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Original article

In vitro toxicity of local anaesthetics and corticosteroids on supraspinatus tenocyte viability and metabolism

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Summary *Background/Objective:* The purpose of this study was to evaluate supraspinatus tenocyte viability and metabolism in explants exposed to various local anaesthetics and corticosteroids. Our hypothesis was that the tendons exposed to these common injectates would have significantly decreased cell viability and metabolism compared with controls.
Methods: Supraspinatus tendon explants were obtained from dogs, placed in a culture media, and randomly assigned to one of the following groups: culture media only (control), 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, 0.0625% bupivacaine, betamethasone acetate (5 mg), methylprednisolone acetate (40 mg), or triamcinolone acetonide (40 mg). Cell viability was determined on Days 1 and 7 after culture treatment using calcein AM (live cell) and Sytox Blue (dead cell) stains. Tissue metabolism was assessed on Days 1 and 7 using the resazurin blue metabolic assay. Significant differences were evaluated using a one-way analysis of variance with Tukey *post hoc* analysis.
Results: Compared with the controls, there were significant decreases in cell viability noted at Days 1 and 7 in tenocytes exposed to 1% lidocaine, betamethasone, and methylprednisolone. Significant decreases in cell metabolism were also noted at Days 1 and 7 in those groups. Treatment with 0.125% bupivacaine, 0.0625% bupivacaine, and triamcinolone demonstrated no decrease in cell viability or metabolism when compared with controls at any time point.
Conclusion: This data confirms that peritendinous injection of commonly used local anaesthetics and corticosteroids results in significant supraspinatus tenotoxicity *in vitro*. Further *in vivo* studies are required before making definitive clinical recommendations.

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Introduction

Local anaesthetic and corticosteroid injections have been widely used as a treatment option for many musculoskeletal conditions. Intra-articular injections are often used to treat arthritic joints whereas extra-articular or peritendinous injections are often used to treat inflammatory conditions [1—3]. Recent studies, however, have suggested potential toxicity at the cellular level after routine use of certain injection agents [4—7]. While the acute clinical result is often relief of symptoms, the potential for toxicity at the cellular level and associated long term morbidity has not yet been fully elucidated.

Numerous studies have been conducted which have demonstrated chondrotoxic properties of local anaesthetic and corticosteroid agents [4—13]. Some of these studies have shown that even a single exposure to anaesthetics or corticosteroids may result in loss of chondrocyte viability. These studies have demonstrated damaging effects of anaesthetic agents alone or in combination with corticosteroids. While there have been many recent investigations documenting chondrotoxicity of local anaesthetics and cortisone derivatives, there have been less studies demonstrating similar toxic effects on tenocytes and tendons [14—18]. Rotator cuff pathology, in particular, is frequently treated clinically with steroid injections, and the deleterious effects of various steroid derivatives on rotator cuff tendons have been shown in previous animal models [1,3,19,20]. Considering the current usage frequency of steroid and combination steroid and anaesthetic injections, it is valuable to further evaluate the effects of multiple commonly used agents.

The purpose of this *in vitro* study was to evaluate the toxicity of commonly used clinical doses of both local anaesthetics and corticosteroids on tenocytes in a canine supraspinatus tendon explant model. This explant model preserves the extracellular matrix and cell heterogeneity of the tissues in an effort to optimally mimic *in vivo* conditions. Our hypothesis was that the tendons exposed to routinely used local anaesthetics and corticosteroids at clinically relevant concentrations would have significantly decreased cell viability and metabolism compared with controls.

Methods

Tissue culture and harvest

All procedures were approved under the Institutional Animal Care and Use Committee policies and procedures for the use of canine cadaveric tissues. Seven adult (age = 2—4 years and mean weight = 28.6 kg), purpose-bred, intact female mongrel canine cadavers were obtained immediately after euthanasia that was performed for reasons unrelated to this study. All shoulder joints used were free of intra- and extra-articular pathology based on complete gross examination. Supraspinatus tendon samples were harvested from the shoulder under sterile conditions. Tissue explants, 4-mm thick, were prepared using a dermal biopsy punch (Fray Products, Buffalo, NY, USA) and were sliced in half to observe the viability of tenocytes across the thickness of the specimen.

The tendon tissue explants were cultured in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in media containing Dulbecco's modified Eagle's medium with high glucose (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 1% insulin—transferrin—selenium, penicillin, streptomycin, amphotericin B, L-ascorbic acid, L-glutamine, and nonessential amino acids. Explants ($n = 7$/group) were cultured for 24 hours prior to assignment to one of the following treatment groups: 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, 0.125% bupivacaine, 0.0625% bupivacaine, betamethasone acetate (5 mg), methylprednisolone acetate (40 mg), and triamcinolone (40 mg). The concentration for each treatment group, shown in Table 1, was based on the average volume of injectate used for treatment of equivalent human pathology and the volume of drug required to obtain the desired concentration for explant culture [21]. 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 tendon explants was assessed after 1 day 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, USA). 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 recorded at 4× magnification using an

Table 1 Amount of medication used for each treatment subset.

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

Olympus F view II camera and Micro Suite Basic Edition software (Olympus, Tokyo, Japan). Subjective assessment of viability was performed by six investigators blinded to the treatment and averaged to ensure consistent overall assessment. Each tendon tissue explant was given a score from 0 (0% viability) to 5 (100% viability). The scores from all investigators were averaged to obtain a mean tenocyte subjective viability score for each explant.

Cell metabolic activity assay

The metabolic activity of tendon explants was assessed using the Alamar Blue assay (Sigma–Aldrich, St. Louis, MO, USA); this is a fluorescent metabolic assay used for Days 1 and 7 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, USA).

Statistical analysis

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

Results

Cell viability

Viability scores for tendon explants cultured for 1 day with 1.0% lidocaine, 0.25% bupivacaine, betamethasone, and methylprednisolone were significantly lower ($p \leq 0.001$) than those for controls. Viability scores of tendon explants cultured for 1 day with 0.5% lidocaine, 0.125% bupivacaine, 0.0625% bupivacaine, and triamcinolone were not significantly different from those of controls ($p = 0.12–0.78$). On Day 7 of culture, viability scores for tendons cultured with 1.0% lidocaine, betamethasone, and methylprednisolone remained significantly lower ($p \leq 0.001$) than those for controls. While the viability scores for tendons cultured with 0.25% bupivacaine remained numerically lower than those for controls on Day 7, this difference was not statistically significant ($p = 0.24$). Furthermore, viability scores for tendon explants cultured for 7 days with 0.5% lidocaine, 0.125% bupivacaine, 0.0625% bupivacaine, and triamcinolone were not significantly lower than those for controls ($p = 0.12–0.78$; Figure 1).

Cell metabolic activity assay

On Day 1 of culture, the level of metabolic activity, measured by the level of fluorescence in the media of

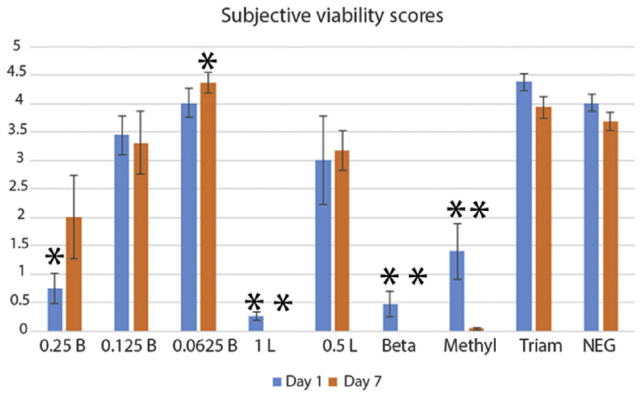

Figure 1 Tenocyte cell viability scores (viable cells/μm^2). Mean viable cell density on Days 1 and 7. 0.25 B = 0.25% bupivacaine; 0.125 B = 0.125% bupivacaine; 0.0625 B = 0.0625% bupivacaine; 1 L = 1% lidocaine; 0.5 L = 0.5% lidocaine; Beta = betamethasone; Methyl = methylprednisolone; NEG = negative control; Triam = triamcinolone. * Significantly lower viable cell density compared with the negative control.

tendon explants cultured with 1% lidocaine, 0.5% lidocaine, 0.25% bupivacaine, betamethasone, and methylprednisolone, was significantly lower ($p \leq 0.001–0.003$) than that for controls. There was no significant difference in the level of tissue metabolic activity between tendon explants cultured with 0.125% bupivacaine, 0.0625% bupivacaine, or triamcinolone ($p = 0.26–0.31$) and controls on Day 1 (Figure 2). On Day 7 of culture, the level of metabolic activity of tendon explants cultured with 1% lidocaine, betamethasone, and methylprednisolone remained significantly lower ($p \leq 0.001$) than that for controls. However, in agreement with the viability scores, the level of metabolic activity of tendon explants cultured with 0.5% lidocaine and 0.25% bupivacaine was not significantly different ($p = 0.1$) than that for controls on Day 7. Furthermore, there was still no significant difference in the level of metabolic activity between tendon explants cultured with

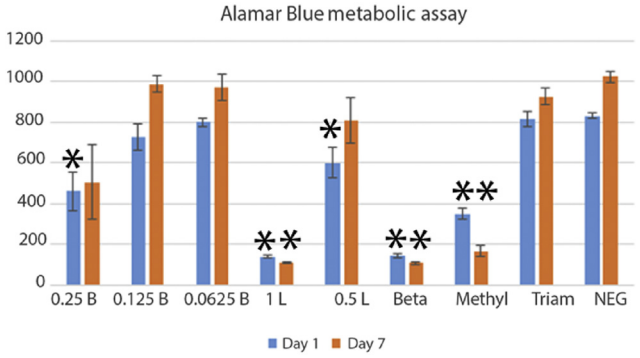

Figure 2 Level of tenocyte metabolic activity using the Alamar Blue metabolic assay. Mean media fluorescence level on Days 1 and 7 of culture post-treatment. 0.25 B = 0.25% bupivacaine; 0.125 B = 0.125% bupivacaine; 0.0625 B = 0.0625% bupivacaine; 1 L = 1% lidocaine; 0.5 L = 0.5% lidocaine; Beta = betamethasone; Methyl = methylprednisolone; NEG = negative control; Triam = triamcinolone. * Significantly lower viable cell density compared with the negative control.

0.125% bupivacaine, 0.0625% bupivacaine, or triamcinolone ($p = 0.07–0.47$) and controls on Day 7 (Figure 2).

Discussion

The purpose of this *in vitro* study was to evaluate the toxicity of commonly used clinical doses of both local anaesthetics and corticosteroids on tenocytes in a canine supraspinatus tendon explant model. The present study demonstrated that 1% lidocaine, betamethasone, and methylprednisolone had significantly negative effects on cell viability and metabolism at both the time points tested. In contrast, 0.125% and 0.0625% bupivacaine and triamcinolone did not differ significantly from controls with respect to cell viability or cell metabolism at either time point. The remaining agents (0.5% lidocaine and 0.25% bupivacaine) showed mixed results.

The toxic effects of local anaesthetics and corticosteroids have been documented by a number of studies in recent years [4–11]. The negative effects of these agents have been reported to occur in intra- and extra-articular tissues, with chondrocytes being most frequently studied.

While the chondrotoxic effects of anaesthetics and corticosteroids have been well documented, there have been fewer studies demonstrating their effects on soft tissues, particularly tendons. Piper et al [14] demonstrated varying levels of toxicity of lidocaine, ropivacaine, and dexamethasone on bovine tenocytes in culture. In their study, lidocaine was noted to have increasingly negative effects on tenocyte viability as the concentration increased. Ropivacaine alone was not found to have significantly negative toxic effects; however, when both the anaesthetics were combined with dexamethasone, they were noted to have significantly increased toxicity to tenocytes. In a separate study, Sherb et al [16] demonstrated a decrease in the cultured tenocyte cell proliferation and extracellular matrix component production after exposure to bupivacaine alone. Yang et al [22] performed an *in vitro* study evaluating the effects of a combination of 1% lidocaine with triamcinolone on rat patellar tendon-derived tenocytes. They noted significantly decreased cell viability and a markedly decreased expression of tenocyte-related genes in the tenocytes treated with the medication combination versus controls. They concluded that a combination injection of 1% lidocaine with triamcinolone should be used with caution in the treatment of tendon disorders [22].

Sung et al [23] recently studied cytotoxic effects of ropivacaine, bupivacaine, and lidocaine on cultured human rotator cuff tenofibroblasts. They studied six anaesthetic subgroups (two different concentrations for each agent) and exposed the tenofibroblasts to each of these agents over increasing duration. They used 0.2% and 0.75% ropivacaine, 0.25% and 0.5% bupivacaine, and 1% and 2% lidocaine. They noted significantly decreased cell viability with increased anaesthetic concentrations and exposure times. They also noted increases in cellular factors that could promote cell death, such as increased production of intracellular reaction oxygen species and increased activation of mitogen-activated protein kinases and caspase-3/7. Of the subgroups studied, the lowest concentration of ropivacaine (0.2%) was noted to be the least toxic. They concluded that the greatest margin of safety for the anaesthetics studied was found in the lowest concentrations and as such higher concentrations should be used with caution.

Scutt et al [15] and Wong et al [17] independently studied the effects of dexamethasone exposure on tenocytes. Scutt et al [15] injected variable levels of dexamethasone around rat tail tendons and noted decreases in tenocyte proliferation and collagen synthesis as dexamethasone concentrations increased. Wong et al [17] studied the response of human hamstring tendons to two dexamethasone concentrations (10μM and 100μM) and noted decreases in cell viability of 35% and 45%, respectively. The conclusions of each of these studies were that dexamethasone could have potentially negative effects on tenocyte cell proliferation and viability.

Beitzel et al [24] conducted an *in vitro* study to evaluate the effects of methylprednisolone, ketorolac tromethamine, and platelet-rich plasma (PRP), alone and in combinations, on human chondrocytes and tenocytes isolated from the samples of biceps tendon. They noted significantly increased tenocyte cell viability with PRP treatment and combined PRP and ketorolac tromethamine treatment compared with controls. Cell viability was significantly decreased after exposure to methylprednisolone and methylprednisolone combined with PRP compared with controls, with the addition of PRP slightly mitigating the decrease in viability. They concluded that tenocyte exposure to methylprednisolone decreases cell viability, with the addition of PRP only partially reversing the negative effect [24].

Similar to the studies by Piper et al [13], Yang et al [22], and Sung et al [23], we noted significantly deleterious effects of 1% lidocaine. However, we noted that 0.5% lidocaine had a less deleterious effect on cell viability than 1% lidocaine. This is in agreement with data from Piper et al who noted that increasing concentrations of lidocaine had increasingly negative effects on cell viability. Similar to Sherb et al [16] and Sung et al [23], we also noted negative effects of a higher concentration (but common clinical dose) of bupivacaine (0.25%). In contrast to their studies, however, we also studied lower concentrations (0.125% and 0.0625%) of bupivacaine and found that the tenocyte exposure to these lower concentrations did not differ significantly from controls. This data is clinically relevant as it indicates that using lower concentrations of these injectates would be better tolerated long term. Our results are also in accordance with those of previous studies in regards to the influence of corticosteroids. We noted significantly negative effects of betamethasone and methylprednisolone, which was also noted by Piper et al, Farkas et al [11], and Beitzel et al [24]. In contrast to the study performed by Yang et al [22], however, we did not note significantly negative effects of triamcinolone on tenocyte viability or metabolism. This difference was partly because they studied the effects of a combination exposure of triamcinolone with 1% lidocaine, whereas we studied the effects of each agent individually.

Study limitations

This is an *in vitro* model using canine tendon explants and thus cannot be broadly extrapolated to all settings. Another limitation is that this study examined the effects of

individual anaesthetics and corticosteroids, not the combination of agents, which are most commonly used clinically. Our rationale for testing individual agents was to detect a baseline effect of each individual agent independently. This would further allow us to formulate potentially stronger hypotheses on the effects of various combinations of these agents in subsequent *in vivo* studies. By using canine tendon explants, we were able to examine a wide spectrum of agents in a valid translational model with sufficient numbers to allow for delineation of the individual effects of each independent agent. While a tendon explant model preserves tissue architecture, extracellular matrix, and cell phenotype, placing the tendon in culture alters its environment and removes biomechanical forces with potential resultant effects on cell viability, physiology, and metabolism. While this limits the direct application of the data to clinical implementation, the results are valid for comparison across treatments as all explants were treated in the same manner, and controls consistently showed maintenance of cell viability, and phenotype and tissue architecture throughout the explant. This experimental design allowed us to provide unique data while adhering to the ethical use of animals and the universal mandate to "reduce, refine, and replace" with respect to animal research. As such, these data provided important *ex vivo* data to justify and guide the design of subsequent *in vivo* research.

Conclusion

The results of this study contribute novel data to the growing body of evidence suggesting that peritendinous injection of commonly used local anaesthetics and corticosteroids results in significant supraspinatus tenotoxicity *in vitro*. Further *in vivo* studies are required before definitive clinical recommendations may be made.

Conflicts of interest

All contributing authors declare no conflicts of interest with this study and no external funding was used.

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