Original Article

Effects of a hyaluronic acid and low molecular weight heparin injection on osteoarthritis in rabbits

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ABSTRACT: An osteoarthritis (OA) model was created in the knees of rabbits by injecting them with 0.3 mL of sterile papain solution in order to evaluate the effects of a hyaluronic acid (HA) and low molecular weight heparin (LMWH) injection on osteoarthritis. HA-LMWH, LMWH, and HA were injected into animals once weekly. After 5 weeks of treatment, the animals were sacrificed and the effects of the injections on osteoarthritis were evaluated by histological assessment. HA levels in cartilage and the levels of IL-1β and TNF-α expression in synovial fluids were determined. As shown by histological observation, recovery of the synovium and cartilage of animals injected with HA-LMWH was better than that in animals injected with HA or LMWH. HA levels in cartilage of animals injected with HA-LMWH were much higher than those of the control group. The levels of IL-1β expression in synovial fluids of animals injected with HA-LMWH were lower than those in other animals. The levels of TNF-α expression in synovial fluids of animals injected with HA-LMWH were much lower than those in the controls. In conclusion, HA-LMWH injection had a favorable anti-inflammatory and therapeutic effect on experimental OA.

Keywords: Hyaluronic acid, osteoarthritis, low molecular weight heparin, IL-1β, TNF-α

1. Introduction

Osteoarthritis (OA) is among the most frequent and symptomatic medical problems for the middle-aged and elderly. The exact etiology, pathogenesis, and progression of this disease have yet to be determined (*1*). Studies have indicated that inflammation of the synovium may play an important role in its pathogenesis (*2*,*3*).

Hyaluronic acid (HA) is a glycosaminoglycan (GAG) composed of D-glucuronic acid and D-*N*-acetylglucosamine with versatile biological activity, such as matrix construction, water retention and lubricating action, and wound healing. HA and its derivatives have been widely utilized in the medical field. HA is a major component of synovial fluid and plays a central role in the formation of the synovial joint (*4*). High molecular weight HA has been used in the treatment of human (*5*-*7*) and animal OA (*8*-*11*). Intra-articular HA treatment of the knee of patients with OA has been shown 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 due to inflammation and to protect the degradation of cartilage (*12*).

Heparin is a sulfated glycosaminoglycan that is the most widely studied natural anticoagulant (*13*). A number of physiological effects have been ascribed to heparin since its discovery, many of which are independent from its first-described and best-characterized activity as an anticoagulant. Heparin is believed to possess many forms of biological activity that include the ability to modulate embryonic development, neurite outgrowth, tissue homeostasis, wound healing, metastasis, cell differentiation, cell proliferation, and inflammation (*14*). The potential anti-inflammatory effects of heparin are supported by several modestly-sized clinical trials that have included patients with rheumatoid arthritis, bronchial asthma, and inflammatory bowel disease, and these effects have been corroborated by its prevention of macroscopic inflammatory lesions in animal models. Recent studies have indicated that heparin and related glycosaminoglycans can modulate the activity of a number of inflammatory cells, including T-cells and neutrophils (*15*).

Low molecular weight heparins (LMWH) have been developed by several manufacturers and have advantages in terms of pharmacokinetics and convenience of administration. LMWH has been shown to be at least as effective and safe as unfractioned heparin and has

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replaced the latter in many applications (*16*). LMWH also has a marked inhibitory effect on inflammation in animal models and clinical practice (*17,18*).

Combinations of HA and NSAIDs have been used to treat OA patients. Although NSAIDs are effective at mitigating inflammation, many reports have noted that they may accelerate disease progression. In order to avoid the adverse effects of NSAIDs and obtain a drug with favorable anti-inflammatory activity to treat OA, the current study prepared an injection of HA and LMWH and evaluated its effect on OA in rabbits.

2. Materials and Methods

2.1. *Preparation of injection*

HA with M_r of 1.56×10^6 was obtained from a bacterial strain of *Streptococcus zooepidemicus* and was provided by Shandong Freda Biochem Co., Ltd. (Ji'nan, China). LMWH with M_r of 4.25×10^3 was purchased from Hangzhou Jiuyuan Gene Engineering Co., Ltd. (Hangzhou, China).

An injection of HA and LMWH (HA-LMWH injection) was prepared according to the following steps: 2.0×10^5 anti-FXa IU of LMWH was dissolved in 100 mL of distilled water containing 0.73 g of NaCl. One gram of HA was added to the solution and left to swell for several hours. Then, the compound solution was sterilized with flowing steam.

An injection of LMWH was prepared by dissolving 2.0×10^5 anti-FXa IU of LMWH in 100 mL of distilled water containing 0.73 g of NaCl followed by flowing steam sterilization. One g of HA was added to 100 mL of PBS (pH 7.4) followed by flowing steam sterilization to prepare an injection of HA.

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 the Center for Drug Safety Evaluation of Shandong Province were housed individually in cages. Osteoarthritis was induced through injection with 0.3 mL of sterile papain solution into the knees (1 mL of the solution containing 4.0 mg of papain and 50 mg of cysteine hydrochloride) under general anesthesia. After osteoarthritis induction, 34 rabbits were randomized into four groups: a control group, a 'HA-LMWH' group, a 'LMWH' group, and a 'HA' group. After 7 days, rabbits in the control group (n = 8) were injected with 0.3 mL of NS in the knees. Rabbits in the 'HA-LMWH' group (n = 9), 'LMWH' group (n = 8) and 'HA' group (n = 9) were respectively injected with HA-LMWH, LMWH, or HA in the knees. All animals were injected once weekly. The animals were injected continuously for 5 weeks.

2.3. *Histological evaluation*

Seven days after the last treatment, the animals were sacrificed and both knees were collected. Synovial fluids were also collected. Routine histological methods, involving fixation in 10% formaldehyde, were followed by decalcification in 10% nitric acid. Standard haematoxylin-eosin staining was performed, and the specimens were assessed by an independent pathologist who was experienced in the examination of osteoarthritis specimens.

2.4. *Biochemical evaluation*

HA levels in cartilage of animals in different groups were collected after sacrifice and evaluated by radioimmunoassay. The levels of IL-1β and TNF-α in synovial fluids were determined with an enzyme-linked immunosorbent assay kit (RapidBio Lab., Shanghai, China).

2.5. *Statistical analysis*

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

3. Results

As shown in Figure 1, the structure of the normal synovial membrane was intact. Lamination of the synovial membrane of animals in the control group disappeared. Some of the epithelial cells swelled and displayed hyaloid degeneration or shedding. There was slight vascular proliferation in the synovial membrane and focal ischemic necrosis in the synovial cavity. The fibrous tissue swelled and large amounts of capillaries expanded. The synovial membrane of animals in the 'HA-LMWH' group was in the early stages of recovery. The synovial membrane recovered in patches and in layers, similar to normal tissue. The proliferation of capillaries was obvious. The 'LMWH' group had large amounts of fibroblasts. The infiltration of inflammatory cells was not obvious. In the 'HA' group, the epithelial cells proliferated in patches. There was slight vascular proliferation. Recovery of the synovial membrane of animals in the 'HA-LMWH' group was better than that of animals in other groups.

As shown in Figure 2, the normal chondrocytes were vacuolar and regularly aligned. In the control group, there was slight focal hyperplasia of the fibrous tissue on the surface of the cartilaginous tissue. The chondrocytes obviously shrank. There was focal chondronecrosis and the strip-funicular fibrous tissue was more abundant than normal tissue. In the 'HA-LMWH' group, large amounts of capillaries proliferated around the margin of the cartilage. There were some chondrocytes in the early stage of proliferation and some mature chondrocytes.

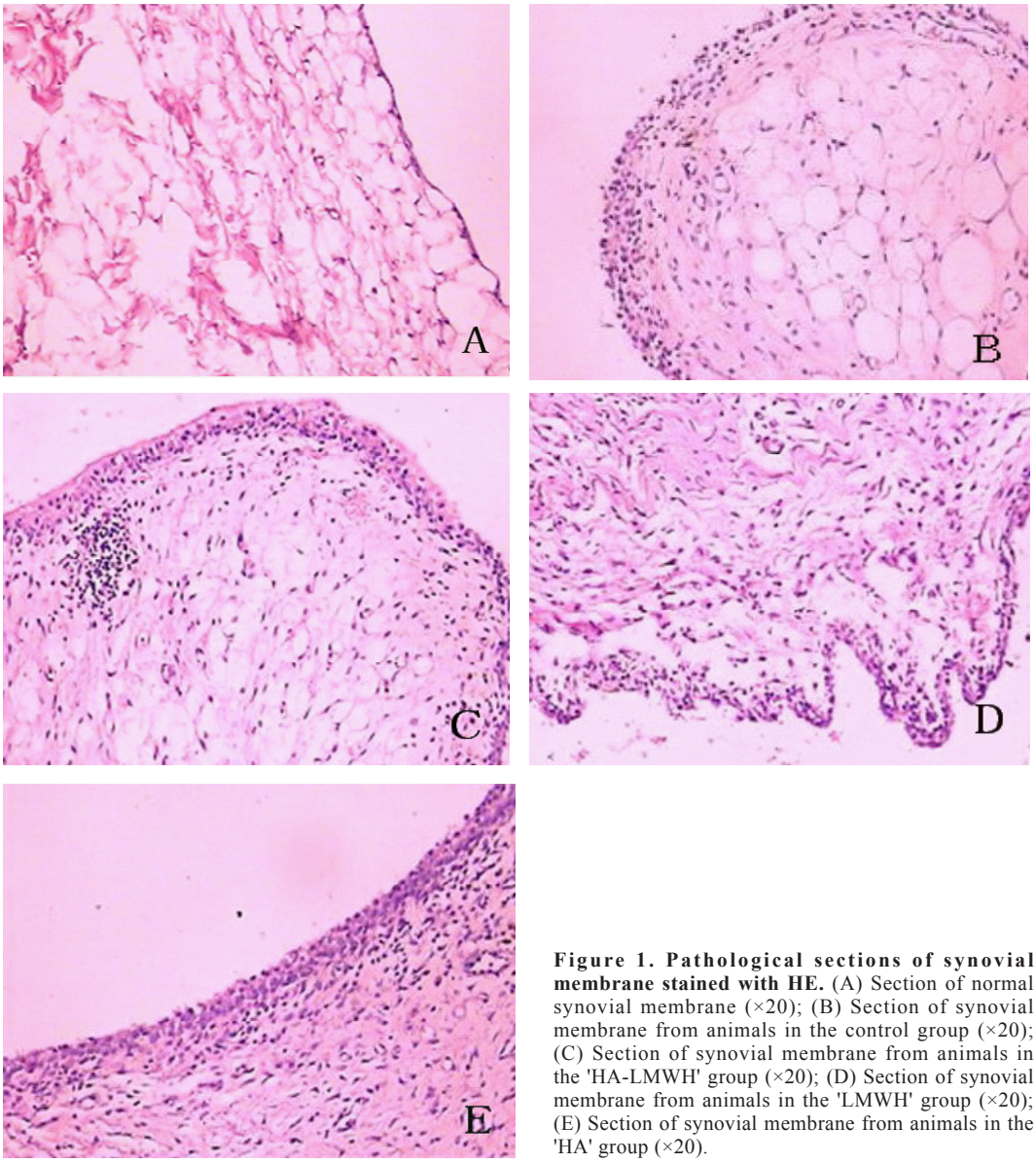

Figure 1. Pathological sections of synovial membrane stained with HE. (A) Section of normal synovial membrane (×20); (B) Section of synovial membrane from animals in the control group (×20); (C) Section of synovial membrane from animals in the 'HA-LMWH' group (×20); (D) Section of synovial membrane from animals in the 'LMWH' group (×20); (E) Section of synovial membrane from animals in the 'HA' group (×20).

There was a slight increase in the amount of neutral GAG on the surface of the cartilage. In the 'LMWH' group, there was no obvious proliferation of chondrocytes and little acid GAG. The chondrocytes were irregularly aligned. There was little granulation tissue. In the 'HA' group, the chondrocytes proliferated in patches. On the surface of the cartilaginous tissue there was punctiform proliferation of fibrous tissue.

HA levels in cartilage and levels of IL-1β and TNF-α expression in synovial fluids of animals are shown in Table 1. HA levels were much higher in the cartilage of animals in the 'HA-LMWH,' 'LMWH,' and 'HA' groups than those of the control group ($p < 0.05$). Meanwhile, the levels of IL-1β expression in synovial fluids of animals in the 'HA-LMWH' and 'HA' groups were much lower than those of the control group ($p < 0.05$). The level of IL-1β expression in synovial fluids of animals in the 'HA-LMWH' group was lower than that of the 'LMWH' and 'HA' groups ($p < 0.05$). The levels of TNF-α expression in synovial fluids of animals in the 'HA-LMWH,' 'LMWH,' and 'HA' groups were also much lower than those of the control group ($p < 0.05$), and there was no difference in the levels of TNF-α expression among the groups receiving injections.

4. Discussion

OA is not a simple wear-and-tear phenomenon but is an active process that is part of the reparative response to injury (*19*). The disease affects not only cartilage but has also been shown to cause damage to the entire joint structure, including the subchondral bone, synovium, and joint capsule. The exact cause of OA is not yet known. Studies have indicated that inflammation of the synovium may play an important role in the pathogenesis of OA (*2*,*3*). Pro-inflammatory cytokines, particularly IL-1β and TNF-α, are synthesized by synoviocytes, chondrocytes, and infiltrating leukocytes during the disease process (*20*).

Evidence suggests that intra-articular administration

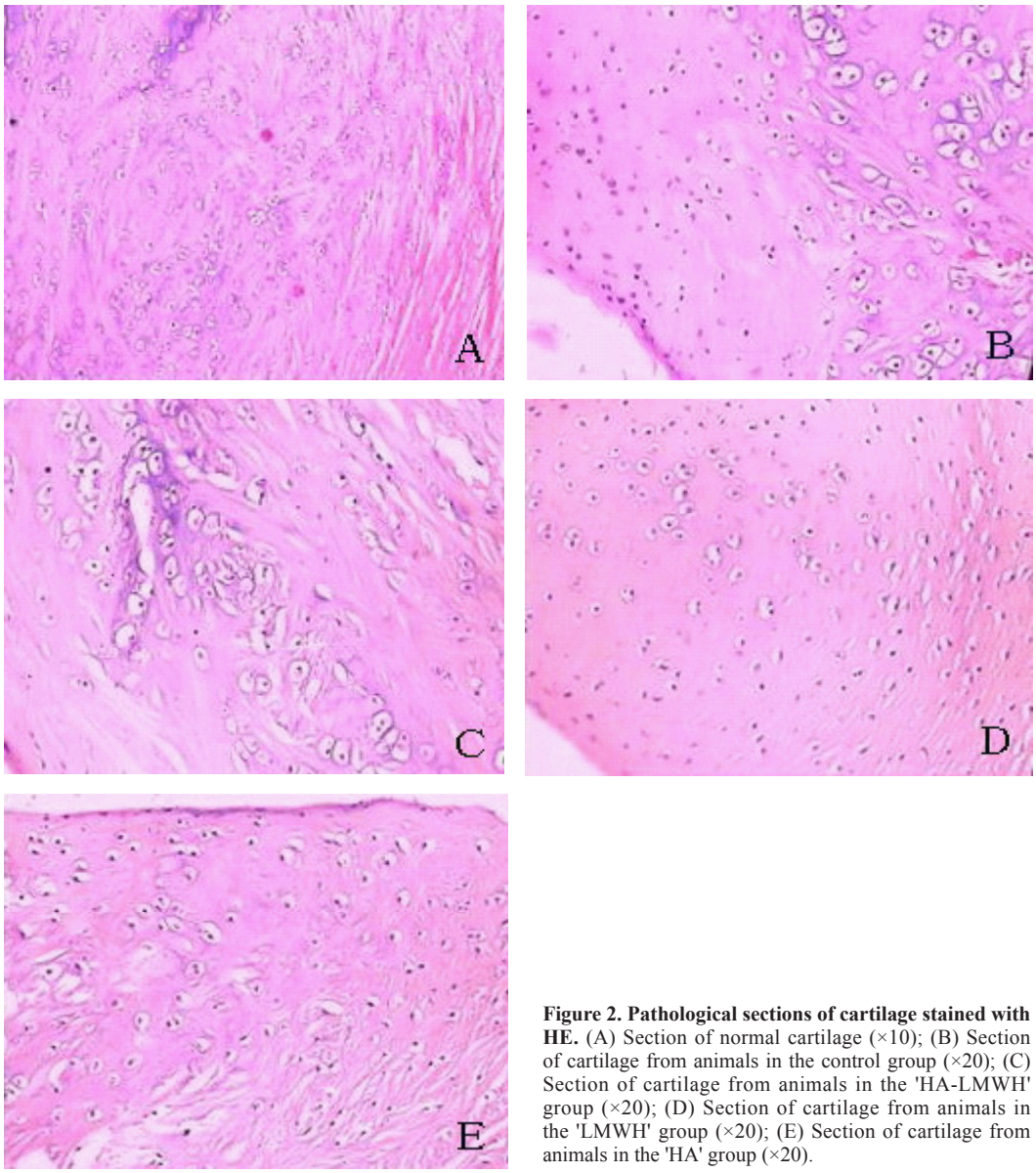

Figure 2. Pathological sections of cartilage stained with HE. (A) Section of normal cartilage (×10); (B) Section of cartilage from animals in the control group (×20); (C) Section of cartilage from animals in the 'HA-LMWH' group (×20); (D) Section of cartilage from animals in the 'LMWH' group (×20); (E) Section of cartilage from animals in the 'HA' group (×20).

Table 1. HA levels in cartilage and levels of IL-1β and TNF-α expression in synovial fluids of animals in different groups

Groups	HA (pg/mg)	IL-1β (pg/mL)	TNF-α (pg/mL)
Control	7.89 ± 0.84	153. 08 ± 52.33	71.26 ± 9.17
'HA-LMWH'	13.63 ± 1.75*	49. 90 ± 16.64*	50.22 ± 6.79*
'LMWH'	17.81 ± 2.90*	109. 29 ± 14.39	43.54 ± 7.99*
'HA'	16.93 ± 1.01*	79. 82 ± 11.35*	50.71 ± 7.05*

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

of HA improves symptoms of OA in selected patients and has few adverse effects (*21*). Anti-inflammatory effects suggest a potential mechanism of HA in osteoarthritis. LMWH has been widely used as an anticoagulant to treat diseases that feature thrombosis as well as for prophylaxis in situations that lead to a high risk of thrombosis (*22*). LMWH also possesses anti-inflammatory activity supported by experimental data and clinical application (*23,24*).

The current study prepared HA-LMWH and injected it into rabbits to treat osteoarthritis. The levels of IL-1β expression in synovial fluids of animals injected with HA-LMWH were lower than those in other groups, to some extent indicating that the inflammation of the synovium was attenuated by injection of HA-LMWH. The levels of TNF-α expression in synovial fluids of animals injected with HA-LMWH, HA, or LMWH were lower than those of the control group, indicating the anti-inflammatory effects of injecting HA-LMWH, HA, and LMWH. However, there was no difference in the TNF-α levels of the three groups receiving injections. The effect of HA-LMWH injection on pro-inflammatory cytokines involved in the process of osteoarthritis still needs to be explored.

There are reports indicating that proteoglycan levels in the cartilage of OA patients or animals decreased in comparison to normal levels (*25,26*). In the current study, the HA levels of the groups receiving injections

were much higher than those of the control group, indicating that injection of HA, LMWH, or HA-LMWH may promote the production of HA in cartilage, thus improving the symptoms of OA.

5. Conclusion

HA-LMWH injection had a favorable effect on experimental OA. However, the effect and mechanism of HA-LMWH injection on OA must still be further explored.

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