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Hyaluronan Protects Bovine Articular Chondrocytes against Cell Death Induced by Bupivacaine under Supraphysiologic Temperatures

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Abstract

Background—Bupivacaine and supraphysiologic temperature can independently reduce cell viability of articular chondrocytes. In combination these two deleterious factors could further impair cell viability.

Hypothesis—Hyaluronan may protect chondrocytes from death induced by bupivacaine at supraphysiologic temperatures.

Study Design—Controlled laboratory study.

Methods—Bovine articular chondrocytes were treated with hyaluronan at physiologic (37°C) and supraphysiologic temperatures (45°C and 50°C) for one hour, and then exposed to bupivacaine for one hour at room temperature. Cell viability was assessed at three time points: immediately after treatment, six hours later, and twenty-four hours later using flow cytometry and fluorescence microscopy. The effects of hyaluronan on the levels of sulfated glycosaminoglycan in the chondrocytes were determined using Alcian blue staining.

Results—(1) Bupivacaine alone did not induce noticeable chondrocyte death at 37°C; (2) bupivacaine and temperature synergistically increased chondrocyte death, that is, when the chondrocytes were conditioned to 45°C and 50°C, 0.25% and 0.5% bupivacaine increased the cell death rate by 131% to 383% in comparison to the phosphate-buffered saline control group; and, (3) addition of hyaluronan reduced chondrocyte death rates to approximately 14% and 25% at 45°C and 50°C, respectively. Hyaluronan's protective effects were still observed at six and twenty-four hours after bupivacaine treatment at 45°C. However, at 50°C, hyaluronan delayed but did not prevent the cell death caused by bupivacaine. One-hour treatment with hyaluronan significantly increased sulfated glycosaminoglycan levels in the chondrocytes.

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This study was performed at Tulane University.

One or more of the authors has declared a potential conflict of interest as specified in the AJSM Conflict of Interest statement.

Conclusions—Bupivacaine and supraphysiologic temperature synergistically increase chondrocyte death and hyaluronan may protect articular chondrocytes from death caused by bupivacaine.

Clinical Relevance—This study provides a rationale to perform pre-clinical and clinical studies to evaluate whether intra-articular injection of a mixture of bupivacaine and hyaluronan after arthroscopic surgery may protect against bupivacaine's chondrotoxicity.

Keywords

anesthetics; bupivacaine; chondrocyte viability; hyaluronan; Orthovisc

Introduction

Bupivacaine has been used in orthopaedic patients as a local analgesic agent after arthroscopic procedures either in a continuous intra-articular infusion or a single intra-articular injection.¹ Central nervous system depression, cardiac arrhythmias, and cardiac arrest are systemic toxicities that have been described when bupivacaine is given in intravenous infusion,¹⁹ but its local toxicity to articular chondrocytes has only been investigated recently.²⁶ Several studies have suggested that bupivacaine caused significant decreases in chondrocyte function and viability after short exposures and that its chondrotoxicity was dose and time dependent.^{5, 11, 12} A recent study showed that bupivacaine induced chondrotoxicity in a monolayer culture but not in cartilage plugs.²⁹ Bupivacaine is considered as a potential contributor for chondrolysis after arthroscopy.²⁸

Horstman et al¹⁷ noted that chondrocytes were damaged at temperatures as low as 45°C during arthroscopic surgery. Edwards et al¹⁰ found that temperatures generated by the use of radiofrequency devices are high enough (>70°C) to cause chondrocyte death as deep as 2,000 µm under the articular surface. Supraphysiologic temperatures resulting from the use of radiofrequency or other thermal devices during arthroscopic surgeries have been associated with postarthroscopic glenohumeral chondrolysis.²⁸

Orthovisc® is a bacterially-derived high molecular weight (1.0–2.9 million daltons) sodium hyaluronate dissolved in physiologic saline with a concentration of hyaluronan of 15 mg/mL (manufactured by Anika Therapeutics, Inc., Woburn, Massachusetts; distributed by DePuy Mitek, Raynham, Massachusetts). It is indicated in the treatment of knee pain in osteoarthritis patients. At least two studies have suggested that Orthovisc® is effective and well tolerated by intra-articular injection.^{21, 32}

Given that both bupivacaine and hyaluronan can be injected intra-articularly to relieve pain and that bupivacaine is likely toxic to articular chondrocytes, we reason that hyaluronan may be able to protect chondrocytes from bupivacaine's cytotoxicity because high molecular weight hyaluronan has been shown to decrease the toxicity of benzalkonium to human epithelial cells.²⁵ If this is true, it is plausible to inject a mixture of bupivacaine and hyaluronan after arthroscopic procedures. In these cases, bupivacaine can provide a short-term pain relief while hyaluronan provides long-term viscosupplementation and pain relief. The idea of intra-articular injection of a mixture of bupivacaine and hyaluronan has never been tested before. Therefore, we conducted the present in vitro study, the objective of which was to test our hypothesis that hyaluronan may protect articular chondrocytes from bupivacaine-induced cell death at supraphysiologic temperatures. We formulated this hypothesis based on two previous reports that addition of hyaluronan to cultured human chondrocytes can enhance the survival of chondrocytes³ and chondrocytes pretreated with hyaluronan can decrease mitochondrial DNA damage, preserve ATP levels, and increase cell viability.¹⁴

MATERIALS AND METHODS

Cell Culture

Normal bovine articular chondrocytes were isolated from twelve stifle joints (equivalent to human knee joints) of 3-week-old calves obtained from an abattoir. The bovine articular chondrocytes were cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, California) in a humidified incubator with 5% CO₂ at 37°C. The chondrocytes used in this study were cultured in monolayer for approximately two weeks.

Treatment of Cells in Suspension

To assess the effects of different dosages of bupivacaine (Hospira, Inc., Lake Forest, Illinois) and hyaluronan (Orthovisc®) on chondrocytes, approximately 0.3 million bovine articular chondrocytes were suspended in phosphate-buffered saline (PBS) and placed into 12×75-mm plastic non-permeable test tubes. The cells were centrifuged at 1,000 revolutions per minute for five minutes. After discarding the supernatant, 0.3 mL of PBS or treatment solution (with hyaluronan but without bupivacaine) was added to each tube. The cells were re-suspended by gently pipetting and kept in suspension by means of gentle vortexing once every five minutes. Each control or treatment group consisted of three tubes. The twelve experimental groups are listed in TABLE 1. The control and treatment groups (only PBS and hyaluronan were added, but no bupivacaine) were incubated at 37, 45, and 50°C for one hour and then bupivacaine was added and mixed by pipetting. Of note, hyaluronan was added to the cells first because hyaluronan was very viscous and difficult to be mixed with the cells, while bupivacaine was easily mixed. The samples were treated at room temperature for another one hour to mimic the simultaneous exposure of the cells to hyaluronan and bupivacaine. After treatment, the cells were washed three times with PBS and processed for flow cytometry analysis.

Flow Cytometry Analysis of Short-time Cell Viability

The treated chondrocytes were stained with a solution containing 0.8-μM calcein AM (acetoxymethyl ester) and 0.8-μM ethidium homodimer-1 in PBS for twenty minutes in the dark at room temperature. The reagents were obtained from the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, Oregon). Live cells emit green fluorescence while dead cells emit red fluorescence,³³ which can be discriminated by flow cytometry analysis as previously reported.⁶ Percentages of live and dead cells were obtained by counting 10,000 cells per sample using a Becton-Dickinson Life Science Research II (BD LSRII) flow cytometry analyzer (Becton, Dickinson and Company, San Jose, California).

Microscopic Analysis of Long-time Cell Viability

To examine the cell viability six hours and twenty-four hours after the treatment, the control and treatment groups (1) to (12) were treated as described above (see TABLE 1). At the end of treatment, the cells were washed three times with PBS in the test tubes to remove hyaluronan and/or bupivacaine. The cells were re-suspended in 0.5 mL of DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and placed in twenty-four-well plates in a humidified incubator with 5% CO₂ at 37°C. Six or twenty-four hours later, the cells were stained in the twenty-four-well plates with 0.8-μM calcein AM and 0.8-μM ethidium homodimer-1 for twenty minutes in the dark at room temperature. The stained cells in the center of each well were photographed with a fluorescence microscope (Nikon AZ100) equipped with a digital camera (Nikon DS-Qi1Mc) using NIS-Elements Basic Research 3.0 software (Nikon Instruments Inc., Melville, New York).

Analysis of sulfated glycosaminoglycan Levels

In order to determine the mechanisms of how hyaluronan provides protection to chondrocytes, we analyzed the levels of sulfated glycosaminoglycan in chondrocytes treated with hyaluronan. Bovine articular chondrocytes were plated in twenty-four-well plates at a density of 300,000 cells per well in 0.5 mL of DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Twenty-four hours later, the culture medium was replaced with 0.3 mL of control or treatment solutions and incubated in a humidified incubator with 5% CO₂ at 37°C. The control and treatment groups (triplicate wells per group) include:

1. Control group: basal medium containing DMEM with 1% FBS, 50 µg/mL ascorbic acid, 100 µg/mL sodium pyruvate, and 1% penicillin/streptomycin.
2. 10% PBS + 90% basal medium.
3. 25% PBS + 75% basal medium.
4. 50% PBS + 50% basal medium.
5. 10% hyaluronan + 90% basal medium.
6. 25% hyaluronan + 75% basal medium.
7. 50% hyaluronan + 50% basal medium.

At one, twelve, or twenty-four hours after treatment, the control and treatment solutions were siphoned off. Each well was washed three times with PBS and the cells were fixed by 0.5 mL of 2% paraformaldehyde for 15 minutes. After washing three times with PBS, the cells were stained with Alcian blue dye (Sigma-Aldrich, St. Louis, Missouri) according to a published protocol.¹⁶ Briefly, the cells were stained with 0.5 mL of 1% Alcian blue in 0.1 M HCl (pH 1.0) for 30 minutes. Then, the cells were washed five times with distilled water. To quantify the intensity of Alcian blue staining, the cells were rinsed with 50 mM Tris-HCl (pH 7.4). 0.3 mL of dissolving solution containing 4 M guanidine-HCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), and 50 mM Tris-HCl (pH 7.4) was added to each well and placed on a shaker for twenty-four hours at room temperature. The absorbance of the dissolved dye in each well was read at 600-nm wavelength with a DU 800 spectrophotometer (Beckman Coulter, Inc., Brea, California).

Statistical Analysis

The experiments were performed three times independently. The means and standard deviations of 9 samples (triplicate samples per group in each experiment, thus total samples per group = 9) were compared between the control group and each treatment group, using the two-tailed Student *t* test. The level of significance was set at $P < 0.05$.

RESULTS

Effects of temperature and bupivacaine on short-time chondrocyte viability

Based on the LIVE/DEAD staining protocol, flow cytometry analysis separated the live cells (Quadrant 4) from the dead cells (Quadrant 1) (Figure 1). Quadrant 2 contained some cells in the early phase of death, whose cell membranes were broken (thus stained with ethidium) but still retained some esterase activity (thus stained with calcein AM). Unstained cells with intact membranes were located in Quadrant 3 (Figure 1). Thus, the cells within Quadrants 1 and 2 were counted as dead cells. At 37°C (Figure 1A–D), the number of dead cells from each representative treatment groups appeared less than the number of dead cells from the matched groups treated at 45°C (Figure 1E–H) and 50°C (Figure 1I–L).

Detailed analysis of the percentages of dead cells treated with 0.25% or 0.50% bupivacaine for one hour, with or without hyaluronan, was shown in Figure 2. Calculation of the percentages was based on analysis of 10,000 cells per sample, thus the denominator was 10,000. At 37°C, the chondrocyte death rate in the PBS control group was 3.6%, while the cell death rates of the treatment groups varied from 3.3% in the 0.25% bupivacaine treatment group to 9.1% in the 50% hyaluronan + 0.50% bupivacaine treatment group (Figure 2). There was no statistically significant difference between the control group and any treatment group (P values ranged from 0.055 to 0.854) except that the 50% hyaluronan + 0.50% bupivacaine treatment group had a significantly higher death rate than the control group ($P = 0.015$).

At 45°C, the PBS control group had a cell death rate of 9.2%, which was significantly higher than the cell death rate at 37°C ($P = 0.001$) (Figure 2). In comparison with the PBS control group, when the cells were treated with 0.25% and 0.50% bupivacaine, the cell death rates were 21.2% ($P = 0.028$) and 44.3% ($P < 0.001$), respectively; and, when the cells were treated with 10%, 25%, and 50% hyaluronan alone, the cell death rates were 6.6% ($P = 0.058$), 6.2% ($P = 0.025$), and 5.4% ($P = 0.013$), respectively. In comparison with the 0.25% bupivacaine group, when the cells were treated with 0.25% bupivacaine plus 10%, 25%, and 50% hyaluronan, the cell death rates were 15.8% ($P = 0.277$), 10.8% ($P = 0.049$), and 12.6% ($P = 0.108$), respectively. In comparison with the 0.50% bupivacaine group, when the cells were treated with 0.50% bupivacaine plus 10%, 25%, and 50% hyaluronan, the cell death rates were 40.8% ($P = 0.759$), 23.3% ($P = 0.017$), and 14.4% ($P = 0.001$), respectively.

At 50°C, the PBS control group had a cell death rate of 24.8%, which was significantly higher than the cell death rate at 37°C and 45°C ($P = 0.001$ and $P = 0.005$, respectively) (Figure 2). In comparison with the PBS control group, when the cells were treated with 0.25% and 0.50% bupivacaine, the cell death rates were 72.4% ($P < 0.001$) and 61.5% ($P < 0.001$), respectively; and, when the cells were treated with 10%, 25%, and 50% hyaluronan alone, the cell death rates were 15.4% ($P = 0.056$), 15.5% ($P = 0.064$), and 20.1% ($P = 0.312$). In comparison with the 0.25% bupivacaine group, when the cells were treated with 0.25% bupivacaine plus 10%, 25%, and 50% hyaluronan, the cell death rates were 45.4% ($P = 0.004$), 32.6% ($P < 0.001$), and 24.6% ($P < 0.001$), respectively. In comparison with the 0.50% bupivacaine group, when the cells were treated with 0.50% bupivacaine plus 10%, 25%, and 50% hyaluronan, the cell death rates were 55.9% ($P = 0.523$), 43.0% ($P = 0.027$), and 25.7% ($P < 0.001$), respectively.

Effects of temperature and bupivacaine on long-time chondrocyte viability

After the chondrocytes were treated with hyaluronan and bupivacaine at different temperatures, they were placed back into culture plates. Six or twenty-four hours later, the cells were stained with the LIVE/DEAD kit and examined under a fluorescence microscope. Representative photomicrographs of the cells after six hours in culture are shown in Figure 3. We found that healthy live cells stained green with spindle or polygonal shapes and were attached to the culture plate (Figure 3A and H, indicated by an arrow). The cells that stained green with a round shape and detached from the culture plate were “dying”; live cells (Figure 3F and L, indicated by an arrow). Dead cells stained red or orange/yellow due to a mixture of red and green colors (Figure 3J, indicated by an arrow). Most cells from the PBS control group and the representative treatment groups that were treated at 37°C appeared as healthy live cells (Figure 3A to D). For the chondrocytes treated at 45°C, most of the cells from the PBS control group, the 50% hyaluronan group, and the 0.50% bupivacaine + 50% hyaluronan group were still healthy live cells (Figure 3E, G, and H), whereas most of the cells from the 0.50% bupivacaine group appeared as “dying” live cells (Figure 3F). For the chondrocytes treated at 50°C, the majority of the cells from the PBS control group, the 50% hyaluronan group, and the 0.50% bupivacaine + 50% hyaluronan group became “dying” live

cells (Figure 3I, K, and L), whereas most of the cells from the 0.50% bupivacaine group appeared as dead cells (Figure 3J, the orange cells indicated by an arrow), accompanied by a few “dying” live cells (Figure 3J, the scattered green round cells).

Twenty-four hours after treatment, most cells from the PBS control group and the representative treatment groups that were treated at 37°C still appeared as healthy live cells (Figure 4A to D). For the chondrocytes treated at 45°C, most of the cells from the PBS control group, the 50% hyaluronan group, and the 0.50% bupivacaine + 50% hyaluronan group were still healthy live cells (Figure 4E, G, and H, indicated by an arrow in H), whereas approximately half of the cells from the 0.50% bupivacaine group appeared as “dying” live cells (Figure 4F, the green round cells indicated by an arrow), with the remaining half becoming dead cells (Figure 4F, the red cells in the background). For the chondrocytes treated at 50°C, approximately half of the cells from the PBS control group, the 50% hyaluronan group, and the 0.50% bupivacaine + 50% hyaluronan group became “dying” live cells (Figure 4L, the green round cells indicated by an arrow) and the other half of the cells appeared dead (Figure 4I, K, and L, the red cells in the background). In contrast, almost all of the cells from the 0.50% bupivacaine group were dead (Figure 4J, the red cells indicated by an arrow).

Effects of hyaluronan on synthesis of sulfated glycosaminoglycan in chondrocytes

We used the method of Alcian blue staining to determine the relative levels of sulfated glycosaminoglycan in the chondrocytes. The intensities of Alcian blue staining were shown as absorbance values at 600-nm wavelength (Figure 5). After one-hour in culture, the control group had an absorbance of 0.26. In comparison with the control group, the groups treated with 10%, 25%, and 50% PBS had an absorbance of 0.23 ($P = 0.499$), 0.27 ($P = 0.793$), and 0.30 ($P = 0.376$), respectively. In contrast, the groups treated with 10%, 25%, and 50% hyaluronan had an absorbance of 0.37 ($P = 0.351$), 0.49 ($P = 0.043$), and 0.61 ($P = 0.017$), respectively. After twelve hours in culture, the absorbance of the control group and 10% PBS treatment group remained unchanged, whereas the absorbance of the 25% and 50% PBS treatment groups decreased slightly. Treatment with 10%, 25%, and 50% hyaluronan did not significantly increase the absorbance in comparison with the control group ($P = 0.559$, $P = 0.701$, and $P = 0.104$, respectively). However, the 50% hyaluronan treatment group had significantly higher absorbance in comparison to the 50% PBS treatment group ($P = 0.005$). After twenty-four hours in culture, the absorbance values were similar among the control group, the PBS treatment groups, and the 10% hyaluronan treatment group. However, the 25% and 50% hyaluronan treatment groups had significantly higher absorbance values in comparison with the 25% and 50% PBS treatment groups ($P = 0.006$ and $P < 0.001$, respectively).

Discussion

In this in vitro study, we wanted to test a hypothesis that hyaluronan may protect against bupivacaine-induced chondrocyte death at supraphysiologic temperatures. Our rationale for performing this study was based on analysis of the published literature reports. First, several recent studies have reported that local anesthetics are toxic to articular chondrocytes when injected intra-articularly.^{5, 11, 12, 26, 29} Second, supraphysiologic temperatures can be achieved during arthroscopic procedures when using a radiofrequency device. Temperatures greater than 45°C can cause damage to chondrocytes and there is a sharp increase in the death rate of chondrocytes between 50°C and 55°C.^{17, 31} Third, our pilot study showed that bupivacaine induced more chondrocyte deaths at supraphysiologic temperatures than at a physiologic temperature. Bupivacaine is often injected into the joint immediately following the arthroscopic procedure, in order to relieve post-operative pain.^{8, 9} Thus, it is possible to encounter a clinical situation in which a combination of bupivacaine and supraphysiologic

temperature acts on the articular chondrocytes. In the present study, we found that 0.25% and 0.50% bupivacaine did not increase chondrocyte death rate in comparison to the phosphate-buffered saline control group when the cells were treated at 37°C, which is consistent with a previous report². Of note, a mixture of hyaluronan and 0.50% bupivacaine increased the chondrocyte death rate by 5.5% at 37°C, compared to the control group. We think that this increase might be caused by pipetting the cell suspensions during mixing bupivacaine with the cells in hyaluronan solutions, as the hyaluronan solutions were viscous. In contrast, when the cells were conditioned to 45°C and 50°C, 0.25% and 0.50% bupivacaine significantly increased the cell death rate by 131% to 383% in comparison to the phosphate-buffered saline control group ($P = 0.028$ or $P < 0.001$). The cell death rate was higher than the sum of the cell death rates caused by temperature alone and bupivacaine alone at 45°C and 50°C. Therefore, bupivacaine added to the chondrocytes at supraphysiologic temperatures may synergistically increase the chondrocyte death rate. To our best knowledge, this is the first time that a synergistic chondrotoxicity of bupivacaine and supraphysiologic temperature has been reported.

Next, we tested if hyaluronan could protect chondrocytes from bupivacaine's toxicity. We chose Orthovisc® because several studies have suggested that Orthovisc® is effective and well tolerated by intra-articular injection^{18, 21, 24, 32}. To treat osteoarthritis of the knee, two mL (containing 30 mg hyaluronan) of Orthovisc® is injected into the patient's knee joint once a week, for a total of 3 or 4 injections. Thus, we calculated that the concentration of hyaluronan in the patient's knee joint is approximately 40% to 50% (volume/volume %) because the injected hyaluronan is diluted by two to three mL of synovial fluid in the osteoarthritic knee joints²⁰. In cases where two mL of hyaluronan is mixed with two mL of bupivacaine in the injections, the intra-articular concentration of hyaluronan is predicted as 29% (i.e., 2 mL hyaluronan mixed with 2 mL bupivacaine and 3 mL synovial fluid) to 33% (i.e., 2 mL hyaluronan mixed with 2 mL bupivacaine and 2 mL synovial fluid). The synovial fluid volume may be increased after intra-articular surgery, thus further diluting the hyaluronan concentration. Therefore, in our in vitro experiments, we chose dosages of 10%, 25%, and 50% hyaluronan, which would cover the range of intra-articular concentrations of hyaluronan in the patients. We found that 25% and 50% hyaluronan significantly inhibited supraphysiologic (45°C) temperature-induced chondrocyte death ($P = 0.025$ and $P = 0.013$, respectively). For the chondrocytes at 50°C, hyaluronan alone slightly decreased the cell death rate but it was not statistically significant ($P > 0.05$). However, we found that 25% and 50% hyaluronan significantly inhibited 0.25% or 0.50% bupivacaine-induced cell death at 45°C ($P = 0.017$ and $P = 0.001$, respectively) and 50°C ($P = 0.027$ and $P < 0.001$, respectively). 10% hyaluronan significantly inhibited 0.25% bupivacaine-induced cell death at 45°C ($P = 0.004$) but not at 50°C ($P = 0.523$). These results suggest that hyaluronan inhibits bupivacaine-induced chondrocyte death at supraphysiologic temperatures in a dose-dependent manner. Because 25% and 50% hyaluronan were effective dosages in this in vitro study, we predict that intra-articular concentrations of 29% to 33% hyaluronan should be effective clinically.

In addition, we found that 50% hyaluronan's inhibition of bupivacaine-induced chondrocyte death lasted for six and twenty-four hours when the cells were initially treated at 45°C. However, when the chondrocytes were exposed to bupivacaine at 50°C, 50% hyaluronan could keep the cells alive up to six hours. After twenty-four hours, the majority of cells had died. Similar findings have been observed with 25% hyaluronan. We suspect that the injury to chondrocytes by bupivacaine at 50°C is too severe to be rescued by hyaluronan, though hyaluronan can keep the cells alive up to six hours. This result underscores the importance of avoiding injection of bupivacaine into a joint with a temperature 50°C, because once the damage occurs, it is difficult to maintain chondrocyte viability.

Although we only tested Orthovisc® in this study, we believe other hyaluronan preparations may have similar protective effects. For example, Brun et al³ reported that addition of hyaluronan to cultured human chondrocytes can enhance the survival of chondrocytes and even restore cell viability after oxidative cell injury. It is not clearly understood how hyaluronan protects chondrocytes. The molecular mechanisms may involve binding of hyaluronan to its receptor, CD44.³ Misra et al²³ has demonstrated that hyaluronan-CD44 interaction stimulates cell survival via a signaling pathway involving ErbB2, phosphoinositide 3-kinase/AKT, beta-catenin, and cyclooxygenase-2/prostaglandin E2 in colon carcinoma cells. It has also been reported that the chondrotoxicity of local anesthetics is associated with mitochondrial dysfunction that leads to a decrease in energy production and ultimately to cell death.¹⁵ Chondrocytes pretreated with hyaluronan can decrease mitochondrial DNA damage, preserve ATP levels, and increase cell viability.¹⁴ We found that hyaluronan induced chondrocytes to maintain higher levels of sulfated glycosaminoglycan within one hour. It is not clear why the overall levels of sulfated glycosaminoglycan were decreased at the twelve-hour and twenty-four-hour points of time, including in the PBS treatment groups and the hyaluronan treatment groups. We suspect that sulfated glycosaminoglycan was under rapid catabolism when the cells were limited in nutrients under the culture conditions used. Nevertheless, it has been reported that sulfated glycosaminoglycan plays an important role in cellular adhesion and cellular survival.^{22, 27, 30} Therefore, induction of sulfated glycosaminoglycan synthesis may be one of the mechanisms that hyaluronan utilizes to protect chondrocytes.

We acknowledge that this study has several limitations. First, the monolayer cultures are different from the chondrocytes in an anatomical joint. The in vivo cellular environment of the cartilage matrix can provide better protection against the harmful effects of bupivacaine.^{4, 13} It is possible that, in animal joints or human joints, bupivacaine's toxicity may be reduced by the endogenous protective factors in the articular cartilage. Whether a combination of bupivacaine and supraphysiologic temperature may cause any synergistic harm to cartilage shall be tested in animal joints. In addition, whether hyaluronan can protect against bupivacaine's chondrotoxicity awaits further research in pre-clinical and clinical studies before any clinical applications can be recommended. Second, this study did not test the role of pH and epinephrine in bupivacaine's chondrotoxicity. It has been reported that low pH and epinephrine contained in bupivacaine formulations are involved in bupivacaine's toxicity.⁷ However, a recent study found that pH values as low as 3.8 do not cause chondrocyte death, neither bupivacaine formulations with or without epinephrine.² Third, the exact mechanisms of hyaluronan's protective effects are not quite clear. Hyaluronan may provide survival signals to chondrocytes through several mechanisms as discussed above. Further research shall be conducted to pinpoint which mechanism is involved in the context of this study.

In conclusion, our findings demonstrated that bupivacaine and supraphysiologic temperature synergistically increase chondrocyte death and hyaluronan may protect articular chondrocytes from death caused by bupivacaine. Thus, this study provides a rationale to perform pre-clinical and clinical studies to evaluate whether intra-articular injection of a mixture of bupivacaine and hyaluronan after arthroscopic surgery may protect against bupivacaine's chondrotoxicity.

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What is known about the subject

Intra-articular injection of bupivacaine following joint surgery is a common practice. Bupivacaine may cause chondrocyte death; intra-articular injection of bupivacaine, particularly through intra-articular pumps, may lead to chondrolysis. Intra-articular temperature may become as high as 70°C with the use of radiofrequency devices and supraphysiologic temperature is harmful to chondrocytes.

What this study adds to existing knowledge

This study demonstrates a synergy between increased temperature and bupivacaine in regards to chondrotoxicity and provides evidence that hyaluronan has a protective effect against bupivacaine toxicity in bovine articular chondrocytes.

Figure 1.

Representative scatter plots of flow cytometry analysis. Bovine articular chondrocytes were treated with hyaluronan and/or bupivacaine at 37°C, 45°C, and 50°C, and then stained with the LIVE/DEAD kit. The abscissa shows calcein fluorescence and the ordinate shows ethidium fluorescence. Quadrant 1 (Q1) represents dead cells, quadrant 2 (Q2) represents newly dead cells, quadrant 3 (Q3) represents live cells without calcein staining, and quadrant 4 (Q4) represents live cells.

Figure 2.

Bar graph illustrating the in vitro effects of hyaluronan and/or bupivacaine on viability of bovine articular chondrocytes. The bars represent the mean percentages of the dead cells immediately after treatment at the indicated temperatures. Error bars represent the standard deviations. The asterisks indicate significant differences between the denoted groups and the 0.25% bupivacaine alone group (2) or 0.50% bupivacaine alone group (3).

Figure 3.

Representative photomicrographs of bovine articular chondrocytes in in-vitro culture six hours after treatment with hyaluronan and/or bupivacaine at 37°C, 45°C, and 50°C. The cells were stained with the LIVE/DEAD kit and photographed under a fluorescence microscope. Arrows in A and H point to the green cells with spindle or polygonal shapes that are attached to the culture plate; these cells are defined as healthy live cells. Arrows in F and L point to the green cells with a round shape that are detached from the culture plate; these cells are defined as “dying” live cells because they have already lost their anchorage on the plate – the first step to cell death. Arrow in J points to the orange cells with a round shape that are defined as dead cells; these cells have been stained with a mixture of red and green colors; entry of ethidium (for red color) indicates their cell membranes are broken.

Figure 4.

Representative photomicrographs of bovine articular chondrocytes in in-vitro culture twenty-four hours after treatment with hyaluronan and/or bupivacaine at 37°C, 45°C, and 50°C. The cells were stained with the LIVE/DEAD kit and photographed under a fluorescence microscope. Arrow in H points to the green cells with spindle or polygonal shapes that are attached to the culture plate; these cells are defined as healthy live cells. Arrows in F and L point to the green cells with a round shape that are detached from the culture plate; these cells are defined as “dying” live cells. Arrow in J points to red/orange cells with a round shape that are defined as dead cells.

Figure 5.

Bar graph illustrating the in vitro effects of hyaluronan on the levels of sulfated glycosaminoglycan in bovine articular chondrocytes. The levels of sulfated glycosaminoglycan are represented by the intensities of Alcian blue staining shown as absorbance values (the ordinate) at 600-nm wavelength. The bars represent the mean absorbance values. Error bars represent the standard deviations. The single asterisk indicates significant differences between the denoted groups and the basal medium control group; the double asterisk indicates significant differences between the denoted groups and the corresponding PBS treatment groups.

TABLE 1

Experimental Groups:^a

Treatment	1	2	3	4	5	6	7	8	9	10	11	12
Phosphate-buffered saline	x											
0.25% bupivacaine		x					x	x	x			
0.50% bupivacaine			x							x	x	x
10% hyaluronan				x			x			x		
25% hyaluronan					x			x			x	
50% hyaluronan						x			x			x

^aX, indicates that the group was treated with; all of the treatment reagents were prepared in phosphate-buffered saline; group (1) was the control group.