Local intra-articular injection of sodium hyaluronate delays articular cartilage degeneration after traumatic osteoarthritis

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Abstract

BACKGROUND: Sodium hyaluronate is an effective treatment for osteoarthritis, but the underlying mechanism remains unclear. There is evidence that abnormal expressions of matrix metalloproteinase (MMP)-1, -3 and -9 and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 show great effects on osteoarthritis.

OBJECTIVE: To assess the influence of intra-articular injection of sodium hyaluronate on expressions of MMPs-1, 3, 9 and tissue inhibitor of TIMPs-1, 2 in the rabbit cartilage after osteoarthritis.

METHODS: Twenty-four mature New Zealand white rabbits were divided into normal control, model, and sodium hyaluronate groups. The model and sodium hyaluronate groups underwent unilateral anterior cruciate ligament transection, and rabbits in the sodium hyaluronate group received 0.3 mL of 1% sodium hyaluronate *via* intra-articular injection at 4 weeks after modeling, once a week for 5 weeks. At 11 weeks following surgery, the rabbits were killed and the cartilage was harvested to extract total RNA. mRNA expressions of MMPs-1, 3, 9 and TIMPs-1, 2 in the cartilage were analyzed using real-time PCR for each group.

RESULTS AND CONCLUSION: Compared with the model group, the range and extent of cartilage damage was reduced in the sodium hyaluronate group ($P < 0.01$), and Mankin scores were noticeably decreased ($P < 0.05$). In the cartilage, mRNA expressions of MMPs-1, 3, 9 were enhanced and mRNA expressions of TIMPs-1, 2 were down-regulated in the model group. However, the mRNA expression levels of MMPs-1, 3, 9 and TIMPs-1, 2 in the articular cartilage were not obviously changed in the sodium hyaluronate group. These results suggest that MMPs-1, 3, 9 and TIMPs-1, 2 are involved in the progression of osteoarthritis and the therapeutic mechanism of sodium hyaluronate is not realized through the down-regulation of their expressions during development of osteoarthritis. Sodium hyaluronate for treatment of osteoarthritis is a complex process and the underlying mechanisms require further investigation.

Subject headings: Tissue Engineering; Osteoarthritis; Cartilage, Articular; Matrix Metalloproteinases
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INTRODUCTION

Osteoarthritis is a joint disease characterized as chronic and progressive cartilage destruction, resulting in recurrent pain and gradual loss of articular function. The initial causes of osteoarthritis are still obscure and therefore, the mechanism of cartilage degradation has been extensively investigated.

The extracellular matrix of articular cartilage is composed of type II collagen and proteoglycan. Matrix metalloproteinase (MMP)-1 causes cleavage in type II collagen. MMP-3 degrades several extracellular matrix molecules, including cartilage proteoglycan and type II collagen. In addition, MMP-3 also activates MMP-1 and facilitates pathological degradation of collagen. Increase in MMP-1 and MMP-3 has been found to be associated with an absence of variation for tissue inhibitor of metalloproteinase (TIMP)-1, 2. It is becoming more and more evident that MMPs play an important role in cartilage destruction. TIMP is not elevated in osteoarthritis cartilage and synovium as much as MMPs. Many studies have shown that the imbalance between the activities of MMPs and TIMPs is thought to be important in the progression of osteoarthritis[1-4].

Intra-articular injection of sodium hyaluronate has been widely used in the treatment of osteoarthritis. Sodium hyaluronate is a large linear glycosaminoglycan composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine, linked *via* the 1–4 positions of the sugar rings, and it is one of the important components of cartilage matrix and

has a protective effect on the progression of osteoarthritis. Sodium hyaluronate is also reported to inhibit the expression of MMP-1 and MMP-3 induced by interleukin-1β[5], and reduce cartilage degradation induced by neutrophil leukocytes[6].

However, there is no systematic report about the effect of sodium hyaluronate on MMPs-1, 3, 9 and TIMPs-1, 2 expressions in rabbit experiment osteoarthritis. So this study detected their mRNA expression and assessed the effect of sodium hyaluronate on their expression in rabbit osteoarthritis.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed at the Experimental Center, Wuhan University Renmin Hospital, China between June 2013 and December 2014.

Materials

Twenty-four mature New Zealand white rabbits about 2.2–2.8 kg were bought from the Animal Center of Medical College of Wuhan University (license No. SYXK (E) 2010-0057) and randomly divided into normal control, model, and sodium hyaluronate groups.

Reagents and instruments:

Reagents and instruments	Source
Sodium hyaluronate	Bausch & Lomb FREDA
ABI 7900HT Fast Real-Time PCR System	ABI Company, USA
Trizol	Invitrogen Technologies, USA
RT-PCR kit	TaKaRa, China

Methods

Grouping

Animals in the model and sodium hyaluronate groups were anesthetized intravenously with ketamine hydrochloride (1.0 mg/kg), and received unilateral anterior cruciate ligament transection. A medial parapatellar incision was made on the skin and a medial arthrotomy was performed. The patella was dislocated and the knee placed in full flexion. The anterior cruciate ligament was visualized and transected without destroying the articular cartilage. The knee was irrigated with physiological saline. Then the capsule and skin were closed respectively. Postoperatively the animals were caged (60 cm× 60 cm×40 cm) without any immobilization and maintained under the same environmental condition. During the first 5 days after the operation, each rabbit received an intramuscular injection of 200 000 units of penicillin every day to prevent infection. At 4 weeks after surgery, rabbits in the model group were not injected after unilateral anterior cruciate ligament transection, and rabbits in the sodium hyaluronate group received 0.3 mL of 1% sodium hyaluronate via intra-articular injection, once a week for 5 weeks.

Macroscopic observation

All animals were killed and knees were harvested at 11 weeks after modeling. Degenerative changes of femoral condyles in the cartilage were observed under dissecting microscope and the osteoarthritis severity was graded on a scale of 0–4[7], as follows: 0=surface smooth with normal color; 1=surface rough with minimal fibrillation or a slight yellowish discoloration; 2=cartilage erosion extending into the superficial or middle layers; 3=cartilage ulceration extending into the deep layers; 4=cartilage depletion with subchondral bone exposed.

Microscopic observation

At 11 weeks post-operation, tissues from eight rabbits of each group were dissected, fixed in neutral buffered 4% formaldehyde in 0.1 mol/L PBS (pH 7.4) for 48 hours, decalcified in 10% ethylenediaminetetraacetic acid for 3–4 weeks, dehydrated in graded alcohols, and embedded in paraffin. Sections, 5 μm thick, were stained with both hematoxylin and eosin for general morphology, or with 0.1% safranin-O and fast green to demonstrate sulfated proteoglycans (PGs). The area of positively stained safranin-O metachromasia, indicative of PGs, and the total area of the joint cartilage in frontal sections were measured. Each data point represented results obtained from measurements of two sections from each of three individual animals belonging to the same group. Regenerative cartilage was histologically scored using Mankin's histopathology grading system[8]. The grading system used a 14-point score. Higher points indicate severer cartilage degeneration.

Real-time quantitative PCR

At 11 weeks post-operation, the total RNA extracted were used the Trizol reagent according to the manufacturer's protocol. The RNA samples were quantified by $A_{260\ nm}$. Then RNA was reverse transcribed to cDNA using RT-PCR kit according to protocol. The cDNA was analyzed immediately or stored at –20 ℃. Quantitative PCR amplification was performed by ABI 7900HT Fast Real-Time PCR System and the SYBR Green I fluorescent dye method was used to quantify cDNA. The sequences are shown as **Table 1**.

The final volume of the real-time PCR reaction was 10 μL and included SYBR Premix Ex Taq 5 μL, primer (10 μmol/L) 0.2 μL each, 50×ROX Reference Dye 0.2 μL, template 1 μL, adjusted to 10 μL with distilled water. PCR cycling conditions consisted of an initial denaturing step for 10 seconds at 95 ℃, and then 40 cycles of 5 seconds at 95 ℃, 30 seconds at 60 ℃. A stable and reliable standard curve was established using synthesized oligonucleotides resembling cDNA fragments in 5-fold decrements as template. The glyceraldehyde-3-phosphate dehydrogenase from the same sample was used as internal control and the

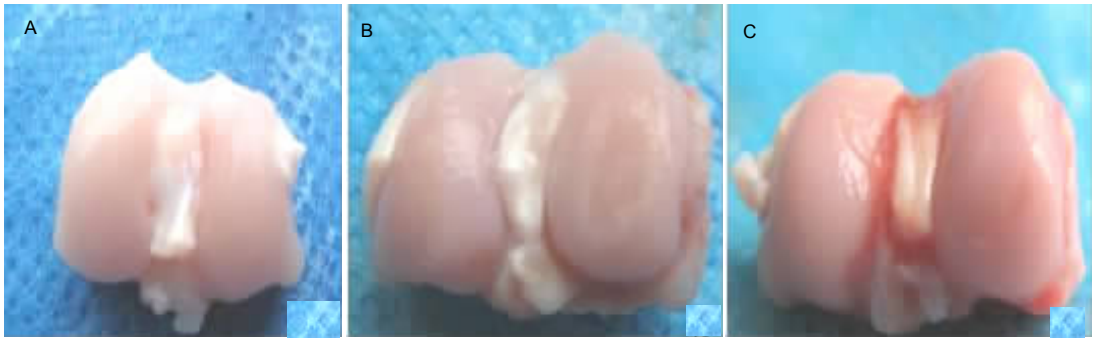

Figure 1 Representative macroscopic appearance of the articular cartilage from the femoral condyles of each group
Note: (A) Normal control group: the femoral condylar articular cartilage was normal, and no osteophyma was observed. (B) Model group: cartilage tissues with ulceration, fibrochondrogenesis and obvious osteophyma were observed. (C) Sodium hyaluronate group: the articular cartilage was anabrotic and osteophyma was reduced.

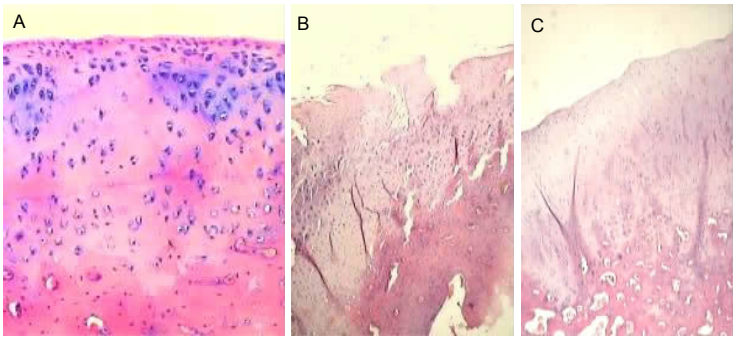

Figure 2 Hematoxylin-eosin staining of the cartilage of the femoral condyle (×100)
Note: (A) Normal control group: the normal cartilage had no evident changes. (B) Model group: conspicuous inflammation, cartilage fibrillation and fissures were visible. (C) Sodium hyaluronate group: sodium hyaluronate could inhibit loss of cartilage.

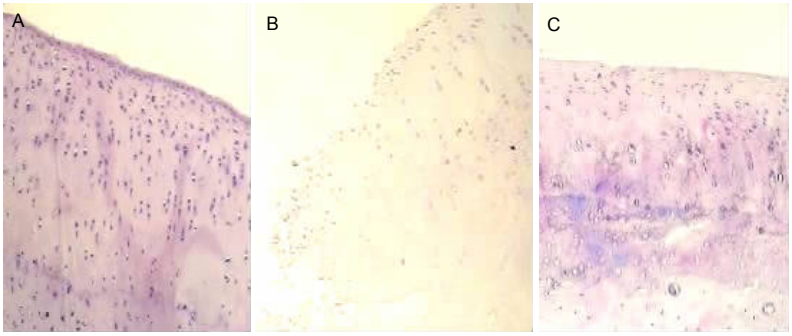

Figure 3 Safranin-O staining of the cartilage of the femoral condyle (×100)
Note: (A) Normal control group: the normal cartilage showed no evident changes. (B) Model group: conspicuous inflammation and severe loss of proteoglycans were found. (C) Sodium hyaluronate group: sodium hyaluronate could inhibit loss of proteoglycans.

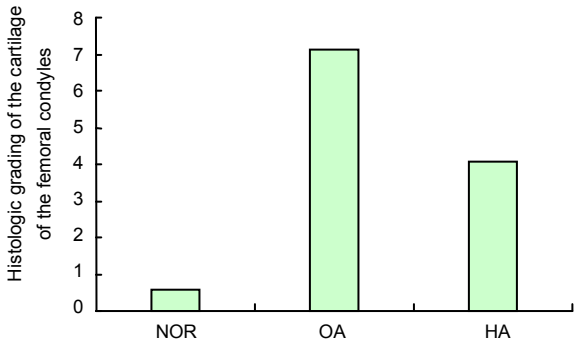

Figure 4 Histologic grading of the cartilage of the femoral condyles
Note: There was a significant difference in the Mankin's scores between the model and normal control groups ($P < 0.01$). Compared with the model group, the Mankin's scores were reduced significantly in the sodium hyaluronate group. NOR: Normal control group; OA: model group; HA: sodium hyaluronate group.

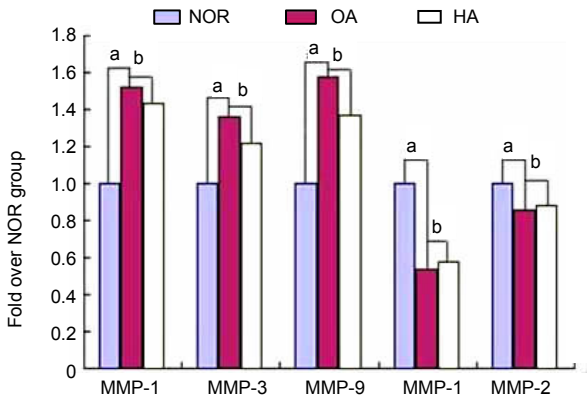

Figure 5 Relative expressions of metalloproteinases (MMPs-1, 3, 9) and tissue inhibitors of MMPs (TIMPs-1, 2) in the normal control group (NOR), the model group (OA) and sodium hyaluronate group (HA)
Note: [a]$P < 0.05$, *vs*. the normal control group; [b]$P > 0.05$, *vs*. the model group.

Table 1 Details of the primers used in this study

Gene	Primers sequence (5'–3')	Product size (bp)
MMP-1	Upstream: GGG GAC TGA GGA GGA GAC GGA	115
	Downstream: GGC CAG CAC CAG GAG TAG CAG	
MMP-3	Upstream: TGG CGT TCC TGA TGT TGG TC	107
	Downstream: TCT TGG CAG ATC CGG TGT G	
MMP-9	Upstream: CAG CTA CGA CAA GGA CAA GCT CTA	104
	Downstream: AGA CGA AGG GGA AGA CAC ACA	
TIMP-1	Upstream: CAA CTG CGG AAC GGG CTC T	235
	Downstream: AGG CGA GAT GGC GGC TCT	
TIMP-2	Upstream: GCC AAA GCG GTC AGC GAG AA	168
	Downstream: CAG GGA CAC GCC GCA CAC	
GAPDH	Upstream: CCA CTT TGT GAA GCT CAT TTC CT	140
	Downstream: TCG TCC TCC TCT GGT GCT CT	

Note: MMP-1: matrix metalloproteinase-1; MMP-3: matrix metalloproteinase-3; MMP-9: matrix metalloproteinase-9; TIMP-1: tissue inhibitor of metalloproteinase-1; TIMP-2: tissue inhibitor of metalloproteinase-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

relative contents of the copy numbers of the target gene's mRNA were calculated, through which we could determine the gene expression level and its trend of change. Specificity of each reaction was controlled by melting curve analysis. A negative PCR control containing water instead of cDNA was performed. Real-time PCR was conducted in triplicate in three independent experiments.

Main outcome measures

Gross morphology of articular cartilage under the anatomical microscope; morphology of articular cartilage under the optical microscope; mRNA expression of MMPs-1, 3, 9 and TIMPs-1, 2.

Statistical analysis

All values were expressed as mean±SEM. Statistical analysis was performed using SPSS 12.0 software. The Kruskal-Wallis *H*-test was used for gross scores. One-way analysis of variance was used for intergroup comparison. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Quantitative analysis of experimental animals

Twenty-four New Zealand white rabbits were included in the final analysis without dropouts.

Macroscopic morphology

In the normal control group, the cartilage on the condyles of rabbits was macroscopically normal, and there were no osteophytes (**Figure 1A**). In the model group, cartilage lesions, cartilage ulceration, fibrocartilage proliferation and osteophytes were evident (**Figure 1B**). Rabbits in the sodium hyaluronate group presented a marked reduction in the severity of lesions and osteophytes on the condyles, and the degenerative changes of cartilage were characterized as roughness and erosion (**Figure 1C**). As shown in Table 2, the macroscopic scores under dissecting microscope at 11 weeks after modeling indicated that the osteoarthritis severity in the sodium hyaluronate group was significantly lower than that in the model group ($P < 0.01$).

Table 2 Macroscopic scores of cartilage tissue injury in each group (n=8, n)

Group	Scores				
	0	1	2	3	4
Normal control	7	1	0	0	0
Model	0	0	1	3	4
Sodium hyaluronate	3[a]	4[a]	1	0[a]	0[a]

Note: [a]$P < 0.01$, *vs.* model group.

Microscopic morphology

Cartilage from normal rabbits had a normal histologic appearance. But cartilage specimens from the model group had morphologic changes, including cartilage fibrillation and fissures, and a loss of Safranin 0 staining (**Figures 2, 3**). In the sodium hyaluronate group, the lesions on the condyles were significantly reduced compared with the model group ($P < 0.05$). This difference was largely due to a decrease in the severity of structural changes and loss of Safranin 0 staining (**Figure 4**).

Real time-PCR analysis

A significant difference of MMPs-1, 3, 9 expressions was observed in all three groups. The mRNA expressions of MMPs-1, 3, 9 in the cartilage of the sodium hyaluronate group were reduced compared with the model group, but there was no significant difference between the two groups. TIMPs-1, 2 mRNA expressions were detected in all samples. Compared with the other two groups, a significant decrease in TIMP-1 was found in the model group ($P < 0.05$), and the TIMP-2 expression was also decreased obviously, but no significant difference was found ($P > 0.05$). The sodium hyaluronate had no effect on either TIMP-1 or TIMP-2 expression in the cartilage of osteoarthritis rabbits. These results indicated that therapeutic mechanism of sodium hyaluronate was not through inhibiting their expressions during development of osteoarthritis (**Figure 5**).

DISCUSSION

Osteoarthritis is a joint disease that involves degeneration of articular cartilage and intra-articualr inflammation manifested by synovitis[9]. And its onset is not ordinarily definable; the development of the disease is difficult to study[10]. Animal models of osteoarthritis are a necessary means for studying the pathological mechanism of osteoarthritis and evaluating the curative effects of drugs. The anterior cruciate ligament transaction model of osteoarthritis in animals is frequently used in recent years, because it is simple to operate, produces minimal surgical trauma, and can present the whole degenerative progress of the articular cartilage, the animal models show early cartilage degeneration, have reproducible degeneration sites, and exhibit gradually aggravated cartilage degeneration with time.

The anterior cruciate ligament transaction models of osteoarthritis in animals were used in this study. Gross observation and optical microscope results showed that after osteoarthritis induction by anterior cruciate ligament transaction in animals, articular cartilage damage, ulceration and obvious osteophyma were observed. Moreover, the articular cartilage presented with fibrous changes with gaps. Safranin O staining was negative. Mankin score was significantly higher in the model group than in the normal control group. Compared with the model group, after intra-articular injection of sodium hyaluronate, the degree of cartilage injury was obviously decreased, osteophyma was obviously reduced, and Mankin scores were noticeably decreased, suggesting that sodium hyaluronate can reduce articular cartilage degeneration.

And it is now generally agreed that both mechanical and biochemical factors play an important role in its progression. The process involves many biochemical mediators such as interleukin-1, nitric oxide, and MMPs and TIMPs. Among these mediators, MMPs can directly destroy nearly all components of the cartilage matrix, and they have a major impact on osteoarthritis degeneration[11-12]. In this study, the mRNA expressions of MMPs-1, 3, 9 were enhanced, especially MMP-3, which showed MMPs-1, 3, 9 were involved in the degeneration of cartilage in osteoarthritis rabbits.

TIMP is a glycoprotein, and inhibits all MMPs on a 1:1 basis by forming high-affinity complexes. MMPs have been found to be significantly increased in the cartilage and synovium of osteoarthritis. TIMP is not elevated in osteoarthritis cartilage and synovium as much as MMPs. In the present study, TIMPs-1, 2 mRNA expressions were down-regulated, especially TIMP-1, which demonstrated a significant imbalance between MMPs and TIMPs joined in the pathologic degradation of the cartilage matrix. An imbalance between the activities of MMPs and TIMPs is thought to be important in the progression of osteoarthritis[13-14].

Intra-articular injection of sodium hyaluronate is now widely used in the treatment of osteoarthrits[15-19]. Injection of exogenous sodium hyaluronate can inhibit prostaglandin E2 synthesis and protect against proteoglycan depletion and cytotoxicity induced by oxygen-derived free radicals, interleukin-1, and mononuclear cell-conditioned medium, and against other alterations. Takahashi *et al* [10] showed that sodium hyaluronate treatment had no effect on either MMP-3 or TIMP-1 expression in the cartilage at all grades of osteoarthritis.

Results from this study showed that compared with the model group, articulator condylar injury and osteophyte formation were noticeably reduced, and articular cartilage degeneration was remarkably alleviated, but the mRNA expression levels of MMPs-1, 3, 9 and TIMPs-1, 2 in the articular cartilage were not obviously changed in the sodium hyaluronate group. These findings suggest that the therapeutic mechanism of sodium hyaluronate is a lack of down-regulation of MMPs-1, 3, 9 and TIMPs-1, 2 in the articular cartilage during early development of osteoarthritis. Sodium hyaluronate for treatment of osteoarthritis is a complex process and the underlying mechanisms require further investigation.

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关节腔内注射透明质酸钠可以延缓创伤性骨关节炎的软骨退变

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文章亮点：

1 透明质酸钠是一种线性大分子黏多糖，由葡糖醛酸和 N-乙酰葡萄糖胺的双糖单位反复交替连接而成。透明质酸钠是软骨基质的重要重要成分之一，对骨关节炎有重要保护作用。有研究报道关节腔内注射透明质酸钠可以抑制白细胞介素 1β 诱导的基质金属蛋白酶 1 和基质金属蛋白酶 3 的表达，减少由中性白细胞诱导的关于软骨退变。

2 实验构建单侧前交叉韧带切断骨关节炎模型，通过关节腔内注射透明质酸钠，观察骨关节炎关节软骨内基质金属蛋白酶 1，3，9 及其抑制剂 1，2 表达的变化及透明质酸纳其表达的影响。文章结果显示透明质酸纳可以有效减轻骨关节炎软骨退变程序，但其作用机制并不是通过抑制基质金属蛋白酶 1，3，9 及其抑制剂 1，2 的表达发挥作用。

关键词：
组织构建；软骨组织工程；透明质酸钠；关节炎；骨关节炎；软骨退变；关节内注射；基质金属蛋白酶 1；基质金属蛋白酶 3；基质金属蛋白酶 9；基质金属蛋白酶组织抑制剂 1；基质金属蛋白酶组织抑制剂 2；国家自然科学基金
主题词：
组织工程；骨关节炎；软骨，关节；基质金属蛋白酶类
基金资助：
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摘要

背景：透明质酸钠是一种治疗骨关节炎的有效手段，但其机制尚不清楚。有研究表明基质金属蛋白酶 1，3，9 和基质金属蛋白酶组织抑制剂 1，2 表达失调对骨关节炎有重要影响。

目的：观察关节腔内注射透明质酸钠对兔创伤性骨关节炎模型软骨中基质金属蛋白酶 1，3，9 和基质金属蛋白酶组织抑制剂 1，2 表达的影响。

方法：采用单侧前交叉韧带切断法构建创伤性骨关节炎模型兔，造模后 4 周关节腔注射体积分数 1%透明质酸钠 0.3 mL，每周 1 次，连续 5 周，设为透明质酸钠组，同时设模型组和正常对照组作对比。术后 11 周麻醉处死动物，获取软骨并提取总 RNA，用实时聚合酶链反应分析各组软骨内基质金属蛋白酶 1，3，9 和基质金属蛋白酶组织抑制剂 1，2 mRNA 表达。

结果与结论：与模型组相比，透明质酸钠组经关节腔内注射透明质酸钠软骨损伤范围和程度均减轻($P < 0.01$)，软骨组织学评分明显降低($P < 0.05$)。模型组软骨内基质金属蛋白酶 1，3，9 mRNA 表达增强，基质金属蛋白酶组织抑制剂 1，2 mRNA 表达下调，而透明质酸钠组软骨中基质金属蛋白酶 1，3，9，基质金属蛋白酶组织抑制剂 1，2 mRNA 的表达并无显著变化。结果说明，基质金属蛋白酶 1，3，9 和基质金属蛋白酶组织抑制剂 1，2 参与了创伤性骨关节炎软骨退变过程，虽然关节腔内注射透明质酸钠可以延缓创伤性骨关节炎软骨退变，但透明质酸钠并不是通过调节上述因子的表达来发挥修复作用的，具体机制有待进一步研究证实。

作者贡献：文章所有作者均参与文章的设计及实施。

利益冲突：文章及内容不涉及相关利益冲突。

伦理要求：实验过程中对动物的处置应符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

学术术语：透明质酸钠与组织工程的关系？透明质酸钠是一种线性大分子黏多糖，由葡糖醛酸和 N-乙酰葡萄糖胺的双糖单位反复交替连接而成。透明质酸钠是软骨基质的重要重要成分之一，对骨关节炎关节软骨修复有重要保护作用。

作者声明：文章为原创作品，无抄袭剽窃，无泄密及署名和专利争议，内容及数据真实，文责自负。

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