in patches, similar to normal tissue. Recovery of synovial membrane was better than other groups. On the whole, the recovery of the synovial membrane of animals in the 'CS-HA' group was best among all groups.

As shown in Figure 2A, the normal chondrocytes were vacuolar and regularly aligned. The sclerotin was intact and the tissue structure was clear. As shown in Figure 2B, in the control group, the chondrocytes were aligned intensively and the thickness of the fibrocartilage increased. There was obvious karyopyknosis. The gap between the lacunas was enlarged and some cells were broken and dissolved. The cartilage matrix was torn as small gaps along the direction at which the collagen fibers were spread. As shown in Figure 2C, the amount of chondrocytes increased in the 'CS' group. Some of the chondrocyte nuclei shrank and the gap between the lacunas was enlarged. The sclerotin was intact, the tissue structure was distinct and the structure layers were obvious. As shown in Figure 2D, in the 'HA' group, the structure of the cartilage was distinct. There was obvious proliferation of chondrocytes. Some of the chondrocyte nuclei shrank and the gap between the lacunas was enlarged. As shown in Figure 2E, in the 'CS-HA' group, the amount of chondrocytes increased. Some of the chondrocyte nuclei shrank and the gaps between the lacunas were enlarged. The sclerotin was intact, the tissue structure was distinct and the structure layers were obvious. In a word, the recovery of the cartilage of animals in the drug-treated groups was better than that of control animals, but there was no obvious difference among the three treated groups.

The results of biochemical evaluation are shown in Table 2. The results showed that the expression levels of IL-1β, TNF-α, TIMP-1 and NO in synovial fluid of

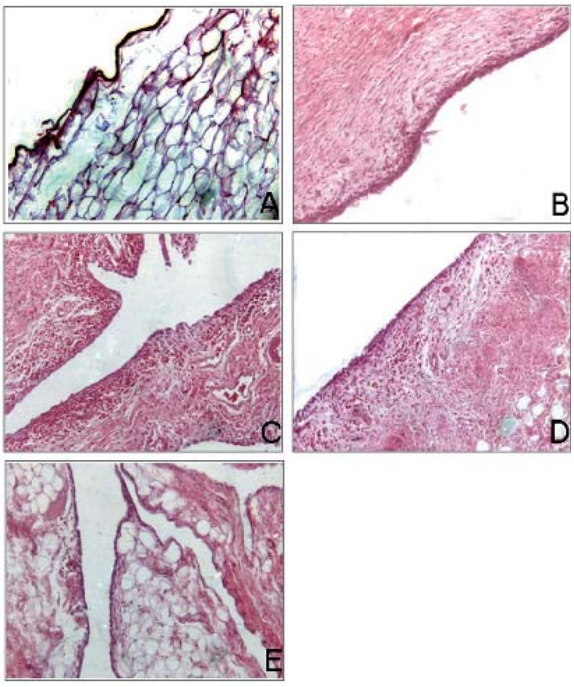

Figure 1. The pathological sections of synovial membrane stained with HE in different groups. A, normal synovial membrane (×200); **B**, control group (×200); **C**, 'CS' group (×200); **D**, 'HA' group (×200); **E**, 'CS-HA' group (×200).

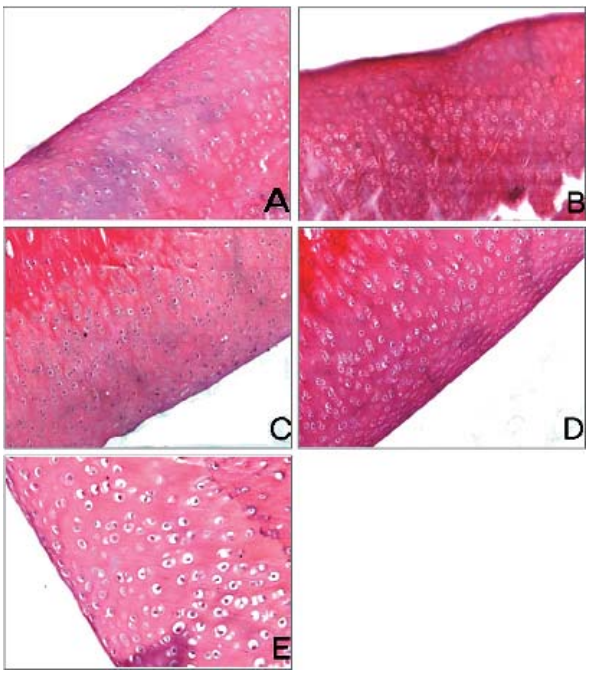

Figure 2. The pathological sections of cartilage stained with HE in different groups. A, normal cartilage (×200); **B**, control group (×200); **C**, 'CS' group (×200); **D**, 'HA' group (×200); **E**, 'CS-HA' group (×200).

Table 2. Expression levels of IL-1β, TNF-α, TIMP-1 and NO in different groups

Groups	IL-1β (pg/mL)	TNF-α (pg/mL)	TIMP-1 (ng/mL)	iNOS (U/mL)	NO (μmol/L)
Control	73.89 ± 5.29	66.20 ± 6.79	4.59 ± 0.38	22.85 ± 3.46	229.40 ± 43.86
CS-HA	45.37 ± 5.09**	51.35 ± 5.49*	9.43 ± 1.20*	16.58 ± 1.53*	125.69 ± 22.00*
CS	52.23 ± 2.68*	48.92 ± 4.72*	7.12 ± 0.97*	16.63 ± 2.24*	106.70 ± 10.29*
HA	58.46 ± 5.50*	47.54 ± 3.20*	7.40 ± 0.63*	21.76 ± 2.88	116.63 ± 18.37*

* Compared to the control group, $p < 0.05$; ** Compared to the control group, $p < 0.01$.

the treated groups were different from control group ($p < 0.05$). iNOS expression levels in synovial fluid of the 'CS-HA' and 'CS' groups were lower than control group, indicating that CS could inhibit the expression of iNOS. IL-1β level in synovial fluid of the 'CS-HA' group was much lower than that of control group. However, there was no obvious difference in the expression levels of the biochemical molecules among the three treated groups.

4. Discussion

SYSADOA are compounds which have been prescribed as drugs in European countries for many years. In Europe, the publication of the EULAR Recommendations for the treatment of Knee osteoarthritis in 2003 listed oral CS as evidence 1A and strength of recommendation A which represents the highest level for a therapeutic strategy (*16*). The benefits of CS for the treatment of osteoarthritis occurs through three main mechanisms: *i*) stimulation of extracellular matrix (ECM) (proteoglycan, CS, hyaluronan) production of chondrocytes; *ii*) suppression of inflammatory mediators (myeloperoxidase, *N*-acetyl glucosaminidase, collagenase, hyaluronidase, elastase), and *iii*) inhibition of cartilage degeneration (*17*).

Nevertheless, the benefit of CS is not accepted by all guidelines, and there is continuing controversy as to the efficacy of these agents as modifying drugs (*18*). A meta-analysis of five placebo-controlled RCTs yielded results that CS might have smaller beneficial effects than expected (*19*).

In this study, we intended to explore the effects of CS injection and CS-HA compound injection on osteoarthritis in rabbits. We found that after 5 intra-articular injections, the recovery of the synovial membrane of animals in the 'CS-HA' group was best among the animals treated with injections. However, the Mankin score of CS-HA treated animals had no obvious difference compared with that of HA treated animals, and the Mankin score of the CS treated group was not different than control. Although the recovery of the cartilage of animals in the drug-treated groups was better than that of control group, there was no obvious difference among the three treated groups. These results suggest that the intra-articular application of CS-HA injection shows no obvious advantage over routine intra-articular HA therapy.

As shown in Table 2, the expression levels of inflammatory factors such as IL-1β, TNF-α and TIMP-1 in synovial fluid of animals in three treated groups were all different from the control group, indicating intra-articular treatment of osteoarthritis with HA, CS or CS-HA injections inhibited joint inflammation. However, the inflammation inhibitory effects of CS and CS-HA were not better than HA. Overexpression of iNOS might increase the level of NO and damage the cartilage of osteoarthritis patients (*20*). In our study, CS and CS-HA injections showed inhibitory effects on the expression of iNOS. However, the NO levels of 'CS', 'HA' and 'CS-HA' groups were not significantly different.

In conclusion, CS-HA injection showed no obvious advantage over HA injection in osteoarthritis treatment.

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