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Research Paper

In vitro effects of sodium hyaluronate on the proliferation and the apoptosis in chondrocytes from patients with Kashin-Beck disease and osteoarthritis ☆

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Abstract

Objective: To identify the *in vitro* effects of sodium hyaluronate(HA) on the proliferation and the apoptosis of chondrocytes from patients with Kashin-Beck disease(KBD) and osteoarthritis(OA). **Methods:**Samples of articular cartilages from KBD and OA patients, as well as healthy volunteers(6 subjects in each of the 3 groups) were dissected, digested with collagenase and the cells cultured in monolayers. Chondrocytes from each sample were assigned to an untreated group and two HA-treated groups: H0(no HA), H100(HA, 0.1 g/L) and H500(HA, 0.5 g/L). The first passage chondrocytes were used to observe proliferation using the MTT assay, and apoptosis by flow cytometry through Annexin V/PI staining. **Results:**HA promoted proliferation of chondrocytes in all the three groups, and in KBD and OA groups, for cells cultured for 4 and 6 days, H500 significantly promoted the cell proliferation. The apoptotic rates of both KBD and OA group chondrocytes were in the order H500 < HA100 < H0. **Conclusion:**Sodium hyaluronate administration has a dose-dependent *in vitro* effect to promote proliferation and inhibit apoptosis of chondrocytes from patients with KBD and OA.

Keywords: sodium hyaluronate; Kashin-Beck Disease(KBD); osteoarthritis; chondrocyte; proliferation; apoptosis

INTRODUCTION

Kashin-Beck disease(KBD) is an chronic endemic osteochondropathy involving degeneration and necrosis, mainly in the growth plate and the articular cartilage[1-2] resulting in growth retardation, secondary osteoarthrosis and disability in advanced stages[3]. The disease affected over 0.72 million patients with 104 million people at risk in China, and 28 372 of these patients were younger than 13 years of age[4]. The etiology of the disease is still under debate, although 3 major environmental hypotheses have been proposed since KBD was discovered in 1849:endemic selenium deficiency, serious cereal contamination by mycotoxin-producing fungi, and high humic acid levels in the drinking water[5-7]. and recently, HuaYin[8] proposed that Kashin-Beck disease may be caused by a virus. Osteoarthritis(OA) is characterized by degradation and loss of articular cartilage, hypertrophic bone changes with osteophyte formation, subchondral bone remodeling, and chronic inflammation of the synovial membrane at the clinical stage of the disease[9-10]. This is the leading cause of physical disability and impaired quality of life in industrialized nations. Risk factors for OA are mainly

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age, gender, genetics, trauma, obesity, knee alignment, and an imbalance of physiologic processes resulting in inflammatory cascades on a molecular level. The effects on the three joint compartments manifest as articular cartilage breakdown, osteophyte formation, subchondral sclerosis, and alterations of the synovium on both morphologic and biochemical levels. Clinically, KBD and OA manifest as chronic pain, joint destruction, disability, depression, and social isolation. Generally, KBD is a special type of OA, and has similar pathological changes, such as the destruction of collagen and the loss of aggrecan.

Currently available pharmacological therapies target palliation of pain and include analgesics(acetaminophen, cyclooxygenase-2-specific inhibitors, nonselective nonsteroidal anti-inflammatory drugs, tramadol, opioids), intra-articular therapies(glucocorticoids and sodium hyaluronate), and topical treatments(capsaicin, methylsalicylate)[11].

Intra-articular treatment with HA has become widely accepted in the armamentarium of therapies for OA pain. HA is responsible for the viscoelastic properties of synovial fluid, which contains a lower concentration and molecular weight(MW) of HA in osteoarthritic joints than in healthy ones[12]. Thus, the goal of intra-articular therapy with HA is to help replace synovial fluid that has lost its viscoelastic properties. The efficacy and tolerability of intra-articular HA for the treatment of pain associated with OA of the knee have been demonstrated in several clinical trials[13], and also in KBD patients[14-15]. Up to now, many experiments showed HA can enhance proliferation of chondrocytes, maintaining their phenotype, glycosaminoglycan and type II collagen synthesis and maturation of collagen[14,16-17], suppressing both MMP-1 and RANTES production[18-19]. However, little is known about HA in chondrocytes from patients with KBD, and there are few comparisons of the predominant mechanism affecting intra-articular hyaluronate in these two diseases. In this paper, we present evidence that HA promotes the proliferation of chondrocytes and inhibits apoptosis in chondrocytes from patients with KBD and OA, particularly at the higher dosage(0.5 g/L).

MATERIALS AND METHODS

Antibodies and reagents

MTT, Trypsin, type II collagenase and mouse monoclonal antibodies against human recombinant type II collagen were purchased from Sigma Chemical Co. (St. Louis, MO, USA.), D MEM/F-12 from Gibco Co. (USA), and FBS from Minghai(Lanzhou, China). The horseradish peroxidase-diaminobenzidine(DAB) immunostaining kit was purchased from Zymed(San Francisco, CA, USA), and HA was purchased from Shanghai Qisheng Biological Preparation Co, Ltd. (Shanghai,China). Annexin Ⅴ-PI was purchased from Jingmei biotechnology engineering Co, Ltd.(ShenZhen, China).

Cartilage samples and groups

The articular cartilage samples were collected from subjects in the KBD, OA and the healthy control groups. In the KBD group(three females and three males, ages from 35 to 47 years old), articular cartilages were collected from knee joints in the diseased areas by joint debridement at hospitals in Shaanxi province. The KBD adults who were selected were diagnosed as being first or second degree, based on the national diagnosis criteria of Kashin-Beck disease in China[20]. In the OA group(three females and three males, ages from 57 to 73 years old), articular cartilages were collected from the patients in the same areas by total knee arthroplasty in Xi'an hospital, Shaanxi province. OA patients were diagnosed according to the Criteria of American College of Rheumatology Subcommittee on Osteoarthritis Guidelines(1995). Six articular cartilage samples in the control group were collected from the fresh cadaver knees donated with a fairly large amount of cartilage from two females and four males aged from 27 to 39 years. The health status of knee cartilage in cadaver donors, who died in traffic accidents, was diagnosed by histological examination with HE staining to exclude genetic bone and cartilage diseases, osteoarthritis, rheumatoid arthritis and KBD. This investigation was approved by the Human Ethics Committee, Medical College of Xi'an Jiaotong University. All patients provided an informed consent.

Cultivation of articular chondrocytes

Within 4 h after operation or 12 h of death, cartilage tissues were transported in Dulbecco's Modified Eagle Media Nutrient Mixture F-12(DMEM/F-12, Gibco Co, USA). After rinsing with phosphate buffered saline (PBS) three times, chondrocytes were prepared from cartilage by overnight digestion using 0.2% trypsin 1 h, 0.1% hyaluronidase 1 h, and 0.25% collagenase 8h(type II, Gibco, 1 ml of solution per 100 mg of tissue) in sequence. Finally, the isolated cells were collected by centrifugation(1 000 r, 5 min) and washed two times with the culture medium. The suspended cells were cultured in DMEM/F-12 with 10% FCS(Minghai, Lanzhou, China), and 1% penicillin/streptomycin. The cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 and the culture medium was changed every 2d. After culture for 2~3 weeks, the cells were passaged using 0.2% trypsin+0.05%EDTA. All experiments were performed using first passage chondrocytes to avoid chondrocyte dedifferentiation.

Treatment with hyaluronic acid

At the time of subdividing the cultures, chondrocytes were divided into three groups, one group that received no sodium hyaluronate(HA) and two groups that received HA in the basic medium as follows:(a)H0(no HA), (b)H100(HA, 0.1 g/L), (c)H500(HA,0.5 g/L). All cultures were treated every other day for 6 d.

Toluidine blue staining

For toluidine blue staining, the first passage chondrocytes were seeded on 8 mm^2 P-L-polylysine-coated coverslips in 24-well plates at a density of 1×10^4 cells/well, and allowed to expand in DMEM/ F12 medium for 6d. The medium was replaced every other day. Cells were then fixed with 4% paraformaldehyde for 20 min, and rinsed 4 times with PBS for 5 min. The coverslips were fixed in 4% paraformalin and stained for 30 min with 0.04%(w/v) toluidine blue in 0.1 mol/L sodium acetate buffer(pH 4.0) to visualize the tissue proteoglycans(aggrecan). This was followed by incubation in 50% ethanol for 1 min and in 70% ethanol for 1 min. The coverslips were finally sealed with neutral gum.

Immunocytochemical staining

Chondrocytes cultured on coverslips were prepared as described for toluidine blue staining, fixed with 4% paraformaldehyde, and rinsed with PBS. Immunocytochemistry was performed following the protocol of a strept-avidin-biotin-peroxidase complex(SABC) kit. The cells were permeabilized with 0.2% Triton X-100 for 10 min. After endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, the cells were incubated with 5% normal bovine serum in PBS(20 min, 37℃) and washed with PBS. Cells were incubated with primary antibodies of types II collagen (diluted 1:100) or the control serum overnight at 4℃, and then with biotinylated sheep anti-mouse IgG at room temperature (RT) for 20 min. After washing with PBS, cells were incubated with SABC, and the peroxidase reaction was visualized with 0.3% DAB buffer containing 0.1% hydrogen peroxide. Finally, cells were stained with hematoxylin, washed with distilled water, dehydrated through an ethanol series(80%, 95%, and 99.9%) and xylene, sealed by neutral gum and photographed using a Nikon light microscope.

Chondrocyte viability and proliferation

Chondrocyte viability and proliferation were determined at the different experimental times in triplicate by the 3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide(MTT, Sigma Chemical Co, St. Louis, MO, USA) -mitochondrial reduction method based on the Mosmann original protocol. Chondrocytes were seeded at a density 1×10^4 cells/well into 96-well culture plate, and 48 h after plating(time 0), the culture medium was replaced with 0.2 ml of fresh medium containing HA at the following concentrations:(a)H0(without HA), (b)H100(HA, 0.1 g/L), (c)H500(HA,0.5 g/L). At least three matrices were analyzed for each condition at 2, 4 and 6 d incubation. After 2, 4 and 6 d, 20 μl MTT(5 g/L) was added in every well and the chondrocytes were incubated for 4 h at 37℃ in a 5% CO_2 incubator. The unreduced MTT and medium were then discarded, and the MTT formazan crystals were dissolved with 150 μl of DMSO. Plates were shaken for 10 min, and the optical density(OD) measured at 490 nm.

Flow cytometric analysis of apoptosis by Annexin V/PI staining

Chondrocytes from KBD, OA and the control groups were seeded onto six-well plates at a density of 2×10^5 cells/well in DMEM/ F-12 containing 10% FCS. After 48 h(time 0), the culture medium was replaced with 2.0 ml of fresh medium, with or without HA. All medium was replaced every other day for 6 d. Cells were collected by trypsinization, washed twice with cool PBS before adding 5 μl Annexin-FITC and 10 μl PI while avoiding light. Cells were incubated at room temperature for 15 min, and buffer added to 200 μl prior to detection by flow cytometry.

Statistical analysis

All statistics are presented as Mean and SD values, and data were analyzed with SPSS 17.0 statistical software. One-Way ANOVA tests and LCD(least significant difference) were used followed by post hoc tests to determine the significance of differences between individual group means. $P < 0.05$ was considered statistically significance.

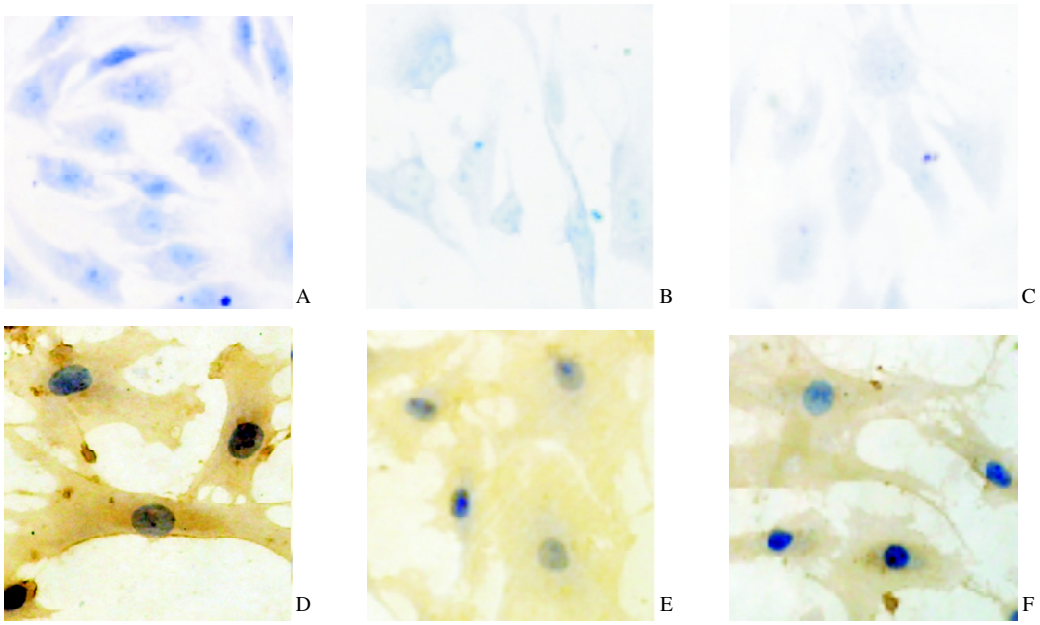
RESULTS

Identification of the chondrocyte

Toluidine blue staining and the pericellular staining for type II collagen were positive in all groups(***Fig. 1***). Our cells had the morphological features of chondrocytes, verifying the preservation of the chondrocyte phenotype. While figure 1 also shows toluidine blue staining and the collagen II staining of KBD(***Fig. 1 B,E***) and OA(***Fig. 1 C,F***) group cells, it was weaker than that of the control group cells(***Fig. 1 A,D***), respectively. This indicated the synthesis and secretion of aggrecan and collagen II was decreased in KBD and OA group chondrocytes.

The effect of HA on chondrocyte proliferation

From the ***Table 1*** and ***Fig. 2***, the chondrocyte proliferation rate is observed to increase with the HA concentration and the duration of culturing when compared to the control group(H0). Based on the



Toluidine blue staining(A-C) and collagen II Immunocytochemical staining(D-F) of condrocytes from patients with KBD(B and E) and OA (C and F) were weaker than that of the control(A and D).

Fig. 1 The results of toluidine blue staining and the pericellular staining for type II collagen(200×)

Table 1 **The comparison of the chondrocyte proliferation following different HA dosages, cultured for different periods** ($\bar{x} \pm s$)

Groups	*n*	Cultured times		
		2d	4d	6d
Control				
H0	6	0.100 5 ± 0.018 71	0.116 1 ± 0.020 61	0.136 3 ± 0.027 45
H100	6	0.103 0 ± 0.024 18	0.114 3 ± 0.032 86	0.137 1 ± 0.023 56
H500	6	0.112 9 ± 0.041 52	0.139 7 ± 0.048 60*	0.151 3 ± 0.030 75
KBD				
H0	6	0.106 0 ± 0.023 77	0.119 0 ± 0.020 68	0.128 2 ± 0.016 76
H100	6	0.109 7 ± 0.022 85	0.123 0 ± 0.024 07	0.128 8 ± 0.023 61
H500	6	0.122 6 ± 0.040 32	0.142 8 ± 0.046 21	0.179 4 ± 0.081 15*
OA				
H0	6	0.117 0 ± 0.030 85	0.148 0 ± 0.019 43	0.208 4 ± 0.034 25
H100	6	0.124 9 ± 0.029 04	0.167 4 ± 0.018 99	0.202 1 ± 0.030 87
H500	6	0.135 7 ± 0.029 09	0.196 1 ± 0.041 84*	0.244 1 ± 0.048 61*

Compared with HO group, $^*P < 0.05$.

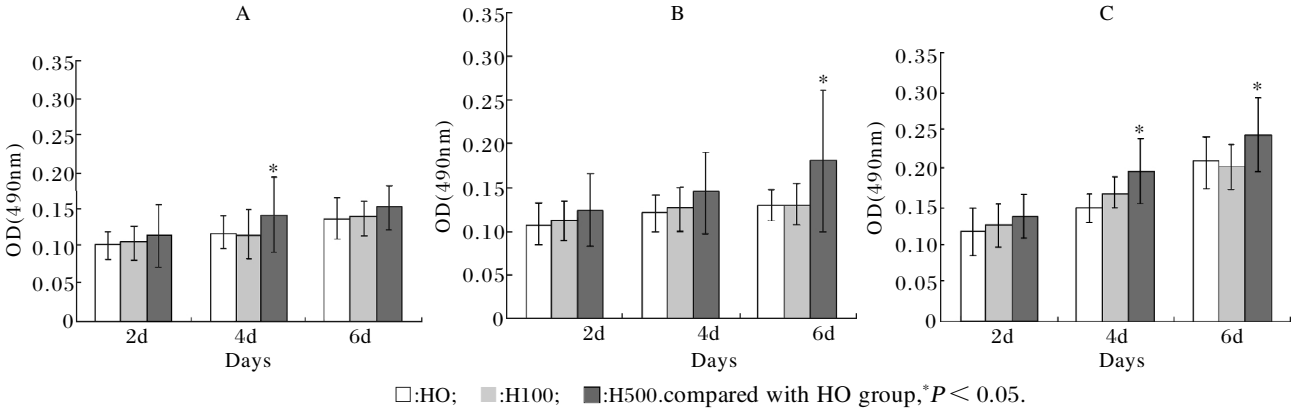


Fig. 2 The comparison of chondrocyte proliferation at the different HA dosages at 2, 4 and 6 d of the control(A), KBD(B) and OA(C) groups using an MTT assay

statistical analysis, proliferation of chondrocytes cultured from KBD patients for 6 d and from OA patients for 4 and 6 d in the HA500 were significantly higher than cells from the control subjects in H100. This indicated that the 0.5 g/L, but not the 0.1 g/L, HA dose promoted the chondrocyte proliferation in KBD and OA groups at the culture periods of 4 and 6 d.

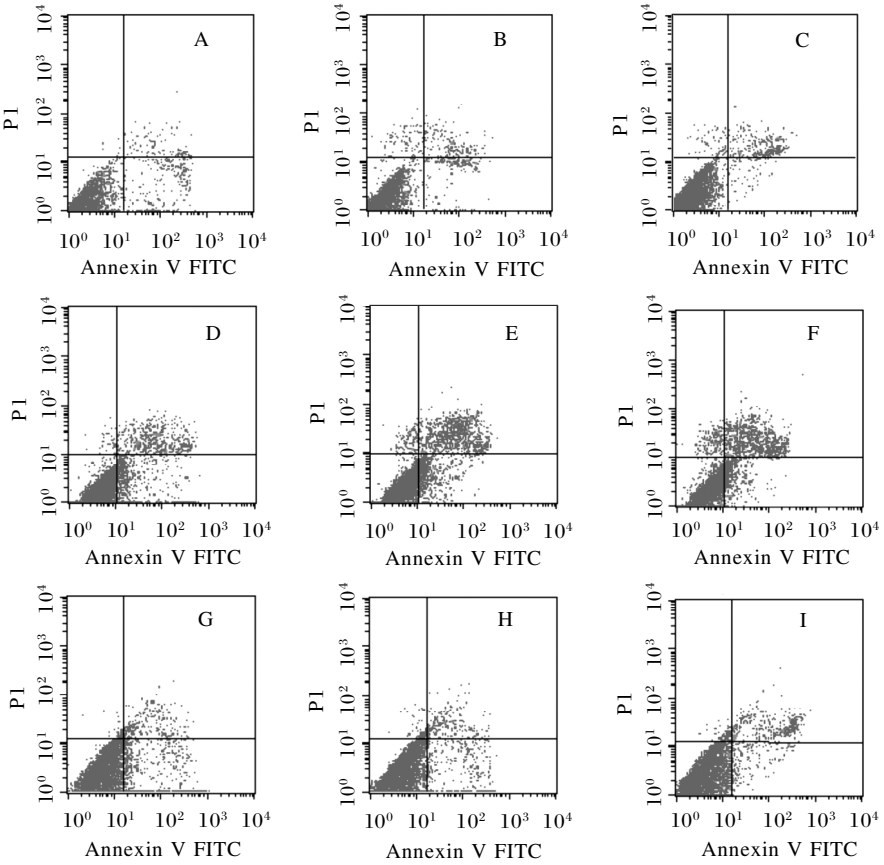
The effect of HA on apoptosis of chondrocytes

The apoptotic rates of chondrocytes cultured from both KBD and OA in H100 and HA500 were significantly lower than when they were cultured in the absence of HA. The apoptotic rates of both KBD and OA patient cells were significantly less with the H500 treatment than the H100 treatment (in the KBD group the mean difference=−2.58, $P < 0.05$; in the OA group the mean difference=−2.87, $P < 0.05$, shown in ***Table 2, Fig.3***).

Table 2 **Apoptotic rates of chondrocytes cultured in different HA dosages for 6d** ($\bar{x} \pm s$)

Groups	*n*	H0	H100	H500
Control	6	7.128 3 ± 1.2437 8	6.011 7 ± 1.5422 5	4.045 0 ± 1.2038 7$^{*\#}$
KBD	6	12.860 0 ± 2.1585 3$^{\triangle}$	10.458 3 ± 1.1427 1$^{*\triangle}$	7.876 7 ± 1.3455 2$^{*\#\triangle}$
OA	6	12.316 7 ± 1.0708 8$^{\triangle}$	10.898 3 ± 1.3918 2$^{\triangle}$	8.028 3 ± 0.9112 9$^{*\#\triangle}$

$^{*}P < 0.05$ H100 and H500 versus H0 group; $^{\#}P < 0.05$ H100 versus H500 group; $^{\triangle}P < 0.05$ KBD and OA versus Control group.



In each plot, the lower left(LL) quadrant represents viable cells, the upper left(UL) quadrant indicates necrotic cells, the lower right(LR) quadrant denotes early apoptotic cells, and the upper right(UR) quadrant represents necrotic or late apoptotic cells. The the lower right(LR) quadrant were regard as apoptotic cells and were used to calculate apoptosis rate.

Fig. 3 The comparison of the apoptosis of chondrocytes cultured at the dosages of H0, H100 and H500 *in vitro* in control(A, B, C), KBD (D, E, F) and OA (G, H, I) groups by flow cytometric analysis, respectively

DISCUSSION

The low aggrecan synthesis, the low collagen II expression and the excess apoptosis in cartilage are major features of Kashin-Beck Disease(KBD) and osteoarthritis (OA). In this paper, we present evidence that sodium hyaluronate has a role in promoting the proliferation and inhibiting apoptosis of chondrocytes from patients with KBD and OA *in vitro*, particularly at an HA dose of 0.5 g/L.

Hyaluronan/hyaluronic acid(HA) is an unbranched

glycosaminoglycan composed of repeating disaccharide units of[D-glucuronic acid-N-acetyl-D-glucosamine]. It is widely distributed from some microorganisms to all animals, being conserved throughout evolution, perhaps because hyaluronan is required for the protection of cells/tissues. Generally, hyaluronan has anti-inflammatory actions in various tissues, and these actions are partly mediated by activation of hyaluronan receptor CD44 and the hyaluronan binding protein, RHAMM[21]. In articular joints, HA plays important roles. Hyaluronan acts as both a lubricant and shock absorber and coats the surface of the articular cartilage, protects the cartilage and blocks the loss of aggrecan from the cartilage matrix into the synovial space, maintaining the normal cartilage matrix[12]. Similarly, hyaluronan may also help prevent invasion of inflammatory cells into the joint space[22]. Exogenous hyaluronan may facilitate the production of newly synthesized hyaluronan. HA in the synovial fluid binds to chondrocytes via the CD44 receptor, supporting a role for HA in healthy cartilage, and can relieve joint pain. This effect may be mediated through the attenuation of prostaglandin E2(PGE2) and bradykinin synthesis[23]. Meanwhile, hyaluronan can play a protective role by inhibiting inflammatory mediators such as TNF-α, IL-1β, MMP-3 and promoting the production of TIMP-1.

Previously some researchers have found that hyaluronan can promote the proliferation of chondrocytes[24-25], while others have reported that HA has no effect on proliferation of chondrocytes[26-27]. In this experiment, sodium hyaluronate administration in chondrocytes cultured in vitro promoted the proliferation of chondrocytes, and at the higher dosage used (0.5 g/L) it was particularly effective in promoting the proliferation of OA and KBD patient chondrocytes. In vitro experiments indicate that hyaluronan administration can enhance the synthesis of extracellular matrix proteins, including chondroitin and keratin sulfate, and at the same time hyaluronan has also been shown to suppress cartilage damage by fibronectin fragments *in vitro* and *in vivo*. This may promote the proliferation of chondrocytes[28].

Apoptosis of chondrocytes is a potentially important feature of osteoarthritic cartilage degeneration[29-32]. Some investigations demonstrated that 500~730kd hyaluronan exerts an anti-apoptotic effect on anti-FAS-induced chondrocyte apoptosis by binding to its specific receptors(CD44 and ICAM-1). Furthermore, this hyaluronan fraction may be able to slow down chondrocyte apoptosis in OA by regulating the processes of cartilage matrix degradation[33]. In our experiment, in the absence of HA the KBD and OA groups had a higher number of apoptotic chondrocytes when compared with the control group. This result confirmed the findings of previous studies. Sodium hyaluronate administration in this experiment inhibited the apoptotic chondrocytes cultured in the control, KBD and OA groups treated with 0.5 g/L HA, and in the KBD group treated with 0.1 g/L HA. This suggests that while 0.50 g/L HA was required to inhibit apoptosis in chondrocytes from OA patients, a lower dose was effective in chondrocytes from KBD patients. Campo *et al.*[25] proposed that hyaluronan plays an anti-apoptosis role through inhibition of NF-κB nuclear translocation, proinflammatory cytokine reduction, and diminution of both NO generation and caspase-3 activation. Zhou *et al.*[34] found that HA appeared to inhibit IL-1b-induced apoptosis of chondrocytes through abolishing NO production and protecting mitochondrial function. It would be interesting in the future to study whether or not sodium hyaluronate administration inhibited chondrocyte apoptosis through the anti-Fas pathway[35].

As a conclusion, sodium hyaluronate administration promotes the proliferation of chondrocytes and inhibits the apoptosis of chondrocytes from patients with KBD and OA *in vitro*, and the HA dosage of 0.5 g/L has the more significant effect.

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