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In Vitro Toxicity of Local Anesthetics and Corticosteroids on Chondrocyte and Synoviocyte Viability and Metabolism

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Abstract

Objective: There is growing concern that intra-articular injection of local anesthetic and/or corticosteroids may cause significant morbidity, including potential toxicity to chondrocytes and synoviocytes, after even a single exposure. We demonstrate that full thickness canine chondral and synovial samples exposed to various local anesthetics and corticosteroids exhibit decreased loss of cell viability compared with prior *in vitro* studies using monolayer culture, due to the protective effects of intact extracellular matrix and cell heterogeneity. *Methods*: Full-thickness cartilage and synovial explants were obtained from canine cadavers and exposed in culture media to the following for 24 hours: 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupvacaine, 0.0625% bupivacaine, betamethasone acetate, methylprednisolone acetate, triamcinolone acetonide, or culture media only (control). Cell viability was determined on days 1 and 7 of culture using a microscopic live-dead and alamar blue metabolic assays. *Results*: Complete loss of chondrocyte and synoviocyte viability was noted in the 1% and 0.5% lidocaine group, 0.25% and 0.125% bupivacaine group, betamethasone group, and methylprednisolone groups after 1 and 7 days of culture. Treatment with 0.0625% bupivacaine and triamcinolone demonstrated no decrease in cell viability or metabolism when compared to negative control. *Conclusions*: In this canine explant model, 1% and 0.5% lidocaine, 0.25% and 0.125% bupivacaine, betamethasone acetate, and methylpresdnisolone acetate were severely chondrotoxic and synoviotoxic after a single exposure, despite intact extracellular matrix. In contrast, chondrocytes and synoviocytes exposed to 0.0625% bupivacaine and triamcinolone remained viable after treatment. Further *in vivo* study is needed before definitive recommendations can be made.

Keywords

chondrocyte, synoviocyte, toxicity, local anesthetic, corticosteroid

Introduction

Intra-articular injections of local anesthetics and corticosteroids are widely used for the acute treatment of inflammatory and arthritic joint conditions.[1-3] Such injections are useful in a variety of settings, including both peri-operatively and in the ambulatory setting.[2,4,5] Despite widespread use, there has been growing concern over the potential toxicity of these substances. While acute exposure often demonstrates clinical relief of symptoms without obvious detrimental effects, the prospect of intra-articular toxicity and long term morbidity from even one time use of these agents is still hotly debated and not yet proven.

Several in vitro studies have been performed which have demonstrated chondrotoxic properties of local anesthetic agents.[2-4,6-12] These studies have shown that even brief exposure to anesthetics or corticosteroids may result in loss of chondrocyte viability. Of the agents most commonly used in the clinical setting, lidocaine and bupivicaine are the best described. However, the majority of these studies have been performed on monolayer cell cultures that do not replicate the extracellular matrix and cell heterogeneity found in either intact tissue samples or in vivo. Additionally,

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no prior studies have also evaluated the effects of these agents on live synoviocytes from full thickness explants.

The purpose of this study is to evaluate the in vitro toxicity of clinically relevant doses of local anesthetics and cortisone derivatives on chondrocytes and synoviocytes in a canine explant culture system. This type of system preserves the extracellular matrix and cell heterogeneity of the tissues to better replicate in vivo conditions. Our hypothesis is that the culture system may have a protective effect against the toxicity of agents demonstrated in prior in vitro models, and that the overall effect on cell viability would be negligible for clinically relevant doses of these common medications.

Table 1. List of Treatments.

Group	Media	Drug
Negative control	7 mL	
0.25% Bupivicaine	7 mL +	5 mL
0.125% Bupivicaine	7 mL +	2.5 mL
0.0625% Bupivicaine	7 mL +	1.25 mL
1.0% Lidocaine	7 mL +	2 mL
0.5% Lidocaine	7 mL +	1 mL
Betamethasone 5 mg	7 mL +	1.25 mL
Methylprednisolone (depo) 40 mg	7 mL +	1 mL
Triamcinolone (kenalog) 40 mg	7 mL +	1 mL

Methods

Tissue Culture and Harvest

All procedures were approved under the institution's Animal Care and Use Committee policies and procedures for use of canine cadaveric tissues. Seven canine cadavers with grossly normal shoulder joints were obtained immediately after euthanasia performed for reasons unrelated to this study. Full-thickness cartilage samples were harvested from the humeral head and synovial samples were harvested from the joint capsule of the shoulder under sterile conditions. Four millimeter tissue explants were prepared using a dermal biopsy punch (Fray Products, Buffalo, NY).

The cartilage and synovial tissue explants were cultured in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) in media containing Dulbecco's modified Eagle's medium with high glucose (Gibco, Invitrogen, Carlsbad, CA) supplemented with 1% ITS, penicillin, streptomycin, amphotericin B, L-ascorbic acid, L-glutamine, and nonessential amino acids. Explants (n = 7 per group) were cultured for 24 hours prior to assignment to one of the following treatment groups: 0.25% bupivacaine, 0.125% bupivacaine, 0.0625% bupivacaine, 1% lidocaine, 0.5% lidocaine, betamethasone acetate, methylprednisolone acetate, and triamcinolone. The concentration for each treatment group was based on the average volume of synovial fluid found in a human knee joint (7 mL)[13] and the volume of drug required to obtain the desired concentration (**Table 1**). Explants were cultured in 1 mL of treatment or control media and incubated at 37°C with 5% CO_2 at 95% humidity for either 24 hours or 7 days.

Cell Viability

Cell viability in cartilage and synovial explants were assessed after 1 and 7 days of culture by fluorescent microscopy using the fluorescent stains calcein AM (excitation 495 nm; emission 515 nm) to stain live cells and Sytox Blue (excitation 633 nm, 635 nm, emission 658 nm) to stain nonviable cells (Life Technologies, Carlsbad, CA). At the time of tissue collection on each day, the explants were incubated in the stain for 30 minutes at room temperature. Tissue images were taken at 4× magnification using an Olympus F view II camera and Micro Suite Basic Edition software (Olympus, Tokyo, Japan). For cartilage explants, live and dead cells were counted using a custom in-house validated cell counting program. The area of the cartilage images was determined using Microsuite and chondrocyte viable cell density (VCD) was reported as the number of live cells divided by the area of the tissue (μm^2). For the synovial explants, subjective assessment of viability was performed by 5 investigators blinded to treatment. Each synovial tissue explant was as given a score from 0 (0% viability) to 5 (100% viability). The scores from all observers were averaged to give a mean synoviocyte subjective viability score (SVS) for each explant.

Cell Metabolic Activity Assay

The metabolic activity of cartilage and synovial explants was assessed using the alamar blue assay (Sigma Aldrich), a fluorescent metabolic assay, after 1 and 7 days of culture. Resazurin is converted to a fluorescent compound, resorufin, by metabolically active cells. The degree of fluorescence detected in the media provides a quantitative measure of the number of viable cells in a tissue. Resazurin (100 µL) was added to the media of each explant and incubated overnight at 37°C. A 200 µL sample of the media was transferred to a black 96-well plate and the level of fluorescence in the media was measured (excitation 530 nm, emission 590 nm) using a Synergy HT plate reader (BioTek, Winooksi, VT).

Statistical Analysis

Statistical analysis was performed using SigmaPlot v12.0 (Systat Software Inc., San Jose, CA). A one-way analysis of variance using Tukey *post hoc* comparisons was used for the detection of statistically significant differences between the control and treatment groups, with significance set at $P < 0.05$.

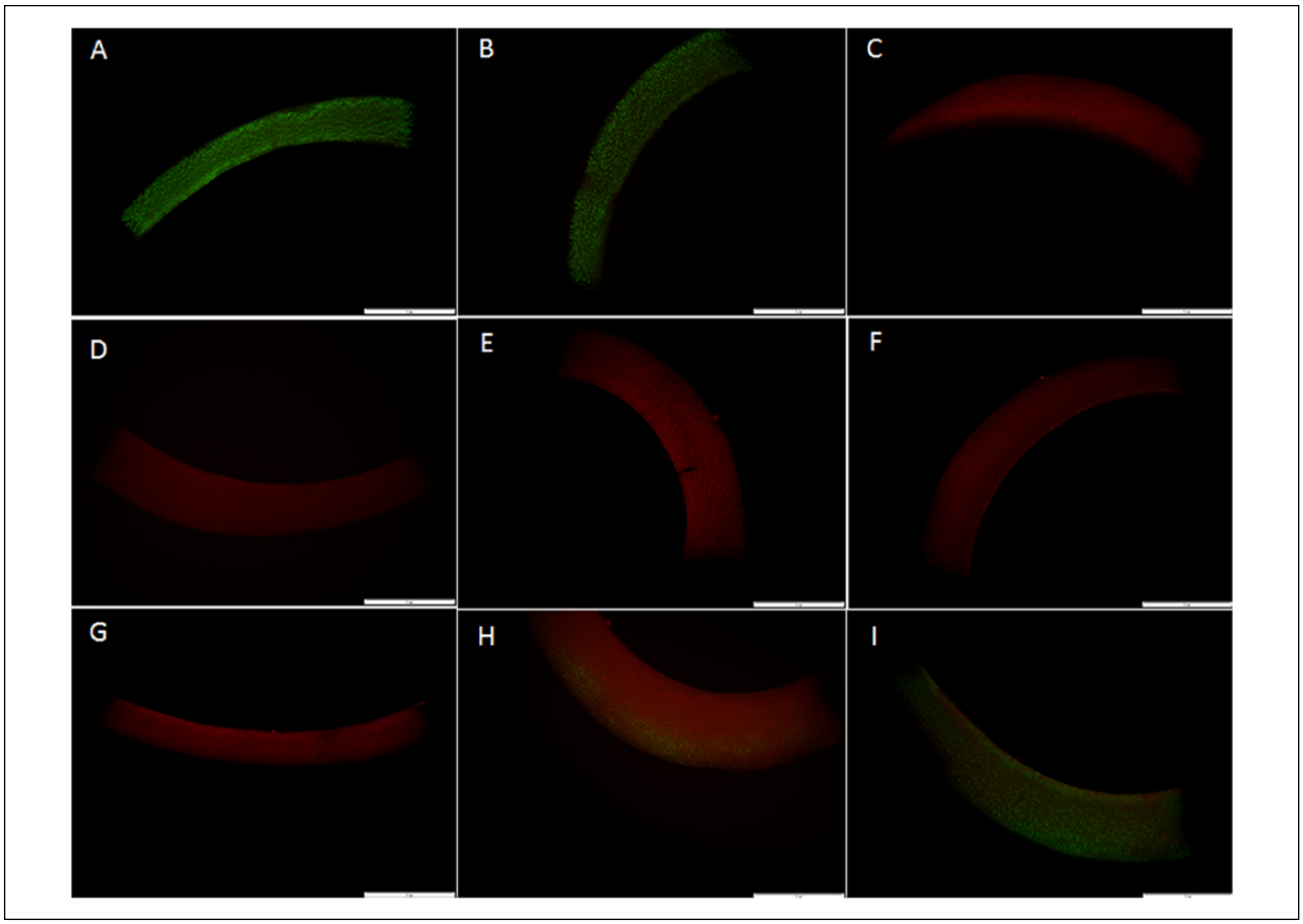

Figure 1. Viable cell density for articular cartilage: (**A**) negative, (**B**) triamcinolone, (**C**) methylprednisolone, (**D**) betamethasone, (**E**) 1.0% lidocaine, (**F**) 0.5% lidocaine, (**G**) 0.25% bupivicaine, (**H**) 0.125% bupivicaine, and (**I**) 0.0625% bupivicaine.

Results

Cell Viability

The viable cell density of cartilage explants cultured for 1 or 7 days with 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, betamethasone, and methylprednisolone was significantly ($P \leq 0.001$-0.002) lower than the negative control. However, the viable cell density of the cartilage explants exposed to 0.0625% bupivacaine ($P = 0.181$-0.687) and triamcinolone ($P = 0.205$-0.687) was not significantly different than the negative control after 1 and 7 days of culture (**Figs. 1** and **2**).

The subjective viability score of synovial explants cultured for 1 or 7 days with 1.0% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, betamethasone, and methylprednisolone were significantly ($P \leq 0.001$-0.007) lower than the negative control. The subjective viability score of synovial explants cultured with 0.0625% bupivacaine ($P = 0.072$-0.498) and triamcinolone ($P \leq 0.636$-0.868) exhibited little to no apparent decrease in cell viability when compared with negative control after 1 and 7 days of exposure (**Figs. 3** and **4**).

Cell Metabolic Activity Assay

A statistically significant ($P < 0.001$) decrease in cell metabolism, as measured by level of fluorescence in the media, of cartilage explants cultured for 1 or 7 days with 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, betamethasone, and methylprednisolone compared with the negative control. There was not a significant difference in the level of cartilage tissue cell metabolism between the 0.0625% bupivacaine ($P = 0.523$) or triamcinolone groups ($P = 0.153$) and the negative control on either day (**Fig. 5**).

A statistically significant ($P \leq 0.001$-0.019) decrease in cell metabolism of synovial explants cultured for 1 or 7 days with 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine group, 0.125% bupivacaine, betamethasone, and methylprednisolone compared with the negative control. There was not a significant difference in the level of synovial tissue

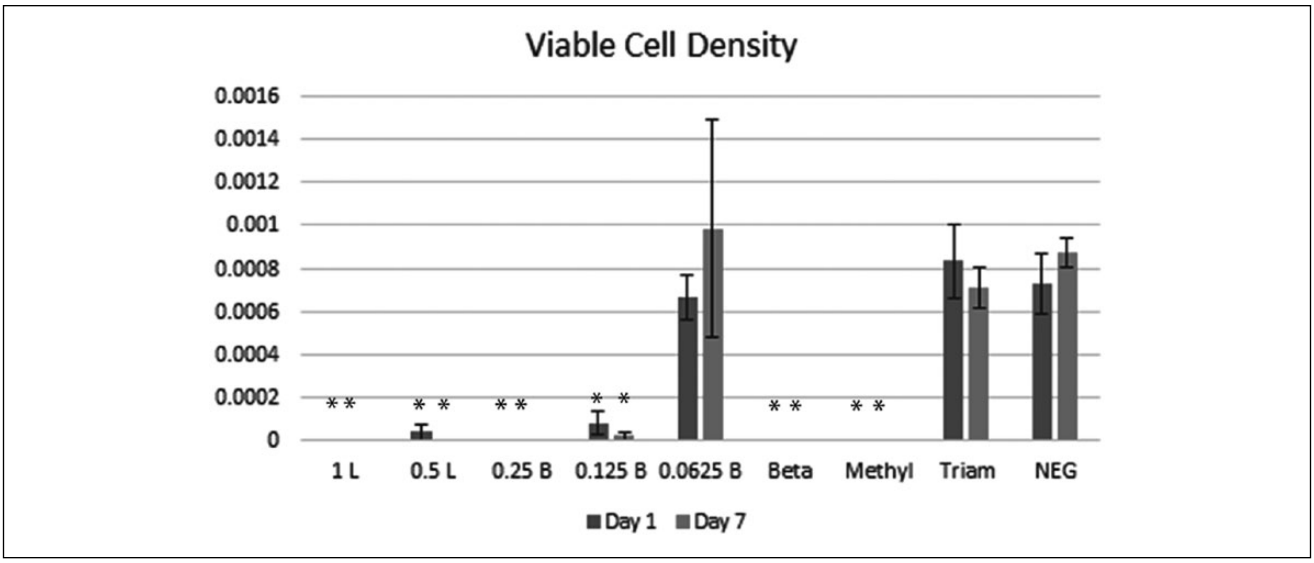

Figure 2. Cartilage tissue viable cell density (viable cells/μm^2): Mean viable cell density on days 1 and 7. 1 L, 1% lidocaine; 0.5 L, 0.5% lidocaine; 0.25 B, 0.25% bupivicaine; 0.125 B, 0.125% bupivicaine; 0.0625 B, 0.0625% bupivicaine; Beta, betamethasone; Methyl, methylprednisolone; Triam, triamcinalone; NEG, negative control. *Significantly lower viable cell density compared with the negative control.

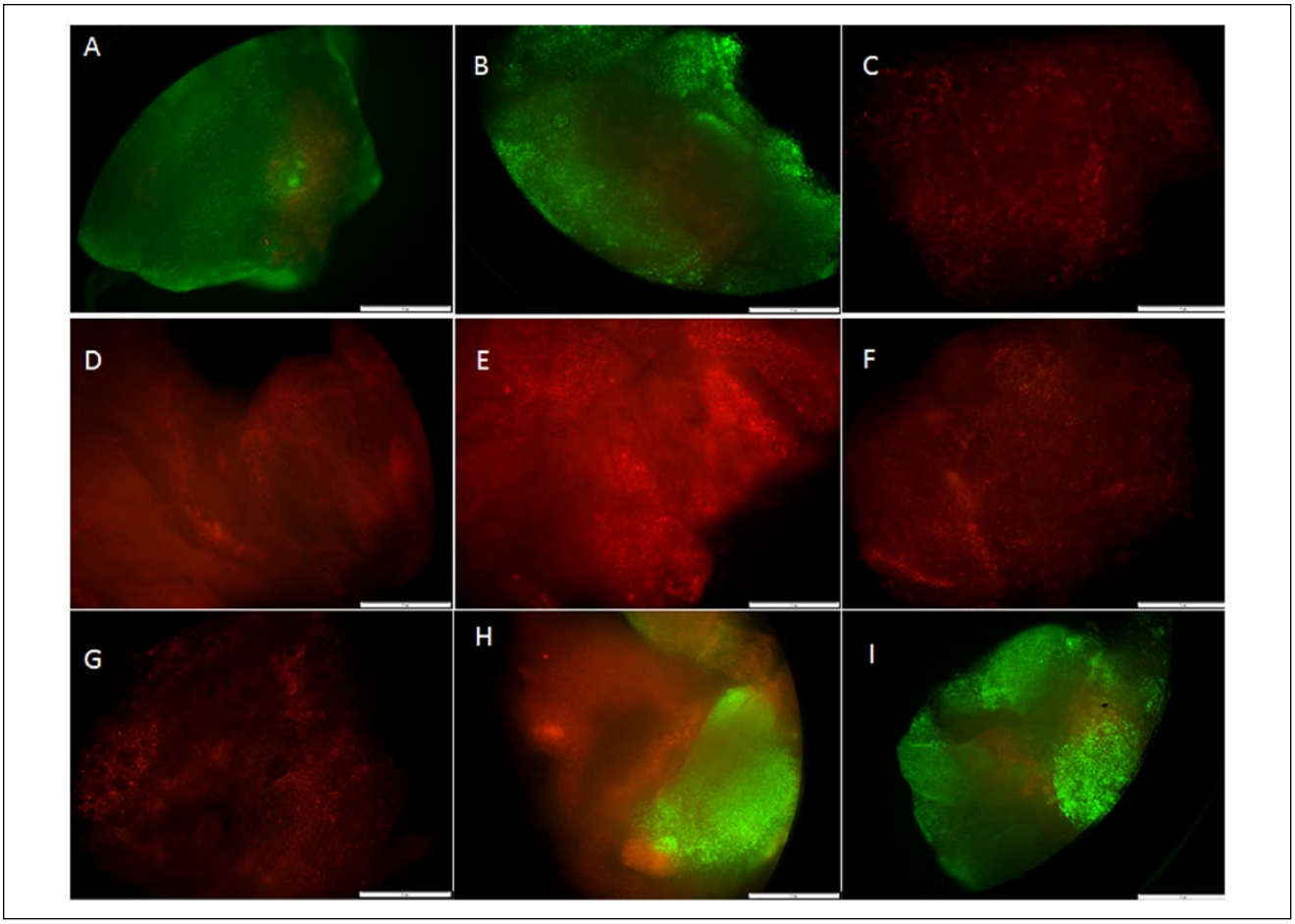

Figure 3. Viable cell density for synovium: (**A**) negative, (**B**) triamcinolone, (**C**) methylprednisolone, (**D**) betamethasone, (**E**) 1.0% lidocaine, (**F**) 0.5% lidocaine, (**G**) 0.25% bupivicaine, (**H**) 0.125% bupivicaine, and (**I**) 0.0625% bupivicaine.

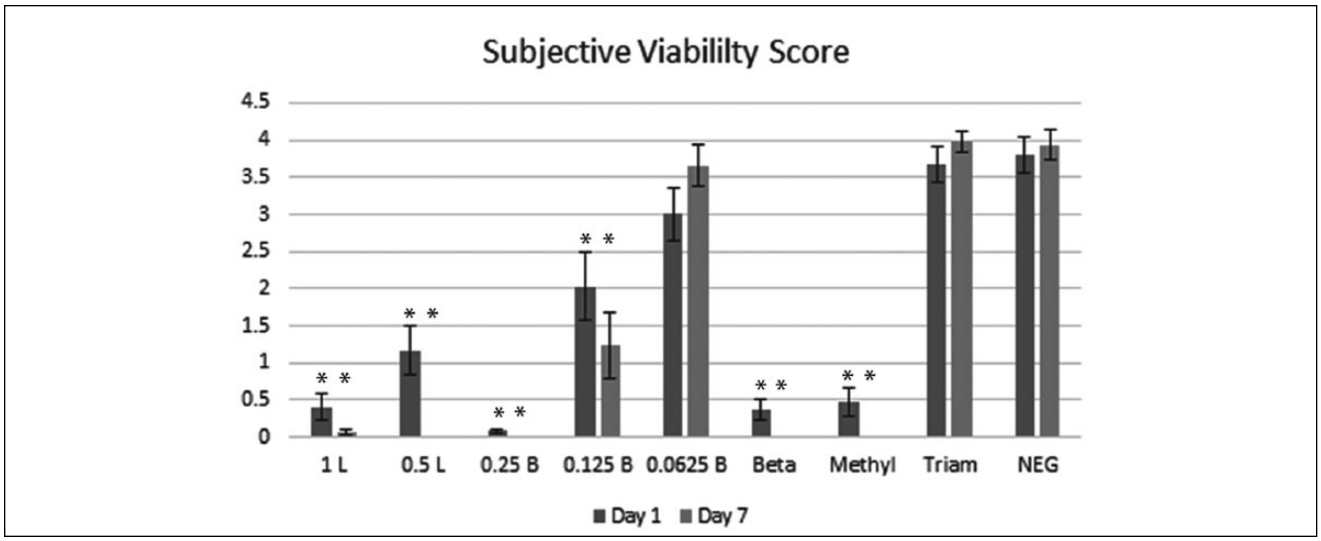

Figure 4. Synovial tissue subjective viability score (0-5): Mean subjective viability score from 0 (0% viable) to 5 (100% viable) of 5 observers blinded to treatment group. 1 L, 1% lidocaine; 0.5 L, 0.5% lidocaine; 0.25 B, 0.25% bupivicaine; 0.125 B, 0.125% bupivicaine; 0.0625 B, 0.0625% bupivicaine; Beta, betamethasone; Methyl, methylprednisolone; Triam, triamcinalone; NEG, negative control. *Significantly lower subjective viability score compared with the negative control.

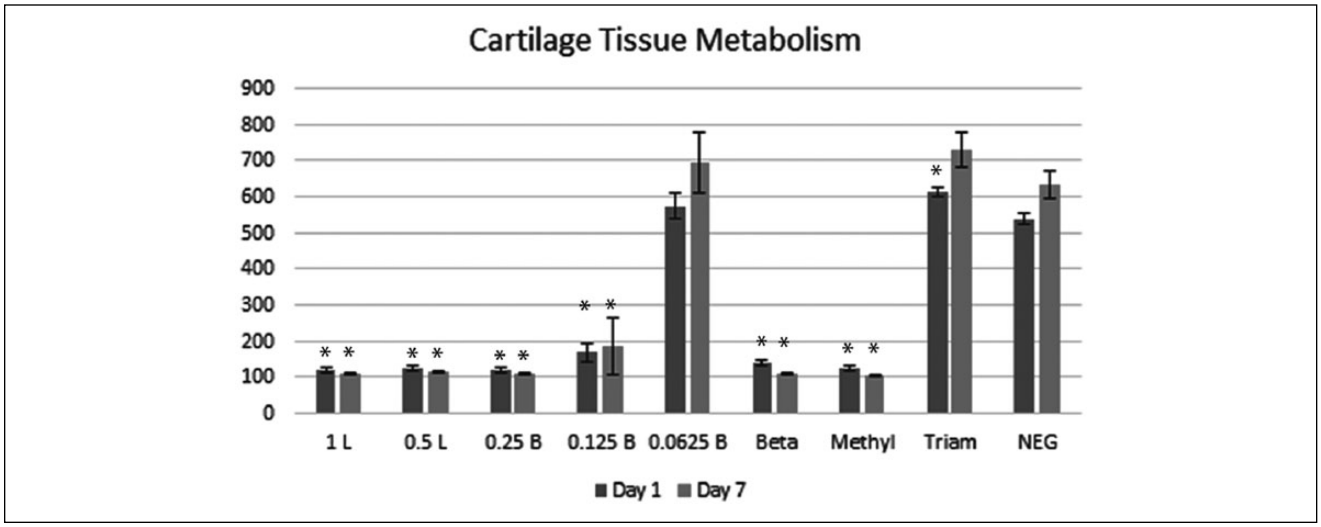

Figure 5. Level of cartilage tissue metabolism using the resazurin assay. Mean media fluorescence level on days 1 and 7 of culture post treatment. 1 L, 1% lidocaine; 0.5 L, 0.5% lidocaine; 0.25 B, 0.25% bupivicaine; 0.125 B, 0.125% bupivicaine; 0.0625 B, 0.0625% bupivicaine; Beta, betamethasone; Methyl, methylprednisolone; Triam, triamcinalone; NEG, negative control. *Significantly lower metabolic activity compared with the negative control.

cell metabolism between the 0.0625% bupivacaine ($P \geq 0.225$) or triamcinolone groups ($P \geq 0.817$) and the negative control on either day (**Fig. 6**).

Discussion

This study demonstrates that clinically relevant concentrations of commonly used local anesthetics and corticosteroid derivatives are highly toxic to normal full thickness articular cartilage and synovial explants using an *in vitro* canine explant culture model. Of the agents tested, only triamcinolone and 0.0625% bupivacaine did not have potentially detrimental effects compared with controls with respect to cell viability and cell metabolism for chondrocytes and synoviocytes. All other agents tested (1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, betamethasone, methylprednisolone) demonstrated near complete cell death and decreased cell metabolism within 24 hours of exposure to normal tissues. Our explant model using both cartilage and synovium with intact extracellular matrix and heterogeneous

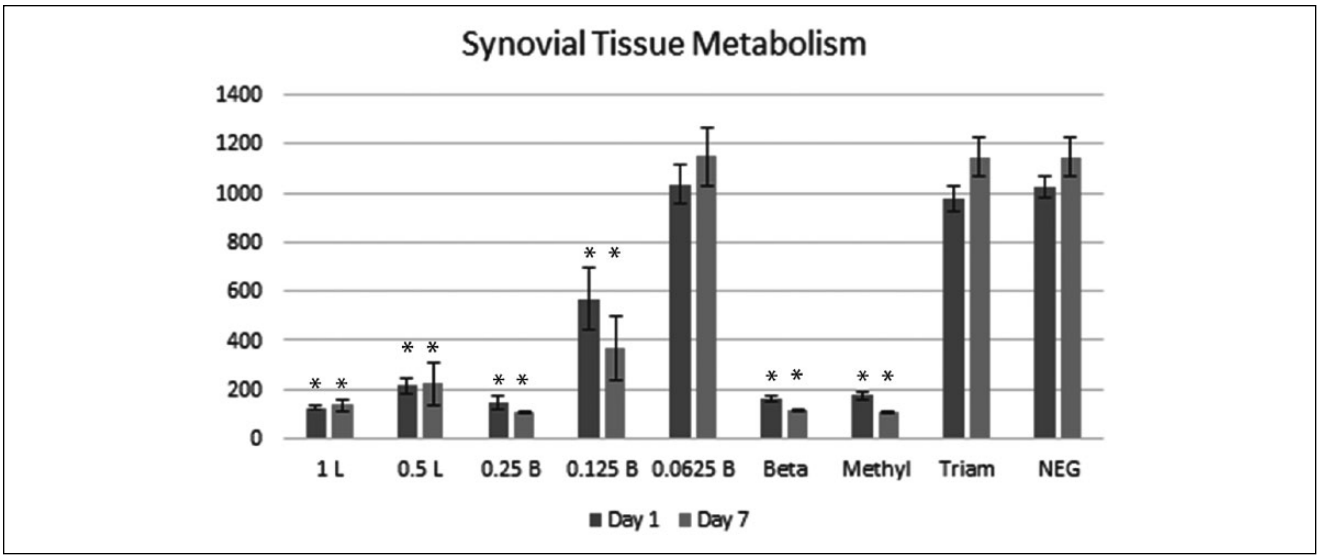

Figure 6. Level of synovial tissue metabolism using the resazurin assay. Mean media fluorescence level on days 1 and 7 of culture posttreatment. 1 L, 1% lidocaine; 0.5 L, 0.5% lidocaine; 0.25 B, 0.25% bupivicaine; 0.125 B, 0.125% bupivicaine; 0.0625 B, 0.0625% bupivicaine; Beta, betamethasone; Methyl, methylprednisolone; Triam, triamcinalone; NEG, negative control. *Significantly lower metabolic activity compared with the negative control.

cell types did not show any protective effects in comparison to previously reported monolayer culture models with regard to detrimental effects of single agent exposure on chondrocyte viability or cell metabolism.

The toxic effects of local anesthetics on chondrocytes have been the subject of study by a number of groups over the past several years. In an *in vitro* study using bovine articular chondrocytes, Chu *et al*.[9] showed that even brief exposure to local anesthetic—in this case, 0.5% bupivacaine—mimicking a single injection, may cause a decrease in cell viability. Others have produced results consistent with these findings. Baker *et al*.[14] in 2011 found that the introduction of magnesium as a local anesthetic helped to reduce toxic effects on articular chondrocytes to be no more toxic than normal saline solution. A systematic review by Baker and Mulhall[15] also suggests that more long-range work is needed to establish the precise mechanism of toxicity and whether or not single bolus administration results in long-term deleterious outcomes. A 6-month cartilage tissue loss *in vivo* study from Chu *et al*.[7] in 2010 suggested bupivacaine toxicity after a single intra-articular injection. Farkas *et al*.[3] suggest that the adverse effect glucocorticoids and local anesthetics have on articular chondrocytes make it a questionable method for the treatment of osteoarthritis. Data from Breu *et al*.[16] data suggest that chondrocyte toxicity is much more prevalent in degenerative compared to intact cartilage.

While the effects of local anesthetics and cortisone derivatives on chondrocytes have been the focus of several studies, to our knowledge, the effect of these agents on synoviocytes has not been as well evaluated in the literature and further clinical evaluation is needed. Anz *et al*.[4] examined the effects of bupivicaine and morphine on cartilage-synovium co-cultures, and found a near 100% loss of tissue viability on exposure of 0.5% bupivicaine to the culture, with no gross chondrotoxicity of samples exposed to morphine. The viability assays in the study by Anz *et al*.[4] focused solely on examination of the chondrotoxicity of those agents; however, the results suggest similar susceptibilities in synovial tissue as cartilaginous tissue concerning the toxic effects of anesthetics and corticosteroids. A 2013 study by Braun *et al*.[6] examined the effects of 0.5% bupivacaine with and without epinephrine on cell cultures of fibroblast-like synoviocytes. Cultures treated with bupivicaine and epinephrine exhibited loss of cell viability compared with negative control, while samples exposed to bupivacaine alone did not. The authors suggest that a rise in matrix metalloprotease noted after exposure to these treatments may provide a mechanism for indirect cartilaginous injury.[6]

The data yielded by the present study are consistent with the existing body of literature and contributes to the growing concern that certain clinically relevant doses of local anesthetics and corticosteroids demonstrate toxicity toward articular tissue following single exposure. However, there were some discrepancies between this study and others. In contrast to our results, a 2008 study by Chu *et al*.[8] found that exposure of bovine and human chondrocytes to 0.125% bupivacaine did not result in a significant decrease in cell viability, compared with control. Additionally, in a study conducted in 2011 by Syed *et al*.,[12] triamcinolone alone and in combination with bupivicaine, demonstrated significant chondrotoxicity when tested against human monolayer cell

cultures and articular plugs. The discrepancy may be explained by the differing susceptibilities of joint types (the present study used humeral head tissue, while the other used femoral), properties of different species (the present study used canine samples, while the other used human patients undergoing arthroplasty), or relative concentrations of agents used. In our study, triamcinolone was the only corticosteroid that demonstrated no loss of cell viability or decreased cell metabolism when compared with controls. As well, our results reveal loss of synoviocyte viability on exposure to 0.25% and 0.125% bupivacaine, in contrast to the 2013 Braun *et al*.[6] study, which found no statistically significant toxicity of 0.5% bupivacine exposure.

The mechanism of toxicity is as yet unclear. Potential explanations include an incompatibility between synovial fluid and anesthetic rather than any direct toxicity of the anesthetics themselves, matrix metalloprotease release, or possibly mitochondrial dysfunction stemming from mtDNA damage, leading to chondrocyte cell apoptosis.[1,5,17] Further investigation is necessary to clarify these and other important issues. Variations in exposure time and expanding the duration of the investigation may shed further light on the long-term toxicity of these agents. Additionally, future work may investigate the toxicity of alternative agents of analgesia, including other long-acting local anesthetics (i.e., ropivicaine), injectable nonsteroidal anti-inflammatory medications (i.e., toradol), and morphine derivatives. Definitive evidence of chondrocyte and synoviocyte toxicity of local anesthetics and cortisone derivatives must also be demonstrated using *in vivo* models, and using combination treatments to best represent common orthopedic practice. This is the subject of a recently published investigation at our institution using an *in vivo* canine model.[18]

The limitations of the present study include the use of normal canine tissues and only 2 short-term assessment time points. Dogs have been validated as an appropriate model for joint pathology based on anatomy, biomechanics, physiology, and clinical disorders seen and treated.[18-21] The use of normal, healthy articular cartilage and synovial tissue was designed to limit variability and to provide a "best case scenario" for this testing in that healthy, intact tissue should be most resistant to detrimental effects associated with the injectates. Based on the significant and rapid effects noted on these healthy tissues, use in pathologic joints is even more concerning. The degree of toxicity noted within the first 7 days of exposure on normal tissues suggests that the acute effects noted are clinically relevant. Furthermore, this study examined the effects of local anesthetics and corticosteroids only when used individually, not in conjunction with one another. Investigations that have examined these drugs in combination have demonstrated consistent findings of chondrotoxicity.[2,6] In the clinical setting, intra-articular injections often may comprise more than one pharmaceutical; therefore, the toxicities of these combinations should be further investigated.

The results of the quantitative cell metabolism additive test were mostly consistent with those of the qualitative cell viability assay. Exceptions include the chondrocyte and synoviocyte treatment groups exposed to low-dose bupivacaine, which appeared to suffer an estimated 50% loss of cell viability by qualitative inspection, but had no statistically significant decrease in cell metabolism. This result is likely due to dynamics of the indicator dye, and the overnight incubation of the tissue explant in the indicator dye. The level of fluorescence detected in the media was at the maximum level for the amount of dye added to the media for all samples that obtained a high reading. Therefore, if the media were assessed after a shorter incubation time, it is likely that there would have been a difference in the level of fluorescence detected in the media of high viability and lower viability samples. The way the assay was performed in this study was to differentiate between viable and nonviable tissues.

In conclusion, this study demonstrates that single exposure of canine cartilage and synovial explants to lidocaine (0.5% and 1.0%), bupivicaine (0.125%, 0.25%), betamethasone, and methylprednisolone results in significant chondrotoxicity and synoviotoxicity. These findings are similar to toxicity studies using monolayer cell cultures. Therefore, the existence of intact extracellular matrix and heterogeneous cell types does not appear to confer any additional resistance to the toxic effects of these agents. Conversely, low-dose bupivicaine (0.0625%) and triamcinolone demonstrated no loss of cell viability compared with controls. Future *in vivo* investigation is warranted to validate the findings of this, and other, *in vitro* studies. Until that time, clinicians should continue to use the best available evidence to make treatment decisions regarding the use of these injections in their patients.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by our institutional review board.

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