©Journal of Sports Science and Medicine (2014) **13,** 516-521
http://www.jssm.org

Research article

Reduction of Environmental Temperature Mitigates Local Anesthetic Cytotoxicity in Bovine Articular Chondrocytes

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Abstract
The purpose of this study was to assess whether reducing environmental temperature will lead to increased chondrocyte viability following injury from a single-dose of local anesthetic treatment. Bovine articular chondrocytes from weight bearing portions of femoral condyles were harvested and cultured. 96-well plates were seeded with 15,000 chondrocytes per well. Chondrocytes were treated with one of the following conditions: ITS Media, 1x PBS, 2% lidocaine, 0.5% bupivacaine, or 0.5% ropivacaine. Each plate was then incubated at 37°C, 23°C, or 4°C for one hour and then returned to media at 37°C. Chondrocyte viability was assessed 24 hours after treatment. Chondrocyte viability is presented as a ratio of the fluorescence of the treatment group over the average of the media group at that temperature (ratio ± SEM). At 37°C, lidocaine (0.35 ± 0.04) and bupivacaine (0.30 ± 0.05) treated chondrocytes show low cell viability when compared to the media (1.00 ± 0.03) control group ($p < 0.001$). Lidocaine treated chondrocytes were significantly more viable at 23°C (0.84 ± 0.08) and 4°C (0.86±0.085) than at 37°C ($p < 0.001$). Bupivacaine treated chondrocytes were significantly more viable at 4°C (0.660 ± 0.073) than at 37°C or 23°C (0.330 ± 0.069) ($p < 0.001$ and $p = 0.002$ respectively). Reducing the temperature from 37°C to 23°C during treatment with lidocaine increases chondrocyte viability following injury. Chondrocytes treated with bupivacaine can be rescued by reducing the temperature to 4°C.

Key words: Clinical hypothermia, local anesthetics, articular cartilage, osteoarthritis, chondrolysis.

Introduction

Osteoarthritis is characterized by articular cartilage degeneration, pain, and loss of joint motion. Annually, approximately 30 million Americans suffer from osteoarthritis, costing more than $100 billion a year in treatment (Brown et al., 2006). Local anesthetic injections are effective for treatment and diagnosis of joint pain, and are commonly used in the clinic, both pre- and post-operatively. To use of these drugs outside of local administration or in nerve blocks are not indicated by the FDA. That is, use of local anesthetics intra-articularly is an off label use that now carries a warning for potential risk of chondrolysis by the FDA (Beyzadeoglu et al., 2012). Clinicians have observed cases of chondrolysis following treatment with local anesthetics to manage postoperative pain (Anakwenze et al., 2010; Noyes et al., 2012; Piper et al., 2011). *In-vitro* studies have confirmed that these intra-articular local anesthetic injections, in particular bupivacaine and lidocaine, are cytotoxic to chondrocytes (Braun et al., 2011; Chu et al., 200; Dragoo et al., 2010; 2012; Lo et al., 2009b; Miyazaki et al., 20116; Piper and Kim, 2008). These studies examined various cell sources, formulations, combinations with other drugs, and dose responses to better characterize the toxicity of local anesthetics to chondrocytes (Braun et al., 2011; Chu et al., 2006; Dragoo et al., 2010, 2012; Lo et al., 2009b; Miyazaki et al., 2011; Piper and Kim, 2008). However, they do not adequately propose ways of mitigating the adverse effects caused by intra-articular local anesthetic injections.

Although the exact mechanism has not yet been described, it has been proposed that local anesthetics lead to cell death by disrupting mitochondrial function in chondrocytes (Grishko et al., 2010). Therefore, one possible way of mitigating the cytotoxic effects of local anesthetics would be to reduce cellular metabolism. Moderate hypothermia has been used clinically for neuronal or myocardial protection following acute spinal trauma, surgical ischemia, and cardiogenic shock (Dietrich et al., 2011; Schmidt-Schweda et al., 2013; Zierer et al., 2012). *In-vitro* cell culture experiments and *in-vivo* small animal models have confirmed the benefit of therapeutic hypothermia to improving histological and functional outcomes in these injury models (Herlambang et al., 2011; Li et al., 2012; Lo et al., 2009a). Kocaoglu et al. demonstrated that cartilage explants submerged in low temperature saline reduced cellular metabolism (Kocaoglu et al., 2011).

Active cooling to the knee joint has shown a reduction in intra-articular temperatures to 20-25°C after 1 hour (Warren et al., 2004). The purpose of this study was to determine whether reducing the environmental temperature during treatment would reduce local anesthetic cytotoxicity in bovine articular chondrocytes. The intent of our study is to model active cooling of the joint following intra-articular injections with local anesthetics, such as those performed in clinic or post-operatively, and measures the viability of chondrocytes following such a procedure. We hypothesize that reduction of environmental temperature to 23°C and 4°C, inducing hypothermia, during treatment with local anesthetics would decrease chondrocyte death when compared to chondrocytes treated under normal physiological temperatures (37°C).

Methods

Chondrocyte isolation and culture

Articular chondrocytes were harvested by removing cartilage from weight bearing portions of juvenile bovine femoral condyles (Rancho Veal; Petaluma, CA). Chon-

Received: 21 June 2013 / Accepted: 26 March 2014 / Published (online): 01 September 2014

drocyte isolation was performed approximately four hours post-mortem. Cartilage was minced and washed in sterile phosphate buffered saline (PBS) (Hyclone®; Logan, Utah) treated with 250μg·mL^{-1} Amphotericin B (MP Biomedicals; Solon, OH) and 1x Penicillin-Streptomycin-Glutamine antibiotic (Pen-Strep) (Hyclone®; Logan Utah). Chondrocytes were isolated by digesting cartilage in digestion media consisting of 500 mL 1:1 Dulbecco's Modified Eagle Media (DMEM):F-12 (Hyclone®; Logan Utah), 50 mL Fetal Bovine Serum (FBS) (Axenia BioLogix; Sacramento, California), and 100 mg collagenase-P (Roche; Mannheim, Germany). The digested cartilage was filtered and the remaining chondrocytes in suspension were centrifuged at 500G for 10 minutes. After discarding the supernatant and re-suspending the chondrocytes in a mixture of 10% FBS Media (500 mL 1:1 DMEM:F12, 50 mL FBS, 5mL 100x Amphotericin B, 5mL Pen-Strep), cells were counted using a hemacytometer.

Chondrocytes were then plated on flat-bottomed clear 75cm^2 flasks at a concentration of 7.5x10^5 cells·mL^{-1} and allowed to grow to confluence. Twenty-four hours following plating, cells were treated with ITS supplemented media until use. ITS media was prepared by combining 500 mL 1:1 DMEM:F12, 25 mg L-ascorbic acid 2-phospate sesquimagnesium salt hydrate (Sigma Aldrich; St. Louis, MO), 5 mL 100x Pen-Strep antibiotic (Hyclone ®; Logan Utah), 5 ml Amphotericin B (MP Biomedicals; Solon, OH), 500 mg bovine serum albumin (BSA) (Sigma Aldrich; St. Louis, MO), 50 mg sodium pyruvate (Mediatech; Manassas, VA), 5 mL 1M HEPES Buffer (University of California, San Francisco Cell Culture Facility; San Francisco, CA), and 5 mL of insulin transferrin selenium (ITS) premix (BD Biosciences; San Jose, CA).

Seeding

Chondrocytes were removed from flasks with trypsin, counted, and transferred to 96-well plates for treatment. Chondrocytes were seeded at a density of 15,000 cells per well onto three plates. Each plate tested five conditions, described below, in replicates of six. The appropriate volume for each well was calculated using the concentration found by methods described in the previous section. Additional ITS Media was added to each of the occupied wells to bring their total volume to 100 μL. Each plate was incubated at 37^{o}C for 48 hours to allow the cells to rest and adhere to the wells.

Treatment

After 48 hours, the media was removed from the wells and chondrocytes were treated with 70μL of one of the following five conditions: DMEM F-12 Media with ITS Supplement, PBS, 2% lidocaine (20 mg·mL^{-1} lidocaine HCl, 6 mg·mL^{-1} sodium chloride) (APP Pharmaceuticals; Schaumburg, IL), 0.5% bupivacaine (5 mg·mL^{-1} bupivacaine HCl, 8.1 mg·mL^{-1} sodium chloride) (Hospira Inc.; Lake Forest, IL) and 0.5% Naropin® (5 mg·mL^{-1} ropivacaine) (APP Pharmaceuticals; Schaumburg, IL). Each plate was treated for one hour at one of three temperature environments; 37°C, 23°C (room temperature), or 4°C. Following treatment, the conditions were removed from the wells and replaced with 70 μL of ITS media. Chondrocytes were allowed to recover by incubating for 24 hours at 37°C. This experiment was conducted two more times to confirm the results collected in each experiment.

Quantification

After 24 hours, media was removed with from all wells and replaced with 50μL of a 1:10 solution of PrestoBlue® (Invitrogen; Frederick, MD) in 10% FBS media (500 mL 1:1 DMEM:F12, 50 mL FBS, 5mL 100x Amphotericin B, 5mL Pen-Strep). Briefly, PrestoBlue is a non-cytotoxic cell viability fluorescence assay. The reagent measures viability by testing the cell's ability to reduce nicotinamide adenine dinucleotide (NAD^+). Following 10 minutes of incubation at 37°C, the plate was read on the Synergy2 plate reader machine (BioTek Instruments, Inc.; Winooski, VA) with an excitation frequency of 535 nm and emission frequency of 595 nm, producing a fluorescence intensity read out in arbitrary units. The average of the blank well readouts on each plate was calculated and subtracted from each experimental well. Next, the average from the media control group in each plate was calculated and used to normalize each experimental condition. Therefore, each sample was presented as a ratio adjusted fluorescence over the average fluorescence of the media control.

Live/dead staining

To confirm the results from PrestoBlue®, chondrocytes were visualized to assess chondrocyte viability. Following staining with LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen; Frederick, MD), representative images of chondrocytes under each treatment were taken using fluorescence microscopy. The stain was prepared by adding 5 μL calcein AM and 20 μL ethidium bromide homodimer-1 to 10 mL of 1x PBS. 100 μL of the Live/Dead stain was added to each well and allowed to incubate at room temperature and protected from light for 35 minutes. Employing fluorescence microscopy, live cells were visualized using a 5x objective under a FITC filter (~494 nm) and dead cells were visualized under a Rhodamine filter (~517 nm).

Statistical analysis

The presented in this study is a summary of three independent repeats of this experiment, each using different batches of cultured chondrocytes for each trial. Statistical analysis was conducted using the computer software R (The R Foundation for Statistical Computing). Data was analyzed for statistically significant differences between all conditions using an ANOVA Test. A Post-Hoc Tukey HSD Test was conducted in order to make pair-wise comparisons between conditions.

Results

Cell viability

Cell viability was presented as a ratio of treatment conditions against the negative control at each respective temperature of media alone (mean ratio±SEM). At 37°C, chondrocytes had an average viability ratio of 1.000 ±

0.029 in media, 0.880 ± 0.058 in PBS, 0.349 ± 0.040 in 2% Lidocaine, 0.300 ± 0.051 in 0.5% bupivacaine, and 0.870 ± 0.082 in 0.5% ropivacaine. At room temperature, chondrocytes had an average viability ratio of 1.000 ± 0.036 in media, 0.871 ± 0.045 in PBS, 0.842 ± 0.075 in 2% lidocaine, 0.326 ± 0.069 in 0.5% bupivacaine, and 0.931±0.06 in 0.5% ropivacaine. Finally at 4°C, chondrocytes had an average viability ratio of 1.000 ± 0.033 in media, 0.790 ± 0.079 in PBS, 0.86 ± 0.085 in 2% lidocaine, 0.660 ± 0.073 in 0.5% bupivacaine, and 0.980 ± 0.096 in 0.5% ropivacaine (Figure 1).

At 37°C, an ANOVA test showed a significant difference ($p < 0.001$) when comparing all conditions against each other. Conducting a Tukey HSD test for pair-wise differences showed that there were no statistically significant difference between Media, PBS, and 0.5% ropivacaine treatments. Chondrocytes treated with 2% lidocaine and 0.5% bupivacaine were significantly less viable when compared to media, PBS, or ropivacaine ($p < 0.001$).

At room temperature, the ANOVA test again indicated a statistically significant difference among all treatments ($p < 0.001$). Similar to the 37°C temperature group, pair-wise comparisons showed no difference between media, PBS, and ropivacaine treated chondrocytes. Also, chondrocytes treated with bupivacaine were significantly less viable than the media, PBS, and ropivacaine ($p < 0.001$) treated chondrocytes. However, the lidocaine treated chondrocytes incubated at 23°C demonstrated a cell viability that was not significantly different from that of chondrocytes treated with media, PBS, and ropivacaine ($p = 0.324$, $p = 0.996$, and $p = 0.814$, respectively).

Finally, chondrocytes treated at 4°C had significantly different cell viabilities ($p = 0.013$). Compared to the media only group, PBS, lidocaine, and ropivacaine treated chondrocytes did not show a statistically significant difference. Bupivacaine treated chondrocytes were significantly less viable from media and ropivacaine treated chondrocytes ($p = 0.018$ and 0.034 respectively).

Additionally, each treatment was evaluated across all temperature conditions. Cell viability for chondrocytes treated with PBS and ropivacaine do not significantly change with decreasing temperature. Chondrocytes treated with 2% lidocaine experience significantly greater viability with decreasing temperature ($p < 0.001$). Compared to a 37°C environment, 2% lidocaine treated chondrocytes were significantly more viable at 23°C and 4°C ($p < 0.001$). There was no statistically significant difference between room temperature and 4°C. Cell viability also changes with decreasing temperature when chondrocytes are treated with bupivacaine ($p < 0.001$). Chondrocytes were significantly more viable when incubated at 4°C than at 37°C ($p < 0.001$) or 23°C ($p = 0.002$). There were no statistically significant differences in cell viability between 37°C and 23°C with bupivacaine.

To assess how viability in media changed with temperature, all corrected fluorescent values for media were normalized to an average of the fluorescence of the media group at 37°C of all three trials. This produced a ratio of 1.000 ± 0.077 at 37°C, 0.960 ± 0.066 at 23°C, and 0.870 ± 0.113 at 4°C. Although there appears to be a trend of decreasing chondrocyte viability with decreasing temperature, an ANOVA revealed that there were no statistically significant differences between the control groups at various temperatures ($p = 0.188$).

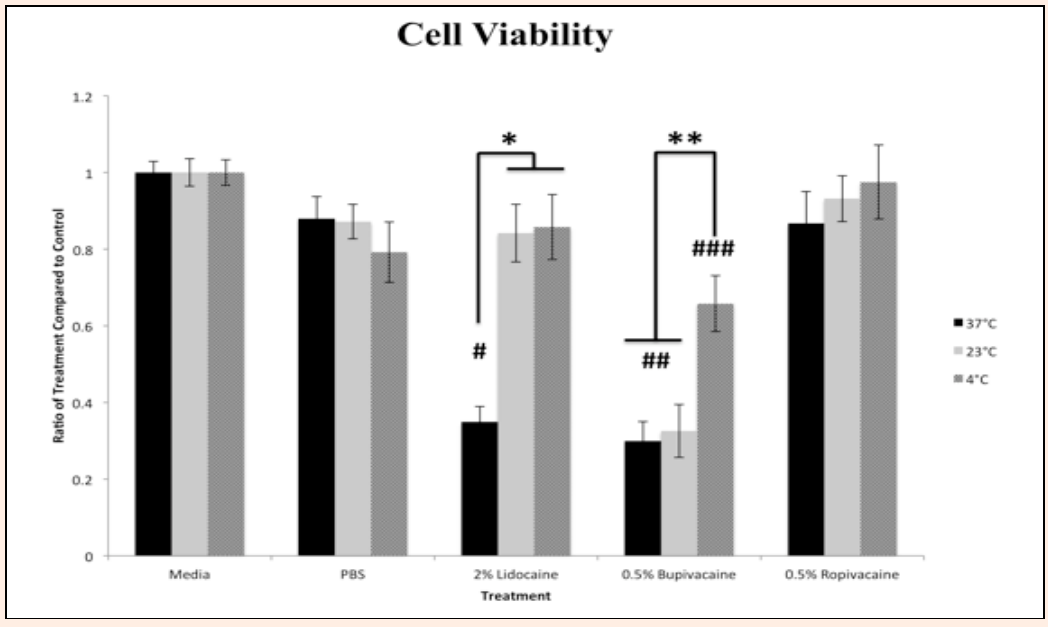

Figure 1. Comparison of cell viability for all treatments relative to media only control. * denotes statistically significant less chondrocyte viability at 37°C compared to 23°C and 4°C for chondrocytes treated with lidocaine ($p < 0.001$). ** denotes significant difference chondrocyte viability between the 37°C and 23°C conditions from the 4°C condition ($p < 0.001$ and $p = 0.002$ respectively). # denotes statistically significant difference between lidocaine treated chondrocytes and chondrocytes treated with media, PBS, or ropivacaine at 37°C ($p < 0.001$). ## denotes significant difference between media and bupivacaine at both 37°C and 23°C ($p < 0.001$). ### denotes significant difference between the media and ropivacaine groups from the bupivacaine treatment groups at 4°C ($p = 0.018$ and $p = 0.034$ respectively).

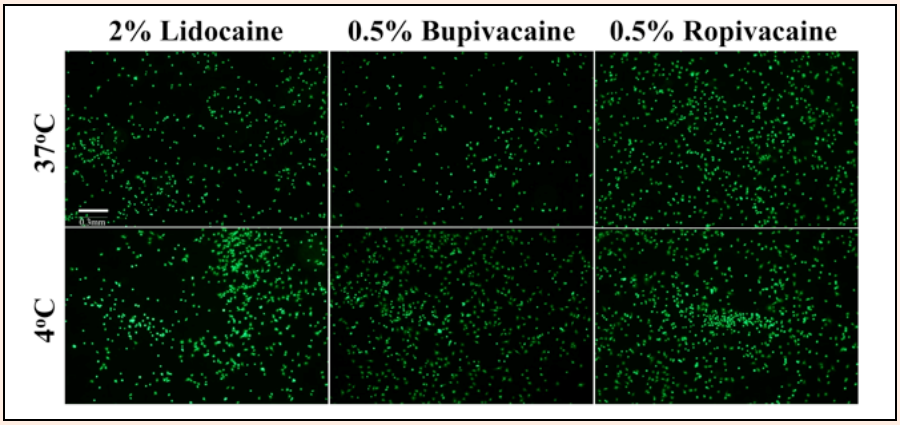

Figure 2. Viable chondrocytes stained with Calcein AM, visualized at 5x magnification under a FITC filter. Chondrocytes were treated with 2% lidocaine, 0.5% bupivacaine, or 0.5% ropivacaine and incubated at 37°C or 4°C. Briefly, when chondrocytes are treated with lidocaine or bupivacaine, there appears to be a higher density of viable chondrocytes when incubated at 4°C compared to chondrocytes incubated at 37°C. Viable cell density appears to remain constant for chondrocytes treated with ropivacaine across both 37°C and 4°C. Results from fluorescent microscopy appears to be consistent with results from PrestoBlue® fluorescence assay. Scale bar = 0.3mm.

Fluorescence microscopy

Representative pictures of chondrocytes stained with LIVE/DEAD and visualized with fluorescence microscopy at 5X magnification. It appears that the PrestoBlue® interferes with the visualization of the ethidium bromide. Additionally, dead cells tend to come off the bottom of well plates, therefore during wash steps it is possible that these chondrocytes are aspirated out. However, the PrestoBlue® and Calcein AM (live) stain did not appear to interfere with one another. Under a FITC filter, live chondrocyte distribution was assessed at 5x magnification. The density of viable chondrocytes treated with media and PBS did not appear to change dramatically with reducing temperature. Figure 2 highlights chondrocyte viability at 37°C and 4°C following treatments with various local anesthetics. Chondrocytes treated with lidocaine or bupivacaine appeared to be at higher density, therefore more viable, when incubated at 4°C compared to chondrocytes incubated at 37°C. Viable cell density remained similar for chondrocytes treated with ropivacaine across both 37°C and 4°C.

Discussion

This study investigated the effect of temperature reduction on chondrocytes treated with bupivacaine, lidocaine, or ropivacaine. Much work has been performed to characterize the toxic effects of local anesthetics on articular cartilage (Braun et al., 2011; Chu et al., 2006; Dragoo et al., 2010; 2012; Grishko et al., 2010; Lo et al., 2009b; Miyazaki et al., 2011; Piper and Kim, 2008). Chu et al. (2006) demonstrated that bupivacaine was toxic to bovine articular chondrocytes in monolayer. Similarly, Miziyaki showed that treating bovine articular chondrocytes in monolayer with lidocaine caused high cell death (Miyazaki et al., 2011). Our results support that 24 hours after a 1-hour treatment at 37°C, 2% lidocaine and 0.5% bupivacaine caused significant reduction in chondrocyte viability. Our results were also consistent with Piper and Kim, who showed that ropivacaine was significantly less cytotoxic than an equivalent concentration of bupivacaine when comparing chondrocyte viability 24 hours after treatment (Piper and Kim, 2008).

The exact mechanism underlying chondrocyte death following treatment with local anesthetics remains largely unknown. Dragoo et al. (2010) suggested that preservatives and lower pH caused by drug formulations containing epinephrine led to chondrocyte death following treatment with lidocaine and bupivacaine. The local anesthetics used in this present study did not contain any preservatives or epinephrine. Yet, the authors of this study still observed a significant reduction in chondrocyte viability when these cells were treated with bupivacaine and lidocaine. Furthermore, Dang et al. (2011) showed significantly lower chondrocyte viability after treating human articular chondrocytes with 1:300,000 epinephrine compared to treatment with 0.9% normal saline, suggesting that treating chondrocytes with epinephrine does in fact have deleterious consequences. Bogatch et al. (2010) suggested from their study that chemical incompatibility between local anesthetics and cell culture media or synovial fluid was responsible for a decrease in chondrocyte viablity. Again, without co-treatment of culture media or synovial fluid, the authors in this present study observed significant cell death following treatment with local anesthetics. This would suggest that another mechanism may potentially be responsible for this observed cytotoxicity.

Grishko et al. (2010) sought to identify a potential mechanism for local anesthetic cytotoxicity by lengthening recovery time following treatment with bupivacaine, lidocaine, and ropivacaine at various concentrations. The study correlated chondrocyte death with mitochondrial dysfunction in a dose dependent manner, especially 120 hours after treatment (Grishko et al., 2010). Taking into consideration the possibility that mitochondrial dysfunction was a major contributor to chondrocyte death following local anesthetic treatment, the authors of this study postulated that mitochondrial protection may be a way to

increase chondrocyte viability following insult. Kocaoglu and colleagues (2011) showed with cartilage explants that chondrocytes metabolism can be retarded by treating explants in irrigation fluids at 24°C and 4°C. Thus, we hypothesized that by reducing environmental temperature during treatment with local anesthetic, reducing cellular metabolism, could improve chondrocyte viability after injury.

Chondrocytes treated with 2% lidocaine maintain significantly more viability after incubation at 23°C or 4°C. Furthermore, at these temperatures chondrocytes are not significantly less viable than chondrocytes treated with media, PBS, or ropivacaine. Bupivacaine treated chondrocytes demonstrate improved viability at 4°C, but are still significantly less viable than chondrocytes treated in media or ropivacaine at the same temperature. Finally, ropivacaine treated chondrocytes become more viable, although not significantly, as temperature was reduced.

Based upon these results, it appears that articular chondrocytes under duress from single dose local anesthetic treatment can be rescued by reducing the environmental temperature during treatment. Other fields have used therapeutic hypothermia or cryotherapy to protect various tissues during periods of acute insult (Lo et al., 2009a; Dietrich et al., 2011; Herlambang et al., 2011; Li et al., 2012; Stalman et al., 2011; Zierer et al., 2012). Li et al. (2012) concluded that rodent cardiomyocytes show improved contractility with hypothermia during ischemia, followed by reperfusion at normal physiologic temperature. This is similar to our model in which chondrocytes are in hypothermic conditions during treatment and then allowed to recover at normal conditions (37°C).

Clinically, moderate hypothermia, in which the patient's core temperature is reduced 3-5°C, has been used to preserve neuronal function and improve patient outcome in spinal trauma, neurosurgery, and cardiovascular surgery (Dietrich et al., 2011; Schmidt-Schweda et al., 2013; Zierer et al., 2012). Stalman et al. (2011) used a compression cuff and cryotherapy following knee arthroscopy, finding that this treatment reduced the energy requirements of hypermetabolic synovial tissue. Furthermore, this study found that this compression/cooling device was able to reduce the temperature of the intra-articular space by approximately 5°C and the joint capsule by about 8°C (Stalman et al., 2011). Warren et al. (2004) were also able to show that icing the joint reduced the intra-articular temperature to about the 20-25°C range as well. Stalman and co-authors (2011) also show that without cooling, the temperature throughout the joint increases overtime after knee arthroscopy. Recently, Piper and Kim (2012) showed that articular chondrocytes treated with bupivacaine at an increased temperature had reduced chondrocyte viability as compared to those treated at 37°C.

In all, this present study demonstrated that, *in-vitro*, articular chondrocytes can be protected from the cytotoxic effects of local anesthetics by reducing environmental temperature. Furthermore, these results are supported by the literature looking at the efficacy of therapeutic hypothermia in other fields. Clinically, cooling the joint immediately after intra-articular injection could protect chondrocytes and surrounding cartilage tissue from additional insult. Future studies need to be conducted to confirm these finding in other models. Of particular interest will be how temperature affects both chondrocyte and extracellular matrix health *in-vitro* and *in-vivo* following local anesthetic treatment. Finally, better understanding of the mechanism for local anesthetic cytotoxicity is required and will provide better insight in to how therapeutic hypothermia can provide clinical cartilage protective treatments.

It should be noted that there are some limitations to this study. First, bovine chondrocytes from non-skeletally mature animals were used, and it is possible that these chondrocytes are more robust than older specimens. Furthermore, human articular chondrocytes, particularly those from osteoarthritic patients, may not be able to recover as well from insult as the chondrocytes used in this study. Additionally, the chondrocytes used in this study were cultured in monolayer, which deviates from the 3-D extra-cellular matrix rich structure that chondrocytes are found *in-vivo*. Without as much protective extracellular matrix, cells in monolayer may show increased sensitivity to the environment. Although the findings in this study, along with work done by other groups, would suggest that changes in mitochondrial function play a major role in cytotoxicity following, this has yet to be substantially confirmed (Grishko et al., 2010). More experiments need to be performed to better define the mechanism behind this injury, which may lead to therapeutic interventions.

Conclusion

This study suggests that reducing the temperature from 37°C to 23°C during treatment with lidocaine increases chondrocyte viability following injury. Furthermore, chondrocytes treated with bupivacaine can be rescued by reducing the temperature to 4°C. Our studies suggest that clinically physicians should consider active cooling of the joint following intra-articular injection to protect the chondrocytes from injury during treatment with lidocaine. Furthermore, physicians should do their best to avoid using bupivacaine, especially because its cytotoxic effects cannot be mitigated at physiologically attainable temperatures. Finally, when possible, it is our suggestion that ropivacaine, which in this study consistently showed no significant deleterious effects, be used clinically when injecting local anesthetics into the joint.

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Key points

- Confirm that local anesthetics, specifically bupivacaine and lidocaine, are toxic to chondrocytes in monolayer
- Chondrocyte viability significantly improved for chondrocytes treated with bupivacaine when the environment was cooled to 23°C.
- Chondrocyte viability significantly improved for chondrocytes treated with bupivacaine or lidocaine when the environment was cooled to 4°C
- It is the recommendation of the authors that physicians should be wary of the risks of injecting local anesthetics into the intra-articular space.
- Active cooling of the joint could potentially protect the articular cartilage from insult following treatment with local anesthetics.

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