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Sorbitol-modified hyaluronic acid reduces oxidative stress, apoptosis and mediators of inflammation and catabolism in human osteoarthritic chondrocytes

John-Max Mongkhon · Maryane Thach · Qin Shi · Julio C. Fernandes · Hassan Fahmi · Mohamed Benderdour

Abstract

Objective and design Our study was designed to elucidate the precise molecular mechanisms by which sorbitol-modified hyaluronic acid (HA/sorbitol) exerts beneficial effects in osteoarthritis (OA).

Methods Human OA chondrocytes were treated with increasing doses of HA/sorbitol ± anti-CD44 antibody or with sorbitol alone and thereafter with or without interleukin-1beta (IL-1β) or hydrogen peroxide (H_2O_2). Signal transduction pathways and parameters related to oxidative stress, apoptosis, inflammation, and catabolism were investigated.

Results HA/sorbitol prevented IL-1β-induced oxidative stress, as measured by reactive oxygen species, p47-NADPH oxidase phosphorylation, 4-hydroxynonenal (HNE) production and HNE-metabolizing glutathione-*S*-transferase A4-4 expression. Moreover, HA/sorbitol stifled IL-1β-induced metalloproteinase-13, nitric oxide (NO) and prostaglandin E2 release as well as inducible NO synthase expression. Study of the apoptosis process revealed that this gel significantly attenuated cell death, caspase-3 activation and DNA fragmentation elicited by exposure to a cytotoxic H_2O_2 dose. Examination of signaling pathway components disclosed that HA/sorbitol prevented IL-1β-induced p38 mitogen-activated protein kinase and nuclear factor-kappa B activation, but not that of extracellular signal-regulated kinases 1 and 2. Interestingly, the antioxidant as well as the anti-inflammatory and anti-catabolic effects of HA/sorbitol were attributed to sorbitol and HA, respectively.

Conclusions Altogether, our findings support a beneficial effect of HA/sorbitol in OA through the restoration of redox status and reduction of apoptosis, inflammation and catabolism involved in cartilage damage.

Keywords Chondrocytes · Osteoarthritis · Hyaluronic acid/sorbitol · Oxidative stress · Apoptosis · Inflammation

J.-M. Mongkhon · M. Thach · Q. Shi · J. C. Fernandes · M. Benderdour (✉)
Orthopaedics Research Laboratory, Hôpital du Sacré-Cœur de Montréal, Room K-3045, 5400 Gouin Blvd. West, Montreal, QC H4J 1C5, Canada
e-mail: mohamed.benderdour@umontreal.ca

Q. Shi · J. C. Fernandes · M. Benderdour
Department of Surgery, Université de Montréal, Montreal, QC H4J 1C5, Canada

H. Fahmi
Osteoarthritis Research Unit, Research Centre of the University of Montreal Hospital Center (CR-CHUM), Notre-Dame Hospital, 1560 Sherbrooke Street East, Montreal, QC H2L 4M1, Canada

Introduction

Osteoarthritis (OA) is a frequent musculoskeletal disease and a major source of disability in the elderly [1]. Knee OA is the foremost cause of consultations for OA-related symptoms [2]. It is well-established that during joint inflammation and degeneration, as in OA, important macromolecules are lost from the extracellular matrix (ECM), including type II collagen [3]. This phenomenon represents deregulation of chondrocyte metabolism due to the actions of inflammatory cytokines, such as interleukin-1beta (IL-1β), responsible for the downregulation of collagen [4] as well as proteoglycan biosynthesis [5] and stimulation of their degradation [6].

Current OA management combines pharmaceutical and non-pharmacological strategies [7, 8], including

intraarticular injections of hyaluronic acid (HA), aimed at decreasing pain and improving joint function [9]. Despite the long history of this therapy, known as viscosupplementation, and numerous studies of its action, the mechanisms responsible for HA's clinical outcome are not clearly recognized. Intraarticular HA injections may progress to the recovery of desirable viscoelastic behavior of OA-altered synovial fluid (SF) [10]. Other biological functions of exogenous HA in OA include analgesia via interaction with pain receptors [11], promotion of endogenous HA production and various anti-inflammatory effects [12–14]. Most of these effects could be mediated by interaction between HA and its receptors, CD44 and hyaluronan-mediated motility receptor [13, 14].

It has been reported that HA counteracts IL-β-induced inhibition of collagen biosynthesis at both the transcriptional and post-transcriptional levels [15]. The mechanism of HA's post-transcriptional impact on collagen biosynthesis is seen in certain experimental conditions on analysis of prolidase activity. Intraarticular HA administration still offers potent and well-tolerated therapy to OA patients. In addition to its unique hygroscopic and rheological properties, HA binds to its cellular receptor CD44, initiating cell signaling. It is well-established that HA-dependent signaling affects chondrocyte proliferation and differentiation [16] as well as glycosaminoglycan and collagen synthesis [17].

The present study was undertaken to evaluate the influence and potential molecular mechanism of HA/sorbitol on IL-1β- and hydrogen peroxide (H_2O_2)-dependent stimulation of catabolic and inflammatory responses, oxidative stress and apoptosis in cultured human chondrocytes. Clinical investigation has disclosed that HA/sorbitol significantly reduces pain for at least 6 months after the first injection [18, 19]. HA/sorbitol is a commercially available product (Synolis V-A) which contains a unique combination of key ingredients: sodium hyaluronate and sorbitol. It provides lubrication and shock absorption to joints. In addition, sorbitol prevents tissue damage caused by inflammation and helps to protect sodium hyaluronate from degradation [20]. However, the mechanism of HA/sorbitol signaling is poorly defined.

Materials and methods

Specimen selection

Discarded human post-surgery OA articular cartilage was obtained from OA patients ($n = 24$, age 67 ± 9 years mean $\pm$ SD) who had undergone total knee arthroplasty. Informed consent was received from them to study their tissues for research purposes. All patients were evaluated by rheumatologists according to American College of

Rheumatology criteria [21]. These specimens represented moderate to severe OA graded by the Mankin system [22]. Cartilage was mostly taken from femoral regions. Our experimental protocol for research into human tissues was approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal.

OA knee cartilage specimens were sliced and rinsed before chondrocyte extraction by sequential enzymatic digestion [23]. Cartilage samples were digested with 1 mg/ml of pronase (Sigma-Aldrich, Oakville, ON) for 1 h at 37 °C, and then with 2 mg/ml of type IV collagenase (Sigma-Aldrich) for 6 h in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Burlington, ON) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen). Chondrocytes were seeded at high density in culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air until they were confluent and ready for experimentation.

HA/sorbitol and experimental culture conditions

Synolis™ V-A (Anteis SA, Geneva, Switzerland), the specific HA/sorbitol formulation tested, is based on HA of high molecular weight (>2 MDa), of non-animal origin with a high HA concentration (20 mg/ml) combined with a high concentration of sorbitol (40 mg/ml), a free radical scavenger.

First-passage chondrocytes were prepared, as described previously [23]. Briefly, the cells were seeded in 24-well plates at high density (2×10^5 cells/cm^2) and cultured in DMEM (Invitrogen) supplemented with 10 % FBS (Invitrogen), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen) at 37 °C in a humidified atmosphere. To ensure that isolated chondrocytes conserve their original phenotype, type II collagen was measured routinely by real-time polymerase chain reaction. In our experiments, the culture medium was replaced by DMEM containing 1 % FBS 24 h before treatment.

OA chondrocytes were pretreated with increasing HA/sorbitol concentrations (0, 20, 50, 100, 500 μg/ml) for 2 h, followed by incubation with or without 1 ng/ml of IL-1β (R&D Systems, Minneapolis, MN), or 0.5 mM H_2O_2 (Sigma-Aldrich) for 1 or 24 h in 1 % FBS-DMEM. In each experimental condition, the final sorbitol concentration was 0, 40, 100, 200 or 1,000 μg/ml. In another set of experiments, OA chondrocytes were pre-treated or not with 1 μg/ml anti-CD44 antibody and 50 μg/ml HA/sorbitol for 2 h, followed by another incubation for 24 h with or without 1 ng/ml IL-1β (R&D Systems). The cells were also treated with 100 μg/ml sorbitol or 50 μg/ml HA alone (Sigma-Aldrich) for 2 h, followed by another incubation for 24 h in the presence or absence of 1 ng/ml IL-1β (R&D Systems) or 0.5 mM H_2O_2 (Sigma-Aldrich).

Prostaglandin E2 (PGE$_2$), metalloproteinase-13 (MMP-13), and nitric oxide (NO) measurement

Media were collected after chondrocyte incubation for 24 h, and PGE$_2$ and MMP-13 levels were assessed by enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) or enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems), respectively. Detection sensitivity was 9 and 8 pg/ml, respectively. All assays were performed in duplicate. Nitrite, a stable end-product of NO, was quantified in supernatants according to a spectrophotometric method based on the Griess reaction [24]. Absorbance was measured with a micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT).

Cell viability

Chondrocyte viability was evaluated, as described previously [25], by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay in 96-well plates (Fisher Scientific Company, Ottawa, ON), by incubating the cells with 0.5 mg/ml MTT reagent (Sigma-Aldrich) for 15 min at 37 °C. Then, 100 μl of solubilization solution (0.04 M HCl-isopropanol) was added, formazan salt was dissolved, and absorbance was read at 570 nm with a micro-ELISA Vmax photometer (Bio-Tek Instruments).

Protein detection by Western blotting

20 μg of total proteins of chondrocyte lysates, treated under the indicated conditions, were loaded for discontinuous 4–12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON) for protein immunodetection and semi-quantitative measurement [23]. The primary antibodies deployed were rabbit anti-phospho and total p47 NADPH oxidase (pp47-NOX, Sigma-Aldrich), anti-inducible NO synthase (iNOS, Cayman Chemical Company), anti-phospho and total p38 mitogen-activated protein kinase (p38 MAPK, Cell Signaling Technology, Inc., Danvers, MA), anti-phospho and total extracellular signal-regulated kinases 1 and 2 (ERK$^{1/2}$, Cell Signaling Technology, Inc.), anti-phospho nuclear factor-kappaB/p65 (NF-κB/p65, Cell Signaling Technology, Inc.), anti-glutathione-s-transferase A4-4 (Gsta4-4, Sigma-Aldrich), and anti-human β-actin (Sigma-Aldrich). After serial washes, the primary antibodies were revealed by goat anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Cell Signaling Technology, Inc.). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce, Rockford, IL) and exposed to Kodak X-Omat film (Eastman Kodak Company, Rochester, NY).

Cellular level of 4-hydroxynonenal (HNE)-protein adducts

Total cellular levels of HNE-protein adducts were calculated in chondrocyte extracts by in-house ELISA under the conditions indicated [23]. HNE-modified bovine serum albumin served as standard for HNE-protein adduct assay.

Reactive oxygen species (ROS) measurement

Intracellular ROS formation was quantified with MitoSOX^{TM} Red reagent (Invitrogen). Its oxidation by superoxide produces red fluorescence, as described by Bentz et al. [26]. Briefly, chondrocytes were seeded at a density of 2×10^4 cells/well in 96-well black plates (Becton–Dickinson, San Jose, CA). They were pretreated with increasing concentrations of HA/sorbitol 2 h before their exposure to IL-1β. Fluorescence was measured by a fluorescence plate reader at 510-nm absorption and 580-nm emission. The data were expressed as relative MitoSOX^{TM} Red fluorescence.

DNA fragmentation

Cytoplasmic histone-associated DNA fragments were quantified with Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Laval, QC) according to the manufacturer's recommendations. Briefly, chondrocytes (2×10^6 cells) were pretreated for 2 h with increasing HA/sorbitol concentrations (0–500 μg/ml) and then treated with or without 1 ng/ml IL-1β for 24 h. After incubation, the cells were lysed with lysis buffer for 30 min and centrifuged at 200g for 10 min. The supernatant and a mixture of anti-histone-biotin and anti-DNA-peroxidase were added to streptavidin-coated microplates and incubated for 2 h at room temperature. Absorbance was measured at 405 nm after addition of the substrate.

Statistical analysis

All values are expressed as mean ± SD unless indicated otherwise. Multiple comparisons were made by one-way analysis of variance, as required, followed by Bonferroni's multiple-comparison post-test. Statistical analysis was undertaken with GraphPad Prism software, version 4b (GraphPad Software, San Diego, CA). In all tests, the criterion for statistical significance was $P < 0.05$.

Results

HA/sorbitol abolishes ROS generation and pp47-NOX

To test the hypothesis that HA/sorbitol scavenges ROS, OA chondrocytes were pretreated with HA/sorbitol
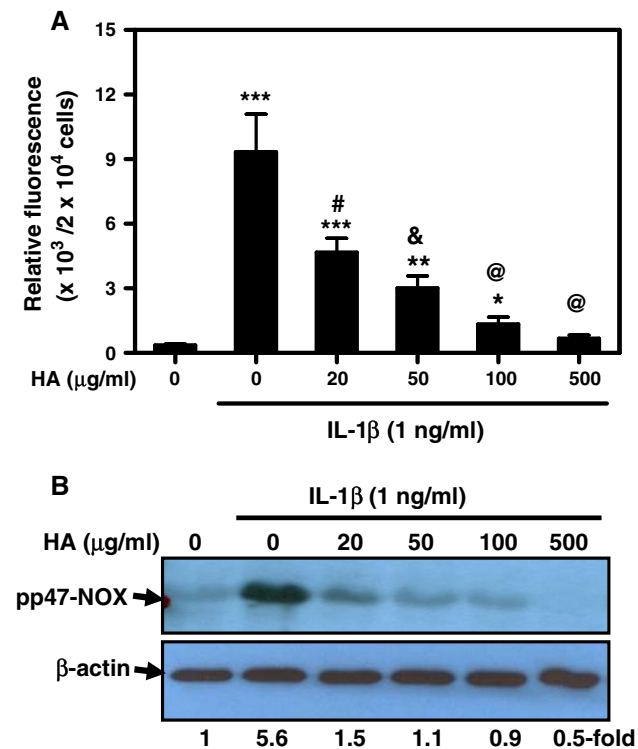
Fig. 1 HA/sorbitol suppresses ROS generation and p47-NOX phosphorylation. Confluent human osteoarthritic (OA) chondrocytes were treated for 2 h with escalating doses of HA/sorbitol (0–500 μg/ml), followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. **a** ROS generation was quantified in cell extracts with MitoSOX™ Red reagent. ROS levels were expressed as relative MitoSOX™ Red fluorescence. **b** p47-NOX protein phosphorylation (pp47-NOX) was analyzed by Western blotting in extracts of human OA chondrocytes treated as described above. The data are mean ± SD of 4 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$: compared to untreated cells; $^\#P < 0.05$, $^\&P < 0.01$, $^@P < 0.001$: compared to IL-1β-treated cells (color figure online)

Fig. 2 HA/sorbitol reduces IL-1β-induced HNE generation and Gsta4-4 downregulation in human osteoarthritic chondrocytes. **a** HNE-protein adducts and **b** Gsta4-4 protein expression were measured by ELISA and Western blotting, respectively, in cellular extracts of chondrocytes treated with various concentrations of HA/sorbitol (0–500 μg/ml) for 2 h in the presence or absence of 1 ng/ml IL-1β for 24 h. The data are mean ± SD of 4 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$: compared to untreated cells; $^\#P < 0.05$, $^\&P < 0.01$, $^@P < 0.001$: compared to IL-1β-treated cells

(0–500 μg/ml) for 2 h, followed by another treatment with 1 ng/ml IL-1β for 24 h. ROS generation was quantified by commercial kit. Our data indicated that relative MitoSOX™ Red fluorescence was stronger in IL-1β-treated cells and reached $9.2 \pm 1.7 \times 10^3/2 \times 10^4$ cells ($P < 0.001$) (Fig. 1a). However, when the cells were treated with both 1 ng/ml IL-1β and 100 μg HA/sorbitol, relative MitoSOX™ Red fluorescence weakened significantly to $1.3 \pm 0.33 \times 10^3/2 \times 10^4$ cells ($P < 0.001$, vs. IL-1β) (Fig. 1a). Finally, an additional experiment was performed to determine the possible ability of HA/sorbitol to inhibit pp47-NOX, a ROS-generating enzyme. Western blotting analysis showed that HA/sorbitol, at a concentration of 20 μg/ml, prevented IL-1β-induced pp47-NOX (Fig. 1b). Collectively, our data indicated that suppression of ROS production and ROS-generating NOX confirmed the antioxidant properties of HA/sorbitol.
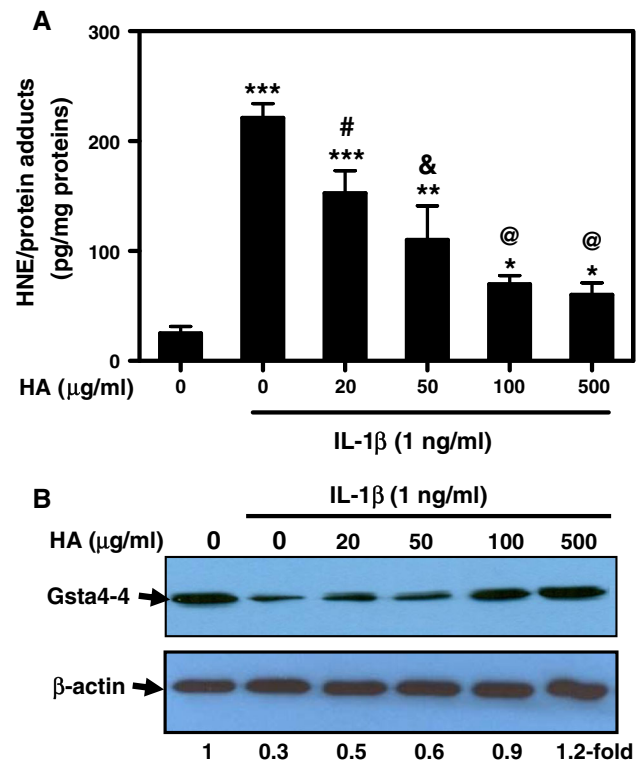
HA/sorbitol prevents HNE production and Gsta4-4 downregulation

It is well-documented that the generation of ROS, such as superoxide anion and hydroxyl radical, plays an important role in initiating lipid peroxidation (LPO). Thus, the purpose of this part of the present study was to investigate whether HA/sorbitol's capacity to inhibit HNE production was attributed to its ability to also prevent ROS generation. To do so, OA chondrocytes were pretreated with HA/sorbitol (0–500 μg) for 2 h, followed by exposure to 1 ng/ml IL-1β for 24 h.

As illustrated in Fig. 2a, escalating HA/sorbitol doses blocked IL-1β-induced HNE production. HA/sorbitol at 50 μg reduced HNE level by 50 % ($P < 0.01$), compared to cells treated with IL-1β alone. These data suggest that inhibition of the LPO process by HA/sorbitol could be related to low ROS levels.
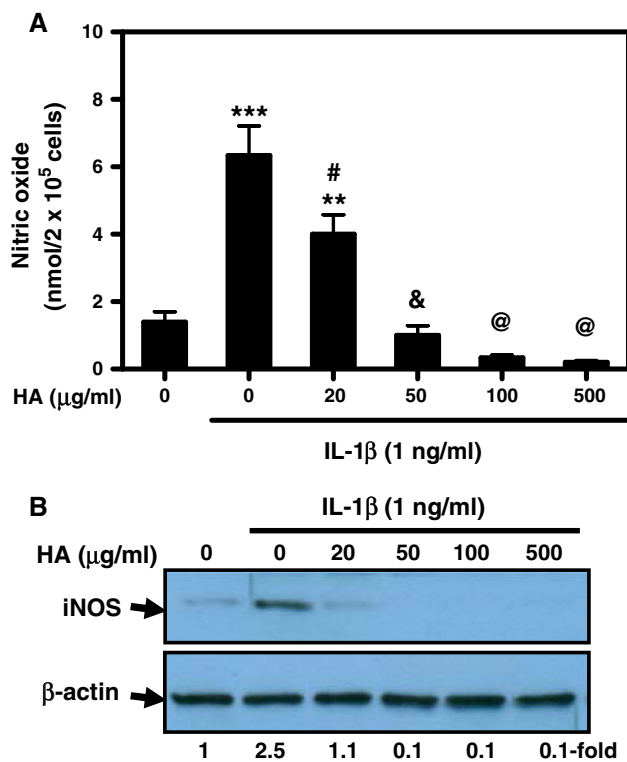
Fig. 3 HA/sorbitol suppresses NO generation and iNOS expression. Confluent human osteoarthritic (OA) chondrocytes were treated for 2 h with 10 escalating doses of HA/sorbitol (0–500 μg/ml), followed by incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. a NO levels were measured in culture medium by the Griess method. b iNOS protein expression was analyzed by Western blotting in extracts of human OA chondrocytes treated as described above. The data are mean ± SD of 4 independent experiments. $**P < 0.01$, $***P < 0.001$: compared to untreated cells; $^{\#}P < 0.05$, $^{\&}P < 0.01$, $^{@}P < 0.001$: compared to IL-1β-treated cells

Fig. 4 HA/sorbitol inhibits IL-1β-induced PGE$_2$ and MMP-13 production in human osteoarthritic chondrocytes. Isolated cells were pretreated with increasing doses of HA/sorbitol (0–500 μg/ml) for 2 h, followed by incubation for 24 h in the presence of 1 ng/ml IL-1β. MMP-13 (a) and PGE$_2$ (b) levels were measured in culture medium by commercial kits. The data are mean ± SD of 4 experiments. Student's unpaired t test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$: compared to untreated cells; $^{\#}P < 0.05$, $^{\&}P < 0.01$, $^{@}P < 0.001$: compared to IL-1β-treated cells

Gsta4-4 exhibits high catalytic efficiency in HNE metabolism. Here, we tested our hypothesis that HA/sorbitol prevents IL-1β-induced Gsta4-4 downregulation in human OA chondrocytes. Our results revealed that Gsta4-4 protein expression was lower in IL-1β-treated than in untreated cells (Fig. 2b). However, 100 μg HA/sorbitol significantly abolished IL-1β inhibition of Gsta4-4 expression in OA chondrocytes. Altogether, our findings confirm that HA/sorbitol restores redox status by preventing Gsta4-4 downregulation in OA chondrocytes.

HA/sorbitol prevents NO and iNOS production in OA chondrocytes

Next, we performed additional experiments to demonstrate that HA/sorbitol averts NO release and inhibits iNOS expression in isolated human OA chondrocytes. Cells were treated for 2 h with increasing doses of HA/sorbitol (0–500 μg) and thereafter with or without 1 ng/ml IL-1β
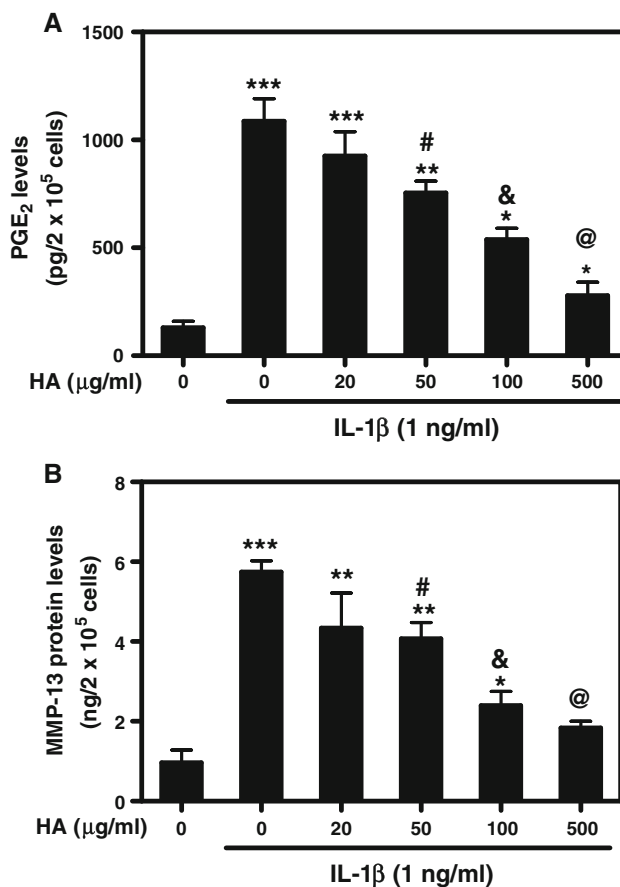
for 24 h. As illustrated in Fig. 3, HA/sorbitol significantly reduced IL-1β-induced NO release (Fig. 3a) and iNOS protein levels (Fig. 3b) in a dose-dependent manner. At 100 μg, it suppressed NO release and iNOS expression by 95 % ($P < 0.001$). Collectively, these data confirm that HA/sorbitol is a potent iNOS inhibitor.

HA/sorbitol abrogates IL-1β-induced PGE$_2$ and MMP-13 production

This part of our study was designed to verify HA/sorbitol's ability to attenuate IL-1β-evoked production of inflammatory and catabolic mediators known to be involved in cartilage damage, such as PGE$_2$ and MMP-13, respectively. When cells were treated with IL-1β, HA/sorbitol prevented PGE$_2$ (Fig. 4a) and MMP-13 (Fig. 4b) release.
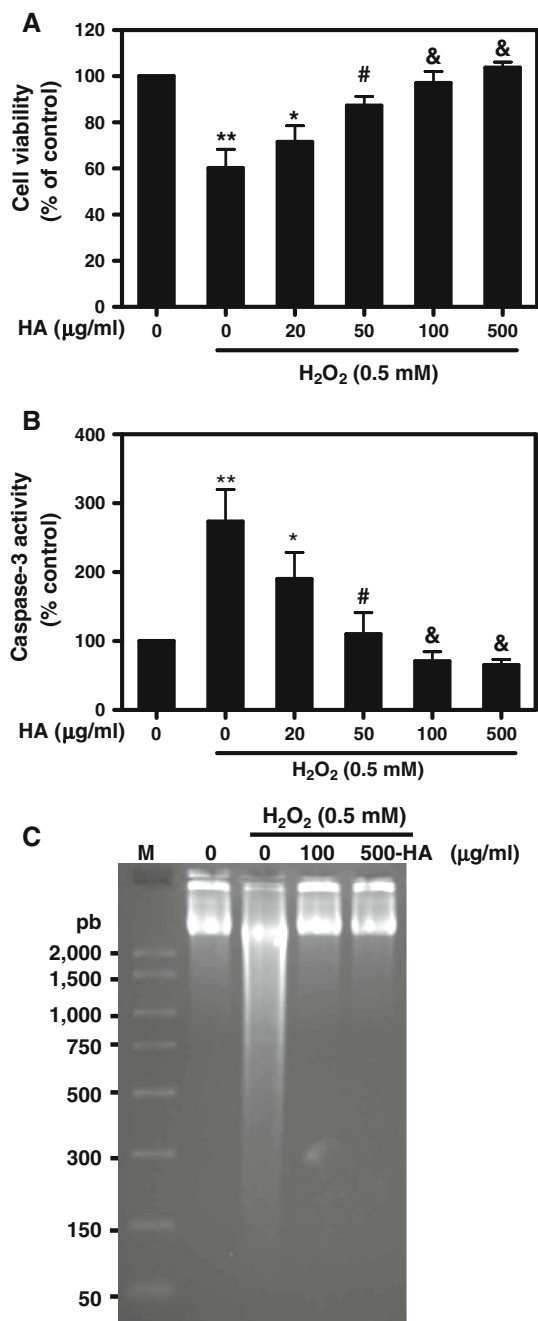
Fig. 5 HA/sorbitol suppresses H_2O_2-induced caspase-3 activation, cell death and DNA fragmentation. Confluent human osteoarthritic (OA) chondrocytes were treated for 2 h with 10 escalating doses of HA/sorbitol (0–500 μg/ml), followed by incubation for 24 h in the presence or absence of H_2O_2 (0.5 mM). **a** Caspase-3 activity was assessed in cellular extracts by commercial kit. **b** Cell viability was analyzed by MTT assay. **c** DNA was extracted after each treatment by commercial kit, and cytoplasmic histone-associated DNA fragments were quantified by kit. The data are mean ± SD of 3 independent experiments. *$P < 0.05$, **$P < 0.01$: compared to untreated cells; #$P < 0.05$, &$P < 0.01$: compared to H_2O_2-treated cells
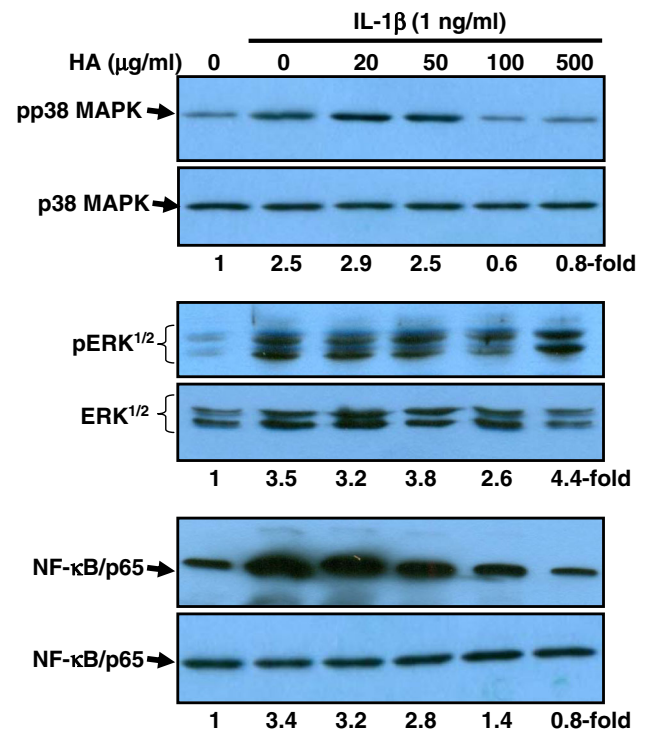

Fig. 6 HA/sorbitol prevents p38 MAPK and p65-NF-κB but not $ERK^{1/2}$ activation. OA chondrocytes were incubated with escalating doses of HA/sorbitol (0–500 μg/ml) for 2 h, followed by a second incubation for 24 h in the presence or absence of 1 ng/ml IL-1β. Cellular extracts were then subjected to Western blotting with specific polyclonal antibodies, anti-phospho and anti-total p38 MAPK, anti-phospho and total NF-κB/p65, and phospho and anti-total $ERK^{1/2}$, as described in "Materials and methods."

At 100 μg, it reduced PGE_2 and MMP-13 levels by 60 % ($P < 0.001$).

HA/sorbitol blocks H_2O_2-induced cell toxicity and death

In the next set of experiments, we evaluated the ability of HA/sorbitol to reduce H_2O_2 cytotoxicity in cultured chondrocytes. Cell viability was assessed with MTT reagent. After 24 h of incubation, pretreatment with 20–500 μg HA/sorbitol for 2 h, before adding 0.5 mM H_2O_2 to culture media, prevented H_2O_2-induced cell death (Fig. 5a) as well as markers of apoptosis, including caspase-3 activation (Fig. 5b) and DNA fragmentation (Fig. 5c). These data suggest that HA/sorbitol probably precludes H_2O_2's effects through direct hydroxyl radical- or HNE-quenching or p47-NOX inactivation.
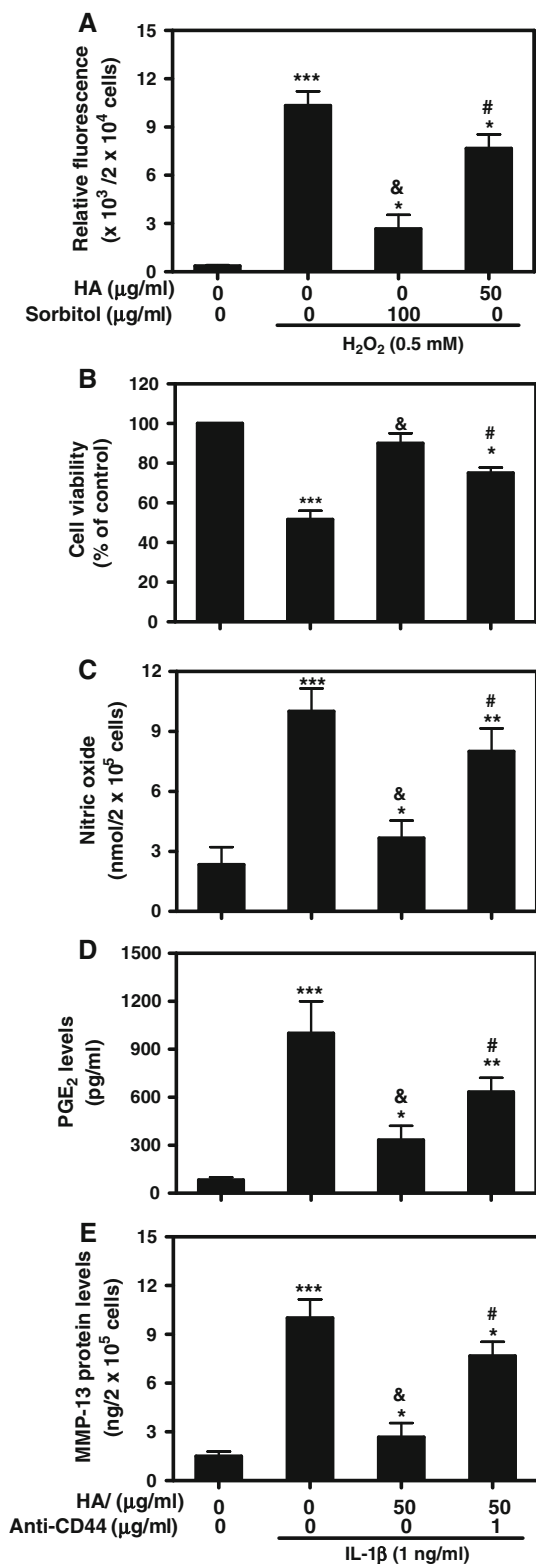
Fig. 7 Distinct effects of sorbitol and HA. **a**, **b** Sorbitol suppresses ROS generation and prevents cell death. Confluent human osteoarthritic (OA) chondrocytes were treated for 2 h with 100 μg/ml sorbitol or 50 μg/ml HA, followed by a second incubation for 24 h in the presence or absence of 1 ng/ml IL-1β or 0.5 mM H_2O_2. ROS generation was quantified in cell extracts with MitoSOX^{TM} Red reagent. Cell viability was measured by MTT assay. **c**, **d** HA/sorbitol blocks NO, PGE_2, and MMP-13 production through the CD44 pathway. Cells were pretreated with 1 μg/ml anti-CD44 antibody and 50 μg/ml HA/sorbitol for 2 h, followed by another incubation for 24 h with 1 ng/ml IL-1β. NO level was assessed in culture media according to a spectrophotometric method based on the Griess reaction. PGE_2 and MMP-13 levels were determined in culture media by EIA and ELISA kits, respectively. The data are mean ± SD of 4 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$: compared to untreated cells; $^{\#}P < 0.05$, $^{\&}P < 0.01$, $^{@}P < 0.001$: compared to H_2O_2- or IL-1β-treated cells

HA/sorbitol inhibits p38 MAPK and NF-κB/p65 activation but not ERK$^{1/2}$

To gain insights into the signaling pathways activated by IL-1β in isolated OA chondrocytes in the presence or absence of HA/sorbitol, we examined the phosphorylation patterns of p38 MAPK, ERK$^{1/2}$ and NF-κB/p65. When cells were incubated with IL-1β and HA/sorbitol for 60 min, p38 MAPK and NF-κB/p65 phosphorylation levels were lower than in cells treated with IL-1β alone (Fig. 6). In contrast, HA/sorbitol had no effect on ERK$^{1/2}$ protein activation evoked by IL-1β.

Distinct effects of sorbitol and HA

Finally, and to highlight the distinct effects of sorbitol and HA, OA chondrocytes were treated with 100 μg/ml sorbitol (∼0.5 mM) or 50 μg/ml HA alone with or without 1 ng/ml IL-1β or 0.5 mM H_2O_2. As illustrated in Fig. 7a, b, sorbitol significantly reduced IL-1β-induced ROS generation and H_2O_2-induced cell death ($P < 0.01$). However, HA alone had a significant but moderate effect, compared to sorbitol ($P < 0.05$). To determine the effect of HA, cells were treated with 1 μg/ml anti-CD44 antibody and 50 μg/ml HA/sorbitol, followed by a second incubation with 1 ng/ml IL-1β for 24 h. As shown in Fig. 7c–e, the addition of anti-CD44 antibody blocked the biological effects of HA/sorbitol, as measured by NO, PGE_2, and MMP-13 determination.

Discussion

HA is a widely distributed tissue ECM component characterized by a repeating disaccharide of *N*-acetylglucosamine and D-glucuronic acid and providing various structural and regulatory functions, including joint lubrication [27]. HA has been administered clinically to treat OA for more than 25 years. Although controversy still surrounds its clinical value [28, 29], numerous clinical trials and meta-analyses have reported durable benefits in knee OA [30–33]. In addition to OA, HA significantly alleviates pain and inflammation in some OA patients [34]. The rheological properties of SF are complex and depend on the molecular weight (MW) of HA and its concentration [35]. Both these HA parameters are significantly decreased in SF from OA and rheumatoid arthritis (RA) compared to normal knees. Low-MW fragments are biologically active in promoting inflammation [36, 37]. Emerging data suggest that high-MW HA has potential to suppress signaling activated by low-MW HA fragments in chondrocytes and various cells through different receptors, including the principal HA receptor CD44 [38].

In this study, we tested HA/sorbitol which contains a unique combination of key ingredients: sodium hyaluronate and sorbitol. Sodium hyaluronate is a natural component of joint SF that provides lubrication and shock absorption. Sorbitol prevents tissue damage caused by inflammation and helps to protect sodium hyaluronate from degradation [20]. It is considered to be a potent scavenger of hydroxyl radicals [39]. We have demonstrated that HA/sorbitol abrogates IL-1β-induced production of catabolic, inflammatory and oxidative stress mediators, such as MMP-13, NO, iNOS, PGE$_2$, ROS and HNE as well as H$_2$O$_2$-induced markers of apoptosis, including caspase-3 activation and DNA fragmentation. Interestingly, our findings showed that the antioxidant as well as the anti-catabolic and anti-inflammatory properties of HA/sorbitol conjugate could be attributed to sorbitol and HA, respectively. All these processes (catabolism, inflammation, oxidative stress and apoptosis) are deemed to be hallmarks of cartilage degradation and synovial inflammation in OA. Upon examination of signaling pathways, we noted that HA/sorbitol prevented IL-1β-induced p38 MAPK and NF-κB/p65 activation, but not that of ERK[1/2].

Our results are in concordance with the literature. In vitro and in vivo studies have demonstrated that HA reduces the expression of MMPs, iNOS and COX-2 [40, 41]. In particular, HA, via its CD44 receptor, suppresses MMP production through p38 MAPK downregulation in IL-1β-stimulated rheumatoid synovial fibroblasts [42] and chondrocytes [43]. Because p38 MAPK activation kindles the expression of various inflammatory genes that cause arthritis, its suppression could protect articular cartilage

from destruction [44]. NF-κB is another important player as it initiates and sustains inflammatory reactions. It regulates many genes, including cytokines, chemokines, and adhesion molecules, that participate in the pathophysiology of synovial inflammation and bone and cartilage degradation [45]. HA can suppress NF-κB activation by fibronectin in RA chondrocytes [46]. With regard to ERK[1/2] activation, our findings are in agreement with those of Julovi et al. [43], who reported that HA was ineffective in counteracting IL-1β-induced ERK[1/2] phosphorylation in both OA and RA chondrocytes. In contrast to our study, Hashizume and Mihara showed that HA suppressed MMP induction by IL-6 in human chondrocytes via ERK[1/2] inactivation through CD44 signaling [47]. CD44 inhibition, by blocking antibody, significantly reverses the inhibitory effect of HA on MMP-13 production. Taken together, HA is likely to suppress the intracellular pathways activated by catabolic stimulators in arthritic joints.

Our investigation into the effect of HA/sorbitol gel on redox status revealed a decrease in HNE and ROS generation as well as Gsta4-4 upregulation. The latter is a major HNE-metabolizing enzyme, and its inhibition induces chondrocyte apoptosis [48]. In particular, the antioxidant capacity of HA/sorbitol conjugate is mostly attributed to sorbitol rather than HA. The results of the present study are in contrast to those of others in that sorbitol can act as an oxidant agent [49, 50]. In those experiments, the authors used very high sorbitol concentrations (0.4–1 M). In regard to HA, our observations are in agreement with previous reports indicating the antioxidant properties of HA in different cell types. Ke et al. [51] demonstrated that HA reduces LPO products and ROS generation but, in contrast, induces the activity of superoxide dismutase, catalase and glutathione peroxidase. The antioxidant mechanisms of HA include also its capacity to inhibit the Fenton reaction via the entrapment of iron ions and to scavenge directly free radical [52]. On the other hand, the impact of extensive ROS and reactive nitrogen species accumulation on HA stability and properties has also been reported. Several authors have demonstrated that HA oxidation by these reactive species is linked with HA cleavage and fragmentation, resulting in reduced HA viscosity [53–55]. Monzon et al. [56] provided evidence that ROS evoke HA fragmentation in normal human bronchial epithelial cells via hyaluronidase 2 (Hyal2) upregulation. Hyal2, a HA-degrading enzyme, could be considered as a key candidate orchestrating inflammatory responses coupled with HA fragmentation, particularly in oxidative stress conditions.

In the present study, we also investigated the effect of HA/sorbitol on IL-1β-induced apoptosis of human OA chondrocytes. We found that HA reverses the process and inhibits apoptosis, as shown by caspase-3 assay and DNA fragmentation analysis. In a similar investigation conducted on

isolated human chondrocytes, Grishko et al. [57] determined that HA has the capacity to protect mitochondria and its genome from the damaging effects of oxidative stress and preserve one of the most essential mitochondrial functions, energy production. Another study showed that HA inhibited IL-1β-induced chondrocyte apoptosis [58]. It has been established that adding HA to media dose dependently reduces the impairment of mitochondrial membrane potential and restores mitochondrial ATP production. In an experimental rabbit model of OA, HA administration is effective in ameliorating damage associated with the OA process, as evidenced by decreased apoptosis.

The chondrocyte death/apoptotic phenomenon in OA is complex and seems related to excessive synthesis of factors having pro-apoptotic activity in cartilage and synovium. Among them, NO and PGE$_2$ seem predominant [59]. Peng et al. [60] demonstrated that HA blocks apoptosis and dedifferentiation of articular chondrocytes caused by NO production in a dose-dependent manner. The inhibitory effects of HA on apoptosis are derived from their ability to block NO-induced inhibition of protein kinase C alpha (PKCα). These results suggest that HA exerts a protective action on cartilage chondrocytes induced by NO, not only by reversing mitochondrial depolarization, but also by blocking PKCα inhibition. Altogether, suppression of NO and inflammatory cytokine activity within joints might be an important mechanism of the clinical action of intraarticular HA injection in OA treatment.

HA's effect appears to be mediated through CD44 receptor binding. A large body of evidence indicates that CD44 receptors may play a critical role in the normal function and survival of many cell types. CD44 can promote resistance to apoptosis in the colonic epithelium via a mitochondria-controlled pathway [61]. In addition, it has been shown that CD44 expression in some cell types, such as stem cells, may provide the means to internalize HA by endocytosis, and one of the functions of internalized HA may be protection of DNA from oxidants [52]. In relation to cartilage biology, it has been determined that CD44 is important in both the normal and abnormal functions of cartilage through its adhesion to HA, which induces a variety of stimulatory signals that regulate chondrocyte proliferation as well as matrix synthesis in the cartilage microenvironment [62].

The present study shows that HA/sorbitol dose dependently suppresses catabolic and inflammatory responses as well as oxidative stress-induced chondrocyte apoptosis in isolated human OA chondrocytes. The suppression of these responses within joints might be a crucial mechanism of clinical HA/sorbitol action by intraarticular injection in OA treatment.

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