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Supraphysiological Temperature Enhances Bupivacaine's Cytotoxic Effects on Bovine Articular Chondrocytes in an In-vitro Study

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Abstract

Purpose—The purpose of this study is to determine the effects of temperature or 0.25% bupivacaine treatment in combination to supraphysiological temperatures on chondrocyte viability.

Methods—Bovine articular chondrocytes in suspension culture were treated with phosphate-buffered saline (PBS) at 20, 37, 40, 42, 45, 47, and 50° C for 15, 30 and 60 minutes; or with PBS at 37, 45, and 50° C for 30 and 60 minutes followed by 0.25% bupivacaine at 20° C for 60 minutes. Chondrocyte viability was analyzed by flow cytometry using the LIVE/DEAD Viability/Cytotoxicity Kit. Annexin-V and ethidium double- staining determined whether apoptosis or necrosis occurred.

Results—Temperatures from 20° C to 42° C did not cause chondrocyte death. Temperatures at or above 45° C caused significant chondrocyte death, particularly at 50° C for 60 minutes, compared to 37° C at 60 minutes ($P < 0.01$). When the chondrocytes were incubated at 50° C, subsequent exposure to bupivacaine significantly increased chondrocyte death, compared to the saline-treated control group ($P < 0.001$). There were additive cytotoxic effects when bupivacaine was combined with supraphysiological temperatures. It was also found that bupivacaine at supraphysiological temperatures caused necrosis of articular chondrocytes.

Conclusions—Temperatures at or above 45° C caused significant chondrocyte death. Bupivacaine treatment in the presence of 45° C and 50° C temperatures significantly increased necrosis of bovine articular chondrocytes in this *in-vitro* study.

Clinical Relevance—Immediate intra-articular injection of bupivacaine after heat-generating procedures may cause damage to the cartilage due to the additive cytotoxic effects of bupivacaine and elevated temperature.

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INTRODUCTION

Bupivacaine has long been used to reduce immediate post-operative pain following arthroscopic procedures,¹⁻³ however, recent studies have implicated bupivacaine as a causative factor in chondrolysis of the joint.^{4,5} Following certain arthroscopic procedures, substantial chondrolysis has been observed in shoulders treated with bupivacaine-containing intra-articular pain pumps,⁶ which led many orthopaedists to discontinue the use of the pumps. However, orthopaedic surgeons have continued to use single intra-articular bupivacaine injections immediately post-operatively, although it remains unclear whether or not a single injection as well as continuous infusion causes chondrolysis.

The reports on bupivacaine's chondrotoxicity vary widely. Recently, Chu, et al., reported 99% of bovine articular chondrocyte death upon exposure of alginate bead-contained chondrocytes to 0.5% bupivacaine in an *in-vitro* study.⁷ The chondrocyte death was mainly in the form of necrosis (approximately 89% of chondrocytes), while approximately 10% of chondrocytes died through apoptosis.⁷ The authors also noted that addition of 0.25% and 0.5% bupivacaine to bovine chondrocytes cultured in alginate beads caused the death of 60% of the chondrocytes in another *in-vitro* study.⁸ In a different *in-vitro* study, Dragoo, et al., reported that 0.25% to 0.5% bupivacaine caused significant death of human articular chondrocytes in 24-hour perfusion culture.⁶ In an *in-vivo* animal study, Dogan, et al., found that intra-articular bupivacaine caused inflammatory changes in rabbit knee joints within 10 days of intra-articular injection of 0.25 ml 0.5% bupivacaine.⁹ In contrast to Dogan, et al., Chu, et al., found no differences in superficial chondrocyte viability or modified Mankin scores between the saline control and bupivacaine groups in rat knee joints from one week to 6 months after intra-articular injection of 0.1 ml 0.5% bupivacaine.¹⁰ The authors did observe a 50% reduction in chondrocyte density in the bupivacaine-treated knees at 6 months, compared with that of the saline-treated controls.¹⁰ The results of these *in-vivo* studies do not support the *in-vitro* results demonstrating that bupivacaine causes immediate chondrocyte death. Grishko, et al., also showed that 0.5% bupivacaine caused insignificant chondrocyte death within 24 hours in an *in-vitro* study.¹¹ Along the same line, Bogatch, et al., recently demonstrated that 0.5% bupivacaine alone or bupivacaine diluted in phosphate-buffered saline (PBS) did not cause significant chondrocyte death in an *in-vitro* study. The authors showed that formation of crystal precipitations occurred and was accompanied by the death of 100 % of the chondrocytes when bupivacaine was mixed with the culture medium, implicating the reaction between the medium and bupivacaine as the cause of chondrocyte death, not bupivacaine alone.¹²

The effect of temperature on chondrocyte viability has been well recognized in the field of orthopaedics. Good, et al., reported that radiofrequency energy generated during shoulder arthroscopy using monopolar or bipolar probes increased the temperature of the synovial fluid to 45° C regardless of the fluid flow rate or the location of the probe within the glenohumeral capsule.¹³ Zoric, et al., reported that the irrigation fluid temperature reached > 80° C after two minutes in a no-flow setting,¹⁴ while Voss, et al., found that temperatures exceeding 45° C were sufficient to induce significant chondrocyte death.¹⁵ It is a common practice to inject bupivacaine into the joint immediately following surgery. Because bupivacaine and supraphysiological temperature individually can cause chondrocyte death, whether or not their combination causes an even greater reduction in chondrocyte viability than either alone has not been studied. We believe such a study is clinically relevant because the rate of chondrolysis is reported to be as high as 24% in some studies.¹⁶ The purposes of this study were: first, to determine if supraphysiological temperatures alone cause chondrocyte death; second, to find out if a combination of bupivacaine and supraphysiological temperature increases cytotoxicity; and third, to identify whether chondrocytes die through necrosis or apoptosis. Our hypothesis is that supraphysiological

temperature alone causes chondrocyte death, yet a combination of bupivacaine and supraphysiological temperature increases chondrocyte death and articular chondrocytes die through necrosis.

METHODS

Cell Culture

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

Treatment of Cells in Suspension Culture

Experiment (1). To test the effects of different temperatures on chondrocyte viability, approximately 0.3 million bovine articular chondrocytes in 0.5 ml of PBS suspension were placed into 12×75 mm test tubes. Each control or treatment group was treated in triplicates (n = 3). A triplicate group was assigned to and incubated at 20° C, 37° C, 40° C, 42° C, 45° C, 47° C or 50° C for 15, 30 or 60 minutes, respectively (Table 1), by placing each triplicate group into temperature-controlled water baths. The temperatures were selected based on a report by Good et al that joint fluid temperature could reach up to 56° C caused by a radiofrequency probe in cadaver shoulder joints.¹³

Experiment (2). To test the effects of bupivacaine on chondrocyte viability under different temperatures, the chondrocyte-containing test tubes were incubated at 37° C, 45° C and 50° C for either 30 or 60 minutes (Table 1). Immediately before being removed from the water baths, each group was treated with either 0.5 ml of normal saline or 0.5 ml of 0.5% bupivacaine hydrochloride (Hospira, Inc., Lake Forest, Illinois), thus the final concentration of bupivacaine was 0.25% (i.e., 0.5 ml of 0.5% bupivacaine diluted in 0.5 ml of cell suspension), and then incubated at room temperature for 60 minutes.

Experiment (3). To determine whether bupivacaine causes apoptosis or necrosis, an independent set of control and treatment groups of the chondrocytes were similarly treated as described in Experiment (2). At the end of the 60-minute incubation with bupivacaine, the chondrocytes were stained with a solution containing the binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂], Annexin-V Fluorescein isothiocyanate (FITC) Conjugate (Invitrogen, Carlsbad, CA; 1:20 dilution), and 0.8 μM ethidium homodimer-1, for 15 minutes in the dark at room temperature. Using flow cytometry analysis, the chondrocyte population that was positive for Annexin-V FITC but negative for ethidium represented apoptotic cells.¹⁷

Flow Cytometry Analysis of Cell Viability

Flow cytometry analysis was performed according to published protocols.^{12, 18} The control and treated chondrocytes in Experiments (1) and (2) were incubated in 0.3 ml of staining solution made of 0.8 μM ethidium homodimer-1 and 0.8 μM calcein AM (acetoxymethyl ester) in PBS for 20 minutes in the dark at room temperature. The reagents were from the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, Oregon). The rates (percentages) of chondrocyte death were obtained by counting the number of red cells (dead cells in Q1 and Q2 quarters of the scatter plot) from a total of 10,000 cells (the denominator n = 10,000) using a BD LSRII analyzer (Becton, Dickinson and Company, San Jose, CA). Similarly, Annexin-V FITC- and ethidium-stained cells were determined by flow cytometry analysis. The Annexin-V binding assay is based on the selective binding of Annexin-V to

phosphatidylserine (PS). PS is normally located on the inner surface of the cytoplasmic membrane. When apoptosis begins, the PS is translocated to the outer surface, allowing Annexin-V to bind to the cells; yet the cell membrane remains intact and can prevent the entry of ethidium, which means that apoptotic cells stain positive for Annexin-V FITC but negative for ethidium.¹⁷

Statistical Analysis

The experiments were performed 3 times independently. The means and standard deviations of 9 samples (pooled from 3 independent experiments) per group ($n = 9$) were compared between the control group and each treatment group, using the two-tailed Student's t test. The level of significance was set at $p < 0.05$. Two way ANOVA was used to examine the statistical significance of differences among multiple groups with different temperatures, time of exposure, and bupivacaine treatment, using a statistical software R (version 2.12). Representative scatter plots from flow cytometry analysis were shown. The bar graphs showed the means and standard deviations (error bars) of 9 samples pooled from the 3 independent experiments.

RESULTS

(1) Supraphysiological Temperature Decreased Viability of Bovine Articular Chondrocytes

As shown in Figure 1, flow cytometry analysis based on LIVE/DEAD staining separated the live cells in Quadrant 4 (Q4) from the dead cells (Q1). Q2 contained “newly” dead cells whose cell membranes were broken down, but still retained some esterase activity. Unstained cells with intact membranes were located in Q3. With supraphysiological temperatures, particularly at $\geq 45^\circ\text{C}$, the total number of dead cells (Q1+Q2) was increased (Figure 1A to G). In the quantitative analysis, there was no significant difference in the rates of chondrocyte death from 37°C to 42°C (Figure 1H). At 45°C , significantly more chondrocyte death occurred, compared to the groups at physiological temperature (37°C), for the 15- and 30- minute treatment groups ($P = 0.007$ and $P = 0.003$). Chondrocyte death increased from 10.1% ($\pm 2.6\%$) to 14.4% ($\pm 3.3\%$) in the 15-minute group and from 11.2% ($\pm 1.3\%$) to 20.0% ($\pm 7.3\%$) in the 30- minute group (Figure 1H). At 47°C , significant chondrocyte death was observed, which was similar to that observed at 45°C . However, at 50°C , the rate of chondrocyte death increased by approximately 8% in the 15- and 30- minute treatment groups and by 31% in the 60-minute treatment group, compared to the 37°C physiological control groups. Two way ANOVA also showed that the rate of cell death not only increased at higher temperatures, but there was an additional increase associated with exposure time at higher temperatures. The increase of death rates was statistically significant ($P < 0.01$).

(2) Combination of Bupivacaine and Supraphysiological Temperature Increased Chondrocyte Death

When the chondrocytes were incubated at supraphysiological temperatures and subsequently treated with bupivacaine, more cell death was observed, particularly at 50°C (Figure 2A to F). At 37°C , bupivacaine treatment for 30 minutes or 60 minutes increased chondrocyte death by approximately 2%, which was insignificant compared to the saline treatment groups (Figure 2G). At 45°C , bupivacaine treatment for 30 minutes caused 12.6% ($\pm 5.0\%$) of chondrocyte death and treatment for 60 minutes caused 11.5% ($\pm 3.5\%$) of chondrocyte death. Both these rates were statistically significantly higher than the rates of chondrocyte death in the saline control groups ($P = 0.04$ and $P = 0.03$, respectively) (Figure 2G). At 50°C , the bupivacaine treated groups demonstrated a chondrocyte death rate of 21.0% ($\pm 5.5\%$) in the 30- minute group and a chondrocyte death rate of 29.7% ($\pm 6.9\%$) in the 60- minute group. These chondrocyte death rates were also statistically significantly higher than the

rates in the saline control groups ($P < 0.001$ and $P < 0.001$, respectively) (Figure 2G). In the saline treatment groups, the rates of chondrocyte death at 50° C were significantly higher than those at 37° C (both $P < 0.001$) (Figure 2G). Two way ANOVA also showed that bupivacaine treatment at 45° C and 50° C significantly increased chondrocyte death rate compared to saline treatment group ($P < 0.05$).

(3) Combination of Bupivacaine and Supraphysiological Temperature Induced Necrosis Instead of Apoptosis

A combination of bupivacaine and 50° C temperature caused obvious cell death (Figure 3A to C). Approximately 1% of cells were apoptotic. These apoptotic cells were stained positive for Annexin-V FITC but negative for ethidium (as shown in Q4). The necrotic cells were stained positive for both Annexin-V FITC and ethidium (as shown in Q2), which consisted of approximately 23% of the examined cells (Figure 3D). The rate in Q2 was significantly higher than the rate in Q4 ($P < 0.001$). At 37° C and 45° C, the rates in Q2 and Q4 were all less than 3%.

DISCUSSION

In this *in-vitro* study, we performed experiments to address three questions: (1) does temperature have any effects on chondrocyte viability? (2) Does a combination of bupivacaine and supraphysiological temperature increase cytotoxicity in articular chondrocytes? And, (3) does chondrocytes die through necrosis or apoptosis?

First, we found that supraphysiological temperatures, particularly at or above 45° C, caused significant chondrocyte death when compared to the physiological temperature (37° C) (Figure 1H). This is consistent to a previous report by Voss, et al.¹⁵ Supraphysiological temperatures cause changes in chondrocyte metabolism, for example, exposure of the cartilage slices to a temperature of 45° C for more than 1 minute significantly decreased proteoglycan synthesis.¹⁹ Significant chondrocyte death has been observed when temperature reaches higher than 45° C.¹⁵ We based our temperature points on previous research that showed the synovial fluid inside the joint easily reaches 45°C during arthroscopic procedures.¹³ Temperature of 50° C and up to 64° C has been measured in cadaver joint fluid.¹³

Second, we demonstrated that 0.25% bupivacaine increased chondrocyte death in combination with the supraphysiological temperatures, particularly at 50° C. We found that at 37° C, 0.25% bupivacaine did not significantly increase chondrocyte death. This finding confirmed the recent report by Bogatch, et al.¹² The rate of chondrocyte death was significantly greater when the temperature was elevated to 50° C under saline treatment. However, with 0.25% bupivacaine treatment, chondrocyte death was further increased compared to the saline treatment alone (Figure 2G). The rate of chondrocyte death at 50° C with 0.25% bupivacaine treatment was higher than the sum of the chondrocyte death rate caused by 0.25% bupivacaine treatment at 37° C and the rate caused by saline treatment at 50° C. Therefore, 0.25% bupivacaine and 50° C temperature have at least an additive (if not synergistic) effect on cytotoxicity in articular chondrocytes. This thermal sensitization effect has been recognized in other fields, for example, elevated temperatures can sensitize mammalian cells to cytotoxic drugs such as those used in chemotherapy.²⁰ The present study is the first to suggest that supraphysiological temperatures can sensitize articular chondrocytes to bupivacaine-induced cytotoxicity. In addition, our findings imply that temperature and time of exposure are important variables in causing chondrocyte death, particularly when local anesthetics are added. Therefore, it would be better not to use local anesthetics immediately following heat-generating procedures; and if local anesthetics are

preferred, sufficient irrigation of the joint to reduce the intra-articular temperature is recommended.

Some studies contend that adding 0.5% bupivacaine to the synovial fluid drops the pH of the fluid enough to decrease chondrocyte viability. The 0.25% bupivacaine solution we used in this study had a pH of 7.0. A recent study conducted by Bogatch, et al demonstrated that any pH > 3.8 is not detrimental to chondrocytes. That study also found that bupivacaine solutions have pH values between 3.9 and 7.0.¹²

Third, we found that bupivacaine at 50° C primarily caused necrosis. During the process of necrosis, the cell membrane is damaged early on, with the cells staining positive for both Annexin-V FITC and ethidium. This was the case in our study, and most of the dead cells were in Q2 (positive for both Annexin-V FITC and ethidium) (Figure 3). Because the cells were treated for 60 minutes, we considered the possibility that apoptosis had reached its end stage, where the cell membrane was no longer intact and therefore allowed ethidium entry. To rule out this possibility, we then performed bupivacaine treatment for 15 minutes and obtained similar results. Chu et al also reported that there was no difference in percentages of necrotic chondrocytes when the cells were exposed to 0.5% bupivacaine for either 15, 30, or 60 minutes.⁷ Thus, bupivacaine treatment at 50° C caused necrosis of bovine articular chondrocytes. The long-term effects of bupivacaine treatment *in vivo* on the functioning of articular chondrocytes are not fully understood.

Bupivacaine is believed to cause its anesthetic effect on nerve cells by altering their membrane fluidity, which disrupts receptor and ion channel function in the cell membrane.²¹ We hypothesize that, since an increase in temperature represents an elevation in kinetic energy, it would make sense that higher temperatures favor membrane fluidity as well. It is speculated that bupivacaine and temperature together have a greater adverse effect on membrane integrity. The current study does not have any data to validate this hypothesis.

Our study was limited by the fact that it was conducted *in vitro* on bovine articular chondrocytes. The advantage of performing the *in-vitro* study is that the experimental conditions (e.g., the temperatures) are easily controlled. The disadvantage is that the *in-vitro* study may not truly mimic the more complicated *in-vivo* conditions. Chu et al has reported that more than 99% chondrocyte death was seen in 0.5% bupivacaine-exposed alginate bead culture.⁷ However, in the osteochondral cores, 0.5% bupivacaine caused 42% chondrocyte death when the joint surface was kept intact, and the chondrocyte death increased to 75% when the articular surface was removed.⁷ Their findings imply that the joint surface and cartilage environment have some chondroprotective effects against bupivacaine's cytotoxicity. Therefore, whether or to what extent our findings are applicable to the clinical situation requires future *in-vivo* studies to verify our findings. Another limitation is that the chondrocytes were treated in a solution without culture medium, thus the cells were deprived of nutrients. We could not treat the cells with bupivacaine while they were in the culture medium, because it has been reported that mixture of bupivacaine and culture medium forms crystals and causes chondrocyte death.¹²

CONCLUSIONS

Temperatures at or above 45° C caused significant chondrocyte death. Bupivacaine treatment in the presence of 45° C and 50° C temperatures significantly increased necrosis of bovine articular chondrocytes in this *in-vitro* study.

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Figure 1.

Effects of temperature on viability of bovine articular chondrocytes. A to G. Scatter plots of flow cytometry analysis in the cells treated for 30 min. The abscissa shows calcein fluorescence and the ordinate shows ethidium fluorescence. Quadrant 1 (Q1) represents the dead cells; quadrant 2 (Q2) represents the newly dead cells; quadrant 3 (Q3) represents live cells without calcein staining; and quadrant 4 (Q4) represents the live cells. H. Quantitative analysis. The bars represent mean percentages of the dead cells after 15-, 30- and 60- minute treatments at the indicated temperatures. Error bars represent the standard deviations. Asterisks indicate significant differences between the indicated groups and the corresponding control groups at 37° C.

Figure 2.

Effects of bupivacaine and temperature on viability of bovine articular chondrocytes. A to F. Scatter plots of flow cytometry analysis in the cells treated for 30 min. The abscissa shows calcein fluorescence and the ordinate shows ethidium fluorescence. Quadrant 1 (Q1) represents the dead cells; quadrant 2 (Q2) represents the newly dead cells; quadrant 3 (Q3) represents live cells without calcein staining; and quadrant 4 (Q4) represents the live cells. G. Quantitative analysis. The bars represent mean percentages of the dead cells after 30 and 60 minutes treatments at the indicated temperatures. Error bars represent the standard deviations. Asterisks indicate significant differences between the indicated groups and the corresponding saline control groups at the same temperature.

Figure 3.

Bupivacaine causes necrosis of bovine articular chondrocytes. A to C. Scatter plots of flow cytometry analysis in the cells treated for 30 min. The abscissa shows Annexin-V FITC fluorescence and the ordinate shows ethidium fluorescence. Quadrants 1 (Q1) and 2 (Q2) represent cells that have undergone necrosis and stained positive with ethidium due to cell membrane breakdown. Quadrant 3 (Q3) represents live cells without any staining. Quadrant 4 (Q4) represents cells that have undergone apoptosis and stained positive with Annexin-V FITC but not ethidium. D. Quantitative analysis. The solid bars represent the mean percentages of apoptotic cells and the open bars represent the mean percentages of necrotic cells. The error bars are the standard deviations. Asterisk indicates significant difference between the necrotic cells and the apoptotic cells at the same temperature.

Experimental design.

Table 1

Temperature (°C)	Exposure Time (min.)	No. per group	Exposure Time (min.)	No. per group	Exposure Time (min.)	No. per group
20	15	3	30	3	60	3
37	15	3	30	3	60	3
40	15	3	30	3	60	3
Experiment (1)	42	3	30	3	60	3
	45	3	30	3	60	3
	47	3	30	3	60	3
	50	3	30	3	60	3
Additional treatment						
37	Saline		30	3	60	3
37	0.25% bupivacaine		30	3	60	3
45	Saline		30	3	60	3
Experiment (2)	45		30	3	60	3
	50		30	3	60	3
	50		30	3	60	3

Note: Experiment (1) was designed to test the effects of temperature on chondrocyte viability; the control group was treated at 37 °C. Experiment (2) was designed to test the effects of temperature in combination to 0.25% bupivacaine; the control groups were treated with saline at each temperature. All experiments were independently performed 3 times.