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# *In vitro* chondrotoxicity of bupivacaine at low concentrations in cultured canine articular chondrocytes

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**Abstract**

**Bupivacaine is a long-acting local anesthetic that is used in various anesthetic and/or analgesic protocols, including intra-articular injection for pain management during orthopedic procedures. The purpose of this study was to evaluate the *in vitro* chondrotoxicity of bupivacaine at concentrations similar to *in vivo* synovial fluid concentrations in canine articular chondrocytes. A controlled *in vitro* experimental study was used. Third passage (P3) chondrocytes in monolayer culture were treated with bupivacaine at 0.25%, 0.125%, 0.062%, 0.031% and 0.016% (2.5, 1.25, 0.62, 0.31 and 0.16 mg/ml, respectively). The control was treated with only growth medium. Chondrocyte viability was evaluated after 1, 6 and 24 hr using the live/dead assay; 3-(4,5-dimehylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay; and Cell Counting Kit-8 (CCK-8) assay. Bupivacaine at 0.125% and 0.25% significantly decreased chondrocyte viability compared to the control at all treatment time-points ($P < 0.001$). Bupivacaine at 0.062% was chondrotoxic at all treatment time-points with the MTT assay ($P < 0.001$) but only after 6 and 24 hr with the live/dead and CCK-8 assays ($P < 0.001$). After 24 hr, 0.031% bupivacaine caused cell clustering with the live/dead assay and significantly decreased cell viability with the MTT ($P < 0.001$) and CCK-8 ($P = 0.001$) assays. Bupivacaine at 0.016% did not significantly decrease cell viability at any of the treatment time-points. These findings indicate that bupivacaine has *in vitro* chondrotoxic effects on canine articular chondrocytes at concentrations that are similar to *in vivo* synovial fluid concentrations, especially with prolonged exposure.**

Key Words: Bupivacaine, Cartilage, Intra-articular, Local anesthetic, Synovial fluid

## Introduction

In veterinary practice, intra-articular administration of local anesthetics is used, especially in dogs and horses, for pain management during arthroscopic surgery, diagnosis of lameness, and for the control of pain associated with joint diseases such as osteoarthritis[4,11,28,31]. The type of local anesthetics used in a protocol, and the specific techniques vary depending on the procedure and availability of drugs. The inclusion of local anesthetics in

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multimodal anesthetic and/or analgesic protocols reduces the doses of systemic anesthetics and/or analgesics required for adequate pain management, and this in turn leads to a decrease in the risks and side effects associated with these drugs[13,20,23,24]. However, *in vitro* and *in vivo* studies have indicated that local anesthetics have chondrotoxic effects in both humans and animals, and may therefore not be safe for intra-articular use[1,6,10,15,17]. Their chondrotoxicity depends on the dose, period of exposure, and the type of drug used[18,19].

Bupivacaine is an amide-type local anesthetic with a slow onset and long duration of action[21]. It has been the most widely used local anesthetic for decades[7]. Local administration of bupivacaine is commonly used as part of a multimodal analgesic plan to minimize postoperative pain and it can be used for different anesthetic technics including intra-articular injection during orthopaedic procedures[3]. A recent survey among veterinarians found that bupivacaine was the most commonly used local anesthetic for peripheral nerve blocks during pelvic limb surgery[30]. Although only one respondent from this survey had used it for intra-articular administration, it is evident that despite the known cytotoxic effects, bupivacaine remains a preferred drug of choice for pain management in veterinary clinical practice. It is therefore necessary to study and understand its effects on cell viability and function in order to identify ways to attenuate or prevent its cytotoxic effects while maintaining the clinical benefits.

Previous *in vitro* studies investigating local anesthetic chondrotoxicity have generally used concentrations that are based on the commercially available drug preparations[2,10,25,29]. However, *in vivo* studies to evaluate synovial fluid concentrations of local anesthetics have shown that after intra-articular injection, the infused drug may be diluted depending on the volume of the synovial fluid present in the joint[3,26]. A study in human patients found that the lidocaine synovial fluid concentration was as low as 0.02% (0.23 mg/ml) 10 to 15 min after a single intra-articular injection with 2% (20 mg/ml) lidocaine[26]. Similarly in dogs, bupivacaine synovial fluid concentrations dropped to 0.36% (3.6 mg/ml) in normal stifles and 0.25% (2.5 mg/ml) in osteoarthritic stifles immediately after a single intra-articular injection of 0.5% (5 mg/ml) bupivacaine[3]. In the same study, the bupivacaine synovial fluid concentration dropped even further to 0.04% (0.4 mg/ml) in normal stifles and 0.06% (0.6 mg/ml) in osteoarthritic stifles 30 min after injection. These studies indicate that the concentration of the local anesthetic drug that intra-articular chondrocytes are exposed to may be lower than what is initially injected; and that the chondrotoxicity may not be as extensive as what has been found in previous *in vitro* studies. Therefore, the objective of this study was to evaluate the *in vitro* chondrotoxic effects of bupivacaine at concentrations that are similar to reported *in vivo* synovial fluid concentrations in canine articular chondrocytes following 1, 6 and 24 hr of treatment.

## Materials and Methods

### *Chondrocyte isolation and culture*

Articular cartilage samples were collected from three dogs that underwent limb amputation at Hokkaido University Veterinary Hospital for reasons unrelated to this study. The samples were obtained from the femoral condyles of a 9-year-old Golden retriever and 13-year-old mixed breed, and humeral condyle of a 12-year-old Shiba Inu. The collection and use of cartilage samples was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval No. 12-0059). After collection, the cartilage was mechanically dissected and incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 0.3% collagenase I (Wako Pure Chemicals Industries, Osaka, Japan) for 18 hr at 37°C and 5% $CO_2$. The chondrocytes were then released from the cartilage by filtration through a 40 µm nylon filter. Primary (P0) chondrocytes were seeded at $1.0 \times 10^4$ cells/$cm^2$ in 100 mm culture dishes (Corning, Lowell, MA, USA). DMEM containing 10 mM HEPES (Dojindo, Kumamoto, Japan), 25 mM $NaHCO_3$

(Wako, Japan), 100 U/ml penicillin G potassium (Wako, Japan) and 73 U/ml streptomycin sulphate (Wako, Japan) and supplemented with 10% fetal bovine serum (FBS: Nichirei Biosciences Inc., Tokyo, Japan) was used as growth medium. At 80-90% confluence, chondrocytes were washed thrice with phosphate buffered saline (PBS) and detached from the culture plate using a solution of 0.5% trypsin (Wako, Japan) and 0.02% ethylenediaminetetraacetic acid (EDTA; Dojindo, Japan) in PBS. Cell number and viability was determined by the trypan blue (Wako, Japan) exclusion assay before they were sub-cultured under the same conditions as described above.

***Chondrocyte treatment***

Third passage (P3) chondrocytes were used for the experimental analyses. For all experiments, chondrocytes were seeded at $2.5 \times 10^4$ cells/cm$^2$ and cultured for 24 hr in DMEM supplemented with 10% fetal bovine serum (10-DMEM) in an incubator at 37°C and 5% $CO_2$, to allow for cell attachment before treatment. The culture medium was removed, and chondrocytes were treated with bupivacaine (Marcain injection 0.5%; Aspen, Tokyo, Japan) which was diluted to concentrations of 0.25%, 0.125%, 0.062%, 0.031% and 0.016% (2.5, 1.25, 0.62, 0.31 and 0.16 mg/ml, respectively) in 10-DMEM. The culture medium (10-DMEM) without bupivacaine was used as the control. Following treatment, chondrocyte viability was evaluated after 1, 6 and 24 hr using the live/dead, 3-(4,5-dimehylthiazolyl-2)2,5- diphenyltetrazolium bromide (MTT), and Cell Counting Kit-8 (CCK-8) assays.

Three different viability tests were used to validate whether the pattern and level of chondrotoxicity would be the same for each method of assessment. The live/dead assay analyses cell membrane integrity and permeability by differential staining with either calcein for live cells or ethidium homodimer (EthD-1) for dead cells. Calcein is well retained within live cells, producing a uniform green fluorescence in live cells whereas EthD-1 enters cells with damaged membranes and produces bright red fluorescence in dead cells. In addition to differentiating between live and dead cells, the live/dead assay can also be used to observe changes in cell morphology and attachment that may occur following treatment. The MTT and CCK-8 assays both measure cellular dehydrogenase activity; however, MTT assay only measures mitochondrial activity whereas CCK-8 involves most of the dehydrogenase in a cell and therefore measures the general cellular metabolic activity.

***Live/dead Assay***

Chondrocytes at a density of $2.0 \times 10^4$ cells per well were plated in 8-well chamber slides (Iwaki, Tokyo, Japan) for 24 hr then treated with 250 µl local anesthetics in 10-DMEM as described above. The live/dead reagent (ThermoFisher Scientific, USA) was prepared by diluting calcein AM and ethidium homodimer (EthD-1) in PBS; resulting in approximately 2 µM calcein AM and 4 µM EthD-1 working solution. After 1, 6 and 24 hr of treatment, 150 µl of this solution was added directly into the wells containing 250 µl 10-DMEM and incubated for 30 min at 37°C in a 5% $CO_2$ incubator. After incubation, the solution was carefully removed from the culture wells and replaced with PBS. Fluorescence imaging was performed using a FLoid Cell Imaging Station (ThermoFisher Scientific, USA) and images were obtained under green (FITC filter) and red (Texas red filter) light. Where necessary, photo contrast and brightness were adjusted using ImageJ software (National Institute of Health, Bethesda, MD, USA).

***MTT Assay***

Chondrocytes were cultured in 96-well plates (Corning, USA) at a density of $8.0 \times 10^3$ cells per well for 24 hr and then treated with 200 µl local anesthetics in 10-DMEM as described above. The treatments were performed in triplicate for each cell source. After 1, 6 and 24 hr of treatment, the culture medium was removed and after washing the cells twice with PBS, 150 µl MTT solution diluted 1:5 in serum-free DMEM was added into each well. The culture plates were then incubated for 3 hr at 37°C and 5% $CO_2$. The MTT solution was then removed from the wells

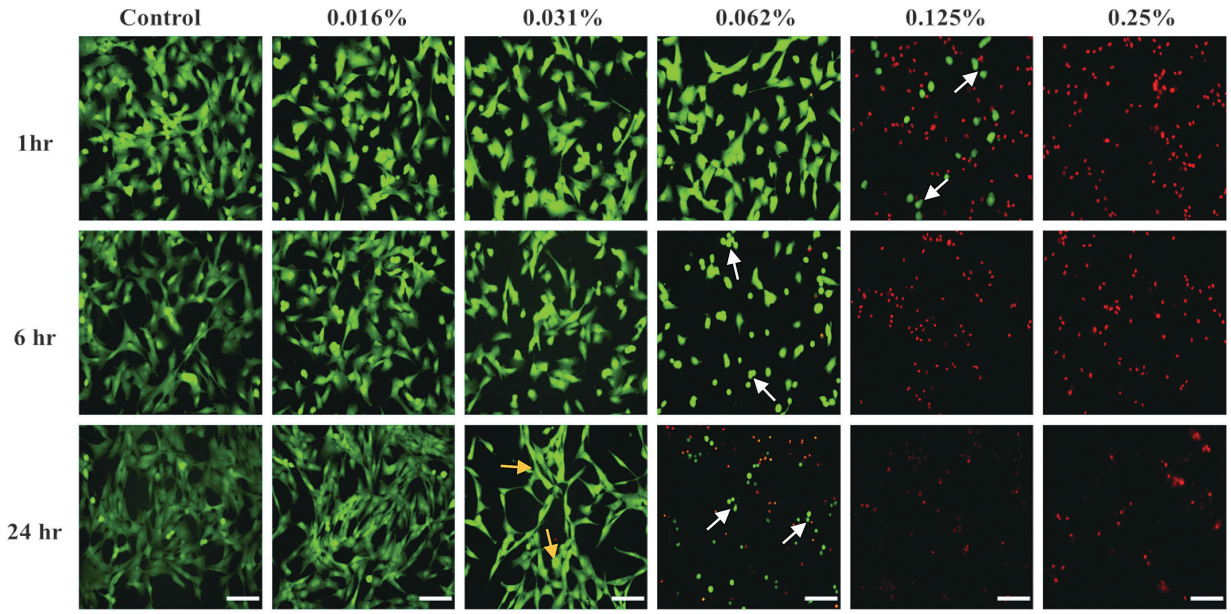


**Fig. 1.** Live cells (green) and dead cells (red) after 1, 6 and 24 hr of treatment with 0.25%, 0.125%, 0.062%, 0.031% and 0.016% bupivacaine. Bupivacaine caused a concentration- and time-dependent reduction in canine chondrocyte viability. The chondrotoxic effects were also visible when live cells became detached from the culture plate (white arrows) or through cell clustering as observed after 24 hr at 0.031% (yellow arrows). Scale bar: 100 µm.

and MTT formazan crystals were dissolved with 100 µl dimethyl sulfoxide (DMSO; Wako, Japan). The absorbance was measured with a microplate reader (Thermo Scientific, Vantaa, Finland) at 570 nm.

### *CCK-8 Assay*

Similar to the MTT assay, $8.0 \times 10^3$ chondrocytes were cultured in 96-well plates (Corning, USA) for 24 hr and the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was used to evaluate cell viability. The treatments were performed in triplicate for each cell source. After treatment with 200 µl local anesthetics in 10-DMEM, the cells were washed twice with PBS then 10 µl of the CCK-8 solution was added to 100 µl 10-DMEM in each well and incubated for 4 hr at 37°C and 5% $CO_2$. Absorbance was then measured with a microplate reader (Thermo Scientific, Vantaa, Finland) at 450 nm.

### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism software version 9.4.1 (GraphPad Software Inc., USA). Analysis of variance (ANOVA) and Dunnett's multiple comparison test were used to compare between the control and treatment groups. $P$-value < 0.05 was considered significant. All results unless specified are presented as mean ± standard deviation (SD).

## Results

### *Bupivacaine at low concentrations compromises cell membrane integrity and morphology in a concentration- and time-dependent manner - Live/dead Assay*

Bupivacaine concentrations of 0.25% and 0.125% were chondrotoxic after 1 hr of treatment (Fig. 1). At 0.125% most of the cells had taken up the EthD-1 stain (red) with the remaining live cells (green) being completely detached from the culture plate (white arrow), whereas at 0.25%, all the cells had taken up the EthD-1 stain. The bupivacaine treatments at 0.016%, 0.031% and 0.062% were similar to the control after 1 hr. After 6 hr, all cells at 0.25% and 0.125% bupivacaine

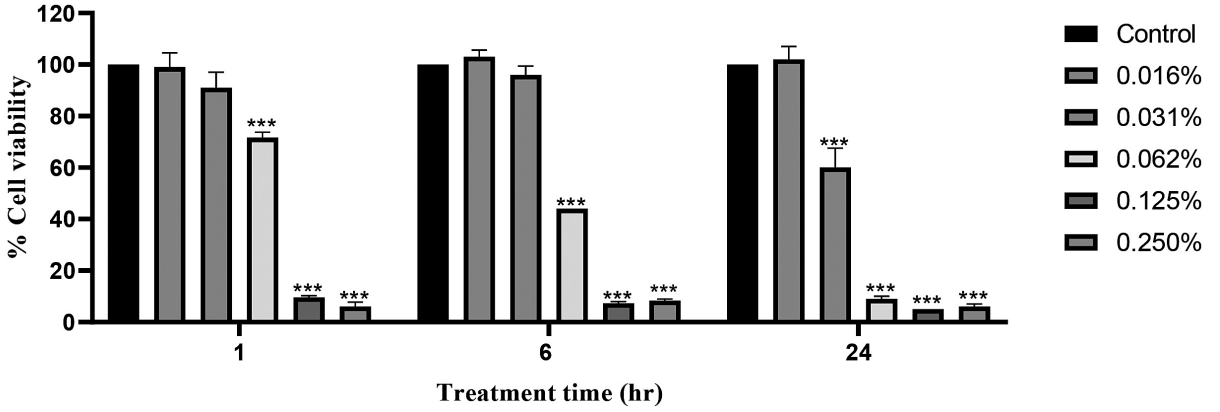


**Fig. 2.** MTT assay results showing the effects of bupivacaine treatment on chondrocyte viability. After 1 hr there was a concentration-dependent effect on cell viability with 0.062%, 0.125% and 0.25% causing a significant reduction in viability (*** $P < 0.001$). Interestingly, after 6 hr and 24 hr, 0.016% bupivacaine caused an increase in cell viability compared to the 1 hr treatment. Bupivacaine at 0.031% also caused a transient increase in viability after 6 hr but after 24 hr, the viability at 0.031% had significantly decreased in addition to the 0.062%, 0.125% and 0.25% treatments (*** $P < 0.001$).

had taken up EthD-1; and at 0.062%, most of the cells had visibly detached from the culture plate (white arrow). Similar to the control, 0.016% and 0.031% did not show any changes after 6 hr. After 24 hr, 0.25% and 0.125% did not only show all the cells taking up the EthD-1 stain, but also a reduction in the number of cells. At 0.062% the number of dead cells increased compared to the 6-hr treatment and all the cells were completely detached. The live cells that did not take up the EthD-1 stain at 0.062% were also visibly smaller in size. At 0.031% the cells did not take up EthD-1 or detach from the plate, however they had a morphological change in their growth pattern after 24 hr with the chondrocytes visibly forming clusters (yellow arrow). For all the bupivacaine treatments except 0.016%, there was a visible progression in these morphological changes and reduction in cell number as the time progressed from 1 to 24 hr of treatment indicating a concentration- and time-dependent effect.

### *Bupivacaine at low concentrations is cytotoxic to canine articular chondrocytes - MTT Assay*

Bupivacaine decreased cell viability in a concentration- and time-dependent manner (Fig. 2) compared to the control (100 % viability). Treatment with 0.25% and 0.125% bupivacaine significantly reduced cell viability (less than 10%) at all treatment time-points ($P < 0.001$ for both). Bupivacaine at 0.062% caused a gradual reduction in cell viability with time, with the chondrocyte viability decreasing from 71.7 ± 2.08% ($P < 0.001$) at 1 hr; to 44% ($P < 0.001$) after 6 hr; and 9 ± 1% ($P < 0.001$) after 24 hr of treatment. With the remaining treatments (0.016% and 0.031% bupivacaine), there was a mild effect on viability after 1 hr of treatment. Viability was 99.0 ± 5.57% for 0.016% bupivacaine ($P = 0.998$) and 91.0 ± 6.08% ($P = 0.504$) for 0.031% bupivacaine. Interestingly, bupivacaine at 0.016% caused a mild increase in cell viability from 99.0 ± 5.57% ($P = 0.998$) after 1 hr, to 103 ± 2.65% ($P = 0.870$) after 6 hr of treatment and 102 ± 5% after 24 hr of treatment. Bupivacaine at 0.031% also caused a mild increase from 91.0 ± 6.08% ($P = 0.504$) at 1 hr to 96.0 ± 3.46% ($P = 0.759$) at 6 hr after treatment. This increase in cell viability was however transient and had receded after 24 hr to 60 ± 7.55% ($P < 0.001$) for 0.031% bupivacaine.

### *Bupivacaine at low concentrations reduces cell viability as validated by the CCK-8 Assay*

The CCK-8 assay showed a similar pattern with the MTT assay except a few variations (Fig.

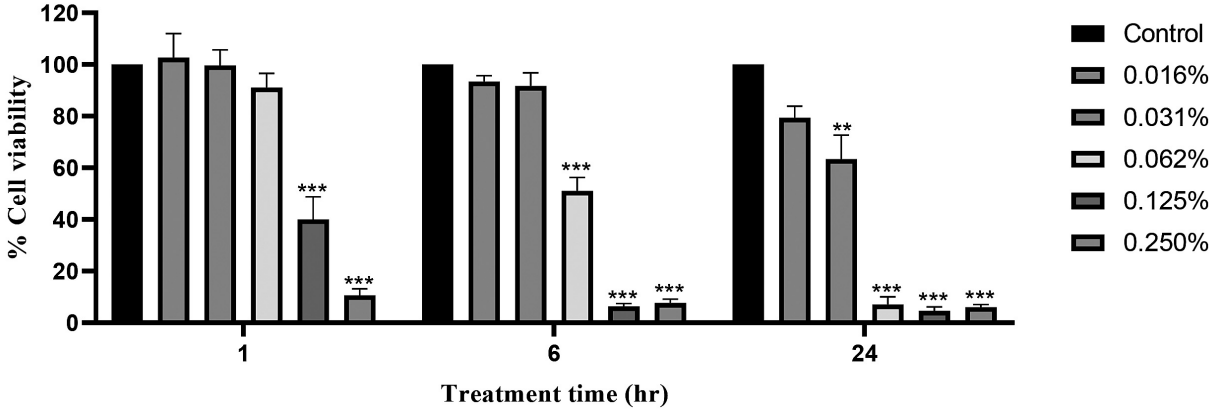


**Fig. 3.** CCK-8 assay results showing the effects of bupivacaine treatment on chondrocyte viability. After 1 hr, bupivacaine at 0.125% and 0.25% caused a significant reduction in viability (*** $P < 0.001$). After 6 hr, 0.062% in addition to 0.125% and 0.25%, caused a significant reduction in viability (*** $P < 0.001$). After 24 hr, all the treatment groups showed a reduction in cell viability with 0.031% being the minimum concentration causing a significant reduction in viability (** $P = 0.001$).

3). All the bupivacaine treatments progressively decreased cell viability as the time of exposure increased. After 1 hr, only 0.125% and 0.25% bupivacaine significantly reduced cell viability to 40 ± 8.72% ($P < 0.001$) and 10.67 ± 2.52% ($P < 0.001$), respectively. The viability decreased even further to 6.33 ± 1.12% ($P < 0.001$) for 0.125% bupivacaine and 7.7 ± 1.53% ($P < 0.001$) for 0.25% bupivacaine after 6 hr; and to 4.67 ± 1.53% ($P < 0.001$) for 0.125% bupivacaine and 6 ± 1% ($P < 0.001$) for 0.25% bupivacaine after 24 hr. Bupivacaine at 0.062% also progressively decreased cell viability to 91 ± 5.57% ($P = 0.63$) after 1 hr, 51 ± 5.29% ($P < 0.001$) after 6 hr, and 7 ± 3% ($P < 0.001$) after 24 hr. Bupivacaine at 0.031% decreased chondrocyte viability to 99.7 ± 6.03% ($P > 0.999$) after 1 hr, 91.7 ± 5.13% ($P > 0.999$) after 6 hr and to 63.3 ± 9.29% ($P = 0.001$) after 24 hr of treatment. At 0.016% bupivacaine, chondrocyte viability gradually decreased from 102.7 ± 9.29% ($P = 0.998$) after 1 hr; to 93.3 ± 2.31 ($P > 0.999$) after 6 hr; and 79.3 ± 4.51 ($P = 0.103$) after 24 hr, although it did not significantly decrease at any of the treatment time-points. Unlike the MTT assay, there was no transient increase in cell viability observed at any of the concentrations or treatment time points with the CCK-8 assay.

## Discussion

Understanding the effects of local anesthetics on articular chondrocyte viability is important in helping to identify ways in which they can be safely included in multimodal anesthetic and analgesic protocols, while avoiding their adverse effects. In the present study, the *in vitro* effects of bupivacaine at low concentrations on canine articular chondrocyte viability after 1, 6 and 24 hr of treatment were evaluated. Bupivacaine affected chondrocyte viability in a concentration- and time-dependent manner, as the level of chondrotoxicity increased with an increase in the concentration of bupivacaine and time of exposure. These findings were similar to previous studies evaluating the *in vitro* chondrotoxic effects of bupivacaine at higher concentrations[5,9,16]; and therefore show that bupivacaine can have *in vitro* chondrotoxic effects at amounts similar to *in vivo* synovial fluid concentrations in canine articular chondrocytes.

Bupivacaine is commercially available in concentrations of 0.25%, 0.5%, and 0.75% (2.5, 5.0 and 7.5 mg/ml, respectively)[3]. In the present study, five bupivacaine concentrations were used, with the highest being a commercially available concentration (0.25%). Bupivacaine was expected to have *in vitro* chondrotoxic effects at this concentration based on previous studies[1,9,27], so

that it could provide a basis for comparison with the other lower concentrations used. Our study found that bupivacaine at 0.25% and 0.125% significantly decreased cell viability ($P < 0.001$) at all treatment time points compared to the control. The *in vitro* chondrotoxicity of bupivacaine at these concentrations has had mixed results with some studies demonstrating that it does not cause significant reduction in chondrocyte viability at 0.125%[9] or at 0.25%[5,12]. However, the findings of the present study were similar to a recent study in which bupivacaine demonstrated chondrotoxic effects in equine chondrocytes at 0.25% and 0.125% after a 30-min and 60-min period of exposure[1]. Another study performed on full-thickness cartilage and synovial explants obtained from canine cadavers also found that 0.25% and 0.125% bupivacaine were chondrotoxic after 24 hr and 7 days after treatment[29].

In the present study, bupivacaine at 0.062% (0.62 mg/ml), 0.031% (0.32 mg/ml) and 0.016% (0.16 mg/ml) were estimated to be within ranges similar to *in vivo* synovial fluid concentrations after intra-articular injection in dogs. This estimation was based on results from the study by Barry *et al.*[3] in which the *in vivo* synovial fluid concentrations of bupivacaine in canine stifles dropped to 0.04% (0.4 mg/ml) in normal stifles and 0.06% (0.6 mg/ml in osteoarthritic stifles 30 min after a single intra-articular injection of 0.5% (5 mg/ml) bupivacaine[3]. A previous study by Rengert *et al.*[26] evaluated the effect of different bupivacaine preparations on canine articular chondrocyte viability using concentrations similar to the *in vivo* synovial fluid concentrations reported by Barry *et al.*[3]. The study demonstrated that low concentrations (of less than 0.25%) of both standard preparation and liposomal bupivacaine did not have chondrotoxic effects after a short-term exposure of 1 hr, indicating that a single intra-articular injection of bupivacaine may not result in chondrotoxic effects[27]. In the current study, three time points were used to evaluate whether bupivacaine would be chondrotoxic after short-term, intermediate, and prolonged exposure, i.e., 1, 6 and 24 hr, respectively. The period of short-term exposure (1 hr) was selected based on previous studies[1,27]; 6 hr was selected as the intermediate period of exposure based on the average duration of action of bupivacaine[4]; and 24 hr was included arbitrarily to evaluate the effect of continuous prolonged exposure to low bupivacaine concentrations on chondrocyte viability. As the study by Barry *et al.*[3] only evaluated the intra-articular bupivacaine concentrations for a maximum period of 30 minutes after a single injection, the *in vivo* concentrations may not remain the same 1, 6 and 24 hr after injection unless used in a protocol with repeated or continuous intra-articular infusion of bupivacaine. The concentrations and time points used in the current study were therefore estimates based on this study.

Bupivacaine at 0.062% did not show chondrotoxic effects with the live/dead assay and it did not significantly reduce cell viability with the CCK-8 assay ($P = 0.630$). However, there was a significant reduction to 71.7 ± 2.08% ($P < 0.001$) with the MTT assay. This was likely because of differences in the sensitivity of the viability tests used. A study by Adler *et al.*[1] on the chondrotoxicity of local anesthetics in equine articular chondrocytes had similar findings with 0.062% bupivacaine reducing cell viability with the MTT assay but not with the Lactate dehydrogenase (LDH) assay after 1 hr of treatment. It has been shown that local anesthetics induce chondrotoxicity by causing mitochondrial dysfunction[16]; therefore, because the MTT assay measures mitochondrial dehydrogenase, it may be more sensitive to changes in mitochondrial activity compared to the CCK-8 and live/dead assays. Previous studies on the *in vitro* chondrotoxicity of bupivacaine in canine articular chondrocytes found that 0.062% bupivacaine did not affect cell viability[27,29]. These mixed results may be due to differences in the experimental designs and the samples used. In the study by Sherman *et al.*[29], full thickness canine cartilage explants were used instead of isolated chondrocytes. On the other hand, Rengert *et al.*[27] compared the bupivacaine treatments with normal saline as the control; however, when the chondrocyte viability was compared with that of cells treated with growth

medium only, bupivacaine at 0.062% caused a significant reduction in viability after 1 hr similar to the findings in this study.

After 6 hr, 0.062% bupivacaine reduced cell viability for all three viability tests. Although the cells did not take up the EthD-1 stain, cell detachment was observed at this concentration with the live/dead assay. Interestingly, with the MTT assay, there was a mild increase in cell viability at 0.016% compared to the control from 99.0 ± 5.57% ($P$ = 0.998) after 1 hr, to 103 ± 2.65% ($P$ = 0.870) after 6 hr of treatment and 102 ± 5% after 24 hr of treatment. Bupivacaine at 0.031% also caused a transient increase from 91.0 ± 6.08% ($P$ = 0.504) at 1 hr to 96.0 ± 3.46% ($P$ = 0.759) at 6 hr after treatment. This may have been due to temporal changes in mitochondrial activity caused by bupivacaine treatment. Under conditions of environmental stress, chondrocytes tend to adapt their metabolism to microenvironmental changes by shifting from one metabolic pathway to another, which can cause metabolic alterations that involve mitochondrial dysfunction[32]. Additionally, chondrocytes producing survival signals following cellular injury can increase intercellular communication in order to prevent cell death[14,22]. These changes can lead to a temporal increase in enzymatic activity which is then detected as an increase in optical density with the MTT assay as was the case in the present study. With the MTT and CCK-8 assays, 0.031% was the minimum concentration showing a significant reduction in cell viability compared to the control ($P$ < 0.001). The CCK-8 assay showed reduction in viability for all bupivacaine concentrations after 24 hr, but 0.016% was not statistically significant ($P$ = 0.103). With the live/dead assay, 0.031% did not take up the EthD-1 stain, but cell clustering was observed after 24 hr. In osteoarthritis, clusters of proliferating cells contain cells in apoptosis[22]. Therefore, the chondrocyte clusters observed in our study may be an indication of early apoptosis caused by injury from treatment with bupivacaine. However, the mechanisms of cell death involved in this case were not investigated further because it was beyond the scope of this study.

The findings of this study show that bupivacaine has *in vitro* chondrotoxic effects at low concentrations that are similar to the concentrations found in synovial fluid after intra-articular injection[3]. However, these chondrotoxic effects are more likely to occur with prolonged and continuous exposure. While bupivacaine at 0.25% and 0.125% caused extensive chondrotoxicity at all time points in the present study, a time-dependent effect was observed at lower bupivacaine concentrations, especially at 0.062% and 0.031%. This was clearly demonstrated with the live/dead assay in which the cells treated with 0.062% bupivacaine were still attached to the surface of the culture plate and had fibroblast-like morphology after 1 hr of treatment (Fig. 1). After 6 hr, the cells appeared spherical and detached, and after 24 hr they had reduced in size and had taken up the EthD-1 stain. These changes in morphology with each time point were an indication of a progressive increase in the chondrotoxic effects of bupivacaine. Bupivacaine at 0.031% similarly only had an effect on chondrocyte morphology after 24 hr of treatment by causing cluster formation, although the cells did not take up the EthD-1 stain. It is also important to note that while the chondrotoxic effects may not be obvious immediately following treatment, local anesthetics can have delayed detrimental effects on cartilage and/or chondrocyte metabolism both *in vitro* and *in vivo*. A study by Grishko *et al.*[16] found a significant decrease in chondrocyte viability and an increase in the number of apoptotic cells 5 days after treatment with lidocaine, bupivacaine, and ropivacaine. Similarly, an *in vivo* study in Sprague-Dawley rats reported a reduction in chondrocyte density six months after a single intra-articular injection of 0.5% bupivacaine[8]. It is therefore necessary to conduct more experimental and clinical studies that can elucidate the mechanisms that are involved in local anesthetic-induced chondrotoxicity.

This study had a number of limitations. Our *in vitro* experimental study does not equate the *in vivo* physiological conditions of a joint environment. The monolayer culture was used as a model and only showed effects on chondrocytes, but it did not account for other

components of a diarthrodial joint such as the extracellular matrix, synovial membrane, and synovial fluid that may affect the dynamics of the joint. In addition, only the immediate effects of bupivacaine on chondrocyte viability were assessed. The biomolecular mechanisms involved or the possibility of delayed effects leading to cell death were not evaluated. Furthermore, The concentrations of bupivacaine used in this study only account for the synovial fluid concentrations determined after a single injection with 0.5% bupivacaine as reported by Barry *et al.*[3]. Since bupivacaine is commercially available in different concentrations, the amount of drug present within the joint may be different from what has previously been reported, depending on the bupivacaine preparation used or the volume of drug injected and whether or not it is repeatedly or continuously infused.

In conclusion, this study demonstrates that bupivacaine at low concentrations reduces cell viability in canine articular chondrocytes *in vitro* in a concentration- and time-dependent manner. Bupivacaine at low concentrations that are similar to *in vivo* synovial fluid concentrations can negatively affect chondrocyte viability especially with prolonged exposure. Further studies are required to evaluate whether these concentrations can be chondrotoxic *in vivo* with prolonged exposure or if they may have delayed deleterious effects on chondrocyte metabolism following intra-articular injection.

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## References

1) Adler DMT, Frellesen JF, Karlsen CV, Jensen LD, Dahm ASQ, Berg LC. Evaluation of the in vitro effects of local anesthetics on equine chondrocytes and fibroblast-like synoviocytes. Am J Vet Res 82, 478–486, 2021.
2) Baker JF, Walsh PM, Byrne DP, Mulhall KJ. In vitro assessment of human chondrocyte viability after treatment with local anaesthetic, magnesium sulphate or normal saline. Knee Surg Sports Traumatol Arthrosc 19, 1043–1046, 2011.
3) Barry SL, Martinez SA, Davies NM, Remsberg CM, Sayre CL, Bachelez A. Synovial fluid bupivacaine concentrations following single intra-articular injection in normal and osteoarthritic canine stifles. J Vet Pharmacol Ther 38, 97–100, 2015.
4) Borer K. Local analgesic techniques in small animals. In Pract 28, 200–207, 2006.
5) Breu A, Rosenmeier K, Kujat R, Angele P, Zink W. The cytotoxicity of bupivacaine, ropivacaine, and mepivacaine on human chondrocytes and cartilage. Anesth Analg 117, 514–522, 2013.
6) Busfield BT, Romero DM. Pain pump use after shoulder arthroscopy as a cause of glenohumeral chondrolysis. Arthroscopy 25, 647–652, 2009.
7) Casati A, Putzu M. Bupivacaine, levobupivacaine and ropivacaine: Are they clinically different? Best Pract Res Clin Anaesthesiol 19, 247–268, 2005.
8) Chu CR, Coyle CH, Chu CT, Szczodry M, Seshadri V, Karpie JC, Cieslak KM, Pringle EK. In vivo effects of single intra-articular injection of 0.5% bupivacaine on articular cartilage. J Bone Joint Surg Am 92, 599–608, 2010.
9) Chu CR, Izzo NJ, Coyle CH, Papas NE, Logar A. The in vitro effects of bupivacaine on articular chondrocytes. J Bone Joint Surg Br 90, 814–820, 2008.
10) Chu CR, Izzo NJ, Papas NE, Fu FH. In vitro exposure to 0.5% bupivacaine is cytotoxic to bovine articular chondrocytes. Arthroscopy 22,

693–699, 2006.
11) Di Salvo A, Chiaradia E, Nannarone S, Della Rocca G. Intra-articular use of analgesic/antinflammatory drugs in dogs and horses. Res Vet Sci 134, 159–170, 2021.
12) Dragoo JL, Braun HJ, Kim HJ, Phan HD, Golish SR. The in vitro chondrotoxicity of single-dose local anesthetics. Am J Sports Med 40, 794–799, 2012.
13) Dutton TAG, Gurney MA, Bright SR. Intra-articular mepivacaine reduces interventional analgesia requirements during arthroscopic surgery in dogs. J Small Anim Pract 55, 405–408, 2014.
14) Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci 22, 12827, 2021.
15) Gomoll AH, Kang RW, Williams JM, Bach BR, Cole BJ. Chondrolysis after continuous intra-articular bupivacaine infusion: an experimental model investigating chondrotoxicity in the rabbit shoulder. Arthroscopy 22, 813–819, 2006.
16) Grishko V, Xu M, Wilson G, Pearsall AW IV. Apoptosis and mitochondrial dysfunction in human chondrocytes following exposure to lidocaine, bupivacaine, and ropivacaine. J Bone Joint Surg Am 92, 609–618, 2010.
17) Kaewpichit P, Charuluxananan S, Srisa-Art M, Sisawat S, Aksornkitti V, Chayudsatid C, Sereemaspun A. Cytotoxic responses of human chondrocytes to bupivacaine, levobupivacaine, and ropivacaine. Asian Biomed (Res Rev News) 12, 169–178, 2018.
18) Karpie JC, Chu CR. Lidocaine exhibits dose- and time-dependent cytotoxic effects on bovine articular chondrocytes in vitro. Am J Sports Med 35, 1622–1627, 2007.
19) Kreuz PC, Steinwachs M, Angele P. Single-dose local anesthetics exhibit a type-, dose-, and time-dependent chondrotoxic effect on chondrocytes and cartilage: a systematic review of the current literature. Knee Surg Sports Traumatol Arthrosc 26, 819–830, 2018.
20) Kushnir Y, Toledano N, Cohen L, Bdolah-Abram T, Shilo-Benjamini Y. Intratesticular and incisional line infiltration with ropivacaine for castration in medetomidine–butorphanol–midazolam sedated dogs. Vet Anaesth Analg 44, 346–355, 2017.
21) Lemke KA, Dawson SD. Local and regional anesthesia. Vet Clin North Am Small Anim Pract 30, 839–857, 2000.
22) Lotz M, Hashimoto S, Kühn K. Mechanisms of chondrocyte apoptosis. Osteoarthritis Cartilage 7, 389–391, 1999.
23) Martin-Flores M, Anderson JC, Sakai DM, Campoy L, Cheong SH, Romano M, Gleed RD. A retrospective analysis of the epidural use of bupivacaine 0.0625-0.125% with opioids in bitches undergoing cesarean section. Can Vet J 60, 1349–1352, 2019.
24) McFadzean WJM, Macfarlane P, Granger N, Murrell JC. Influence of peri-incisional epaxial muscle infiltration with bupivacaine pre- or post-surgery on opioid administration in dogs undergoing thoraco-lumbar hemilaminectomy. Vet J 270, 105622, 2021.
25) Park J, Sutradhar BC, Hong G, Choi SH, Kim G. Comparison of the cytotoxic effects of bupivacaine, lidocaine, and mepivacaine in equine articular chondrocytes. Vet Anaesth Analg 38, 127–133, 2011.
26) Ravnihar K, Marš T, Pirkmajer S, Alibegović A, Koželj G, Stožer A, Drobnič M. The influence of a single intra-articular lidocaine injection on the viability of articular cartilage in the knee. Cartilage 13 (1-suppl), 456S–463S, 2021.
27) Rengert R, Snider D, Gilbert PJ. Effect of bupivacaine concentration and formulation on canine chondrocyte viability in vitro. Vet Surg 50, 633–640, 2021.
28) Schumacher J, Boone L. Local anaesthetics for regional and intra-articular analgesia in the horse. Equine Vet Educ 33, 159–168, 2021.
29) Sherman SL, Khazai RS, James CH, Stoker AM, Flood DL, Cook JL. In vitro toxicity of local anesthetics and corticosteroids on chondrocyte and synoviocyte viability and metabolism. Cartilage 6, 233–240, 2015.
30) Thomson R, Trimble T, Pawson P, Bell AM. A survey-based study into the use of peripheral

nerve blocks for pelvic limb surgery among veterinary professionals with an interest in anesthesia. Top Companion Anim Med 42, 100507, 2021.
31) Van Vynckt D, Samoy Y, Mosselmans L, Verhoeven G, Verschooten F, Van Ryssen B. The use of intra-articular anesthesia as a diagnostic tool in canine lameness. Vlaams Diergeneeskundig Tijdschrift 81, 290–297, 2012.
32) Zheng L, Zhang Z, Sheng P, Mobasheri A. The role of metabolism in chondrocyte dysfunction and the progression of osteoarthritis. Ageing Res Rev 66, 101249, 2021.