Chondrotoxicity of local anesthetics:

In vitro effects of local anesthetics on cell viability and apoptosis in cultured canine articular chondrocytes

（局所麻酔薬の軟骨毒性に関する研究：培養軟骨細胞における局所麻酔薬の細胞障害性とアポトーシスについて）

Carol MWALE

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A Dissertation for the Degree of Doctor of Philosophy (PhD)

Carol MWALE

Laboratory of Veterinary Surgery

Department of Clinical Sciences

Graduate School of Veterinary Medicine

Hokkaido University, Sapporo, Japan

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Abbreviations

%	Percent
µl	Microliter
µm	Micrometer
µM	Micromolar
3-D	Three-dimensional
ANOVA	Analysis of variance
Bup	Bupivacaine
CO_2	Carbon dioxide
CCK-8	Cell counting Kit-8
cm	Centimeter
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	Hour(s)
IV	Intravenous
Lev	Levobupivacaine
min	Minute(s)
ml	Milliliter
mm	Millimeter

MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimehylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Na+	Sodium ions
$NaHCO_3$	Sodium bicarbonate
No.	Number
OA	Osteoarthritis
PBS	Phosphate buffered saline
Rop	Ropivacaine
SD	Standard deviation

List of publications related to the dissertation

The contents of Chapter 1 have been accepted for publication in *The Japanese Journal of Veterinary Research (JJVR)*.

Mwale C, Sunaga T, Wang Y, Bwalya EC, Wijekoon S, Kim S, Okumura M. *In vitro* chondrotoxicity of bupivacaine at low concentrations in cultured canine articular chondrocytes. *Jpn J Vet Res*, in press.

The contents of Chapter 2 have been accepted for publication in *The Journal of Veterinary Medical Science (JVMS)*.

Mwale C, Sunaga T, Wang Y, Bwalya EC, Wijekoon S, Kim S, Okumura M. *In vitro* effects of bupivacaine, levobupivacaine and ropivacaine on cell viability and caspase activity in cultured canine articular chondrocytes. *J Vet Med Sci*, in press.

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1. General introduction

Local anesthetics are a group of drugs that are used to induce analgesia and loss of sensation in restricted areas of the body (Borer, 2006; Grubb & Lobprise, 2020b, 2020a). Their mode of action is through the reversible blockade of voltage-gated sodium channels (**Fig. 1**) in excitable tissues; they bind to open channels and convert these into an inactivated or closed state which inhibits the influx of sodium ions (Na+) into the cell, thereby interrupting neural conduction (Barletta & Reed, 2019; Becker & Reed, 2006; Cox et al., 2003; Taylor & McLeod, 2020). As a result, local anesthetics prevent the propagation of the pain stimulus, making them the only class of drugs that can fully block nociceptive impulses (Barletta & Reed, 2019; Grubb & Lobprise, 2020b), and they are the only means of completely eliminating pain without inducing unconsciousness (Barletta & Reed, 2019).

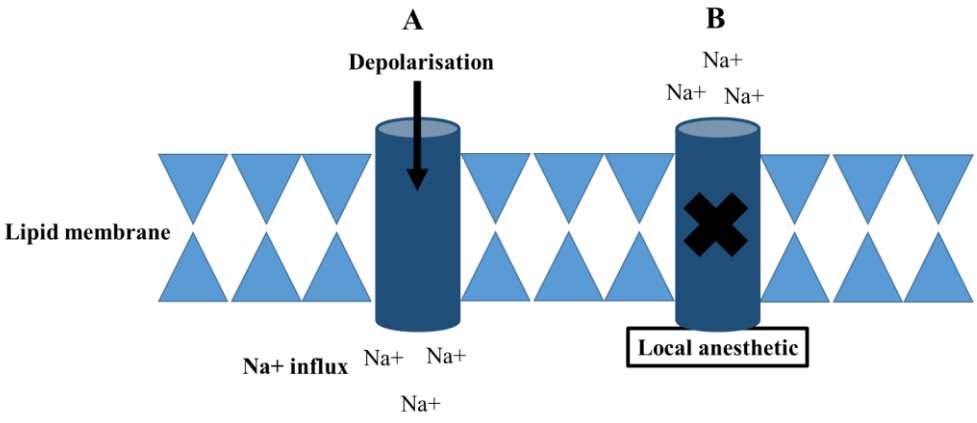

Fig. 1: **Schematic representation of the mode of action of local anesthetics**

A, **B:** Voltage-gated sodium channels along a neuronal lipid membrane. **(A)** shows an open sodium channel with intracellular influx of sodium ions (black arrow) during depolarization, and (**B)** shows a sodium channel blocked following intracellular binding of a local anesthetic which prevents the influx of sodium ions and conduction of nerve impulses.

Local anesthetics are weak bases which are divided into amides and esters based on their structure (Barletta & Reed, 2019). The general structure of all local anesthetic agents consists of three components (**Fig. 2**) including a lipophilic aromatic ring, an intermediate ester or amide chain, and a terminal amine (Barletta & Reed, 2019; Becker & Reed, 2006; Taylor & McLeod, 2020). The structure of the agent affects its clinical properties such as the speed of onset of action, duration of action and the potency of the drug. These properties are an important basis for the selection of the type of agent or drug that will be included in a particular protocol during anesthesia.

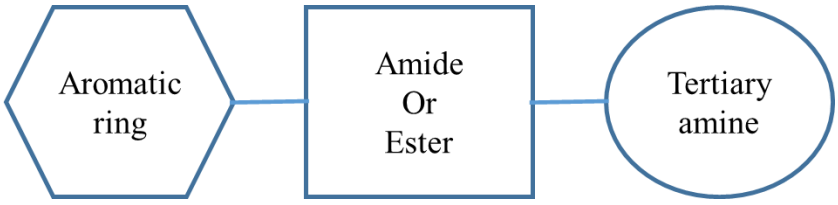

Fig. 2: **Schematic representation of the basic molecular structure of local anesthetic agents.**

Local anesthetics consist of a lipophilic aromatic ring, an intermediate ester or amide chain, and a terminal amine.

Bupivacaine is an amide-type local anesthetic that is highly lipid soluble and also has high protein-binding ability (Barletta & Reed, 2019; Becker & Reed, 2006, 2012). It is a clinically potent, long-acting drug with a slow onset of action. A solution of bupivacaine contains equal amounts of dextrorotatory (R+) and levorotatory (S-) stereoisomers, making it a racemic solution (Athar et al., 2016; Leone et al., 2008). Because of its potency and long duration of action, bupivacaine is commonly used in multiple anesthetic and analgesic protocols (Campagnol et al., 2012; Grubb & Lobprise, 2020a; Martin-Flores et al., 2019; McFadzean et al., 2021; Thomson et al., 2021). Despite its efficacy, the administration of bupivacaine carries

the potential hazard of inadvertent intravascular injection, which can cause life-threatening central nervous system (CNS) and cardiovascular toxicity (Cox et al., 2003). As a result, levobupivacaine and ropivacaine, which are two relatively new long-acting local anesthetics were developed after reports of bupivacaine cardiotoxicity and neurotoxicity (Athar et al., 2016; Kuthiala & Chaudhary, 2011; McLeod & Burke, 2001). Levobupivacaine is the pure S(-)-enantiomer of racemic bupivacaine but is less toxic to the heart and central nervous system (Glaser et al., 2002). Ropivacaine is structurally related to bupivacaine (**Fig. 3**) but it has a propyl (3-carbon side-chain) group on the piperidine nitrogen atom compared to bupivacaine, which has a butyl (4-carbon side-chain) group (Casati & Putzu, 2005; Kuthiala & Chaudhary, 2011). Similar to levobupivacaine, ropivacaine is also a pure S(-)enantiomer from the parent chiral molecule propivacaine, and was developed for the purpose of reducing potential toxicity and improving relative sensory and motor block profiles (Kuthiala & Chaudhary, 2011). For that reason, while levobupivacaine and ropivacaine are both long-acting, amide-type local anesthetics like bupivacaine, they have less potential for toxicity on the cardiovascular and central nervous systems in comparison; and are considered to be safer alternatives for clinical use than bupivacaine (Athar et al., 2016; Casati & Putzu, 2005; Kuthiala & Chaudhary, 2011; Leone et al., 2008).

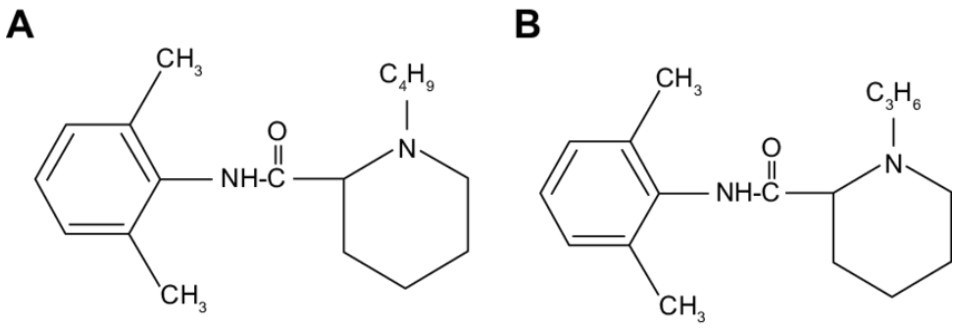

Fig. 3: Comparative molecular structures of (A) bupivacaine and (B) ropivacaine (Ni et al., 2016)**.**

Ropivacaine has a 3-carbon side-chain (propyl group) while bupivacaine has a 4-carbon side-chain (butyl group) on the tertiary amine.

The physiological and clinical characteristics of local anesthetics are determined by the pKa, lipid solubility and protein-binding ability of each drug. For example, the speed of onset, potency, and duration of action is dependent on the pKa, lipid solubility and protein binding, respectively (Taylor & McLeod, 2020). The pKa and protein binding capacity of bupivacaine, levobupivacaine and ropivacaine are similar, while ropivacaine is much less lipophilic (Athar et al., 2016; Grubb & Lobprise, 2020b; Leone et al., 2008). The pKa of a molecule represents the pH at which 50% of the molecules exist in the unionized, lipid-soluble form and 50% in the ionized, water-soluble form (Becker & Reed, 2006). The dissociation of amphipathic local anaesthetics is determined by their pKa and the pH of the tissue into which they are injected (Taylor & McLeod, 2020). It is the unionized lipid soluble molecules that can more easily, thus more rapidly, cross into the cell and the pKa of the drug dictates the proportion of molecules that are in an unionized lipid-soluble state (Grubb & Lobprise, 2020b). Therefore, time for onset of local anesthesia is directly related to the proportion of molecules that convert to the tertiary, lipid-soluble structure when exposed to physiologic pH of 7.4 (Becker & Reed, 2012; Grubb & Lobprise, 2020b). If the pKa of the local anesthetics is greater than the physiologic pH, a greater proportion of the molecules exists in the quaternary, water-soluble form when injected into tissue having normal pH of 7.4 (Becker & Reed, 2006). Thus, local anesthetics with a high pKa, such as bupivacaine, levobupivacaine and ropivacaine that have a pKa of 8.1, have a slow onset of action. Drug potency is based on lipid solubility, with increased solubility equating to increased potency (Grubb & Lobprise, 2020b). Greater lipid solubility enables more rapid diffusion of the drug through lipid membranes to reach their site of action, and gives a greater volume of distribution (Taylor & McLeod, 2020). Local anaesthetics with high protein binding have a longer duration of action and lower bioavailability (Taylor & McLeod, 2020). Protein binding correlates with their affinity for protein within sodium channels and predicts the duration they will sustain neural blockade (Becker & Reed, 2006). Therefore, bupivacaine,

levobupivacaine and ropivacaine having all these characteristics means they are all potent, long-acting drugs with a slow onset of action; and this makes them a desirable choice for clinical applications including intra-articular injection.

Although local anesthetics have many clinical benefits, some of their applications; including intra-articular administration, have raised questions on their safety. While levobupivacaine and ropivacaine have less cardiotoxic and neurotoxic effects compared to bupivacaine, they still have the potential to cause adverse effects in other tissues and cell types. Several human and animal studies have demonstrated that bupivacaine, levobupivacaine and ropivacaine all have chondrotoxic effects following intra-articular administration (Adler et al., 2021; Breu, Rosenmeier, et al., 2013; Kaewpichit et al., 2019; Kürkçüoğlu et al., 2014; Özcan et al., 2017). Therefore, the use of intra-articular local anesthetics is controversial because their chondrotoxicity can potentially lead to more detrimental, rather than beneficial effects. Intra-articular administration of analgesic and anti-inflammatory drugs is a common practice in veterinary medicine, and is used for both lameness diagnosis and management of joint pain (Di Salvo et al., 2021). Intra-articular anesthetics are also beneficial for orthopedic surgical procedures that are associated with severe pain. Previous studies have shown that pre-emptive, perioperative and postoperative administration of intra-articular local anesthetics provides adequate analgesia and reduces the need for interventional administration of opioids (Brioschi et al., 2021; Dutton et al., 2014; Gaesser et al., 2020; Gurney et al., 2012). However, studies on the applications and efficacy of intra-articular local anesthetics in veterinary patients are limited. As a result, recommendations on their clinical use in terms of safe dosage, types of drug to use and appropriate length of treatment remain unclear. Consequently, there are no consensus guidelines recommending an anesthetic of choice for intra-articular use (Holder et al., 2022). Additionally, the margin of safety between chondrotoxic doses and clinically effective doses, or synovial fluid concentrations required to induce analgesia in veterinary

patients remains unknown. As such, their use as intra-articular analgesics is based on the choice and judgement of individual clinicians. However, the use of local anesthetics is so routine, and adverse effects are so infrequent, that providers may overlook many of their pharmacotherapeutic principles (Becker & Reed, 2012), which can result in extra-label use of these drugs and/or extrapolation of protocols from human studies, which may not be safe or effective in animals. It is therefore necessary to have more extensive studies on their efficacy, safety, and adverse effects so as to develop and provide standard recommendations on the appropriate and safe use of intra-articular local anesthetics.

Studying the chondrotoxic effects of local anesthetics is important because they can potentially cause long-term or irreversible harmful effects on cartilage and other joint structures. For instance, the use of pain pumps in humans has been associated with the development of glenohumeral chondrolysis, which is the rapid and irreversible destruction of normal articular cartilage (Matsen & Papadonikolakis, 2013). Animal pain pump models have also shown gross cartilage necrosis after sustained exposure to local anesthetic infusion (Dragoo et al., 2008). Some studies have also indicated that local anesthetics can have delayed deleterious effects (Chu et al., 2010; Grishko et al., 2010; Özcan et al., 2017) which makes them a risk factor in the development of chronic degenerative joint conditions such as osteoarthritis (OA). Articular cartilage has no or very low ability of self-repair, and untreated lesions may lead to the development of osteoarthritis (Becerra et al., 2010). Chondrocyte dysfunction and death is a key characteristic of early osteoarthritis (Piper et al., 2011; Piper & Kim, 2008). Additionally, local anesthetics cause mitochondrial damage (Grishko et al., 2010; Johnson et al., 2004), which can contribute towards OA pathogenesis (Grishko et al., 2009). Therefore, the use of intra-articular local anesthetics can be a risk factor in the development and/or exacerbation of OA. Local anesthetics have also demonstrated cytotoxicity towards mesenchymal stem cells (MSCs) (Breu et al., 2015; Breu, Eckl, et al., 2013; Dregalla et al.,

2014; Kubrova et al., 2021; Zhang et al., 2017). MSCs have the potential for differentiation under specific conditions into other cell types, including cartilage, bone, and adipose tissue (Sasaki et al., 2019). Stem cells are most commonly used in clinical veterinary medicine for therapeutic applications for the treatment of musculoskeletal injuries such as repair of cartilage in horses and dogs (Fortier & Travis, 2011; Prządka et al., 2021). Intra-articular local anesthetics may be applied during stem cell injections to reduce patient discomfort (Kubrova et al., 2021) and this can potentially have a negative impact on the successful application of stem cell therapy for joint diseases. Moreover, local anesthetics have also been shown to have cytotoxic effects on other cell types found within joints including tenocytes, synoviocytes and fibroblasts (Adler et al., 2021; Zhang et al., 2017), which indicates that their adverse effects can potentially extend to other joint structures such as the synovium, ligaments and tendons which can subsequently affect chondrocyte survival. On the other hand, understanding the mechanisms involved in local anesthetic chondrotoxicity can possibly expand their clinical applications and can contribute towards understanding their effects on other cell types. While they are widely known for their blockade of voltage-gated sodium channels, evidence of their effects on other biomolecular processes such as apoptosis demonstrates their potential for other clinical indications in addition to anesthesia and analgesia. For example, local anesthetics have anti-cancer (Chiu et al., 2015; Jayaram et al., 2019) and anti-microbial effects (Adler et al., 2017; Sams et al., 2012), which shows that their cytotoxicity can be beneficial in particular clinical applications. Therefore, understanding the chondrotoxicity of local anesthetics and the mechanisms involved has many clinical implications and can contribute towards advancing their medical use.

The purpose of the present thesis was to evaluate the *in vitro* chondrotoxic effects of long-acting local anesthetics including bupivacaine, levobupivacaine and ropivacaine in cultured canine articular chondrocytes; and to elucidate the biomolecular pathways involved in

local anesthetic induced chondrotoxicity. The aim was to provide recommendations on the safe use of intra-articular local anesthetics in veterinary clinical applications. The first chapter investigated the *in vitro* chondrotoxicity of bupivacaine at low concentrations based on previously determined synovial fluid concentrations following intra-articular injection in dogs. This was a preliminary study to evaluate whether different concentrations of bupivacaine would decrease chondrocyte viability after 1, 6 and 24 hr of treatment. The second chapter evaluated the comparative chondrotoxicity of bupivacaine, levobupivacaine and ropivacaine and assessed their effects on caspase activity. The objectives of these studies were (1) to investigate the *in vitro* effects of long-acting local anesthetics on articular chondrocyte viability and (2) to elucidate the apoptotic pathways involved in their chondrotoxicity.

2. Chapter 1: *In vitro* chondrotoxicity of bupivacaine at low concentrations in cultured canine articular chondrocytes

2.1. Summary

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 the study done in chapter 1 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 0.25% and 0.125% 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.

2.2. 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 (Borer, 2006; Di Salvo et al., 2021; Schumacher & Boone, 2021; Van Vynckt, Samoy, et al., 2012). 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 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 (Dutton et al., 2014; Kushnir et al., 2017; Martin-Flores et al., 2019; McFadzean et al., 2021). 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 (Adler et al., 2021; Busfield & Romero, 2009; Chu et al., 2006; Gomoll et al., 2006; Kaewpichit et al., 2019). Their chondrotoxicity depends on the dose, period of exposure, and the type of drug used (Karpie & Chu, 2007; Kreuz et al., 2018).

Bupivacaine is an amide-type local anesthetic with a slow onset and long duration of action (Lemke & Dawson, 2000). It has been the most widely used local anesthetic for decades (Casati & Putzu, 2005). 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 (Barry et al., 2015). A recent survey among veterinarians found that bupivacaine was the most commonly used local anesthetic for peripheral nerve blocks during pelvic limb surgery (Thomson et al., 2021). 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 (Baker et al., 2011; Chu et al., 2006; Park et al., 2011; Sherman et al., 2015). 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 (Barry et al., 2015; Ravnihar et al., 2021). 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 (Ravnihar et al., 2021). 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 (Barry et al., 2015). 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 if local anesthetics leave the joint compartment as rapidly as suggested by the synovial fluid concentrations, they are unlikely to damage chondrocytes when administered, especially as a single intra-articular injection during clinical applications. It also shows 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.

2.3. Materials and methods

2.3.1. 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 and centrifuged at 400 × g for 5 min then resuspended in growth medium. Primary (P0) chondrocytes were seeded at 1.0×10^4 cells/cm^2 in 100 mm culture dishes (Corning, Lowell, MA, USA). Growth medium containing 10 mM HEPES (Dojindo, Kumamoto, Japan), 25 mM $NaHCO_3$ (Wako, Osaka, Japan), 100 U/ml penicillin G potassium (Wako) and 73 U/ml streptomycin sulphate (Wako) and supplemented with 10% fetal bovine serum (FBS: Nichirei Biosciences Inc., Tokyo, Japan) was used for cell culture. 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) and 0.02% ethylenediaminetetraacetic acid (EDTA; Dojindo) in PBS. After 5-10 min, growth medium containing 10% FBS was added to the culture plate to inhibit the trypsin, and the cells were then centrifuged at 400 × g for 5 min before they were resuspended in growth medium. Cell number and viability was determined by the trypan blue (Wako) exclusion assay before they were sub-cultured under the same conditions as described above.

2.3.2. Chondrocyte treatment

Third passage (P3) chondrocytes were used for all the experimental analyses. For all experiments, chondrocytes were seeded at 2.5×10^4 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 highest bupivacaine concentration (0.25%) was used as the positive control while the culture medium (10-DMEM) without bupivacaine was used as the negative 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.

2.3.3. Live/dead Assay

Chondrocytes at a density of 2.0×10^4 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 (Thermo Fisher Scientific, Waltham, MA, 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 (Thermo Fisher Scientific) 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).

2.3.4. MTT Assay

Chondrocytes were cultured in 96-well plates (Corning) at a density of 8.0×10^3 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 (Dojindo) 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 and MTT formazan crystals were dissolved with 100 µl dimethyl sulfoxide (DMSO; Wako). The absorbance was measured with a microplate reader (Thermo Scientific, Vantaa, Finland) at 570 nm.

2.3.5. CCK-8 Assay

Similar to the MTT assay, 8.0×10^3 chondrocytes were cultured in 96-well plates (Corning) for 24 hr and the CCK-8 (Dojindo) 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) at 450 nm.

2.3.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.4.1 (GraphPad Software Inc., San Diego, CA, USA). Analysis of variance (ANOVA) and Tukey's multiple comparisons test were used to compare between the treatment groups for the MTT and CCK-8 assays. *P*-value < 0.05 was considered significant. All results unless specified are presented as mean ± standard deviation (SD).

2.4. Results

2.4.1. Bupivacaine at low concentrations compromises cell membrane integrity and morphology - Live/dead Assay

Bupivacaine was chondrotoxic in a concentration- and time-dependent manner (**Fig. 4**). Cell death was observed at concentrations of 0.25 and 0.125% after 1 hr of treatment. 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 negative control after 1 hr. After 6 hr, all cells at 0.25 and 0.125% bupivacaine had taken up EthD-1; and at 0.062%, most of the cells had visibly detached from the culture plate (white arrow); while 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 there was also a marked 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 and visibly smaller in size, including the live cells that did not take up the EthD-1 stain. At 0.031% the cells did not take up EthD-1 or detach from the plate after 24 hr, however they had a morphological change in their growth pattern 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 – 6 – 24 hr of treatment indicating a concentration- and time-dependent effect.

2.4.2. Bupivacaine at low concentrations is cytotoxic to canine articular chondrocytes - MTT Assay

Bupivacaine decreased cell viability in a concentration- and time-dependent manner when compared to the negative control, which was considered to have 100 % viability (**Fig. 5**).

Treatment with 0.25 and 0.125% bupivacaine significantly reduced cell viability to less than 10% at all treatment time-points ($P < 0.001$ for both treatments). 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. Cell 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 after 1 hr. Interestingly, bupivacaine at 0.016% increased cell viability from 99.0 ± 5.57% ($P = 0.998$) at 1 hr treatment, to 103 ± 2.65% ($P = 0.870$) after 6 hr of treatment and 102 ± 5% ($P = 0.945$) after 24 hr of treatment; however, this change was not statistically significant. Bupivacaine at 0.031% also increased viability 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.

2.4.3. Bupivacaine at low concentrations reduces cell viability as validated by the CCK-8 Assay

The CCK-8 assay showed a similar pattern to that of the MTT assay with a few variations (**Fig. 6**). 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% gradually 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.

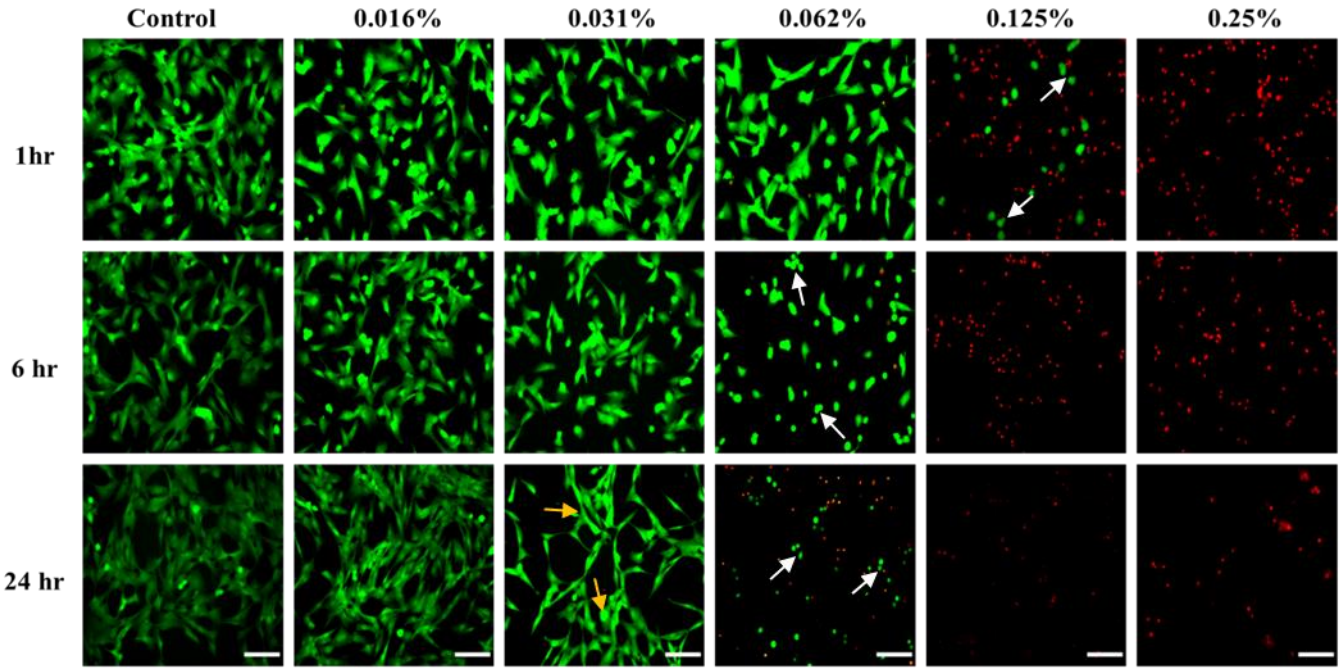

Fig. 4: **Effects of low bupivacaine concentrations on cell membrane integrity and morphology - Live/dead Assay**

The figure shows 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 *in vitro*. 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.

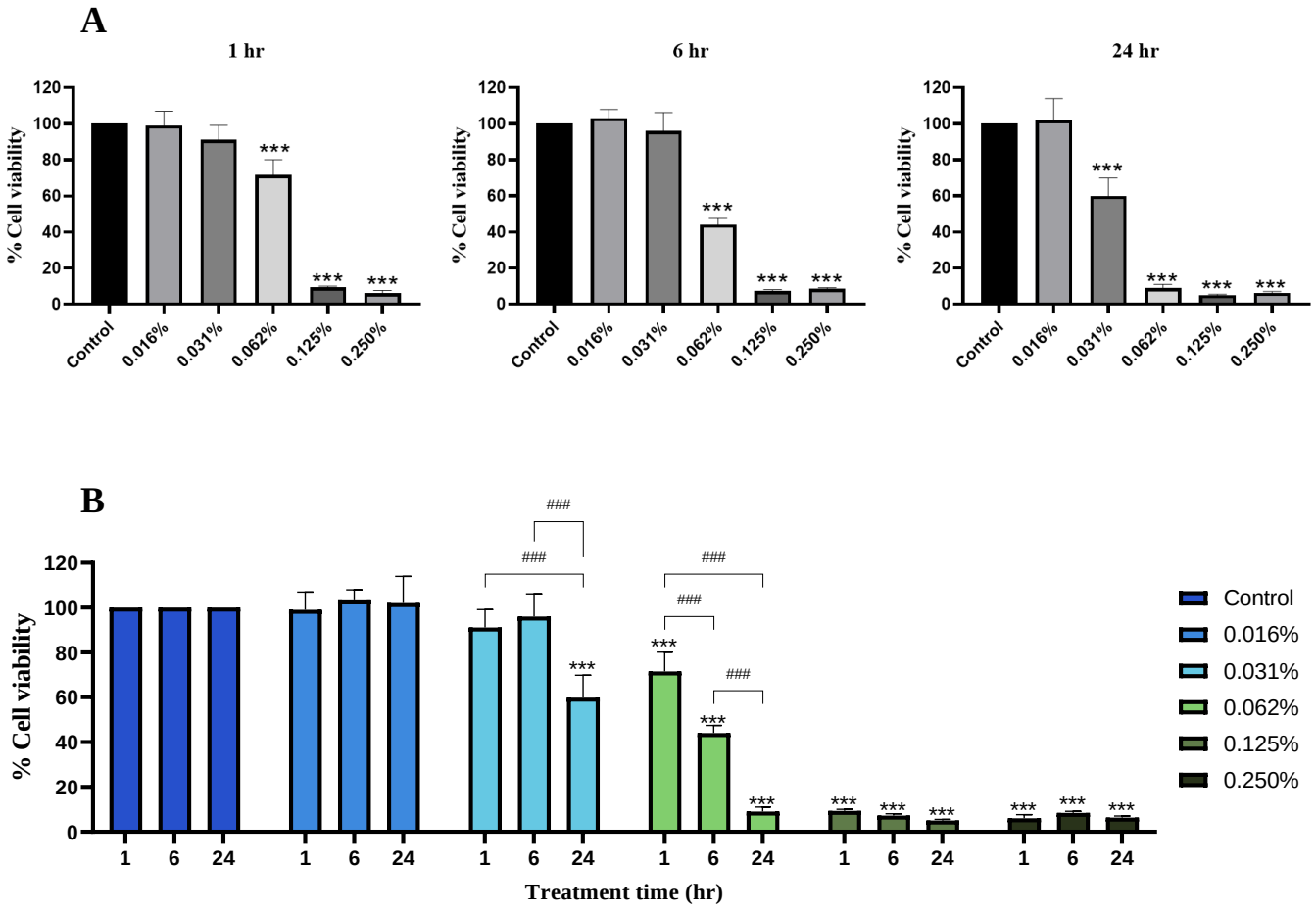

Fig. 5: **MTT assay results showing the effects of bupivacaine treatment on chondrocyte viability.**

(**A**) shows the graphs for each individual treatment time point and (**B**) shows a combination of the results from (**A**). Bupivacaine was chondrotoxic in a concentration- and time-dependent manner. After 1 hr there was a significant reduction in cell viability with 0.062, 0.125 and 0.25% bupivacaine (*** $P <$ 0.001) compared to the negative control (100% viability). After 6 hr and 24 hr, 0.016% bupivacaine caused a non-significant increase in cell viability compared to the 1 hr treatment. At 0.031% bupivacaine 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 (*** $P < 0.001$). There was also a significant difference in chondrocyte viability between the different time-points for the 0.031 and 0.062% bupivacaine treatments ($^{###}P < 0.001$).

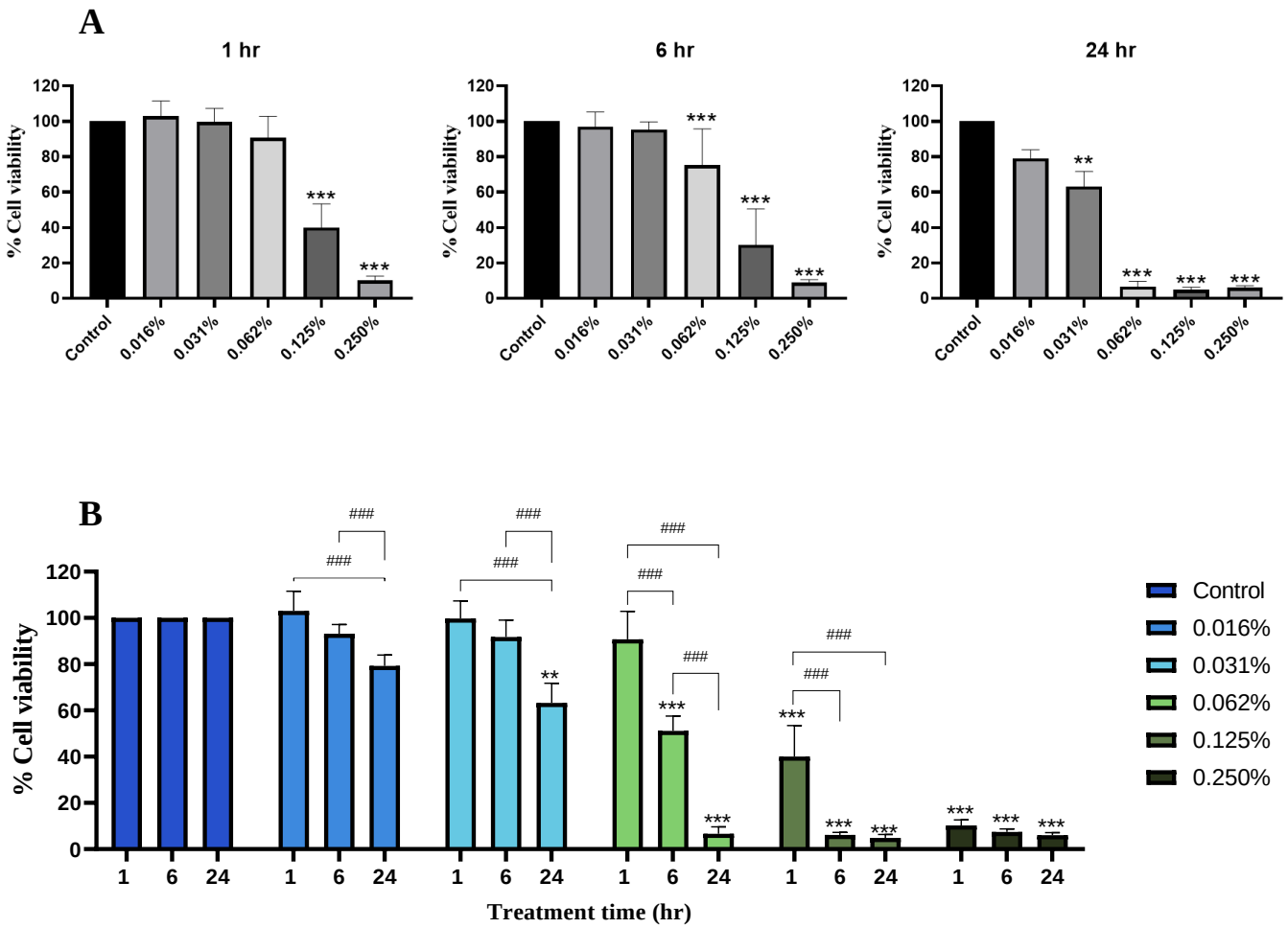

Fig. 6: **CCK-8 assay results showing the effects of bupivacaine treatment on chondrocyte viability.**

(**A**) shows the graphs for each individual treatment time point and (**B**) shows a combination of the results from (**A**). Similar to the MTT assay, bupivacaine was chondrotoxic in a concentration- and time-dependent manner. After 1 hr, bupivacaine at 0.125 and 0.25% caused a significant reduction in chondrocyte viability (*** $P < 0.001$) compared to the negative control. 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 effect (**$P = 0.001$) while there was no significant difference between 0.016% and the negative control. Additionally, there were significant time-dependent differences in cell viability for 0.016, 0.031, 0.062 and 0.125% bupivacaine ($^{###}P < 0.001$).

2.5. 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 (Breu, Rosenmeier, et al., 2013; Chu et al., 2008; Grishko et al., 2010); and therefore show that bupivacaine can have *in vitro* chondrotoxic effects at proportions similar to *in vivo* synovial fluid concentrations in canine articular chondrocytes.

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.

Bupivacaine is commercially available in concentrations of 0.25, 0.5, and 0.75% (2.5, 5.0 and 7.5 mg/ml, respectively) (Barry et al., 2015). 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 (Adler et al., 2021; Chu et al., 2008; Rengert et al., 2021), 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% (Chu et al., 2008) or at 0.25% (Breu, Rosenmeier, et al., 2013; Dragoo et al., 2012). 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 (Adler et al., 2021). 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 (Sherman et al., 2015).

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. (2015) 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 (Barry et al., 2015). A previous study by Rengert et al. (2021) 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. (2015). 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 when administered *in vivo* (Rengert et al., 2021). In the current study, bupivacaine concentrations of less than 0.25% were still able to significantly decrease chondrocyte viability depending on the length of exposure. 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 (Adler et al., 2021; Rengert et al., 2021); 6 hr was selected as the intermediate period of exposure based on the average duration of action of bupivacaine (Borer, 2006); 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. (2015) 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 used in the current study were therefore estimated values and may not be the precise amounts of bupivacaine that would be found within the joint space at each specific time point *in vivo*.

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 ($P = 0.630$) after 1 hr. 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. (2021) 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 (Grishko et al., 2010); 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 (Rengert et al., 2021; Sherman et al., 2015). These mixed results may be due to differences in the experimental designs and the samples used. In the study by Sherman et al. (2015), full thickness canine cartilage explants were used instead of isolated chondrocytes. On the other hand, Rengert et al. (2021) 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% ($P = 0.945$) 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 (Zheng et al., 2021). Additionally, chondrocytes producing survival signals following cellular injury can increase intercellular communication in order to prevent cell death (Ghasemi et al., 2021; Lotz et al., 1999). 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, except for 0.016% which 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 (Lotz et al., 1999). 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 (Barry et al., 2015). 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, a time-dependent effect was observed at lower bupivacaine concentrations as indicated in **Fig. 5 (B)** and **Fig. 6 (B)**. The MTT assay showed a gradual decrease in chondrocyte viability with an increase in the length of treatment time at 0.062% and 0.031%; while 0.016, 0.031, 0.062, and 0.125% bupivacaine showed significant reduction in cell viability between different time points with the CCK-8 assay. This pattern was also 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. 4**). 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. (2010) 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 (Chu et al., 2010). 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. The *in vitro* experimental conditions under which the chondrocytes were treated and analysed do not equate the *in vivo* physiological conditions of a joint environment. During intra-articular administration of local anesthetics, nerve endings that are primarily located in the fibrous joint capsule and ligaments of the joint are the main target for analgesia (Barry et al., 2015), because cartilage is an avascular and aneural tissue (Kheir & Shaw, 2009). Therefore, the response of the chondrocytes to treatment with local anesthetics will also depend on how other structures within the joint are affected by the local anesthetic. In our study, the monolayer culture was used as a model and only showed drug 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. (2015). 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 into the joint.

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.

3. Chapter 2: *In vitro* effects of bupivacaine, levobupivacaine and ropivacaine on cell viability and caspase activity in cultured canine articular chondrocytes

3.1. Summary

The second chapter evaluated the comparative *in vitro* effects of bupivacaine, levobupivacaine and ropivacaine on cell viability and caspase activity in canine articular chondrocytes in order to elucidate whether they would activate the extrinsic or intrinsic pathways of apoptosis. Third passage chondrocytes in monolayer culture were treated with culture medium as the control, or with 0.062% (0.62mg/mL) bupivacaine, 0.062% levobupivacaine and 0.062% ropivacaine for 24 hr. Cell viability was evaluated using the live/dead, 3-(4,5-dimehylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and Cell Counting Kit-8 (CCK-8) assays. Evaluation of caspase-3, caspase-8 and caspase-9 activity was performed using colorimetric assays. The MTT and CCK-8 assays were used to evaluate the effect of caspase inhibitors on local anesthetic chondrotoxicity. All three local anesthetics significantly decreased chondrocyte viability after 24 hr ($P < 0.001$). Bupivacaine significantly increased caspase-3, caspase-8, and caspase-9 activity ($P < 0.001$). Levobupivacaine increased caspase-3 activity ($P = 0.03$), while ropivacaine did not significantly upregulate activity for any of the three caspases. Caspase inhibition did not suppress bupivacaine and levobupivacaine chondrotoxicity whereas inhibition of caspase-8 and caspase-9 attenuated ropivacaine chondrotoxicity. Local anesthetics induced apoptosis through both the extrinsic and intrinsic pathways. The type of caspase activated, the level of caspase activation, and the response to caspase inhibitors was dependent on the type of local anesthetic. Ropivacaine may be a safer choice for intra-articular administration compared to levobupivacaine and bupivacaine.

3.2. Introduction

Intra-articular administration of local anesthetics is used for both diagnostic and therapeutic purposes in veterinary clinical practice (Borer, 2006; Di Salvo et al., 2021). Some of the applications for intra-articular local anesthetics in veterinary patients include diagnosis of lameness and joint diseases (Schumacher & Boone, 2021; Van Vynckt, Samoy, et al., 2012; Van Vynckt, Verhoeven, et al., 2012), anesthesia during joint/arthroscopic surgery,(Borer, 2006; Di Salvo et al., 2021) and management of pain associated with osteoarthritis and other joint diseases (Di Salvo et al., 2021). Despite their efficacy in pain management, local anesthetics can have adverse clinical effects, such as chondrotoxicity following intra-articular injection (Kreuz et al., 2018; Piper et al., 2011). As a result of these adverse effects on chondrocytes, intra-articular administration of local anesthetics is controversial both in human and veterinary medicine, and it is sometimes discouraged due to concerns about their risk/benefit profile (Barletta & Reed, 2019; Di Salvo et al., 2021). However, because local anesthetics play an important and unique role in pain management, it may not be possible to completely eliminate their use in clinical applications. It is therefore necessary to understand their clinical and biomolecular effects on cell viability so as to identify ways in which their cytotoxic effects can be prevented or attenuated.

Bupivacaine, levobupivacaine and ropivacaine are potent, long-acting amide local anesthetics that can be used as part of various anesthetic and/or analgesic protocols, including intra-articular administration. Bupivacaine is commonly used in clinical applications because of its high potency and long duration of action (Casati & Putzu, 2005; Thomson et al., 2021). Levobupivacaine and ropivacaine are analogues of bupivacaine that were developed following evidence of bupivacaine-induced toxicity (Casati & Putzu, 2005; Leone et al., 2008). They are considered to be less toxic than bupivacaine while having similar potency and duration of action (Barletta & Reed, 2019; Casati & Putzu, 2005; Leone et al., 2008). While this may be true for

systemic and cardiac toxicity, both levobupivacaine and ropivacaine have demonstrated chondrotoxicity, especially at high doses and with prolonged exposure (Çevik et al., 2018; Grishko et al., 2010; Gungor et al., 2014; Lo et al., 2009). The cytotoxic effects of local anesthetics appear to occur mainly through mitochondrial damage, which then leads to apoptosis or necrosis depending on the dose and period of time that the cells are exposed to the drug (Grishko et al., 2010; Irwin et al., 2002; Johnson et al., 2004; Werdehausen et al., 2007). However, the pathway of apoptosis involved in local anesthetic-induced chondrotoxicity remains unclear and requires further investigation.

Apoptosis is regulated by caspases, which are a family of proteases, that act in a proteolytic cascade to induce programmed cell death (Eduardo et al., 2018). Initiation of apoptosis occurs through either a cell-extrinsic pathway, regulated by caspase-8; or a cell-intrinsic pathway, regulated by caspase-9 (Parrish et al., 2013). The extrinsic pathway is induced by signals coming from outside of the cell whereas the intrinsic pathway results from changes in the mitochondrial integrity (Kiraz et al., 2016; Parrish et al., 2013). Activation of either caspase-8 or caspase-9 leads to the downstream activation of effector caspases, which then induce apoptosis (Kiraz et al., 2016; Parrish et al., 2013). Caspase-3 is the most well-characterized effector caspase which is activated via both extrinsic and intrinsic apoptotic pathways (Kiraz et al., 2016; Parrish et al., 2013). As such, the objective of this study was to evaluate the effects of bupivacaine, levobupivacaine and ropivacaine on cell viability, and to further elucidate the pathway of apoptosis involved in their chondrotoxicity by evaluating their effects on caspase-3, caspase-8, and caspase-9 activation, and the response to inhibition of these caspases in cultured canine articular chondrocytes. Because of their effects on mitochondrial function, it is likely that local anesthetics primarily induce apoptosis through the intrinsic pathway (Eduardo et al., 2018). Therefore, the hypothesis was that all three local anesthetics would activate the intrinsic pathway of apoptosis through caspase-9 activation.

3.3. Materials and methods

3.3.1. Chondrocyte isolation and culture

Cartilage samples were collected from the joints of three dogs undergoing limb surgery at The Hokkaido University Veterinary Teaching Hospital for reasons unrelated to this study. The samples were obtained from the femoral heads of a 7-year-old Cocker Spaniel and 8-year-old Toy Poodle undergoing femoral head resection; and from the humeral condyle of a 12-year-old Shiba Inu undergoing limb amputation. 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) for 18 hr at 37°C and 5% CO_2. The cartilage samples were then filtered through a 40 μm nylon filter to release the primary (P0) chondrocytes which were then seeded at 1.0×10^4 cells/cm^2 in 100 mm culture dishes (Corning). DMEM containing 10 mM HEPES (Dojindo), 25 mM $NaHCO_3$ (Wako), 100 U/mL penicillin G potassium (Wako) and 73 U/mL streptomycin sulphate (Wako) and supplemented with 10% fetal bovine serum (FBS: Nichirei) was used as growth medium. Chondrocytes were harvested at 80-90% confluence, by washing them three times with phosphate buffered saline (PBS) and they were detached from the culture plate using a solution containing 0.5% trypsin (Wako) and 0.02% Ethylenediaminetetraacetic acid (EDTA; Dojindo) dissolved in PBS. Culture medium containing 10% FBS was used to inhibit the trypsin and the cells were centrifuged at 400 × g for 5 min before they were resuspended in growth medium. The trypan blue (Wako) exclusion assay was used to determine the chondrocyte number and viability before they were sub-cultured until third passage (P3) chondrocytes were obtained.

3.3.2. Chondrocyte treatment

Third passage chondrocytes in monolayer culture were used for all the experiments. The cells were seeded at 2.5×10^4 cells/cm^2 and cultured in an incubator at 37°C and 5% CO_2. Experiments were performed in duplicate for each cell source. DMEM supplemented with 10% fetal bovine serum (10-DMEM) was used as the culture medium. Following culture, the growth medium was removed, and the chondrocytes were treated with 10-DMEM as the control, or with 0.062% (0.62 mg/ml) bupivacaine (Marcaine injection 0.5%; Aspen); 0.062% levobupivacaine (Popscaine injection 0.5%; Maruishi Pharmaceutical Co., Osaka, Japan); and 0.062% ropivacaine (Anapeine injection 1.0%; Aspen). The local anesthetics were diluted to these concentrations using 10-DMEM. The treatment groups were labeled as Control; Bup 0.062% for bupivacaine; Lev 0.062% for levobupivacaine; and Rop 0.062% for ropivacaine.

3.3.3. Live/dead Assay

The live/dead assay was used to visually differentiate between live and dead cells, and to assess morphological changes that occurred following treatment with the local anesthetics. 2.0×10^4 chondrocytes were plated in 8-well chamber slides (Iwaki) for 24 hr then treated with 10-DMEM as the control or with local anesthetics diluted in culture medium as described above. After 24 hr of treatment, the live/dead reagent (Thermo Fisher Scientific) was added to the culture wells and incubated for 30 min at 37°C and 5% CO_2, then the solution was carefully removed from the culture wells and replaced with PBS for viewing. A FLoid Cell Imaging Station (Thermo Fisher Scientific) was used for fluorescent microscopy 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). The live/dead assay analyses cell membrane integrity and permeability by differential staining with either calcein for live cells (green) or ethidium homodimer (EthD-1) for dead cells (red).

3.3.4. MTT Assay

The MTT (Dojindo) assay was used to evaluate the effects of bupivacaine, levobupivacaine and ropivacaine on mitochondrial dehydrogenase activity. Chondrocytes were seeded at a density of 8.0×10^3 per well in 96-well plates (Corning) for 24 hr and treated as described above. After 24 hr, the culture medium was removed and the cells were washed twice with PBS, then MTT working solution 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 and MTT formazan crystals were dissolved with 100 µl dimethyl sulfoxide (DMSO: Wako). The absorbance from each well was measured using a microplate reader (Thermo Scientific) at 570 nm.

3.3.5. CCK-8 Assay

The CCK-8 assay (Dojindo) was used to validate the MTT assay by evaluating the effects of bupivacaine, levobupivacaine and ropivacaine on cellular dehydrogenase activity. Chondrocytes were seeded at a density of 8.0×10^3 per well and cultured in 96-well plates (Corning) for 24 hr and treated as described above. After 24 hr treatment, the medium was removed, and the cells were washed twice with PBS. Cells were then treated with 10 µl of the CCK-8 solution 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) at 450 nm.

3.3.6. Evaluation of caspase activity

After determining their effect on chondrocyte viability, the effects of bupivacaine, levobupivacaine and ropivacaine on caspase-3, caspase-8 and caspase-9 activation were evaluated. Chondrocytes were seeded onto 60 mm culture dishes (Corning) at a density of 52.5×10^4 cells per plate. The cells were cultured in 10-DMEM for 6 days until they reached 90% confluence, with the growth medium being changed every 3 days during culture. The

chondrocytes were then treated with 10-DMEM or bupivacaine, levobupivacaine and ropivacaine for 24hr as described above. After treatment, the medium was removed, and the cells were washed twice with PBS before they were harvested using Accutase solution (Sigma-Aldrich, St Louis, MO, USA). Caspase activity was then determined using the caspase-3, caspase-8 and caspase-9 assay kits (Abcam, Cambridge, UK) according to the manufacturer's instructions. After harvest, the chondrocytes were resuspended in chilled cell lysis buffer for 10 min. Thereafter, the protein concentration of each sample was measured using a protein quantification assay kit (Macherey-Nagel, Dürren, Germany) and adjusted accordingly. Then DTT followed by DEVD-p-NA substrate for caspase-3, IETD-pNA substrate for caspase-8, or LEHD-p-NA substrate for caspase-9 were added to the test sample, respectively. Samples were then incubated at 37°C for 2 hours and the absorbance was measured with a microplate reader (Thermo Scientific) at 405 nm.

3.3.7. Inhibition of caspase activity

The MTT assay and CCK-8 assay were used to evaluate the effect of caspase inhibition on local anesthetic-induced chondrotoxicity. The inhibitor concentrations and were substrates based on previously described protocols (Chen et al., 2012; Huser et al., 2006), with minor modifications. Chondrocytes were seeded at a density of 8.0×10^3 per well in 96-well plates (Corning) and cultured for 24 hr. The cells, except the control, were then pretreated with 50 µM caspase inhibitors in culture medium for 1 hr. The caspase inhibitors used included a caspase-3 inhibitor (Ac-DMQD-CHO; Peptide Institute Inc., Osaka, Japan), caspase-8 inhibitor (Ac-IETD-CHO; Peptide Institute Inc.) or a caspase-9 inhibitor (Ac-LEHD-CHO; Peptide Institute Inc.), respectively. The cells were then treated with 10-DMEM as the control, or with Bup 0.062%, Lev 0.062% and Rop 0.062% combined with the 50 µM caspase inhibitors for 24 hr. The cell viability was evaluated using the same methods described for the MTT and CCK-8 assays above.

3.3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.4.1 (GraphPad Software Inc.). The Shapiro-Wilk test was used to assess normality. Analysis of variance and Dunnett's multiple comparison test was used to compare cell viability and caspase activity levels between the control and the local anesthetic treatments. Comparisons in chondrocyte viability were also made between the local anesthetic treatments with no inhibitors and those treated with local anesthetic combined with caspase inhibitors for each drug. The Dunnett's multiple comparison test or Dunn's multiple comparisons test was used for the latter depending on the normality of the data. *P*-value < 0.05 was considered significant. All results unless specified are presented as mean ± standard deviation (SD).

3.4. Results

3.4.1. Effects of bupivacaine, levobupivacaine and ropivacaine on chondrocyte viability and morphology - Live/dead Assay

All three local anesthetics caused morphological changes after 24 hr of treatment (**Fig. 7**). Bupivacaine was the most chondrotoxic as most of the cells had taken up the EthD-1 stain (red). Even the live cells were completely detached from the culture plate and had a spherical shape (white arrows) instead of the fibroblast-like morphology normally observed in monolayer culture. On the other hand, the cells treated with levobupivacaine and ropivacaine did not take up the EthD-1 stain but they had formed clusters and remained attached to the surface of the culture plate with fibroblast-like morphology (yellow arrows). Although there were no dead cells observed, cluster formation was an indication that the local anesthetics caused metabolic changes leading to changes in the growth pattern.

3.4.2. Effects of bupivacaine, levobupivacaine and ropivacaine on mitochondrial dehydrogenase activity- MTT Assay

All three local anesthetics significantly decreased chondrocyte viability compared to the control (**Fig. 8**). Bupivacaine was the most chondrotoxic with the cell viability decreasing to 11.33 ± 0.58% ($P < 0.001$) compared to the control. Chondrocyte viability was decreased to 61.67 ± 14.29% ($P < 0.001$) for levobupivacaine and to 64 ± 9.85% ($P < 0.001$). Bupivacaine also significantly decreased chondrocyte viability compared to levobupivacaine ($P < 0.001$) and ropivacaine ($P < 0.001$). There was no statistical difference between the levobupivacaine and ropivacaine treatments ($P > 0.999$).

3.4.3. Effects of bupivacaine, levobupivacaine and ropivacaine on cellular dehydrogenase activity - CCK-8 Assay

All treatments with local anesthetics caused a significant reduction in chondrocyte viability after 24 hr (**Fig. 8**). Bupivacaine was the most chondrotoxic with the cell viability decreasing to 8.33 ± 1.53% ($P < 0.001$) compared to the control. Levobupivacaine and ropivacaine decreased chondrocyte viability to 59 ± 2.65% ($P < 0.001$) and 68 ± 6.08% ($P < 0.001$), respectively. Bupivacaine also significantly decreased chondrocyte viability compared to levobupivacaine ($P < 0.001$) and ropivacaine ($P < 0.001$). There was also a significant difference between the levobupivacaine and ropivacaine treatments, as levobupivacaine reduced chondrocyte viability more than the ropivacaine treatment ($P = 0.004$).

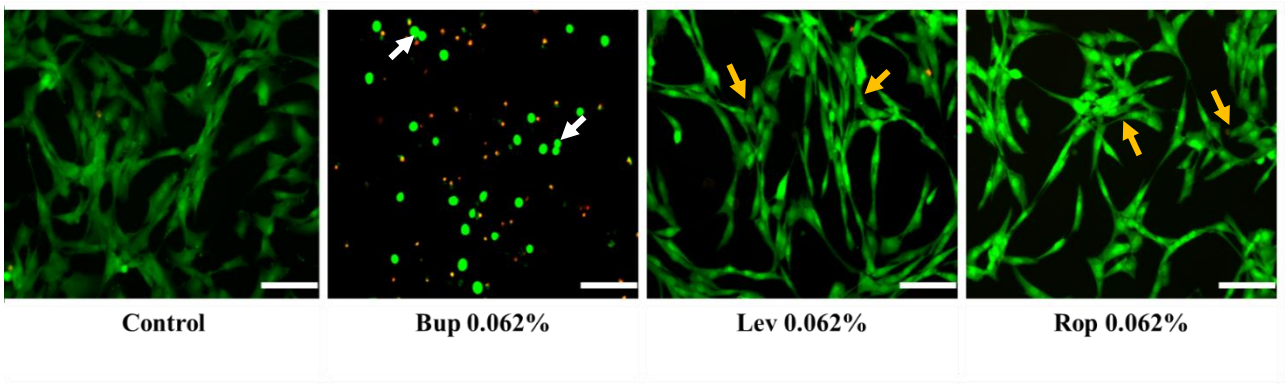

Fig. 7: **Live/dead assay results showing the effects of bupivacaine, levobupivacaine and ropivacaine on chondrocyte viability and morphology.**

Chondrotoxicity was observed through differential staining between live cells (green) and dead cells (red) after 24 hr treatment with 0.062% bupivacaine, levobupivacaine and ropivacaine. The chondrotoxic effects were also visible when live cells became detached from the culture plate (white arrows) or through cell clustering (yellow arrows). Cells treated with bupivacaine were completely detached from the culture plate and had taken up the Eth-D stain. Cells treated with levobupivacaine and ropivacaine formed clusters, indicating a morphological change in response to treatment with the local anesthetics. Scale bar: 100 μm.

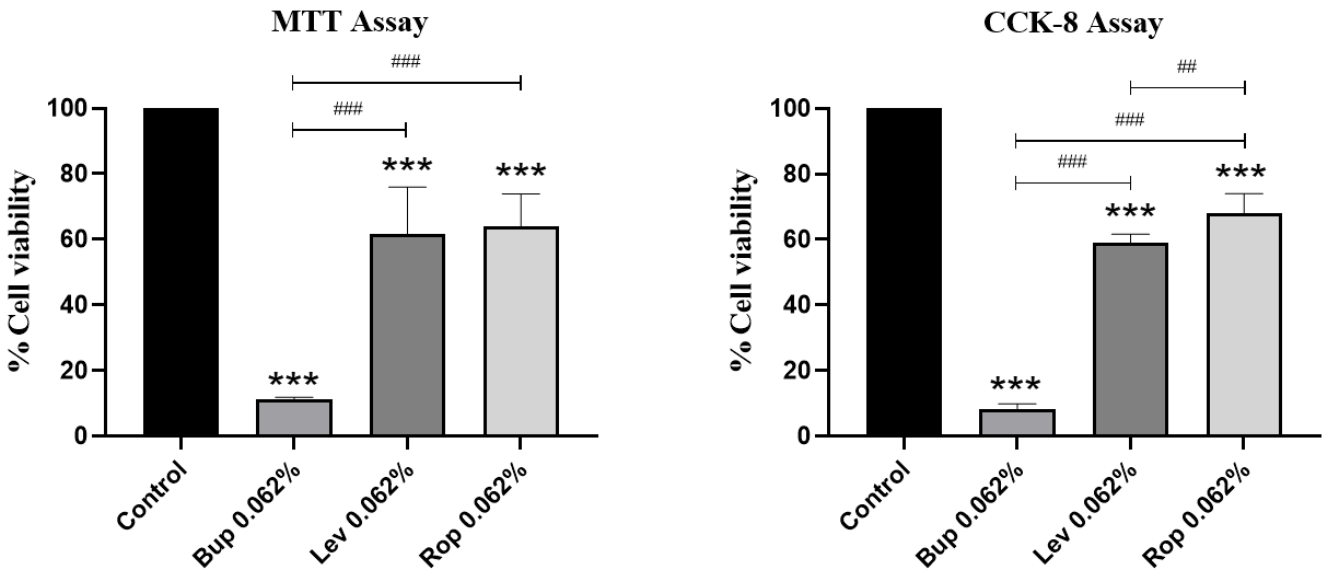

Fig. 8: **MTT and CCK-8 viability assay results showing the effects of bupivacaine, levobupivacaine and ropivacaine on chondrocyte viability.**

All three local anesthetics significantly decreased chondrocyte viability compared to the control after 24 hr (***$P < 0.001$) for both the MTT and CCK-8 assays. Both viability tests also indicated that bupivacaine had the most chondrotoxic effect compared to levobupivacaine and ropivacaine ($^{###}P < 0.001$). With the CCK-8 assay, chondrocyte viability for the ropivacaine treatment group was significantly higher than that of the cells treated with levobupivacaine ($^{##}P = 0.001$).

3.4.4. Effects of bupivacaine, levobupivacaine and ropivacaine on caspase-3, caspase-8, and caspase-9 activity

After determining that all three local anesthetics were chondrotoxic at 0.062%, their effect on caspase activity was evaluated (**Fig. 9**). The level of caspase activity was dependent on the type of local anesthetic and had a similar pattern to the cell viability assays. All three local anesthetics increased caspase-3 activity compared to the control, although ropivacaine did not have a statistically significant effect. Bupivacaine caused the highest increase ($P < 0.001$) while levobupivacaine ($P = 0.03$) and ropivacaine ($P = 0.06$) had comparative levels of caspase-3 activity. Interestingly, bupivacaine significantly increased caspase-8 activity ($P < 0.001$) whereas the caspase-8 levels for levobupivacaine ($P = 0.974$) and ropivacaine ($P = 0.986$) were similar to the control. Bupivacaine caused the highest increase in caspase-9 activity ($P < 0.001$) followed by levobupivacaine ($P = 0.08$), while ropivacaine had the least effect on caspase-9 activity ($P = 0.892$). Bupivacaine significantly upregulated all three caspases compared to levobupivacaine and ropivacaine ($P < 0.001$).

3.4.5. Effect of specific caspase inhibition on local anesthetic-induced chondrotoxicity – MTT assay

Cellular response to inhibition of caspase-3, caspase-8, and caspase-9 was dependent on the type of local anesthetic used. With the MTT assay, ropivacaine was the most responsive to caspase inhibition, while bupivacaine and levobupivacaine chondrotoxicity were not attenuated (**Fig. 10**). Caspase inhibition did not inhibit bupivacaine chondrotoxicity as the cell viability was still severely decreased compared to the control ($P < 0.001$) even with caspase inhibitors. Additionally, there was no significant difference between the bupivacaine only treatment and the groups treated with bupivacaine and caspase-3 ($P = 0.575$), caspase-8 ($P = 0.575$), and caspase-9 ($P = 0.305$) inhibitors. For levobupivacaine, there was no significant difference between the treatments with or without the caspase inhibitors. Caspase-3 inhibition

did not inhibit its chondrotoxicity (P = 0.980), and inhibition of caspase-8 (P = 0.301) and caspase-9 (P = 0.471) only mildly increased chondrocyte viability compared to the levobupivacaine only treatment (without inhibitors); however, this effect was not statistically significant. Meanwhile, caspase inhibition had a notable effect on ropivacaine chondrotoxicity. Inhibition of caspase-3 non-significantly increased the cell viability from 64 ± 9.85% to 70.33 ± 10.97% (P = 0.865), while caspase-8 and caspase-9 inhibition markedly increased chondrocyte viability to 98.33 ± 8.02% (P = 0.013) and 88.33 ± 11.06% (P = 0.070) respectively.

3.4.6. Effect of specific caspase inhibition on local anesthetic-induced chondrotoxicity – CCK-8 assay

The CCK-8 assay showed a similar pattern to the MTT assay (**Fig. 11**). Inhibition of all three caspases did not inhibit bupivacaine chondrotoxicity, although there was a significant difference between the bupivacaine only treatment and the group treated with caspase-9 inhibitor (P = 0.013). Levobupivacaine had a similar response, as the cell viability for the groups treated with caspase-3 (P = 0.981), caspase-8 (P = 0.790), and caspase-9 (P > 0.999) inhibitors was similar to that of the levobupivacaine only treatment. Similar to the MTT assay results, ropivacaine was the most responsive to caspase inhibition. Inhibition of caspase-3 did not increase chondrocyte viability compared to the ropivacaine only group (P = 0.996); whereas inhibition of caspase-8 and caspase-9 significantly increased the chondrocyte viability from 68 ± 6.08% to 90.33 ± 4.5%1 (P = 0.006) and 90 ± 7.55% (P = 0.007), respectively.

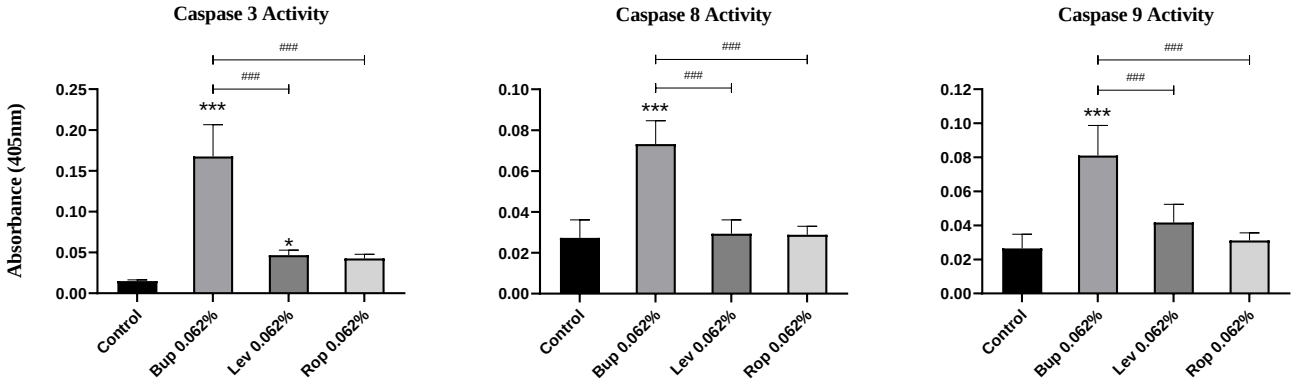

Fig. 9: **Effects of bupivacaine, levobupivacaine and ropivacaine on caspase-3, caspase-8, and caspase-9 activity.**

Bupivacaine significantly increased caspase-3, caspase-8, and caspase-9 activity (***$P < 0.001$). Levobupivacaine induced significant increase in caspase-3 ($P = 0.03$) activity but did not significantly upregulate caspase-8 ($P = 0.95$) and caspase-9 ($P = 0.08$) activity. While ropivacaine had comparative levels of caspase-3 activity with levobupivacaine, it was not significantly different compared to the control ($P = 0.06$). Similarly, ropivacaine did not upregulate caspase-8 ($P = 0.986$) and caspase-9 activity ($P = 0.892$) compared to the control. Bupivacaine significantly upregulated all three caspases compared to levobupivacaine and ropivacaine ($^{###}P < 0.001$).

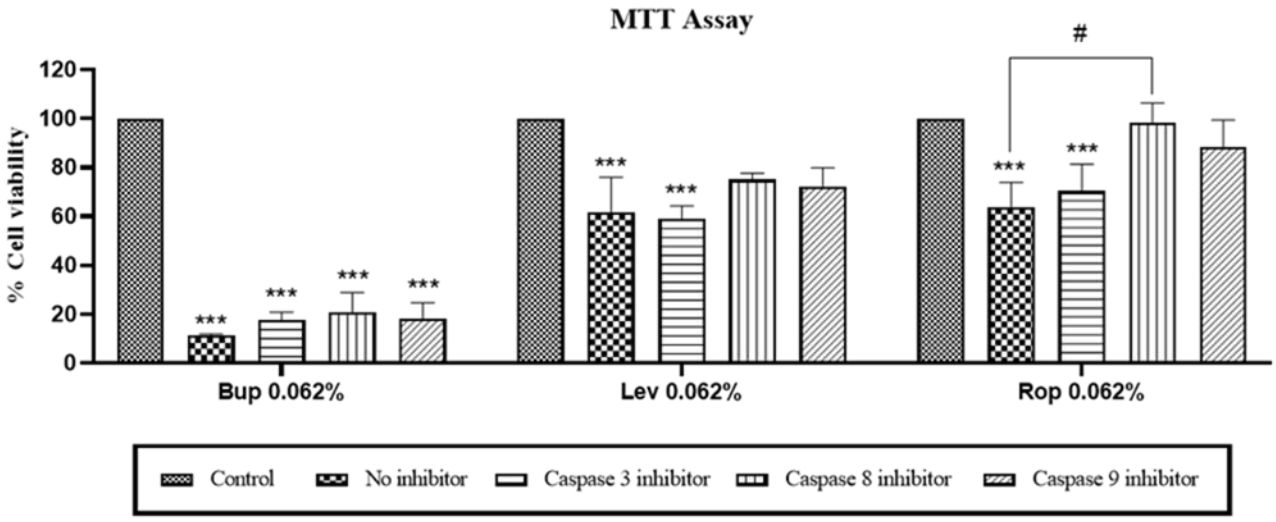

Fig. 10: **MTT viability assay results showing the effects of caspase inhibition on local anesthetic chondrotoxicity.**

Inhibition of caspase-3 did not suppress chondrotoxicity for any of the three local anesthetics as cell viability was still significantly lower than the control (***$P < 0.001$). Caspase-8 and caspase-9 inhibition also did not suppress bupivacaine chondrotoxicity as it was still able to substantially decrease chondrocyte viability compared to the control (***$P < 0.001$). Inhibition of caspase-8 and caspase-9 however mildly attenuated levobupivacaine chondrotoxicity although this change was not significant. Caspase-8 inhibition significantly increased chondrocyte viability compared to the ropivacaine only treatment ($^{\#}P = 0.013$) and Caspase-9 inhibition also markedly decreased ropivacaine chondrotoxicity compared to the ropivacaine only treatment ($P = 0.07$).

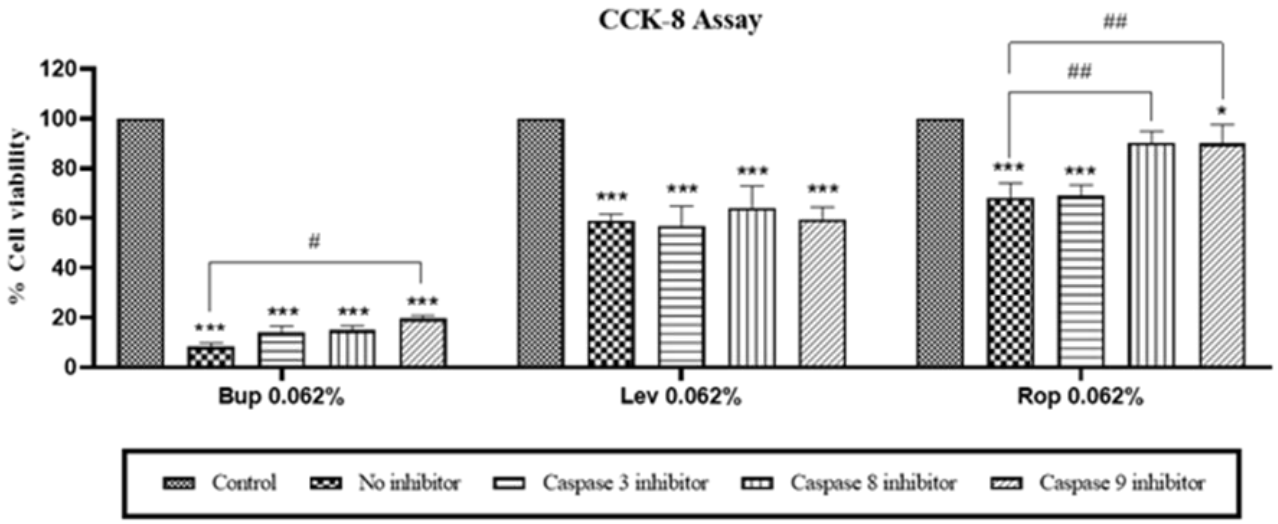

Fig. 11: **CCK-8 viability assay results showing the effects of caspase inhibition on local anesthetic chondrotoxicity.**

Caspase inhibition did not attenuate bupivacaine and levobupivacaine chondrotoxicity as the cell viability for all treatment groups was still significantly lower than that of the control group (***$P <$ 0.001). However, inhibition of caspase-9 significantly increased chondrocyte viability relative to the bupivacaine only treatment ($^{\#}P$ = 0.013). In contrast, caspase inhibition attenuated ropivacaine chondrotoxicity. Although chondrocyte viability was still significantly lower than the control even with the caspase-9 inhibitor (*P = 0.033), chondrocyte viability significantly increased compared to the ropivacaine only treatment following caspase-8 ($^{\#\#}P$ = 0.006) and caspase-9 ($^{\#\#}P$ = 0.007) inhibition. Similar to the MTT assay, caspase-3 inhibition did not attenuate chondrotoxicity for any of the three local anesthetics including ropivacaine.

3.5. Discussion

While many studies have demonstrated the chondrotoxicity of local anesthetics (Adler et al., 2021; Chu et al., 2006; Gomoll et al., 2006; Kaewpichit et al., 2019; Lo et al., 2009), the mechanism of their cytotoxicity has not yet been fully elucidated (Grishko et al., 2010; Kaewpichit et al., 2019). Because local anesthetics are a mainstay in pain management, it is important to understand their effects on cell metabolism and function so as to provide appropriate recommendations for their safe application in veterinary clinical practice. In the second chapter, the results showed that the effects of bupivacaine, levobupivacaine and ropivacaine on chondrocyte viability and caspase activity were dependent on the type of local anesthetic drug that the cells were treated with. These findings suggest that the cell death pathways involved in local anesthetic-induced chondrotoxicity may also be dependent on the type of drug that the chondrocytes are exposed to during treatment.

All three viability assays used in the present study showed a drug-type dependent chondrotoxic effect. Although only cells treated with bupivacaine were detached from the culture plate and had taken up the EthD-1 stain with the live/dead assay (**Fig. 7)**, cluster formation observed with the levobupivacaine and ropivacaine treatments was also a sign of chondrotoxicity. Within the joint, cell organization in clusters can occur in normal articular cartilage, but it can also represent tissue responses to chemical or mechanical injury and presents prominently in cartilage affected by diseases such as osteoarthritis (Lotz et al., 2010). For example, in osteoarthritis, chondrocyte clusters containing proliferative cells that are also in apoptosis are formed in the early stages as a response to changes in the microenvironment (Lotz et al., 1999; Maldonado & Nam, 2013). Therefore, the change in morphology observed with the live/dead assay was likely an early response to chondrocyte injury and/or a sign of early apoptosis due to levobupivacaine and ropivacaine chondrotoxicity in the present study. The MTT and CCK-8 assays were both used to validate the effects of the local anesthetics on

cellular dehydrogenase activity because the MTT assay only measures mitochondrial activity whereas CCK-8 involves most of the dehydrogenase in the cell. All three local anesthetics significantly reduced chondrocyte viability compared to the control ($P < 0.001$), with bupivacaine being the most chondrotoxic compared to levobupivacaine ($P < 0.001$) and ropivacaine ($P < 0.001$). Although the level of chondrotoxicity was similar between levobupivacaine and ropivacaine with the MTT assay ($P > 0.999$), ropivacaine was the least chondrotoxic among the three local anesthetics. This was clearly demonstrated with the CCK-8 assay where the viability of the cells treated with ropivacaine was significantly higher than that of cells treated with bupivacaine ($P < 0.001$) and levobupivacaine ($P = 0.001$). Similar to our findings, previous studies have shown that ropivacaine is generally less chondrotoxic compared to other local anesthetics (Breu, Rosenmeier, et al., 2013; Grishko et al., 2010; Kaewpichit et al., 2019; Piper & Kim, 2008). On the other hand, comparisons between bupivacaine and levobupivacaine have had mixed results. While bupivacaine is generally known to be the most chondrotoxic local anesthetic (Holder et al., 2022; Kreuz et al., 2018), levobupivacaine has been shown to be more chondrotoxic (Cobo-Molinos et al., 2014); less chondrotoxic (Çevik et al., 2018); or having similar effects on chondrocyte viability as bupivacaine (Kaewpichit et al., 2019). Further studies are required to clarify the reasons for these differences; however, this was not the focus of the current study. Because the chondrotoxic effects of bupivacaine, levobupivacaine and ropivacaine have been established in previous studies (Adler et al., 2021; Grishko et al., 2010; Gungor et al., 2014; Kaewpichit et al., 2019), the first part of the experiments was done as a preliminary step to confirm their chondrotoxicity before evaluating the pathways of apoptosis involved through caspase activity.

The ability of local anesthetics to induce chondrocyte death through apoptosis has previously been demonstrated (Breu, Rosenmeier, et al., 2013; Çevik et al., 2018; Grishko et al., 2010). However, studies on whether this chondrotoxicity is through the intrinsic or extrinsic

pathways of apoptosis are limited. In the second chapter, bupivacaine, levobupivacaine and ropivacaine all induced apoptosis as demonstrated by increased caspase-3 activity compared to the control. Although only bupivacaine ($P < 0.001$) and levobupivacaine ($P = 0.03$) caused a significant increase, ropivacaine also markedly increased caspase-3 activity ($P = 0.06$) compared to the control (**Fig. 9)** showing that it had induced apoptosis to a lesser degree than bupivacaine and levobupivacaine. Caspase-8 and caspase-9 activity were evaluated to further assess whether apoptosis was induced through the extrinsic or intrinsic pathways, respectively. Their level of activation was dependent on the type of local anesthetic, as only bupivacaine significantly increased caspase-8 and caspase-9 activity compared to the control ($P < 0.001$); and it also caused the highest increase in their activity compared to levobupivacaine ($P < 0.001$) and ropivacaine ($P < 0.001$). Levobupivacaine appeared to moderately increase caspase-9 activity, but this effect was not statistically different compared to the control ($P = 0.130$) or to ropivacaine ($P = 0.402$). Grishko et al. (2010) showed that lidocaine, bupivacaine and ropivacaine induced apoptosis and mitochondrial damage in human chondrocytes through caspase-3 and caspase-9 cleavage in a drug-type dependent manner (Grishko et al., 2010). Similarly, Tian and Li demonstrated that lidocaine, bupivacaine and ropivacaine all upregulated caspase-3 and caspase-9 activity in human chondrocytes (Tian & Li, 2016). Both of these studies used much higher concentrations of ropivacaine (0.2. 0.5, and 0.75%) which could have induced a much higher response in caspase activity compared to the findings of the current study. Additionally, both studies used western blot to evaluate caspase activity, which was likely more sensitive in detecting caspase activation compared the method used in our analyses. Therefore, these differences in the effect of ropivacaine on caspase activity were likely due to variations in the experimental conditions and types of analyses used between the current and previous studies. Previous information on the effects of levobupivacaine on caspase-3 and caspase-9 activation is however limited. In the present study, bupivacaine significantly

increased caspase-8 activity ($P < 0.001$), unlike levobupivacaine ($P = 0.974$) and ropivacaine ($P = 0.986$) whose levels were similar to the control. This was an interesting finding as it was hypothesized at the beginning of the study that all three local anesthetics used in the second chapter would induce apoptosis through the intrinsic pathway and therefore upregulate caspase-9 activity. Studies on the effects of local anesthetics, particularly levobupivacaine and ropivacaine, on caspase-8 activity in canine articular chondrocytes are limited. However, similar to our findings, a previous study demonstrated that bupivacaine could activate caspase-3, caspase-8, and caspase-9 in a human leukemia cell line (Unami et al., 2003). This effect of bupivacaine on caspase-8 activation demonstrates that it is able to activate both the extrinsic and intrinsic pathways of apoptosis. It is also important to note that apart from directly activating caspase-3, caspase-8 also activates proteins that mediate mitochondrial apoptosis and subsequently activate caspase-9 (Kantari & Walczak, 2011; Tummers & Green, 2017), thereby inducing the intrinsic pathway of apoptosis. As such, there is also a possibility of cross-interaction between intrinsic and extrinsic apoptosis through caspase-8 activity in this case. This effect of bupivacaine on caspase activation in the present study demonstrated that the specific pathways of apoptosis activated, and the level of caspase activation may be dependent on the type of local anesthetic used to treat the cells.

To assess whether caspase inhibition would suppress local anesthetic-induced chondrotoxicity, caspase-3, caspase-8 and caspase-9 inhibitors were used for each local anesthetic. Inhibition of all three caspases did not affect bupivacaine chondrotoxicity although the cell viability relatively increased compared to the treatment without the inhibitors. With the CCK-8 assay (**Fig. 11**), there was a significant difference in cell viability between the bupivacaine only treatment and the treatment with the caspase-9 inhibitor ($P = 0.013$). However, there was still a substantial decrease in chondrocyte viability compared to the control ($P < 0.001$) even after treatment with the inhibitors. Inhibition of caspase-8 and caspase-9

moderately suppressed levobupivacaine chondrotoxicity with the MTT assay (**Fig. 10**), but not with the CCK-8 assay (**Fig. 11**). Nonetheless, caspase inhibition did not completely suppress levobupivacaine chondrotoxicity compared to the control. With severe cellular damage, local anesthetics can induce necrosis, which is irreversible and cannot be blocked (Eduardo et al., 2018), unlike apoptosis which is reversible (Pountos & Giannoudis, 2017). Therefore, these findings could have resulted from bupivacaine and levobupivacaine activating other cell death pathways, such as necrosis, in addition to apoptosis. Ropivacaine was the most responsive to caspase inhibition, with a marked increase in cell viability following caspase-8 and caspas-9 inhibition with both the MTT and CCK-8 assays. Because of its lower cytotoxic potential, it is likely that ropivacaine chondrotoxicity was reversible because it did not cause severe cell damage compared to bupivacaine and levobupivacaine. Inhibition of caspase-3 did not have a marked effect on any of the local anesthetics. Although caspase-3 is the most well characterised effector caspase, other effector caspases including caspase-6 and caspase-7 are also involved in the process of apoptosis (Kiraz et al., 2016; Parrish et al., 2013). Therefore, inhibition of one effector caspase may not have been enough to block other cell death mediators activated by the initiator caspases (caspase-8 and caspase-9). Surprisingly, levobupivacaine and ropivacaine responded to both caspase-8 and caspase-9 inhibition although they did not significantly increase caspase-8 and caspase-9 activity. This suggests that caspase-8 and caspase-9 could be involved in levobupivacaine and ropivacaine chondrotoxicity. However, the colorimetric assays used in the present study may have not been sensitive enough to detect their activation. As such, the experimental design was a limitation for this study. While we were able to show the effects of bupivacaine, levobupivacaine and ropivacaine on chondrocyte viability and caspase activity, apoptosis is a complex process that involves multiple pathways and mediators that were not included in the present study. As such, further investigations are warranted to clarify the biomolecular mechanisms involved in the outcomes observed in this chapter.

The differences between the effects of bupivacaine, levobupivacaine and ropivacaine on caspase activity observed in the second chapter of the present thesis could have been as a result of the differences in their 3-dimensional (3-D) structures. Commercially available bupivacaine is a racemic mixture of dextrorotatory (R+) and levorotatory (S-) stereoisomers; and levobupivacaine is the pure levorotatory enantiomer of bupivacaine (Casati & Baciarello, 2008). Ropivacaine is a derivative of bupivacaine and is also available as the pure S(-)-enantiomer of propivacaine (Casati & Baciarello, 2008; Kuthiala & Chaudhary, 2011). Although the three local anesthetics are structurally similar, significant differences can exist in their interaction with biological receptors, due to differences in spatial configuration, resulting in levobupivacaine and ropivacaine having less cytotoxic effects than bupivacaine (Casati & Putzu, 2005; Leone et al., 2008). Administration of a racemate like bupivacaine can give rise to a range of diverse effects, depending on the pharmacological profile of each enantiomer and its distribution and metabolism (McLeod & Burke, 2001). The biological properties of enantiomers can differ because of interaction with chiral endogenous molecules constituting proteins such as receptors and enzymes (Tucker, 2000). Therefore, while the two enantiomers are exactly similar in physicochemical properties, they can have different affinity for the site of action or the side effects (Athar et al., 2016). Because of this, pure S(-)-enantiomers such as ropivacaine and levobupivacaine are less toxic in comparison to the R(+)-enantiomer or racemic mixtures (Casati & Putzu, 2005; Leone et al., 2008). Therefore, in this case, bupivacaine may have been able to interact with both mitochondrial and plasma membrane cell death receptors and caused more severe cellular damage compared to levobupivacaine and ropivacaine as a result of differences in their conformation.

In conclusion, the findings in the present chapter demonstrated that local anesthetics induce apoptosis through both the extrinsic and intrinsic pathways. The type of caspase activated, and the level of caspase activation is dependent on the type of local anesthetic used.

Bupivacaine was the most chondrotoxic local anesthetic for all the assays used, followed by levobupivacaine and ropivacaine was the least chondrotoxic. These findings suggest that ropivacaine may be safer for intra-articular administration compared to bupivacaine and levobupivacaine during clinical applications. Further studies are required to investigate the specific mechanisms involved in the effects of local anesthetics on apoptosis induction and regulation.

4. General discussion

Although local anesthetics have been used widely for decades, the interaction of local anesthetics with cellular function is a more recent focus of study (Gordon et al., 2010). Local anesthetics play a unique role in clinical pain management and are a potent drug of choice that can be used for both unimodal and multimodal analgesic protocols, as an alternative or adjunct for general anesthetics and systemic analgesics. This is because they can be directly applied to a specific area of interest and exert clinical effects without the need for systemic administration. As a result, they can be used to induce analgesia within a targeted and/or restricted part or region of the body. Additionally, when used as adjuvants, their localised administration reduces the frequency and dosage of other systemic analgesic drugs required for adequate pain management (Borer, 2006). This decreases the likelihood of unwanted adverse- or side-effects associated with systemic administration of drugs such as opioids and anti-inflammatory drugs (Borer, 2006; Grubb & Lobprise, 2020b). Furthermore, local anesthetics are widely considered safe, with adverse events generally limited to very high doses or inadvertent IV administration such as those associated with bupivacaine (Epstein et al., 2015). These properties make local anesthetics an important part of day-to-day procedures and interventions in both human and veterinary clinical practice.

In chapter 1, the chondrotoxicity of bupivacaine at low concentrations was assessed by evaluating its effect on canine articular chondrocyte viability. Bupivacaine was selected because it is one of the most commonly used local anesthetics in both human and veterinary patients and it is generally known to be the most chondrotoxic local anesthetic (Holder et al., 2022; Kreuz et al., 2018). Intra-articular bupivacaine administration is a highly popular but increasingly controversial practice in veterinary and human orthopedics, particularly joint surgery (Barry et al., 2015). While the findings showed that bupivacaine was chondrotoxic at low concentrations, these findings were an estimate of its effects and not an exact reflection of

how it would affect articular chondrocytes under *in vivo* conditions. This is because in addition to chondrocytes, joint structures are made up of multiple cell and tissue types that have complex interactions and can affect the metabolism of drugs within the joint. Chondrocytes are the sole constituent cell of articular cartilage and they are responsible for the synthesis and turnover of the cartilage extracellular matrix (ECM) in which they are embedded (Archer & Francis-West, 2003; Lin et al., 2006). Nutrition of the articular cartilage occurs by diffusion from the synovial fluid through the matrix (Bhosale & Richardson, 2008; Sophia Fox et al., 2009). Therefore, several factors such as drug dispersion through joint tissues, rate of diffusion and clearance of synovial fluid from the joint, viscosity and protein content of the synovial fluid and metabolic processes of surrounding cells and tissues can affect how chondrocytes respond to local anesthetics. Also, it has been demonstrated that analgesia from intra-articular bupivacaine can last up to 6 hr in dogs (Hoelzler et al., 2005), which is a longer period than it takes for the drug to be cleared from the synovial fluid (Barry et al., 2015) following intra-articular injection. It is therefore possible that the local anesthetic drug can persist within the intracellular space long after the drug has been cleared from the joint space and continue exerting cytotoxic effects for an extended period of time. Additionally, commercial preparations of local anesthetics contain additives such preservatives and adjuvants that can potentially affect chondrocyte viability and could not be accounted for in the experimental design used in the first chapter. However, the study was used to investigate whether bupivacaine treatments similar to synovial fluid concentrations would still have chondrotoxic effects, because studies on its chondrotoxicity typically involve cell cultures incubated in solutions containing high bupivacaine concentrations for long durations (Barry et al., 2015). Therefore, the findings in chapter 1 established that low concentrations were chondrotoxic, especially with prolonged exposure.

After determining, the *in vitro* chondrotoxicity of bupivacaine at low concentrations, chapter 2 assessed the comparative chondrotoxic effects of bupivacaine and its two analogues,

levobupivacaine and ropivacaine. This was done to evaluate whether the level of levobupivacaine and ropivacaine chondrotoxicity would be similar to that of bupivacaine at the same concentration and period of exposure. Since bupivacaine has been shown to have an unsatisfactory safety profile, the development of levobupivacaine and ropivacaine was prompted by the need for a wider safety margin, while preserving the desirable pharmacodynamic properties of bupivacaine (Casati & Baciarello, 2008). They both have a clinical profile similar to that of racemic bupivacaine, and the minimal differences reported between the three anesthetics are mainly related to the slightly different anesthetic potency, with racemic bupivacaine > levobupivacaine > ropivacaine (Leone et al., 2008). Therefore, comparing their effects both *in vitro* and *in vivo* is helpful in deciding which drug can be most effective in pain management while having the least probability of inducing adverse effects. The three local anesthetics had differential effects on chondrocyte viability and caspase activity. This was attributed to the differences in the spatial conformation of commercially available preparations of bupivacaine, levobupivacaine and ropivacaine. Bupivacaine exists in two enantiomers, which are mirror images of each other (Taylor & McLeod, 2020). Although structurally identical, enantiomers can exhibit clinical differences including potency and adverse effects (Taylor & McLeod, 2020). This is important because levorotatory (S-) isomers have less potential for systemic toxicity than the dextrorotatory ones (Casati & Putzu, 2005). Therefore, levobupivacaine, the S(-)-isomer of bupivacaine; and ropivacaine, the S(-)-isomer of 1-propyl-2',6'-pipecoloxylidide (the propyl homolog of bupivacaine) are potentially less toxic alternatives to bupivacaine (Stewart et al., 2003). The spatial conformation of local anesthetics may affect the interaction of a molecule with its receptors, determining a greater affinity to certain subtypes compared to others (Casati & Baciarello, 2008). Additionally, ropivacaine's propyl group gives it a lower lipid solubility which results in decreased potential for cytotoxicity (Kuthiala & Chaudhary, 2011; Taylor & McLeod, 2020).

The studies performed as part of the present dissertation were *in vitro* models using cultured chondrocytes; therefore, the experimental conditions were not the same as those of a normal diarthrodial joint in terms of structure and physiology. This may have therefore affected chondrocyte response to treatment with the local anesthetics. As such, *in vivo* clinical studies to clarify the clinical implications of local anesthetic chondrotoxicity and how these adverse effects can be managed are warranted. Additional studies on the synovial fluid concentrations of local anesthetics following intra-articular injection are also necessary to evaluate the minimum amount of drugs required to induce nociception and determine the margin of safety. Additionally, *in vivo* studies would be required to determine safe anesthetic and analgesic protocols involving intra-articular local anesthetics while maintaining clinical efficacy. Further studies to investigate the biomolecular processes involved in local anesthetic chondrotoxicity and the mechanisms responsible for the differences in their level of chondrotoxicity are also warranted.

In conclusion, the *in vitro* chondrotoxicity of local anesthetics in cultured canine articular chondrocytes was dependent on the time of exposure, the concentration of the drug and the type of local anesthetic that the chondrocytes were exposed to in culture. Additionally local anesthetics were able to activate both the intrinsic and extrinsic pathways of apoptosis in a drug-dependent manner. These findings showed that the level of toxicity and the cell death pathways induced by local anesthetics are dependent on the type of drug. Of the three local anesthetics evaluated, bupivacaine was the most chondrotoxic followed by levobupivacaine, and ropivacaine was the least chondrotoxic. As such, ropivacaine may be a safer choice for intra-articular local anesthesia in clinical applications compared to bupivacaine and levobupivacaine.

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