

Supraphysiologic Temperature Enhances Cytotoxic Effects of Bupivacaine on Bovine Articular Chondrocytes in an In Vitro Study

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Purpose: The purpose of this study was to determine the effects of temperature or 0.25% bupivacaine treatment in combination with supraphysiologic temperatures on chondrocyte viability. **Methods:** Bovine articular chondrocytes in suspension culture were treated with phosphate-buffered saline solution at 20°C, 37°C, 40°C, 42°C, 45°C, 47°C, and 50°C for 15, 30, and 60 minutes or with phosphate-buffered saline solution at 37°C, 45°C, 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 with the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). 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 with 37°C at 60 minutes ($P < .01$). When the chondrocytes were incubated at 50°C, subsequent exposure to bupivacaine significantly increased chondrocyte death compared with the saline solution-treated control group ($P < .001$). There were additive cytotoxic effects when bupivacaine was combined with supraphysiologic temperatures. It was also found that bupivacaine at supraphysiologic 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 because of the additive cytotoxic effects of bupivacaine and elevated temperature.

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Bupivacaine has long been used to reduce immediate postoperative pain after arthroscopic procedures¹⁻³; however, recent studies have implicated bupivacaine as a causative factor in chondrolysis of the joint.^{4,5} After 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 postoperatively, although it remains unclear whether a single injection, as well as continuous infusion, causes chondrolysis.

The reports on bupivacaine's chondrotoxicity vary widely. Recently, Chu et al.⁷ reported death of 99% of bovine articular chondrocytes upon exposure of alginate bead-contained chondrocytes to 0.5% bupiva-

caine in an in vitro study.⁷ The chondrocyte death was mainly in the form of necrosis (approximately 89% of chondrocytes), whereas approximately 10% of chondrocytes died through apoptosis. Chu et al.⁸ also noted that the 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. In a different in vitro study, Dragoo et al.⁶ reported that 0.25% to 0.5% bupivacaine caused significant death of human articular chondrocytes in 24-hour perfusion culture. In an in vivo animal study, Dogan et al.⁹ found that intra-articular bupivacaine caused inflammatory changes in rabbit knee joints within 10 days of intra-articular injection of 0.25 mL of 0.5% bupivacaine. In contrast to Dogan et al., Chu et al.¹⁰ found no differences in superficial chondrocyte viability or modified Mankin scores between the saline solution control and bupivacaine groups in rat knee joints from 1 week to 6 months after intra-articular injection of 0.1 mL of 0.5% bupivacaine. They did observe a 50% reduction in chondrocyte density in the bupivacaine-treated knees at 6 months compared with that of the saline solution-treated controls.¹⁰ The results of these in vivo studies do not support the in vitro results showing that bupivacaine causes immediate chondrocyte death. Moreover, Grishko et al.¹¹ showed that 0.5% bupivacaine caused insignificant chondrocyte death within 24 hours in an in vitro study. Along the same line, Bogatch et al.¹² recently showed that 0.5% bupivacaine alone or bupivacaine diluted in phosphate-buffered saline solution (PBS) did not cause significant chondrocyte death in an in vitro study. They 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.

The effect of temperature on chondrocyte viability has been well recognized in the field of orthopaedics. Good et al.¹³ 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. Zoric et al.¹⁴ reported that the irrigation fluid temperature reached greater than 80°C after 2 minutes in a no-flow setting, whereas Voss et al.¹⁵ found that temperatures exceeding 45°C were sufficient to induce significant chondrocyte death.

It is a common practice to inject bupivacaine into the joint immediately after surgery. Because bupiva-

caine and supraphysiologic temperature individually can cause chondrocyte death, whether 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 whether supraphysiologic temperatures alone cause chondrocyte death; second, to find out whether a combination of bupivacaine and supraphysiologic temperature increases cytotoxicity; and third, to identify whether chondrocytes die through necrosis or apoptosis. Our hypothesis was that supraphysiologic temperature alone would cause chondrocyte death; that a combination of bupivacaine and supraphysiologic temperature would increase chondrocyte death; and that 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 containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a 5% carbon dioxide 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 (experiment 1). Each control or treatment group was treated in triplicate (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 (Table 1), by placing each triplicate group into temperature-controlled water baths. The temperatures were selected based on a report by Good et al.¹³ that joint fluid temperature could reach up to 56°C caused by a radiofrequency probe in cadaveric shoulder joints.

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, or 50°C for either 30 or 60 minutes (experiment 2) (Table 1). Immediately be-

TABLE 1. Experimental Design

	Temperature (°C)	Additional Treatment	First Exposure		Second Exposure		Third Exposure	
			Time (min)	No. per Group	Time (min)	No. per Group	Time (min)	No. per Group
Experiment 1	20	—	15	3	30	3	60	3
	37	—	15	3	30	3	60	3
	40	—	15	3	30	3	60	3
	42	—	15	3	30	3	60	3
	45	—	15	3	30	3	60	3
	47	—	15	3	30	3	60	3
	50	—	15	3	30	3	60	3
Experiment 2	37	Saline solution	30	3	60	3	—	—
	37	0.25% bupivacaine	30	3	60	3	—	—
	45	Saline solution	30	3	60	3	—	—
	45	0.25% bupivacaine	30	3	60	3	—	—
	50	Saline solution	30	3	60	3	—	—
	50	0.25% bupivacaine	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 with 0.25% bupivacaine; the control groups were treated with saline solution at each temperature. All experiments were independently performed 3 times.

fore being removed from the water baths, each group was treated with either 0.5 mL of normal saline solution or 0.5 mL of 0.5% bupivacaine hydrochloride (Hospira, Lake Forest, IL); 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). Incubated was then performed 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 (experiment 3). At the end of the 60-minute incubation with bupivacaine, the chondrocytes were stained with a solution containing the binding buffer [10-mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/sodium hydroxide, pH 7.4; 140-mmol/L sodium chloride; and 2.5-mmol/L calcium chloride], annexin V–fluorescein isothiocyanate (FITC) conjugate (Invitrogen, Carlsbad, CA) (1:20 dilution), and 0.8- μ mol/L ethidium homodimer 1 for 15 minutes in the dark at room temperature. By use of 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- μ mol/L ethidium homodimer 1 and 0.8- μ mol/L calcein 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, OR). The rates (percentages) of chondrocyte death were obtained by counting the number of red cells (dead cells in quadrant 1 [Q1] and quadrant 2 [Q2] of the scatter plot) from a total of 10,000 cells, thus the denominator was 10,000, by use of a BD LSRII analyzer (Becton Dickinson, San Jose, CA). Similarly, annexin V–FITC–stained 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; however, 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 by use of the 2-tailed

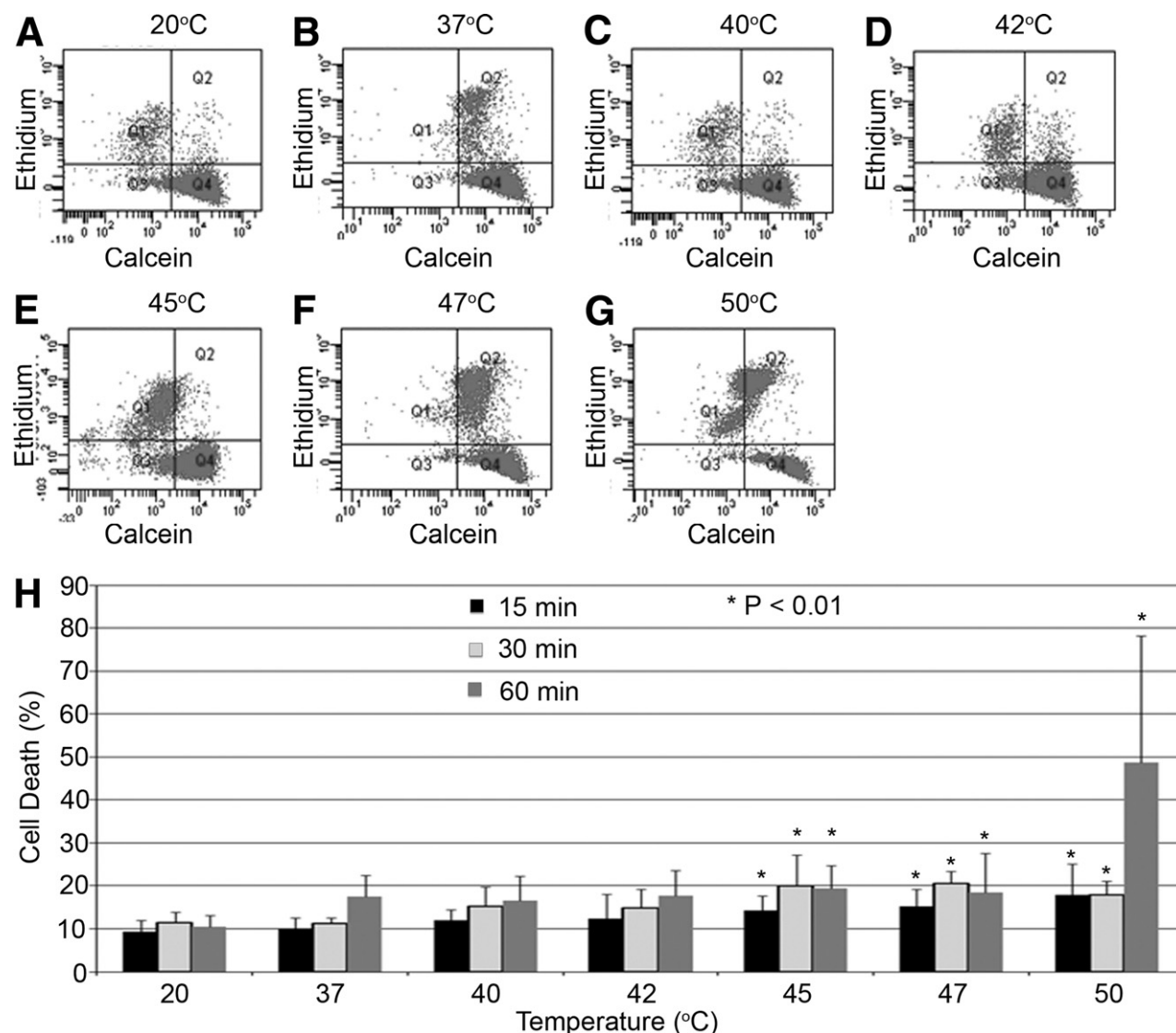


FIGURE 1. Effects of temperature on viability of bovine articular chondrocytes. (A-G) Scatter plots of flow cytometry analysis in cells treated for 30 minutes. The abscissa shows calcein fluorescence, and the ordinate shows ethidium fluorescence. Q1 represents the dead cells, Q2 represents the newly dead cells, quadrant 3 (Q3) represents live cells without calcein staining, and 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. The error bars represent standard deviations. The asterisks indicate significant differences between the indicated groups and the corresponding control groups at 37°C.

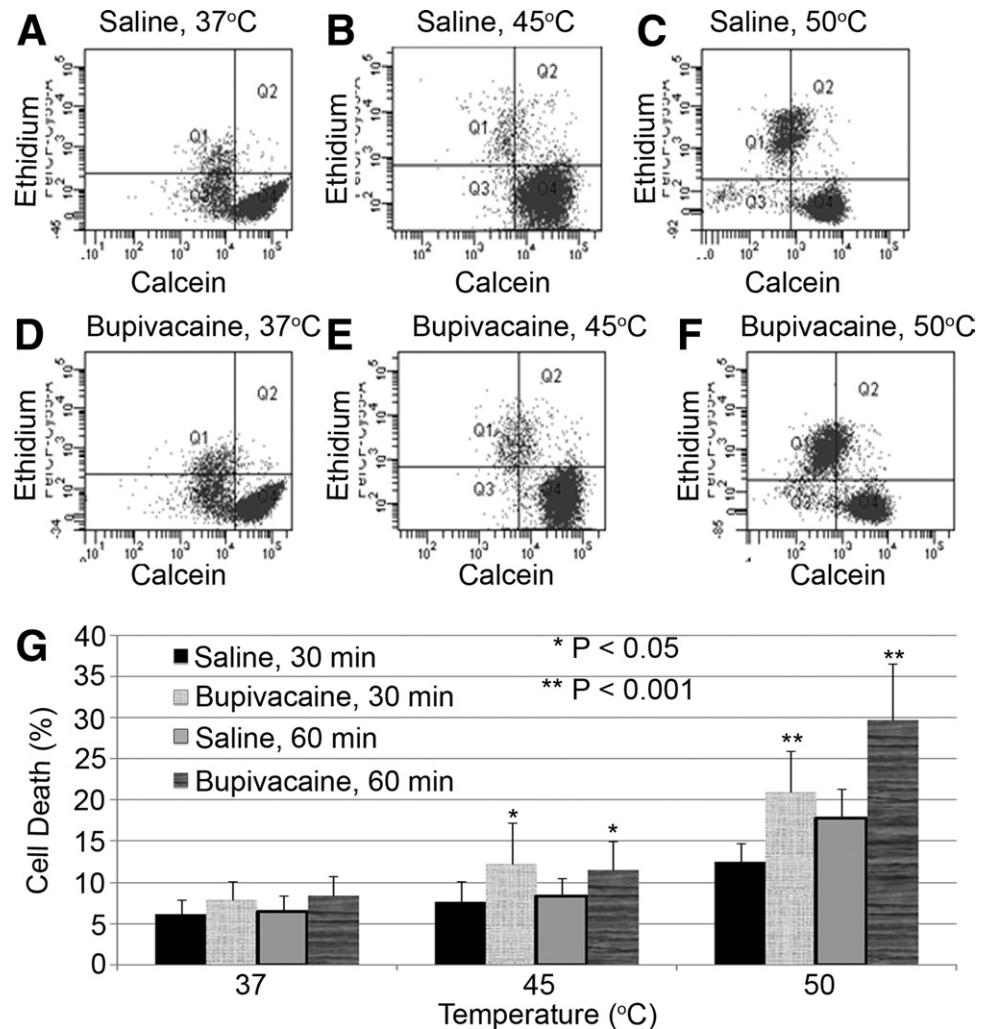
Student *t* test. The level of significance was set at $P < .05$. Two-way analysis of variance (ANOVA) was used to examine the statistical significance of differences among multiple groups with different temperatures, time of exposure, and bupivacaine treatment, by use of R statistical software (version 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

Supraphysiologic Temperature Decreased Viability of Bovine Articular Chondrocytes

As shown in Fig 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.

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



Unstained cells with intact membranes were located in quadrant 3. With supraphysiologic temperatures, particularly at 45°C or greater, the total number of dead cells (Q1 + Q2) was increased (Figs 1A-1G). In the quantitative analysis, there was no significant difference in the rates of chondrocyte death from 37°C to 42°C (Fig 1H). At 45°C, significantly more chondrocyte death occurred compared with the groups at physiologic temperature (37°C) for the 15- and 30-minute treatment groups ($P = .007$ and $P = .003$, respectively). 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 (Fig 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 with

the 37°C physiologic control groups. Two-way ANOVA also showed not only that the rate of cell death increased at higher temperatures but that there was an additional increase associated with exposure time at higher temperatures. The increase in death rates was statistically significant ($P < .01$).

Combination of Bupivacaine and Supraphysiologic Temperature Increased Chondrocyte Death

When the chondrocytes were incubated at supra-physiologic temperatures and subsequently treated with bupivacaine, more cell death was observed, particularly at 50°C (Figs 2A-2F). At 37°C, bupivacaine treatment for 30 or 60 minutes increased chondrocyte death by approximately 2%, which was insignificant compared with the saline solution treatment groups

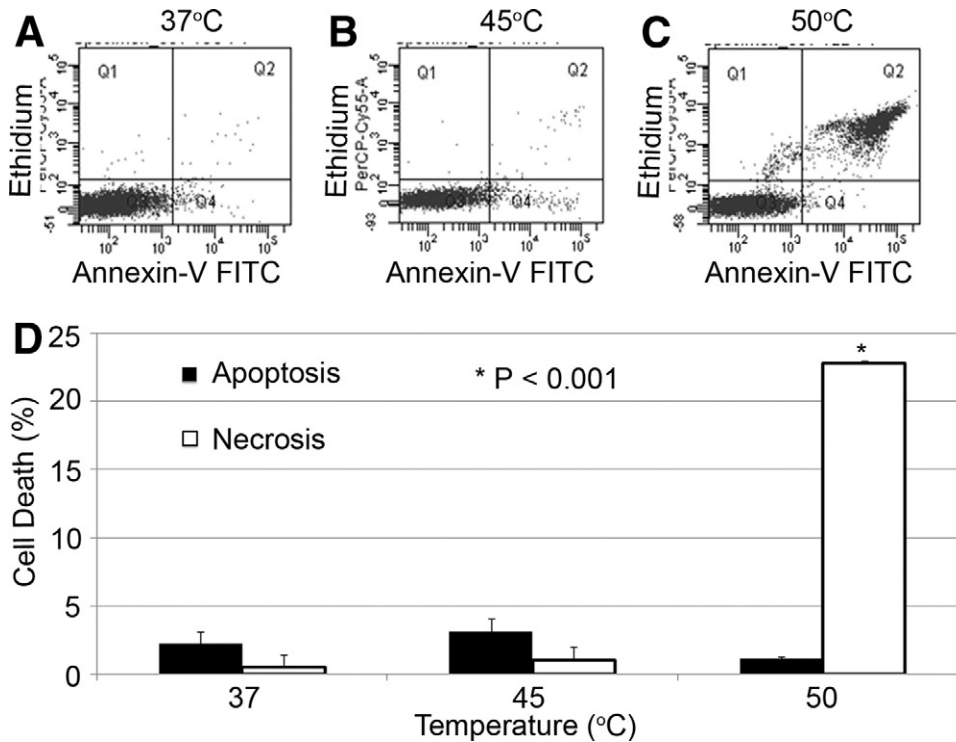


FIGURE 3. Bupivacaine causes necrosis of bovine articular chondrocytes. (A-C) Scatter plots of flow cytometry analysis in cells treated for 30 minutes. The abscissa shows annexin V-FITC fluorescence, and the ordinate shows ethidium fluorescence. Q1 and Q2 represent cells that have undergone necrosis and stained positive with ethidium because of cell membrane breakdown. Quadrant 3 (Q3) represents live cells without any staining. 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 standard deviations. The asterisk indicates a significant difference between the necrotic cells and the apoptotic cells at the same temperature.

(Fig 2G). At 45°C, bupivacaine treatment for 30 minutes caused a 12.6% ($\pm 5.0\%$) rate of chondrocyte death and treatment for 60 minutes caused an 11.5% ($\pm 3.5\%$) rate of chondrocyte death. Both these rates were statistically significantly higher than the rates of chondrocyte death in the saline solution control groups ($P = .04$ and $P = .03$, respectively) (Fig 2G). At 50°C, the bupivacaine treated groups showed a chondrocyte death rate of 21.0% ($\pm 5.5\%$) in the 30-minute group and 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 solution control groups (both $P < .001$) (Fig 2G). In the saline solution treatment groups, the rates of chondrocyte death at 50°C were significantly higher than those at 37°C (both $P < .001$) (Fig 2G). Two-way ANOVA also showed that bupivacaine treatment at 45°C and 50°C significantly increased the chondrocyte death rate compared with the saline solution treatment group ($P < .05$).

Combination of Bupivacaine and Supraphysiologic Temperature Induced Necrosis Instead of Apoptosis

A combination of bupivacaine and 50°C temperature caused obvious cell death (Figs 3A-3C). Approx-

imately 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 (Fig 3D). The rate in Q2 was significantly higher than the rate in Q4 ($P < .001$). 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 3 questions: (1) Does temperature have any effect on chondrocyte viability? (2) Does a combination of bupivacaine and supraphysiologic temperature increase cytotoxicity in articular chondrocytes? (3) Do chondrocytes die through necrosis or apoptosis?

First, we found that supraphysiologic temperatures, particularly at or above 45°C, caused significant chondrocyte death when compared with the physiologic temperature (37°C) (Fig 1H). This is consistent with a previous report by Voss et al.¹⁵ Supraphysiologic temperatures cause changes in chondrocyte metabolism; for example, exposure of the cartilage slices to a temperature of 45°C for more than 1 minute signifi-

cantly decreased proteoglycan synthesis.¹⁹ Significant chondrocyte death has been observed when temperatures reach higher than 45°C.¹⁵ We based our temperature points on previous research that showed that the synovial fluid inside the joint easily reaches 45°C during arthroscopic procedures.¹³ Temperatures of 50°C and up to 64°C have been measured in cadaveric joint fluid.¹³

Second, we showed that 0.25% bupivacaine increased chondrocyte death in combination with the supraphysiologic 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 solution treatment. However, with 0.25% bupivacaine treatment, chondrocyte death was further increased compared with the saline solution treatment alone (Fig 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 solution 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 This study is the first to suggest that supraphysiologic 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 after heat-generating procedures; moreover, 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 decreases 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.¹² showed that any pH greater than 3.8 is not detrimental to chondrocytes. Their study also found that bupivacaine solutions have pH values between 3.9 and 7.0.

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) (Fig 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.⁷ similarly reported that there was no difference in percentages of necrotic chondrocytes when the cells were exposed to 0.5% bupivacaine for 15, 30, or 60 minutes. 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, because 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. Our 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 an *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.⁷ have reported that death of more than 99% of chondrocytes was seen in 0.5% bupivacaine-exposed alginate bead culture. However, in the osteochondral cores, 0.5% bupivacaine caused a 42% chondrocyte death rate when the joint surface was kept intact, and the chondrocyte death rate 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 the 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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