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Chondrocyte death in canine osteochondral explants exposed to 0.5 percent bupivacaine

Geoffrey Stuart Hennig
Louisiana State University and Agricultural and Mechanical College

CHONDROCYTE DEATH IN CANINE OSTEOCHONDRAL EXPLANTS EXPOSED TO 0.5 PERCENT BUPIVACAINE

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Geoffrey Stuart Hennig
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Objective: Our in vitro study evaluated chondrocyte death in canine articular cartilage exposed to 0.5% bupivacaine without (B) and with methylparaben (BP) and compared the viability with intact (SI) and mechanically debrided surfaces (SR). We hypothesized that B or BP would cause chondrocyte death in vitro in a time dependent manner and that chondrocyte death would be increased in SI osteochondral cores compared to SR.

Materials and Methods: Intact cartilage cores with underlying subchondral bone were collected aseptically from both humeral heads of 16 fresh canine cadavers. Joints from each dog were randomly assigned to SI or SR. Ten osteochondral cores were harvested from each humeral head. Synovium and one core were examined to verify joint health. The remaining 9 cores were exposed at random to canine chondrocyte culture media (CCCM), B, or BP for 5, 15, or 30 minutes. The pH of each solution was measured at the beginning and end of each treatment. The treated cartilage was stained with ethidium and calcein and digital images were captured under laser confocal microscopy. Three zones of each core (superficial, middle, deep) were examined. The number of dead/ (live+dead) chondrocytes were counted with digital image editing software. The proportion of dead cells (chondrocyte death) was expressed as a percentage and transformed to follow a normal distribution for analysis.

Results: Superficial zone of SI—The BP had significantly higher %cell death at 5 minutes (47.7%) than B (23.6%) or CCCM (25.4%) but by 30 minutes B (53.8%) and BP (62.5%) was similar while CCCM (20.0%) remained unchanged and significantly lower. Superficial zone of SR—The BP had significantly higher %cell death at 30 minutes (59%) compared to 5 minutes (37.7%); The BP had significantly higher %cell death at 30 minutes (59%) than CCCM (28.9%). The pH was significantly lower for B (5.81-6.52) and BP (5.36-6.36) than CCCM (7.51-7.85).

Conclusions and Clinical Relevance: This study shows a cytotoxic effect of 0.5% bupivacaine with and without preservative on canine articular chondrocytes in a time dependant manner in vitro. Intra-articular administration of 0.5% bupivacaine with or without preservative should be used with caution.

CHAPTER 1: BACKGROUND AND REVIEW OF THE LITERATURE

Bupivacaine is an amide local anesthetic with an intermediate onset of action, a high potency due to its lipid solubility, and a long duration of action. When local anesthetics are combined with systemic analgesics, less systemic analgesics are required and negative side effects of these drugs can be minimized[1,2]. Intra-articular bupivacaine is an effective analgesic in the perioperative period in people, sheep, and dogs[1-4]. It is used as a component of multimodal perioperative pain management after arthrotomy or arthroscopy in dogs [1,2]. Currently accepted intra-articular administration protocols in dogs employ bupivacaine preserved with methylparaben or preservative-free bupivacaine at 0.25% or 0.5% concentrations[1,2,5].

Bupivacaine is used for intra-articular analgesia despite clear understanding of its effects on articular cartilage. Use of intra-articular bupivacaine in dogs has been considered safe due to reports in previous studies[6-8]. In an in vivo study in pigs and dogs, structural damage to chondrocytes was not evident up to 4 to 6 days after intra-articular administration of varying concentrations of bupivacaine in saline solution[6,7], although proteoglycan synthesis was inhibited for at least 3 days[6]. Cartilage degeneration was not apparent following intra-articular administration of bupivacaine in dogs after 1, 14, and 28 days based on histopathologic evaluation[8]. These results are open to interpretation due to the subjective nature of histopathologic evaluation and grading and the assumed correlation between gross appearance and cellular function. To date, no studies have been performed assessing whether bupivacaine causes chondrocyte death.

Despite the aforementioned studies, more recent case reports of complications in people and investigations on bovine, rabbit, and human cartilage have raised concerns that intra-articular administration of bupivacaine may result in chondrolysis and subsequent rapid onset of osteoarthritis[9-16]. In vitro exposure of human, bovine, and rabbit articular cartilage to bupivacaine resulted in chondrocyte death[9,13-16]. In a clinical trial investigating intra-articular administration of 0.5% bupivacaine in rabbits, articular cartilage damage and synovial inflammation were observed over 10 days[9]. In vitro exposure of bovine cartilage to 0.5% bupivacaine significantly increased chondrocyte death[14]. Chondrocyte death was increased to a lesser extent in intact cartilage (26%) compared to cartilage with mechanically disrupted articular surface (75%)[14]. In vitro mixture of bupivacaine with methylparaben preservative and human osteoarthritic synovial fluid resulted in a crystalline precipitate in half of the four samples tested[6]. When human

articular chondrocyte cultures were exposed to 0.25% bupivacaine for 15 minutes, significant time and dose dependent chondrocyte death occurred[15].

Considering that loss of chondrocytes can result in development of chondrosis and osteoarthritis[17], the recent evidence that intra-articular use of bupivacaine results in chondrocyte death in some species is disconcerting[9-16]. Although increased chondrocyte death has been observed after human, bovine and rabbit articular cartilage is exposed to bupivacaine[9,13-16], these results can not be directly extrapolated to dogs due to species variation in cartilage composition and physiology. Furthermore, the effect of the pH or the preservative methylparaben on chondrocyte death in dogs is unknown. The purpose of our in vitro study was to measure chondrocyte death in articular cartilage of dogs exposed to 0.5% bupivacaine with and without methylparaben preservative and to compare the effect on intact and mechanically debrided articular surfaces. We hypothesized that exposure of canine articular cartilage to 0.5% bupivacaine would increase chondrocyte death in a time-dependent manner over 5 to 30 minutes and that chondrocyte death would be increased to a lesser extent in surface intact cartilage cores compared to those with the surface mechanically debrided.

1.1 Bupivacaine

Local anesthetics act by reversibly blocking the propagation of action potential along nerve axons by reversibly binding to voltage-gated sodium channels[18-20]. They are relatively unique in that their application is applied directly to the target tissue, meaning that systemic circulation is not required for the desired effect. Local anesthetics prevent or reduce pain or nocioceptive input by interrupting neural transmission in sensory afferent nerves or tracts after local tissue infiltration, regional nerve blocks, or epidural or intrathecal (subarachnoid) injection[20]. The use of local anesthetics offer additional benefit to the patient by avoiding general anesthesia or reducing the amount of general anesthetics required to perform various procedures.

Bupivacaine has the chemical name of 1-Butyl-2', 6 pipecoloxylidide-HCl (Figure 1.1). It may be dosed at 0.25% for infiltration, 0.5% for nerve and epidural blocks[20]. It has an intermediate onset of action and a duration of 3-10 hours[20]. It has a lipid solubility of 30 (procaine is 1) and an acid dissociation constant (pKa) of 8.1. It is 95% protein bound and has a molecular weight of 288 daltons[20].

1.1.1 History of Local Anesthetics

Cocaine was the first clinically used local anesthetic[20]. This plant alkaloid was extracted from the coca leaf originally found in the Andes Mountains. The local inhabitants of the region

would chew or suck the leaves and achieve a sense of well-being. Gaedicke first extracted erythroxylin (the coca plant is also known as *Erythroxylon coca*) from the coca leaf in 1855. Later, Niemann isolated cocaine in 1860. The first clinical use of cocaine as a local anesthetic was by a Peruvian army surgeon[20]. Koeller first reported cocaine's use as an ophthalmic local anesthesia in 1884[21]. Following its applications as an ophthalmic anesthetic, it was then applied as a peripheral nerve block by Halsted and for spinal anesthesia by Bier[20].

Due to cocaine's addictive and toxic side effects, the first synthetic local anesthetic was developed. Procaine, an ester of para-aminobenzoic acid, was developed by Einhorn in 1904 and led to the formulation of other benzoic acid derived local anesthetics. Other amino-ester local anesthetics were then synthesized including tetracaine in 1932 and chloroprocaine in 1955. In 1943, Lofgren developed lidocaine, an amide derived from diethylaminoacetic acid. More local anesthetics were developed including mepivacaine in 1956, bupivacaine in 1957, prilocaine in 1959, etidocaine in 1971, articaine in 1974, and ropivacaine in the 1980's[19,22,23]. More recently, levobupivacaine, the pure S- (-) enantiomer, has been developed and reportedly is less likely to cause side effects[19]. Levobupivacaine has less affinity for brain and myocardial tissue than either the R- (+) enantiomer or the racemic bupivacaine[24].

1.1.2 Properties of Local Anesthetics

1.1.2.1 Chemistry of Local Anesthetics

Most local anesthetics used clinically have similar chemical structures and physical properties[19,20]. Most of the local anesthetics are weak basic tertiary amines, but a few secondary amines do exist. Amines are composed of a nitrogen atom with three organic groups attached[20]. Anesthetic molecules are generally made up of a hydrophilic end connected to a lipophilic end by an intermediate hydrocarbon chain (Figure 1.2). The hydrophilic end, commonly amino derivatives of ethyl alcohol or acetic acid, is responsible for the water solubility. The aromatic lipophilic end, which is derived from benzoic acid or aniline, contributes to the lipid solubility. Substitution of alkyl groups on the aromatic ring or amine end increases lipid solubility and potency[19].

1.1.2.2 Grouping of Local Anesthetics

Broad categorizations of local anesthetics are based on the molecule's intermediate linkage and are classified as ester-linked (cocaine, procaine, benzocaine, tetracaine, chloroprocaine) or amide-linked (lidocaine, prilocaine, dibucaine, articaine, etidocaine, bupivacaine, mepivacaine, ropivacaine)[19,20]. These broad categories determine how the drugs are

biotransformed. Ester-linked local anesthetics are derived from benzoic acid and amide-linked are derived from aniline. Ester-linked local anesthetics are hydrolyzed plasma cholinesterase and have short half-lives when stored in solution without preservatives. Amides are very stable and cannot be hydrolyzed by plasma cholinesterases. They undergo biotransformation via hepatic microsomal enzymes.

Local anesthetic behavioral characteristics (onset of action, duration of action, and potency) are based on the chemical properties of the molecular structure[19,20]. The potency of a local anesthetic is greatly influenced by the molecule's size and lipid solubility[25]. The smaller the molecule and the more lipophilic it is, the more readily it will penetrate the axonal membrane and bind the sodium channels with greater affinity. Given that the membranes of axons have a high lipid concentration, local anesthetics have a strong effect on these structures. If the methyl group on the lipophilic end of the amide-linked local anesthetic mepivacaine is replaced with a butyl group, the result is bupivacaine. Bupivacaine is 30 times more lipid soluble and 8 times more potent than procaine and 15 times more lipid soluble and 4 times more potent than mepivacaine[19,20].

Local anesthetic duration of activity is believed to be due to its respective protein binding properties and the vasoactivity of the drug[19,20]. For the given local anesthetic, the greater the protein binding affinity to the axonal membrane protein, the longer the duration of local anesthetic activity. More lipid-soluble local anesthetics are relatively water insoluble and highly protein bound. The duration of effect is inversely related to the rate of systemic absorption. The rate of vascular absorption is variable and changes based on the vascularity of the tissue at the injection site and the properties and dose of the local anesthetic. Bupivacaine is highly lipid-soluble, it is slowly washed out from isolated nerves and it is not readily removed by the bloodstream from nerve membranes, which contributes to the long duration of action[19]. Bupivacaine is 16 times more protein bound and its anesthetic duration of effect is 3 to 6 times longer than procaine.

The speed of onset of action of local anesthetics is most likely associated inversely with the lipid solubility and its acid dissociation constant (pKa)[19]. Local anesthetic agents exist in solution in both the charged cationic (+) and the uncharged base forms. The percentage of local anesthetic molecules present in the uncharged non-ionized base form, which is primarily responsible for membrane permeability, decreases with increased pKa at any given tissue pH[26]. It

is suspected that the base is responsible for onset of action because the charged form diffuses readily across the nerve membrane.

1.1.2.3 Mechanism of Action

Local anesthetics block the formation of an action potential by blocking the influx of sodium into the nerve axons[18-20]. The resting membrane potential is the difference between the inside and the outside of the nerve cell and is maintained at -70mV. The resting membrane potential is maintained by the plasma membrane sodium/potassium (Na^+/K^+) pump[18]. This pumps 3 Na^+ out of the cell for every 2 K^+ pumped into the cell. An action potential is generated when a change in membrane permeability permits large amounts of sodium to pass through sodium-gated channels in the cell membrane. The action potential is terminated by closure of the sodium channels and opening of the slow potassium channels. The cell repolarizes as potassium passes out of the cell.

Local anesthetics are believed to act primarily on binding receptors located on sodium channels[27]. Each local anesthetic may have its own way of binding to the sodium channel based on the conformational state of the channel. There are families of channels and in a given tissue there may be multiple forms of sodium channels[28]. For example, the sodium channels in the heart, brain, and axons are not identical[20].

1.1.2.4 Differential Nerve Blockade

Local anesthetics have the ability to preferentially provide analgesia or anesthesia without affecting motor function[20,29,30]. This indicates that sensory nerve fibers may be more sensitive to the effects of local anesthetics than the larger motor fibers. Studies have shown that the level of sympathetic blockade extends further than sensory block which extends further than motor function blockade[31]. This suggests that there is a rank order of sensitivity to local anesthetics, implying that preganglionic sympathetic B nerve fibers are more sensitive than small sensory A-delta nerve fibers, which are more sensitive than large A-alpha motor fibers. The sensitivity of unmyelinated C fibers appears to be quite similar to myelinated fibers[29]. For myelinated axons, local anesthetic concentrations must be high enough to block three consecutive nodes of Ranvier to stop electrical transmission[20]. This does not apply to unmyelinated axons. The fiber groups appear to have individual sensitivities with regard to blockade. It is believed that the frequency of axon discharge is related to the sensitivity of local anesthetic effect. Axons with a higher discharge rate, such as C fibers and A-delta fibers, are

more sensitive to local anesthetics than those fibers with lower discharge rates, such as A-beta fibers[30].

1.1.3 Pharmacokinetics of Local Anesthetics

1.1.3.1 Absorption

Absorption of local anesthetics after extravascular infiltration is dependent on a variety of factors including the particular local anesthetic, the patient's hemodynamic condition, the site of administration, the concentration and dose, the vascularity of the site of administration, and the effect of an added vasoactive drug such as epinephrine[32]. The faster the rate of systemic absorption, the shorter the duration of anesthetic activity and the greater the risk for systemic toxicity[20]. For this reason, vasoconstrictors may be added to the local anesthetic infusions to reduce local blood flow and in turn minimize absorption to maximize effect and decrease the risks of systemic toxicity. Epinephrine is the most commonly utilized vasoconstrictor and may be used at concentrations of 1:200,000 (5μg/ml) or 1:400,000 (2.5μg/ml)[20].

Local anesthetics are not routinely injected intravascularly for the purposes of anesthesia. Exceptions include intravenous regional anesthesia of a distal limb and, more recently, the use of low-dose lidocaine constant rate infusions as adjuncts to inhalant anesthetics[33-36].

In some cases, local anesthetics may have a topical effect[20]. They may be applied to the skin, but are only effective if the integrity of the skin is compromised. They may also be effective at providing analgesia to the mucous membranes and the cornea. Recently, lidocaine dermal patches have gained popularity as part of a post-operative multimodal analgesia plan[37,38].

Reports indicate that following intra-articular injections, bupivacaine is absorbed over several hours, with peak serum concentrations within the first hour[39,40]. Some authors advocate decreasing the bupivacaine concentration from 0.5% to 0.25% when the joint is compromised to decrease the risk of systemic toxicity[41]. Following a single intra-articular injection of 1.5mg/kg of 0.5% bupivacaine in dogs, delayed absorption into the systemic circulation occurred resulting in a peak serum concentration at 11 minutes with the absence of clinical signs of toxicity[42].

1.1.3.2 Distribution

Local distribution of anesthetic at the injection site depends on the volume of agent infused, the tissue resistance to the spread of local anesthetic, and the specific drug employed[20]. To enhance the spread of local anesthetic, especially when instilling local anesthesia to the bony orbit to anesthetize the eye, hyaluronidase may be added to the local anesthetic solution[20]. Systemically, the liver and lungs are the major sites of plasma clearance of local anesthetics.

Extraction fractions explain the amount of local anesthetic extracted from the plasma by a given organ[20]. The liver has a high extraction fraction for most local anesthetics (0.75 for lidocaine). A decrease in hepatic blood flow or function may prolong the plasma high-life. The pH may also affect the local distribution of a local anesthetic. Increasing the pH (more basic) increases the ratio of uncharged to charged molecules in solution. The uncharged molecules readily diffuse to the target tissue increasing the speed of onset of action.

The distribution of amino-ester local anesthetics (procaine, chloroprocaine, tetracaine) in body tissues is limited due to their rapid enzymatic hydrolysis by non-specific plasma pseudocholinesterases[19]. Amide local anesthetics (lidocaine, mepivacaine, prilocaine, bupivacaine, levobupivacaine, etidocaine, ropivacaine) are widely distributed in the body after intravenous bolus injection or a fast rate of vascular absorption[19]. Their pharmacokinetic properties are typically described as a two- or three-compartment model[43,44].

1.1.3.3 Elimination

The liver and lungs are major sites for plasma clearance of local anesthetics. Metabolism converts relatively lipid soluble anesthetics into smaller, more water-soluble agents[19]. For esters, the primary step is ester hydrolysis, catalyzed by nonspecific plasma cholinesterases. The rate of plasma hydrolysis is rapid, yielding half-lives measured in seconds, and is inversely related to toxicity[19,45]. Ester metabolism can be slowed by reduced cholinesterase activity during pregnancy and long-term cholinesterase inhibition via poisons, thereby prolonging the clearance of ester anesthetics and increasing the potential for toxicity[19].

The amino-amide are almost exclusively metabolized by the liver and hepatic degradation, which requires conjugation with glucuronic acid[46]. Cats glucuronidate drugs to a lesser extent that dogs, increasing the risks of developing side effects when given amide local anesthetics[46]. Less than 5% of these agents are excreted unchanged in the urine. Mepivacaine, etidocaine, bupivacaine and ropivacaine also undergo N-dealkylation and hydroxylation. They are further conjugated with glucuronide before excretion from the body in the urine or bile[19,45].

Since the liver metabolizes amide local anesthetics, drug clearance is highly dependent on hepatic blood flow, hepatic extraction, and enzyme function. Amide local anesthetic clearance can be slowed or reduced by factors that decrease hepatic blood flow, such as beta-adrenergic or H_2-receptor antagonists, by hypotension during regional and general anesthesia, or by heart or liver failure[19,45].

1.1.3.4 Toxicity

Toxic reactions to local anesthetics may be categorized as systemic or localized. Such reactions are rarely fatal if recognized and treated early. The most common clinical toxicities involve acute reactions involving the central nervous system (CNS) and/or the cardiovascular system. Bupivacaine is considered to have higher levels of cardiotoxicity than other local anesthetics[47,48] and has been documented to cause deaths related to its cardiotoxicity[49]. It is believed that bupivacaine affects the heart differently than other local anesthetics. Most toxicities are due to accidental intravascular injection of local anesthetics[20,49,50]. Needle aspiration of the vessel prior to injection of the dose may minimize these accidents[20].

Signs related to the CNS typically precede cardiovascular changes when local anesthetic toxicity occurs. In humans, the first signs of CNS toxicity are drowsiness, followed by numbness of the lips, slurring of speech, and fine tremors, and with increasing doses, progression to grand mal seizures. A large enough dose could cause generalized CNS depression. Interestingly, lidocaine has been shown to be effective against tonic-clonic and simple partial status epilepticus when combinations of other anticonvulsant agents failure[51]. Conclusive evidence to explain this finding is lacking. Studies indicate that seizures induced by local anesthetics originate in the limbic brain[52]. Local anesthetics produce CNS signs by interfering with sodium conductance by a similar mechanism to their effects on the peripheral nervous system. Studies suggest that the cardiotoxic effects of local anesthetics, especially bupivacaine, are not only directly toxic to the myocardium, but may be partially due to effects on the brain[53].

Local anesthetics affect the cardiovascular system by a number of ways. They may act directly on the heart or on the peripheral vasculature or indirectly by causing conduction blockade on autonomic fibers[20]. Their main effect is directly on the myocardium by decreasing electric excitability, conduction rate, and the force of contraction[54]. The effects on the vasculature are dose dependant. High concentrations of local anesthetics cause vasodilation while low concentrations may cause vasoconstriction[55]. Inhibition of sodium conductance increases appears to play a major role in the cardiac effects of local anesthetics. Studies suggest that part of the cardiotoxic effects of local anesthetics may be attributed to potassium channel blockade[56]. Lidocaine and bupivacaine are commonly compared when evaluating the cardiotoxic effects[57]. Both cause profound cardiac depression, however, bupivacaine toxicity is more difficult to treat than lidocaine toxicity[49]. Animal studies have shown that resuscitation may be successful if treated immediately after bupivacaine intoxication[50]. More recent studies have

shown that elevated progesterone levels (pregnancy)[58], hyponatremia[59], and diabetes mellitus[60] may potentiate the cardiotoxic effects of local anesthetics.

Local anesthetics have also been reported to cause methemoglobinemia, which occurs when the ferrous iron (Fe^{++}) in hemoglobin undergoes oxidation to the ferric form (Fe^{+++}). Although prilocaine is primarily implicated and is the only clinically used secondary amine local anesthetic, benzocaine, lidocaine, and procaine have also been associated with methemoglobinemia[61].

Local anesthetics have been reported to cause local tissue toxicity, resulting in irritation and lysis of cells. Muscle and nerve cells appear the most susceptible to damage[62-64]. Factors such as pH of the solution or the preservatives in the solutions may contribute to the deleterious effects[20]. The highly lipid soluble, highly potent, and long lasting local anesthetics reportedly cause more tissue damage compared to other local anesthetics[65]. According to electron microcopy studies, the perineurium, Schwann cells, and axons are all structural disrupted by local anesthetics[66].

Allergic reactions to local anesthetics are rare and have only been reported in case reports[67]. Allergic reactions are more likely to occur with ester-linked local anesthetics than with amide-linked agents[20]. The preservatives, such as methylparaben, added to local anesthetic solutions may be responsible for the allergic reactions[68].

More recently, chondrolysis is being recognized as a potential complication associated with intra-articular administration of bupivacaine. Several reports in both humans[10-12,69] and veterinary species[9,13,14] suspect an association between intra-articular administration of 0.5% bupivacaine and increased chondrocyte death.

1.1.4 Bupivacaine as an Intra-articular Anesthetic

1.1.4.1 Intra-articular Bupivacaine in People

Bupivacaine has been used routinely as part of a multi-modal peri-operative anesthetic since arthroscopic day-patient care gained popularity[70-75]. Its properties as a local anesthetic are appealing and despite the published toxicities its use as an intra-articular anesthetic is considered safe. The duration of action of intra-articular bupivacaine is reportedly 2 to 4 hours[39,76,77]. Multi-modal analgesia combines drugs that work through different mechanisms or that target different receptors and is currently recommended for perioperative pain management[78-80]. This methodology takes advantage of the additive or synergistic effects of different analgesics, allowing for lower doses of each drug subsequently minimizing adverse effects[75,78,81].

Administration of intra-articular analgesics in combination with systemic analgesics has been used to prolong the effect of perioperative pain control. Bupivacaine has been shown to be an effective intra-articular analgesic when administered at concentrations of 0.25%[4,76,82-87] or 0.5%[88-91]. Bupivacaine has been shown to be an effective sole intra-articular analgesic[76,85-87,90,91]. It can be administered either pre-operatively[86,87,92,93] or post-operatively[4,76,82-85,88-91,94]. It has been used effectively intra-articularly in combination with non-steroidal anti-inflammatory drugs (NSAIDs)[95], opioids[4,84,89,94,96], and corticosteroids[97]. More recently, bupivacaine has been effectively delivered to offer a longer analgesic effect through a variety of indwelling intra-articular catheters[98-100].

1.1.4.2 Intra-articular Bupivacaine in Dogs

Bupivacaine has been used as an intra-articular analgesic in dogs. Intra-articular analgesia using 0.5% bupivacaine and intra-articular morphine were compared in dogs following stifle surgery for ruptured cranial cruciate ligament in dogs[2]. They found that intra-articular administration of either 0.5% bupivacaine or morphine provided better analgesic control than saline, and bupivacaine showed the greatest effect. Comparing analgesia provided by epidural bupivacaine and morphine to either intra-articular 0.5% bupivacaine or intravenous hydromorphone showed a significantly longer time to first dose of rescue analgesics between the epidural and bupivacaine groups compared to the control group and that more supplemental analgesics were required for the control group[1].

1.2 Cartilage[101-103]

Cartilage is a form of connective tissue specialized for a supportive role in the body. Three basic forms of cartilage exist, each type containing chondrocytes within an amorphous ground substance or matrix. This matrix contains sulfated glycosaminoglycans complexed with protein to form proteoglycan macromolecules. Proteoglycans are electrostatically bound to unit fibrils of collagen, forming a firm but flexible substance. In general, this tissue is devoid of vessels, lymphatics, and nerves.

1.2.1 Cartilage Cells

Two types of cells, the chondroblast and chondrocyte, are recognized as cartilage. The chondroblast is found in growing cartilage. Following formation of the cartilage matrix, the chondroblast becomes the less active chondrocyte. The chondrocytes are located within lacuna and are responsible for the ongoing maintenance of the surrounding matrix.

1.2.2 Cartilage Matrix

The matrix of cartilage is similar to other types of connective tissue but there are some unique properties to cartilage matrix. Collagen forms the matrix framework, with type II collagen predominating. Type I collagen is predominate in fibrocartilage. The ground substance contains the glycosaminoglycans (GAGs), chondroitan sulfate, keratan sulfate, and hyaluronic acid. These substances have an important role in transporting water and electrolytes and in binding water to give hyaline cartilage its resiliency. The GAGs form complexes with proteins to form proteoglycans. Aggrecan is formed when proteoglycans are joined to a hyaluronic aid core with linking proteins. A loose network is formed by proteoglycans bound to collagen fibers, which limits the movement of larger cells. The adhesive molecules chondronectin, anchorin, CII, and fibronectin are involved in the interaction between collagen and chondrocytes. The cartilage matrix is mineralized by hydroxyapatite in the zone of hypertrophy of the physis and the deeper regions of the articular matrix.

1.2.3 Types of Cartilage

Fibrous cartilage, or fibrocartilage, is a dense connective tissue with linear groupings of chondrocytes embedded in a small amount of matrix. This type of cartilage occurs least frequently and is found in the intervertebral discs, the menisci of the stifle, in the cardiac skeleton (the atria and ventricles are connected by fibrocartilage), and in some tendons close to their attachments to bone. There is a predominance of type I collagen fibers in the matrix which is most abundant in the vicinity of the cells. Fibrocartilage lacks a distinct perichondrium and a cellular chondrogenic layer is absent.

Elastic cartilage is very similar to hyaline cartilage, however, it contains large amounts of dense elastic fibers throughout the matrix. The elastic fibers are visible on H&E preparations. It is found in the tissues requiring elasticity, as well s rigidity, in such tissue as the epiglottis, part of the larynx (corniculate and cuneiform cartilages), and the pinna.

Hyaline cartilage is the most common form of cartilage. It forms a large portion of the developing vertebrate skeleton, progresses to form epiphyseal discs, articular cartilage, tracheal components, bronchial components, and other tissues. The ground substance of hyaline cartilage can be separated into a pale staining interterritorial matrix and a darkly staining substance called territorial matrix. The territorial matrix stains darkly due to the higher concentration of sulfated glycosaminoglycans. Chondrocytes are confined to small spaces, or lacunae, within the matrix. Small clusters of chondrocytes form isogenous groups due to cellular division. Cartilage matrix

is usually invested by a perichondrium whose inner layer is chondrogenic. This chondrogenic layer contains cells with the ability to become chondroblasts. The outer portion of the perichondrium is composed of dense irregular connective tissue.

Four morphologically distinct layers are present within articular cartilage[104]. Closest to the joint surface, the two-layered superficial zone is the thinnest layer and consists of discoid chondrocytes with long and flat profiles. These cells are orientated with their long axis parallel to the joint surface and the thin collagen fibrils are aligned parallel to the joint surface. A thin sheet of fibrils with little polysaccharide and no cells covers the articular surface of the superficial zone[103]. Deep to the superficial layer lies the transitional, or intermediate zone, which is a much thicker zone with more spherical cells and larger collagen fibrils that are not organized in a parallel fashion. The next layer is the radial, or deep, zone in which the chondrocytes are organized in a columnar orientation and collagen fibrils are vertically oriented, perpendicular to joint surface. Adjacent to the subchondral bone lies the calcified cartilage. A basophilic tidemark delineates the radial zone from the calcified cartilage.

Variation of the articular cartilage thickness exists and is believed to be due to weight bearing forces. A study evaluating rabbit articular cartilage reported weight-bearing articular cartilage to be thicker and containing a small number of large chondrocytes within a large mass of matrix compared to less-weight-bearing surfaces[105].

All synovial joints contain a synovial fluid that provides lubrication for the hyaline cartilage or fibrocartilage articular surfaces. Fibrocartilage has very few blood vessels or nerves and hyaline cartilage does not have either. The synovial fluid has a secondary function of providing nutrients to and removing waste from the hyaline cartilage. This fluid allows for leukocytes to circulate and phagocytize debris. The amount of synovial fluid of stifles of adult canines ranges from 0.2ml to 2ml, and the volume may be a reflection of the overall health of the dog. Synovial fluid is thought to be a dialysate because the chemical composition resembles tissue fluid. The mucin is thought to be produced by the fibroblasts of the synovial membrane[106]. Synovial fluid also contains salts, albumin, fat, and cellular debris.

Articular cartilage covering the bone surfaces of synovial joints is usually hyaline cartilage. The deeper layers in contact with the bone may be calcified. Lacking vessels and nerves, hyaline cartilage derives its nutrition from the synovial fluid and is capable of some regeneration following injury[107]. The thickness of the cartilage varies depending on the particular joint and the portion of the joint. It is thickest in young, healthy joints and in joints that bear

increased weight. The thickness is proportional to the weight it is subjected to. Disuse atrophy of the hyaline cartilage may occur. Hyaline cartilage has both elastic and compressibility qualities to absorb shock and protect the underlying bone from trauma.

1.2.4 Articular Cartilage Metabolism

Chondrocytes make up a small proportion of cartilage by volume in the mature dog yet they are responsible for synthesizing, maintaining and regulating the pericellular, territorial, and interterritorial matrix[108]. Structural organization, cellularity, DNA content, biochemical composition, the pattern of nutritional supply, repair potential, rate of protein and proteoglycan metabolism, and proliferative activity of chondrocytes differ between immature and mature cartilage[105]. Mature articular cartilage is devoid of lymphatics and blood supply so metabolism is primarily anaerobic[108]. Nutrients, including glucose, oxygen, and amino acids, diffuse from the synovial fluid that surrounds the articular cartilage[109]. Diffusion is dependent on variables such as molecular size, shape, charge of the molecule and on the concentration of proteoglycans in the cartilage[110]. Intermittent weight bearing as occurs while ambulating aids in diffusion via a pumping action, forcing substances out of the cartilage while bearing weight and bringing substances into the cartilage while non-weight bearing[108,111]. Metabolism of the extracellular matrix is influenced by the mechanical environment and cytokines and growth factors produced by synovial cells and chondrocytes[108].

1.2.5 Response to Cartilage Injury

The response of cartilage to injury differs from classic tissue healing because of two important features of the structure of cartilage, the most important feature being it's avascular status[112]. The second difference is that chondrocytes are within a mesh of collagen and proteoglycan and can therefore not migrate to the site of injury from the adjacent healthy cartilage[104]. When partial-thickness injury to the articular surface occurs, a zone of necrosis develops adjacent to the site with ghost cells seen within the chondrocyte lacunae[112,113]. A brief period of mitotic activity and matrix activity follows but rapidly ceases with no healing[112,113]. The healing has not been shown to progress over time, however, these lesions remain stable and rarely progress to osteoarthritis[112].

These conditions are different if the cartilage injury penetrates through the subchondral bone providing a pathway to the highly vascular bone[112]. In this injury scenario, response to injury is similar to that seen elsewhere in the body. The defect is filled with a fibrin plug which traps cells from the blood and bone marrow, including undifferentiated mesenchymal cells[114].

The inflammatory and reparative phases proceed as other tissues would respond to injury and the new tissue in the cartilage defect undergoes a metaplasia to a hyaline-like chondroid tissue[114]. By 2 weeks following injury rounded chondrocytes appear and produce substantial amounts of Type II collagen[104]. However, later in the process, there is still 20-35% Type I collagen present[115], the proteoglycan content decreases significantly, and the tangential layers of collagen in the superficial zone fail to appear[116]. Collagen fibers of the repair tissue are not well integrated with those of healthy cartilage, which is thought to lead to vertical shear stresses between the repaired and healthy cartilage leading to micromotion and potentially to degenerative changes[114]. The chondrocyte lacunae adjacent to the repaired cartilage are devoid of chondrocytes, which could be detrimental to metabolism of the matrix[114].

1.3 Arthroscopic Surgery Irrigating Solutions

With the gaining popularity of arthroscopic procedures in the early to mid-1980's, studies were performed to assess the effect of irrigating solutions and intra-articular analgesics on the articular cartilage and synovium. Systemic analgesics have inherent side effects and risks associated with their use. Local anesthetics, especially bupivacaine, were shown consistently to offer analgesia at the surgical site perioperatively. This allowed for decreased administration of systemic analgesics and gaining popularity of day or outpatient arthroscopic procedures.

Traditionally, normal saline had been used for irrigation during arthroscopy due to its characterization as a physiologic solution based on the ionic concentrations of sodium (Na^+) and chloride (Cl^-). However the pH of this solution was determined to be 5.3, more acidic than normal physiologic pH of 7.4. It also lacked potassium, calcium, phosphate, bicarbonate, acetate, and lactate, supporting the idea that saline is truly not physiologic and possibly not the ideal solution to use for arthroscopic procedures. An in vitro study was performed In efforts to determine the optimum solution for irrigation during arthroscopy by comparing the effects of multiple commercially available solutions on cartilage proteoglycan synthesis[117]. Proteoglycan synthesis is essential to maintain the cartilage matrix and can be assessed through the use of $^{35}SO^4$. Results indicated that Lactated Ringer's solution (LRS) supports chondrocyte metabolism for 8 hours in an ex-vivo organ culture system and does so as effectively as Ham F12 medium.

A simulated arthroscopic procedure in rabbits evaluated the effects of various local anesthetic agents and irrigating solutions on chondrocyte function[118]. Normal saline and Lactated Ringer's solution (LRS) were compared for synovial and cartilage metabolism measured by tissue purine nucleotide content. Also, 0.5% bupivacaine, hydrochloric acid in an amount equal

to that bound by bupivacaine, 0.5% bupivacaine with 1:200,000 epinephrine, 0.25% bupivacaine, and autologous blood were compared. Results of the study revealed evidence of acute stress evidenced by elevated purine nucleotide levels on both cartilage and synovial cells by all solutions in the immediate post-exposure samples, with saline causing more stress than LRS. All bupivacaine solutions and blood caused more stress on the cartilage and synovial cells than either saline or LRS. By one week post-exposure, stress patterns approached similarity to unirrigated control joints. A transient decrease in matrix staining was observed for all variables upon histopathologic examination but there was no evidence of morphologic damage at any time.

The ultrastructure of human hyaline cartilage biopsy specimens was evaluated with scanning electron microscopy following exposure to 1.5% glycine, Synovisol (an irrigating solution used for electrosurgery), saline, Ringer's lactate, or water for 30 and 45 minutes[119]. Scanning electron microscopy revealed that all solutions caused macroscopic changes to the articular cartilage surface. The 1.5% glycine solution showed the fewest changes, followed by Synovisol, then saline and Ringer's lactate, and finally water.

The effects 0.9% NaCl, Ringer's solution, Ringer glucose 5%, Ringer lactate, and Betadine on anatomically intact rat patellar articular cartilage metabolism were compared to M199 culture medium as a control[120]. Following treatment, each sample was radiolabeled with $^{35}SO_4$ as a marker of cartilage metabolism. Findings revealed that only Betadine solution caused macroscopic softening of the cartilage. The Betadine treatment group inhibited chondrocyte metabolism by 55% compared to the M199 culture medium, where as saline and Ringer lactate solutions reduced metabolism by 20%, Ringer glucose by 10%, and Ringer's solution by 5%.

Chlorhexidine has previously been used as an arthroscopic irrigating solution because of its bactericidal effects and its lack of deleterious effects on wound healing. A case series of five men who had diagnostic and/or therapeutic arthroscopy performed on their knees reported the accidental use of a 1% chlorhexidine irrigation solution causing rapid chondrolysis evidenced by radiographic loss of the joint space and histopathologic necrosis, non-specific inflammation, and fibrosis of the cartilage and synovium[121]. Another review of 3 cases of chondrolysis of the knee following arthroscopy that utilized 0.02% chlorhexidine as an irrigating solution was published[122]. All patients in the report developed clinical signs and radiographic evidence of chondrolysis within four months of their initial procedure that was confirmed on arthroscopic and histopathologic evaluation and resulted in the necessity for total knee replacements.

Temperature of arthroscopic solutions has been shown to have an effect on chondrocyte viability. An ex vivo study showed decreased chondrocyte death with arthroscopic solution of 37°C compared to standard room temperature (22°C) solutions[123]. With regard to cartilage thermal injury, A strong relationship exists between increasing temperature and chondrocyte death when chondrocytes are exposed to solutions of varying temperatures, with a sharp increase in chondrocyte death between 50° and 55°C[124].

1.4 Chondrocytotoxicity

Several proposed inciting causes have been elucidated in the etiology of chondrolysis and many of the proposed inciting causes involve a surgical event. Several case reports, case series, and in vitro and in vivo research projects have been published describing chondrolysis following a variety of procedures.

The term idiopathic chondrolysis of the hip was first used 1975 to describe cases of hip chondrolysis of unknown etiology[125]. Since then, multiple case reports and case series have been published[126-132]. This uncommon condition primarily affects prepubertal adolescent women, primarily of African descent. It is characterized by progressive loss of the articular cartilage of the femoral head and decreased range of motion that may progress to ankylosis. Idiopathic chondrolysis is reportedly a form of cartilaginous dysplasia[133] and affected individuals have absence of trauma, infection, rheumatoid arthritis or systemic illness in their histories. Radiographs show narrowing of the coxofemoral joint, premature closure of the proximal femoral and greater trochanteric growth plates, and periarticular osteopenia. Debate exists about the proper treatment of these patients, but the goal of treatment remains to be relief of pain, correction of deformity, and restoration of hip motion[129,134].

The surgery for treatment of slipped capital femoral epiphysis (SCFE) involves the use of pins to stabilize the epiphysis. The findings in seventeen patients with chondrolysis following surgery for treatment of SCFE have been reported[135]. Fourteen of the 17 cases had evidence supportive of pin penetration of the femoral head. One had evidence of pin penetration of the intra-articular femoral neck on postoperative radiographs. The remaining 2 cases both had pins placed within 2mm of the articular cartilage. The authors concluded that there was a relationship between articular pin penetration and chondrolysis in patients with SCFE. A rabbit model to research the association between pin protrusion and chondrolysis was performed results suggested that both enzymatic degradation and mechanical destruction of the articular cartilage occurred[136]. Conclusions were that the mechanical injury of the pins was not as detrimental as

the associated inflammatory response and enzymatic damage. Cartilage at sites remote from the pins appeared to experience more purely enzymatic damage. The articular cartilage lost 30% of its proteoglycan content, however, they found that the joint spaces did not narrow like chondrolysis cases typically do, but instead increased. They concluded that some other factor is necessary to produce chondrolysis. Their theory suggests that the mechanical damage of the pins induces an autoimmune response responsible for the chondrolysis. This is supported by a human case series report of 11 patients with SCFE. It was concluded that transient intra-articular pin penetration did not lead to chondrolysis over the 2 years that cases were followed[137].

Chondrolysis affecting the shoulder following the utilization of gentian violet for color testing has been reported in Japan[138-142]. Gentian violet has also been used as an extrinsic antiseptic and as a treatment for oral thrush in infants. The pathogenesis of the chondrolysis for these cases was unknown at the time of publication. Various concentrations of gentian violet injected into rat knees has been since shown to cause chondrocyte death in the superficial layer of the articular cartilage 7 days post-injection[139].

Rapid lateral femoro-tibial chondrolysis following arthroscopic lateral meniscectomy has also been reported in four cases following arthroscopic lateral meniscectomy in young athletes[143]. At the time of meniscectomy, the cartilage of the lateral compartment was grossly normal. A second arthroscopic procedure was performed after a mean of 6 months due to persistence of signs consistent with chondrolysis. Chondrolysis was diagnosed based in the presence of cartilaginous debris floating in the joint, along with severe cartilage damage in the lateral compartment. The etiology was suspected to be mechanical, but was not definitively known. The authors speculated that the rapid chondrolysis represented an acute form of chronic slow chondrolysis, which is a known complication after lateral meniscectomy. A similar American case report has been published[144].

Rapid chondrolysis has also been reported following accidental intra-articular injection of bone cement into the coxofemoral joint for treatment of a benign acetabular subchondral bone cyst[145]. Contrast imaging of the cyst prior to treatment showed no communication with the joint, however, after injection of a small amount of methylmethacrylate it was noted to have leaked into the joint space so the injection was immediately discontinued. Pain intensified following the treatment so five days after injection arthroscopy was performed and the bone cement present within the joint was ablated. There were no macroscopic cartilage lesions at the time. Pain persisted and 8 weeks post-treatment radiographs revealed loss of the joint space. Chondrolysis

was diagnosed and a total hip replacement was performed. Proposed mechanisms responsible for the chondrolysis include exothermic reaction of the methylmethacrylate and release of free radicals by the cement.

Transfer of the greater trochanter is a surgical technique that is used for overgrowth of the greater trochanter arising from a disturbance of the growth plate of the proximal femur. Chondrolysis of the hip has been reported in three patients following transfer of the greater trochanter[146]. In this report, one patient had Perthes' disease, one had bilateral developmental dysplasia of the hip, and the third had unspecified arthritis of the hip. This was the first report of chondrolysis as a complication of this procedure. Mechanisms proposed by the authors included joint immobilization for 6 weeks and mechanical derangements as a result of increased pressure on the joint surface produced by the descent of the gluteal attachment.

Axillary nerve injury, recurrent instability, capsular obliteration, and adhesive capsulitis are known complications of thermal capsulorrhaphy that have been documented in the literature however, case reports of glenohumeral chondrolysis attributed to thermal capsulorrhaphy have recently been published[147-150]. Two cases of glenohumeral chondrolysis after capsulorrhaphy in young athletes have been attributed to the use of thermal energy[147]. A case of glenohumeral chondrolysis was attributed to a capsular release procedure using bipolar radio frequency probe for treatment of adhesive capsulitis[148]. A case of glenohumeral chondrolysis in a young woman following treatment of a shoulder luxation using monopolar radiofrequency to perform a thermal stabilization procedure has also been reported[149]. A retrospective study evaluated 8 cases of glenohumeral chondrolysis following arthroscopic procedures that utilized thermal energy found no patients had evidence of cartilage damage at the time of initial surgery and no patient received an intra-articular pain pump catheter post-operatively[150]. Open surgical stabilization has not been shown to be associated with chondrolysis so it has been speculated that heating of the joint fluid at the time of arthroscopy played a role in the chondrolysis. Bipolar radiofrequency probes have been shown to raise the temperature inside the joint high enough to kill chondrocytes[123,151,152]. A case report of chondrolysis following debridement of a partial thickness articular cartilage defect that was treated with arthroscopic chondroplasty using bipolar radiofrequency energy has been published[153]. The detrimental effect of elevated temperature on chondrocytes has been proven in the literature[154-156]. Both monopolar and bipolar radiofrequency energy applied to articular cartilage has been shown to cause time-, temperature-, and power-dependent chondrocyte death[151,154,156-159].

Fibronectin and fibronectin fragments are extracellular matrix proteins that have been implicated in chondrocyte cellular breakdown by suppression of proteoglycans[160-171]. Increased levels of fibronectin and fibronectin fragments in the synovial fluid of arthritic patients trigger expression of proteinases and cause subsequent chondrolysis[160]. Small amounts of free fibronectin fragments in bovine cartilage cultures resulted in increased levels of stromelysin, gelatinase, and collagenase activity. It was also shown that fibronectin fragments not only increase matrix metalloproteinases (MMPs), but they suppress proteoglycan synthesis and increase rates of proteoglycan loss from cartilage tissue[168]. It is speculated that a signaling pathway exits that regulates MMP-13 and MMP-1 stimulation by fibronectin fragments[172]. These fibronectin fragments stimulate MMPs such as collagenase-3 (MMP-13) and are capable of degrading the extracellular matrix of cartilage.

Disruption of the cartilage extracellular organization has been shown to lead to chondrolysis[173]. Transmembrane receptors mediate cell matrix activity and these receptors are vital to the maintenance of cartilage homeostasis. A dose-dependent chondrolysis occurs when articular cartilage slices are exposed to hyaluronic acid hexasaccharides. When hyaluronic acid hexasaccharides bind to CD44, the chondrocytes are uncoupled from the matrix, resulting in deleterious changes in matrix structure and modifications in chondrocyte metabolism.

All of these discussed mechanisms of chondrolysis are currently recognized as clinical concerns and are therefore respectable considerations when joint surgery is performed. However, cases of chondrolysis have been documented where these previously discussed causes have been diagnostically and therapeutically ruled-out.

1.5 Bupivacaine-Associated Chondrolysis

With the increasing popularity of arthroscopy and intra-articular local anesthetic injections, it was important to determine bupivacaine's effects on articular cartilage. The first research examining the effect of bupivacaine on cartilage was published in 1985 and evaluated the immediate effects of bupivacaine in isotonic saline solution on pig and canine articular cartilage[6]. Their findings indicated that saline solution has a profound acute effect on articular cartilage, but the ability to incorporate sulfate into proteoglycan was apparently restored to normal 3 days later. Sulfate uptake is a marker of cartilage proteoglycan metabolism and is a reflection of cartilage anabolism[174]. Bupivacaine caused slight additional inhibition of sulfate uptake, which indicates bupivacaine caused greater proteoglycan synthesis inhibition than saline. However, there was a return to normal proteoglycan synthesis by 3 days after treatment. When

bupivacaine containing methylparaben preservative was incubated with human inflammatory synovial fluid, half of the samples formed a crystalline precipitate that was not further characterized.

A study evaluated and compared the short- and long-term histopathologic effects of intra-articularly administered bupivacaine and neostigmine on rabbit stifle articular cartilage and the synovial membranes[9]. The joints that received bupivacaine and neostigmine had significantly more inflammation of the articular cartilage compared to the joints treated with saline. The cartilage inflammation was more pronounced in the neostigmine group at 24 hours when compared to the bupivacaine group, however by 10 days the bupivacaine group showed more evidence of inflammation than the neostigmine group. All joints treated with bupivacaine showed minimal or mild changes in synovial membrane cell hyperplasia.

Reports of shoulder arthroscopy in young athletes that resulted in glenohumeral chondrolysis within 6 months of surgery have been published[10]. At the time of each initial arthroscopic procedure, no cartilage pathology was reported in any of the patients. In two of the three cases, radiofrequency energy was used. Thermal energy could not be excluded as a contributing factor but the authors suggested that these patients potentially had an autoimmune component to the development of chondrolysis. They theorized that the arthroscopy episode potentially triggered this autoimmune response, causing migration of inflammatory cells to the joint. These inflammatory cells could then release chondrolytic substances resulting in cartilage death. However, one case received an intra-articular pain pump catheter in the immediate post-operative period. Immediate post-operative pain management was not described for the other 2 cases. One of these cases received a bupivacaine injection 4 months post-surgery, which was one month prior to a diagnosis of chondrolysis.

A report describes a case of chondrolysis of the ankle of a 21-year-old male following treatment of a soft tissue injury to the ankle that occurred five years previously and progressed to chronic instability[11]. Arthroscopy on the ankle joint was followed by a lateral ankle ligament reconstruction. Postoperative analgesia included an intra-articular bupivacaine continuous pump infusion. No complications were encountered with the surgery or in the 4 months postoperatively, however 11 months post-surgery he represented for ankle pain. Upon the second arthroscopic examination of the joint, chondrolysis was present. The patient signalment and the affected joint were not typical for idiopathic chondrolysis. Speculation arose regarding the use of the intra-articular administration of bupivacaine with a pain pump catheter.

The records of 152 patients that underwent 177 shoulder arthroscopies were reviewed for post-arthroscopic glenohumeral chondrolysis[12,69]. Twelve shoulders in 10 patients developed chondrolysis, and 83% of these required a subsequent procedure. All cases of chondrolysis had been treated post-operatively with an intra-articular pain pump catheter (IAPPC) administering 0.25% bupivacaine and 1:200,000 epinephrine. The pH of the bupivacaine and epinephrine administered was between 3.5 and 5.5 and was administered at a rate of 4.16cc/hour for 2 to 3 days. All patients had negative results on the rheumatologic and infectious work-ups. No other risk factors were identified that could account for the chondrolysis. Of the 30 arthroscopic procedures with IAPPCs, 63% had developed chondrolysis at the time of publication.

An experimental model examining IAPPCs was performed to investigate the potential chondrotoxic effects of a continuous intra-articular infusion of bupivacaine in the rabbit shoulder and the experiment was designed to mimic the clinical application of IAPPC's used in human shoulder surgery[13]. An IAPPC was surgically placed and a constant flow rate of 0.25% bupivacaine, saline, or 0.25% bupivacaine and 1:200,000 epinephrine was administered for 48 hours. Findings revealed that the group with bupivacaine infusions had 50% reduction in articular cartilage $^{35}S0_4$ uptake compared to the saline group. The group with both bupivacaine and epinephrine had a 56% reduction in sulfate uptake when compared to the saline group. When cell viability was quantified with confocal microscopy, the group with bupivacaine infusions had a 32% decrease in cell viability when compared to the saline group. The bupivacaine and epinephrine group had a 20% decrease in cell viability when compared to the saline group. On histological analysis of the cartilage and synovium, both the bupivacaine and bupivacaine and epinephrine groups had significantly worse histological grades on all evaluated parameters when compared to the saline group. The comparatively larger reduction in sulfate uptake than in cell viability suggests that even cells that survived after bupivacaine infusion remained at a decreased metabolic state 5 days after cessation of the infusion.

The effects of 0.5% bupivacaine on the viability of bovine articular chondrocytes in vitro was performed to determine if 0.5% bupivacaine is chondrocytotoxic to articular chondrocytes and if an intact articular surface protects chondrocytes from the effects of short-term 0.5% bupivacaine exposure[14]. Alginate bead cultures were evaluated with flow cytometry and intact cartilage cores were evaluated with fluorescent viability stains and laser confocal microscopy to evaluate chondrocyte death. Following exposure of the chondrocyte cultures to 0.5% bupivacaine for 15, 30, and 60 minutes, 99% chondrocyte death was observed compared to 31%

chondrocyte death was observed following saline exposure. When evaluating the cartilage cores, the saline-treated intact cores had predominantly live chondrocytes near the articular surface. Following exposure to 0.5% bupivacaine for 30 minutes, dead chondrocytes were present extending below the intact articular cartilage surface. The percentage of live chondrocytes for the saline-treated control cores was 74% and for the bupivacaine treated cores was 58%. Again, the study found that the saline-treated cores with the surface removed had predominantly live chondrocytes near the articular surface. However, treatment of these cores with 0.5% bupivacaine resulted in a zone of dead chondrocytes that extended to a depth of 0.5mm to 1.0mm. When quantified with confocal microscopy, the cores with the surface removed preserved 76% live chondrocytes for the saline-treated samples, and when treated with 0.5% bupivacaine decreased chondrocyte viability to 25%. The findings suggest increased chondrocyte death in the superficial regions of both intact articular cartilage and the cartilage with the surface removed following 30 minutes of exposure to 0.5% bupivacaine. The data also suggests that the intact surface of articular cartilage offers some protection from the chondrocytotoxic effects of 0.5% bupivacaine.

The findings in a case series of 4 young patients with rapid-onset glenohumeral chondrolysis following arthroscopy were reported[175]. All 4 patients had normal articular cartilage based on pre-operative MRI and no complications occurred during the surgical procedures. By 6 months after the procedures, all 4 patients had decreased range of motion in the joint and follow-up radiographs revealed narrowing of the joint space. No loose intra-articular bodies or marginal osteophytes were present at the time of imaging. The inciting mechanism of the chondrolysis was not determined but the use of bupivacaine as a post-operative intra-articular analgesic was mentioned as a possibility.

Given the recent evidence that intra-articular bupivacaine may cause chondrocyte death, a group of investigators wanted to determine if lidocaine, an amide local anesthetic similar to bupivacaine, also had similar chondrocytotoxic effects. Using similar methodology as Chu's 2006 in vitro study[14], bovine chondrocyte cell cultures and explants were exposed to 1% and 2% lidocaine and chondrocyte death was assessed[176]. The tissues were treated for 15 to 30 minutes and compared to saline controls. Cell viability was assessed at three different time points: 1 hour, 24 hours, and 1 week after exposure. They also compared chondrocyte viability of articular surface intact explants to explants with the articular surface removed. Results indicated that lidocaine had time and dose-dependent cytotoxic effects on both bovine articular chondrocyte

cell cultures and explants. Cell viability decreased over the week of assessment and chondrocytotoxicity was greater for 2% lidocaine treat chondrocytes compared to 1% lidocaine. In contrast to their bupivacaine study[14], lidocaine did not cause as great an amount of chondrocytotoxicity and an intact surface did not offer an apparent protective effect against lidocaine. However, similar to the bupivacaine study, the cell cultures did exhibit greater chondrocyte death compared to the explants, which suggests a protective effect of the cartilage matrix. Bupivacaine and lidocaine have an acidic pH, much lower than a physiologic pH, so the effects of saline at pH 5.0, pH 7.0, and pH 7.4 on chondrocyte viability were compared. There were no statistical differences between the different saline pH treatments on chondrocyte viability.

With the recent evidence that bupivacaine and lidocaine have been shown to be chondrocytotoxic, other intra-articular analgesics were investigated. Ropivacaine is a long acting aminoamide local anesthetic that has been shown to have fewer systemic side effects than bupivacaine due to its lower lipid solubility and to be an effective intra-articular analegesic[177-181]. Comparing the effects of 0.5% bupivacaine, 0.5% ropivacaine, and saline exposed for 30 minutes on macroscopically normal human cartilage explants and cell cultures, bupivacaine was found to cause significantly greater chondrocyte death than either ropivacaine or saline[16]. There was no difference in viability between the ropivacaine and saline treated groups. Similar to other studies, chondrocyte death was greater in the cell cultures compared to the osteochondral explants[14,176].

After establishing that bovine chondrocytes exposed in vitro to 0.5% bupivacaine caused increased chondrocyte death compared to controls, human cartilage was exposed to bupivacaine in a similar study design[15]. Also, bovine cartilage was exposed to a variety of bupivacaine concentrations for up to 60-minutes and results showed that 0.25% and 0.5% bupivacaine caused time-dependent chondrocytotoxicity in both human and bovine chondrocytes in vitro. The viability of bovine and human chondrocytes exposed to 0.125% bupivacaine for up to 60 minutes was similar to saline exposed chondrocytes. They also found a time-dependent increase in bovine chondrocyte death and rates of apoptosis using flow cytometry and time-lapse confocal microscopy. Cellular death occurred more rapidly with 0.5% bupivacaine compared to 0.25% bupivacaine and faster in the human chondrocytes than the bovine chondrocytes.

The purpose of this in vitro study was to measure chondrocyte death in articular cartilage of dogs exposed to 0.5% bupivacaine with and without methylparaben preservative and to

compare chondrocyte death when the articular surface is intact to when mechanically debrided. We hypothesized that exposure of canine articular cartilage to 0.5% bupivacaine would increase chondrocyte death in a time-dependent manner over 5 to 30 minutes and that chondrocyte death would be increased to a lesser extent in intact cartilage cores compared to those with the surface mechanically debrided.

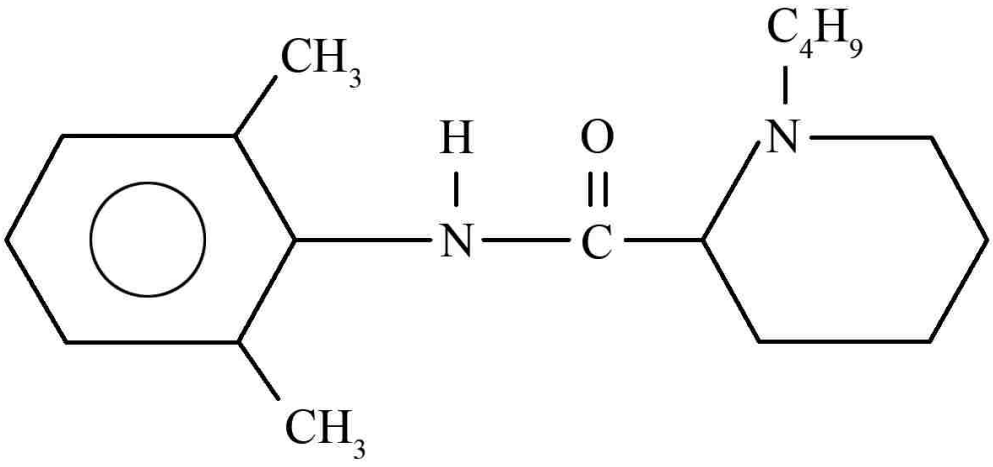

Figure 1.1 Molecular structure of bupivacaine

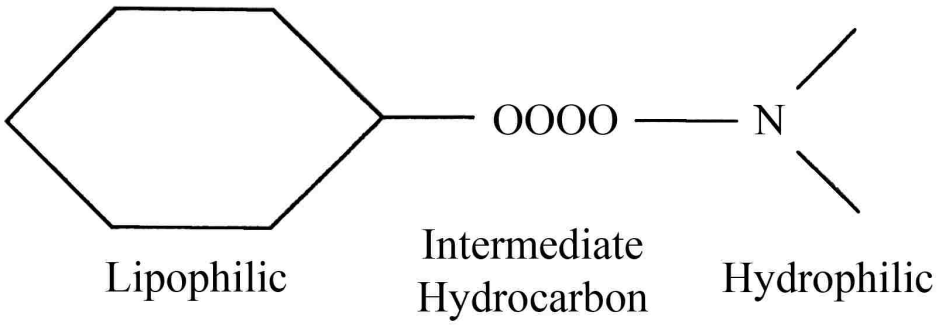

Figure 1.2 Basic molecular structure of local anesthetics.

CHAPTER 2: MATERIALS AND METHODS

2.1 Collection of Phase 1 Tissue Samples

Six mature, fresh, canine cadavers weighing between 9.6kg and 19.8kg (mean 15.8kg) were used immediately after humane euthanasia according to an approved IACUC protocol. Cadavers were excluded if any overt gross joint pathology was present. The glenohumeral joints of each dog were randomly assigned to one of two articular surface preparations prior to collection: surface intact (SI) or surface removed (SR). For SI, the articular surface was preserved. For SR, the cartilage surface was debrided by rubbing a sterilized abrasive pad (Devon® Demolisher™, The Ludlow Company LP, Chicopee, MA) on the articular cartilage surface (Figure 2.1). Then osteochondral cores were aseptically harvested from each glenohumeral joint.

Cores of articular cartilage and the underlying subchondral bone were aseptically harvested to a depth of 6mm by cutting the articular surface of each humeral head in a grid-like fashion of 6mm x 6mm squares with a sterilized jig saw (Figure 2.2). Phosphate buffered saline (PBS) at 37°C was flushed over the cartilage surface during cutting to minimize thermal damage. Final osteochondral core dimensions were 6mm x 6mm x 6mm. The cores were labeled as SI or SR depending on collection site and prior randomization. Cores were washed with PBS and then submerged in sterile 60mm Petri dishes containing 37°C canine chondrocyte culture medium (CCCM; Cell Applications, Inc., San Diego, CA). The cores were maintained at 37°C under a 5% CO_2/95% air atmosphere until further testing was performed (approximately 24 hours).

2.2 Treatment and Evaluation of Phase 1 Tissue Samples

Each glenohumeral joint from 6 dogs was randomly assigned to SI or SR surface preparations. Two cartilage cores from each humeral head of 6 dogs were randomly assigned to one of three treatment solutions containing 15ml CCCM, 0.5% bupivacaine (B; AstraZeneca LP, Wilmington, DE), or 0.5% bupivacaine with methylparaben preservative (BP; Hospira, Inc., Lake Forrest, IL), all with 0.1% methylene blue (MB)[111]. All cores were maintained in the treatment baths at 37°C for either 15 minutes or 30 minutes (Figure 2.3; Table 2.1). After either 15 or 30 minutes, each core was washed with PBS. A 1mm slice was collected from the center of each core with two scalpel blades spaced 1mm apart.

A digital image was taken of each slice under 10x magnification standard light microscopy (Figure 2.4). The depth of stain penetration from the articular surface and the cut

surfaces, and the depth of the articular cartilage to the subchondral bone were measured in micrometers (μm) using a graticule. The dimensions of the unstained portion of the cartilage after treatment determined the size of the unstained central section.

2.3 Collection of Phase 2 Tissue Samples

Ten mature, fresh, canine cadavers weighing between 14.5kg and 27.3kg (mean 20.1kg) were used immediately after humane euthanasia according to an approved IACUC protocol. Cadavers were excluded if any overt gross joint pathology was present. The glenohumeral joints of each dog were randomly assigned to one of two articular surface preparations prior to collection: SI or SR. Surface debridement for SR cores was performed as described for Phase 1. Then osteochondral cores were aseptically harvested from each glenohumeral joint. One synovial tissue sample and one core from each humeral head was randomly selected and fixed in 10% buffered formalin and prepared for standard light microscopy to determine the overall health of the articular cartilage. The SR osteochondral core for histopathologic assessment was collected prior to surface debridement.

Cores of articular cartilage and the underlying subchondral bone were aseptically harvested to a depth of 6mm by cutting the articular surface of each humeral head in a grid-like fashion of 6mm x 6mm squares with a sterilized jig saw (Figure 2.2). PBS at 37°C was flushed over the cartilage surface during cutting to minimize thermal damage. Final osteochondral core dimensions were 6mm x 6mm x 6mm. The cores were labeled as SI or SR depending on collection site and prior randomization. Cores were washed with PBS and then submerged in sterile 60mm Petri dishes containing 37°C CCCM. The cores were maintained at 37°C under a 5% CO_2/95% air atmosphere until further testing was performed (approximately 24 hours).

2.4 Treatment and Evaluation of Phase 2 Tissue Samples

One core from each humeral head was assigned to 1 of 3 treatment solutions (CCCM, B, or BP) and 1 of 3 time periods (5, 15, or 30 minutes) (Figure 2.5, Table 2.2). Cores were treated in 60mm sterile Petri dishes containing 15ml of treatment solution maintained at 37°C. The pH of each treatment solution was recorded with a digital pH probe (Topac, Inc., Cohasset, MA) and meter (Corning, Inc., Corning, NY) at the beginning and end of each timed treatment.

The formalin fixed synovial tissue and articular cartilage from each joint were prepared and stained with hematoxylin and eosin (H&E) for microscopic evaluation and verification that the joints were normal. A board-certified veterinary pathologist blinded to the subsequent treatment of the cores from that joint evaluated the synovial tissue and articular cartilage. Any

pathology was noted and samples were graded for inflammation. Grades of 0 to 4 were assigned as previously described[162,163]: 0) no inflammation; 1) minimal inflammation, minimal congestion and edema; 2) mild inflammation, evidenced by an increase in cell lining thickness and presence of inflammatory cells (neutrophils); 3) moderate inflammation, evidenced by an increase in cell lining thickness, synoviocyte hyperplasia and an increase in inflammatory cells (neutrophils and macrophages); or 4) severe inflammation, evidenced by the marked numbers of inflammatory cells (neutrophils and macrophages), fibrin exudation, increase in cell lining thickness and synoviocyte hyperplasia.

One millimeter thick slices extending from the articular surface through the subchondral bone were taken from the center of each osteochondral core prior to viability staining. The slices were washed with PBS and then stained with 0.4μl calcein AM and 13μl ethidium homodimer-1 (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR) in 1ml PBS for 30 minutes. Living cells with intact plasma membranes and active cytoplasm metabolize calcein and exhibit green fluorescence. Cell membranes of dead, damaged or dying cells are penetrated by ethidium and their nuclei will exhibit red fluorescence. The samples were washed with PBS after staining and mounted (VECTASHELD, Vector Laboratories, Burlingame, CA) on slides prior to imaging.

Imaging was performed within one hour of staining using a laser confocal microscope (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) equipped with fluorescein and rhodamine filters at 10x magnification (Figure 2.6). The laser confocal microscope was used to visualize intact viable tissue by forming serial optical sections through the depth of the specimen[182]. Digital images were acquired of fourteen sequential planes of the center field of view from the articular surface to the subchondral bone of each cartilage slice. One of the fourteen image planes was randomly evaluated for each cartilage slice. The cartilage was divided into three equal zones from the surface to the subchondral bone (superficial zone, middle zone, deep zone) and the live and dead cells were quantified for each zone of each selected image (Figure 2). The cells were counted using digital image editing software (Photoshop CS, Adobe Systems, Inc., San Jose, CA). For each zone of each image, the number of pixels contained in ten cells was counted to calculate the average pixel count for a cell of that zone. This was performed for both the calcein stained cells and the ethidium stained cells. The number of cells in each zone was calculated by dividing the total number of green or red pixels in that zone by the respective

average cell pixel count. The percentage of chondrocyte death (dead cells / live + dead cells x 100) was determined for the 3 zones of each randomly selected image from each tissue slice.

2.5 Statistical Methods

Phase 1: The proportional penetration of MB from the surface of the cartilage was calculated by dividing the depth of penetration by the thickness of the cartilage to the level of the subchondral bone. The proportional penetration was the response variable used to compare the fixed effect of treatment (bath solution) and time. The proportional penetration was normally distributed with failure to reject the null hypothesis of normality at $p<0.05$ using the Shapiro-Wilk statistic. The fixed effect of treatment and time on proportional penetration was evaluated for SI and SR samples using a mixed effect linear model that accounted for the random variance of dog across treatment and time. Where there were significant fixed effects at $p<0.05$, pair-wise comparisons across treatments and time were considered significant with a Scheffe adjustment at $p\leq0.05$. The behavior of SI and SR samples was compared and described. PROC UNIVARIATE, MEANS, and MIXED were used for the analysis (SAS v 9.1, SAS Institute, Cary, NC).

Phase 2: Synovial tissue and cartilage histopathologic grades and viability within and between treatment groups were compared. All data was categorical and was analyzed using Mantel-Haenszel methods, stratifying across time. The frequency of outcomes was compared across treatments with the null-hypothesis of like distributions rejected at $p<0.05$. Where there was a significant difference, ad hoc comparisons were made using Fisher's exact test maintaining type I error at 0.05. The ph of the solution was compared before and after treatment using a paired t-test (parametric) or Mann-Whitney U test (non-parametric) with significance determined at $p<0.05$. The test used was based on the distribution of the data (normal or non-normal), using the Shapiro-Wilk statistic with the null hypothesis of normality rejected at $p<0.05$. The proportion of dead cells (chondrocyte death) was expressed as a percentage and transformed to follow a normal distribution for analysis. The fixed effect of treatments, time and zones were evaluated using a mixed effect linear model including the random variance of dogs across treatments. Where there were significant fixed effects at $p<0.05$, pair-wise comparisons were performed using a Scheffe adjustment to maintain type I error at 0.05. All results were considered significant at $p<0.05$. PROC UNIVARIATE, PROC FREQ, MIXED, T TEST and PROC NPAR1WAY (SAS V9.1) were used for the analysis.

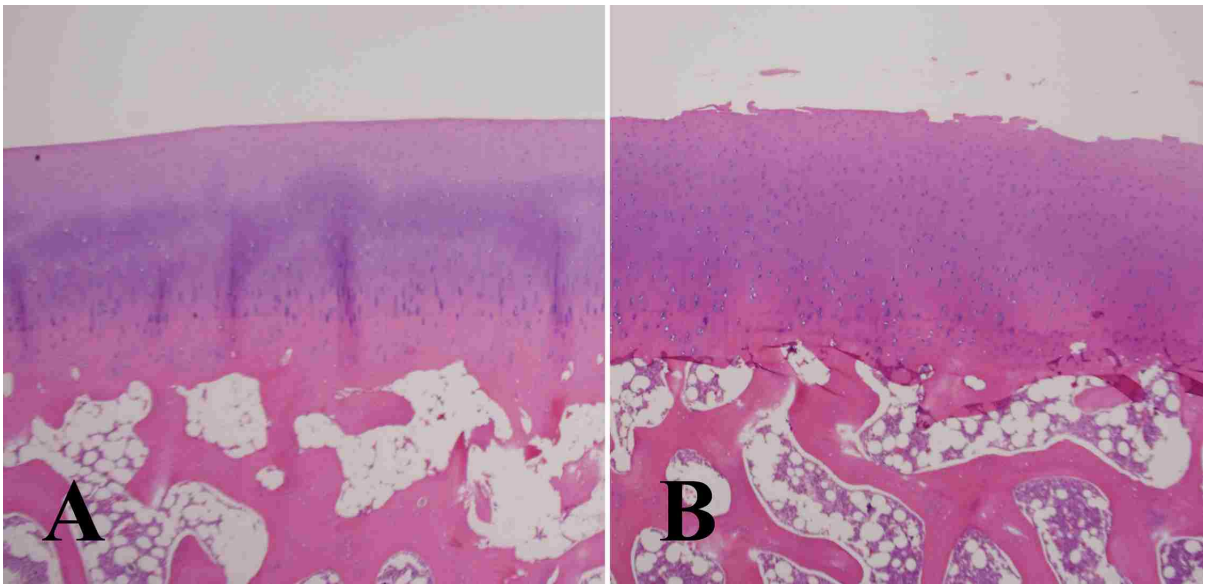

Figure 2.1 Photomicrograph at 10x of H&E stained osteochondral cores with an intact surface (A) and with the surface mechanically debrided (B).

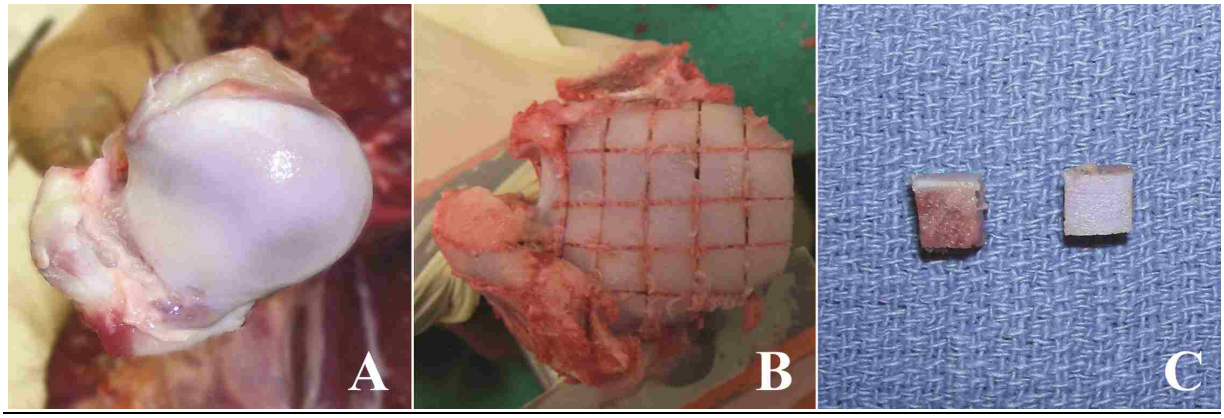

Figure 2.2 Humeral heads (A) were cut in a grid-like fashion using a jigsaw (B) to harvest osteochondral cores with final dimensions of 6mm x 6mm x 6mm (C).

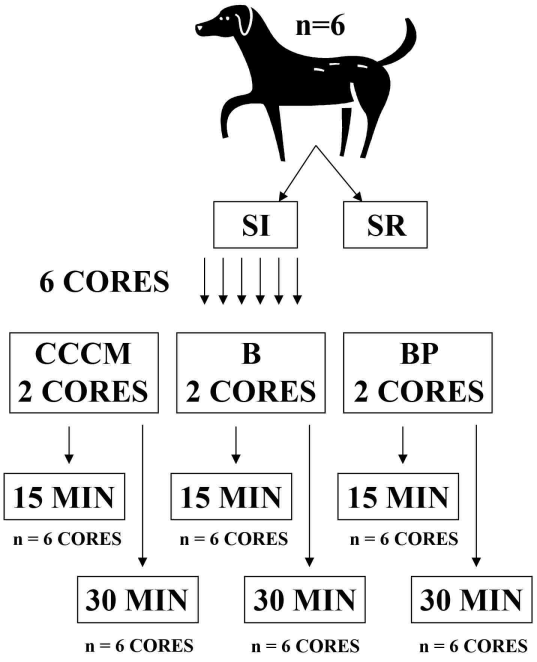

Figure 2.3 Schematic diagram of the experimental design describing the tissue sample collection and treatment distribution for Phase 1.

Table 2.1 Phase 1 osteochondral core treatments and sample size.

15 minutes	CCCM with MB	B with MB	BP with MB
SI	6	6	6
SR	6	6	6
30 minutes	**CCCM with MB**	**B with MB**	**BP with MB**
SI	6	6	6
SR	6	6	6

SI (Surface Intact); SR (Surface Removed); MB (Methylene Blue)
CCCM (Canine Chondrocyte Culture Medium); B (0.5% Bupivacaine without preservative); BP (0.5% Bupivacaine with Preservative)

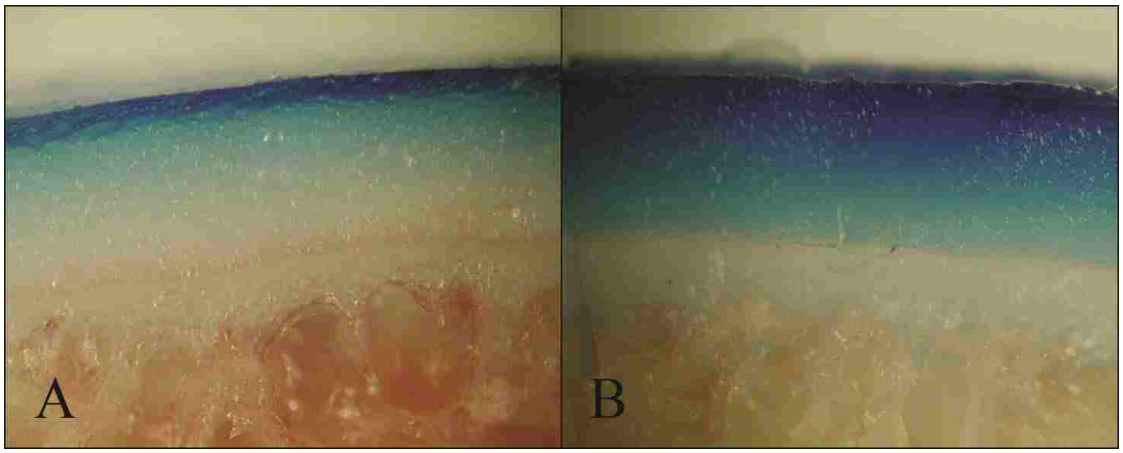

Figure 2.4 Standard light microscopic digital image at 10x of a 1mm slice of a surface intact osteochondral core treated with 0.5% bupivacaine and methylene blue at 15 minutes (A) and 30 minutes (B).

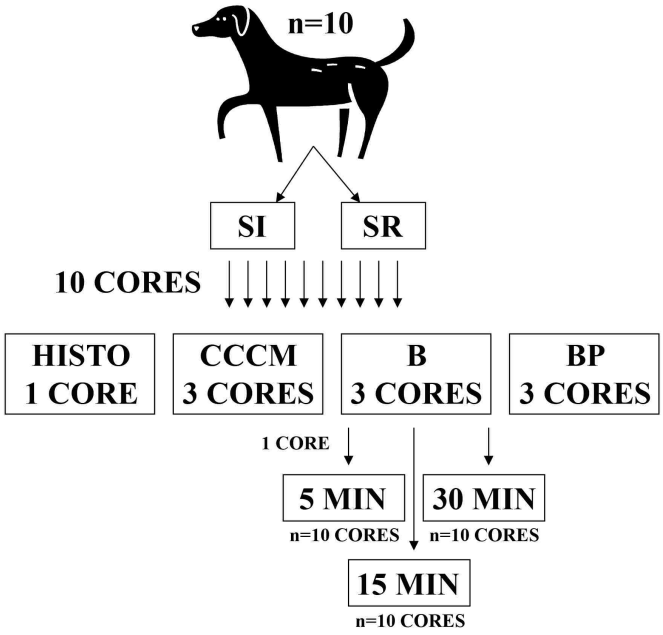

Figure 2.5 Schematic diagram of the experimental design describing the tissue sample collection and treatment distribution for Phase 2.

Table 2.2 Phase 2 osteochondral core treatments and sample size.

SI	**Treatment Time**	**CCCM**	**B**	**BP**
	5 minutes	10	10	10
	15 minutes	10	10	10
	30 Minutes	10	10	10
SR	**Treatment Time**	**CCCM**	**B**	**BP**
	5 minutes	10	10	10
	15 minutes	10	10	10
	30 minutes	10	10	10

SI (Surface Intact); SR (Surface Removed)
CCCM (Canine Chondrocyte Culture Medium)
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

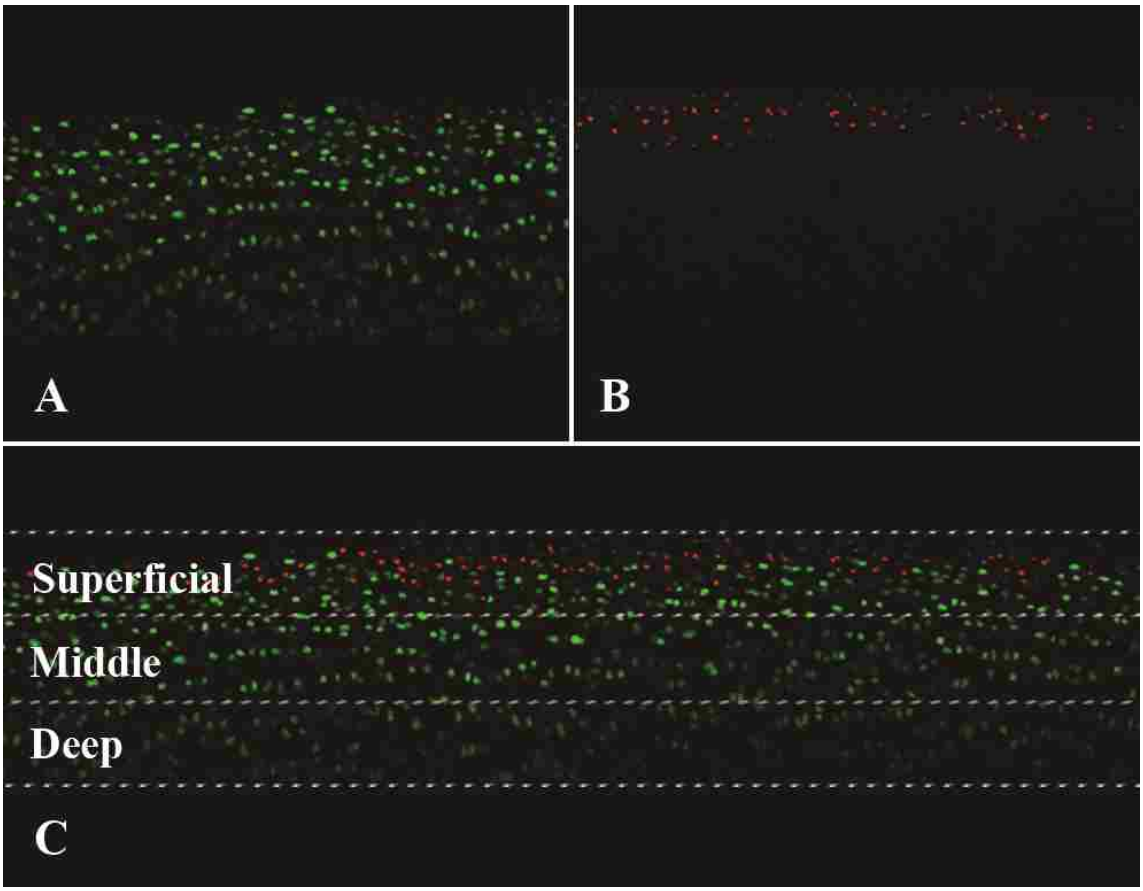

Figure 2.6 Confocal microscopic digital image at 10x of a surface intact osteochondral core treated with canine chondrocyte culture media for 5 minutes stained with calcein AM (A) and ethidium homodimer-1 (B). Images A and B are superimposed in image C showing the greater chondrocyte death that occurred in the superficial zone.

CHAPTER 3: RESULTS

3.1 Phase 1 Results

No gross lesions were present on the articular cartilage of any of the six cadavers. Phase 1 results revealed minimal penetration of MB from the cut surfaces of the cartilage core (Table 3.1). The average penetration from the side cut surface for all treatments ranged from 37 μm to 49 μm with the average core width ranging from 5901 μm to 5925 μm. There was a slight increase in the penetration from the side cut surfaces over time but the remaining stain free core was of more than adequate width for evaluation of surface penetration of treatment solutions. The subchondral bone blocked any penetration of treatment solutions from the deep margin of the core. Thus, the cores were deemed adequate for evaluation of surface penetration since side or deep penetration was not contributing to any staining into the central core.

There was surface penetration on all cores (Table 3.2, Figure 3.1). For SI cores, the mean proportional penetration significantly increased from 15 to 30 minutes for all treatment solutions. For SI cores, there was no significant difference in the mean proportional penetration across treatment solutions at 15 minutes or 30 minutes. For SR cores, the mean proportional penetration significantly increased from 15 to 30 minutes for all treatment solutions except BP. For SR cores, there was no significant difference in the mean proportional penetration across treatment solutions at 15 minutes or 30 minutes.

3.2 Phase 2 Results

No gross lesions were present on the articular cartilage of any of the ten cadavers. Histopathology of the synovium and articular cartilage in Phase 2 samples was normal for 19 of the 20 joints examined. One of the joints from dog 1 had a focal area of grade 1 inflammation of the synovium. The articular cartilage of that joint showed no evidence of histologic disease.

3.2.1 Surface Intact

Comparing chondrocyte death in SI cartilage across zones (Table 3.3; Figures 3.2 & 3.3), chondrocyte death was significantly higher in the superficial zone for all treatment solutions at each time compared to that of the middle and deep zones. In the superficial zone of SI cartilage, BP had significantly higher chondrocyte death at 5 minutes (47.7%) than B (23.6%) or CCCM (25.4%) but by 30 minutes B (53.8%) and BP(62.5%) had similar chondrocyte death while CCCM (20.0%) had significantly less chondrocyte death. In the middle and deep zones of SI cartilage, chondrocyte death did not progress over time for B, BP, or CCCM. In the middle zone

of SI cartilage, BP caused significantly higher chondrocyte death at 30 minutes (27.6%) compared to CCCM (7.6%). In the deep zone of SI cartilage, BP had significantly higher chondrocyte death at 30 minutes (14.1%) compared to B (6.4%).

3.2.2 Surface Removed

Comparing chondrocyte death in SR cartilage across zones (Table 3.3; Figures 3.4 & 3.5), chondrocyte death was significantly higher in the superficial zone for all treatments at each time compared to that of the middle and deep zones. In the superficial zone of SR cartilage, BP had significantly higher chondrocyte death at 30-minutes (59.0%) compared to 5-minutes (37.7%). In the superficial zone of SR cartilage, BP had significantly higher chondrocyte death at 30 minutes (59.0%) than CCCM (28.9%). In the superficial zone of SR cartilage, chondrocyte death did not progress over time for B and CCCM. In the middle and deep zones of SR cartilage, chondrocyte death did not increase over time for any treatment solution or between treatment solutions at any time.

3.2.3 Surface Intact Compared to Surface Removed

In the superficial zone (Table 3.3), chondrocyte death was significantly higher in SR cartilage treated with B (48.2%) and CCCM (42.7%) at 5 minutes than in SI cartilage treated with B (23.6%) and CCCM (25.4%). In the superficial zone, chondrocyte death was significantly higher in SR cartilage at 15 minutes treated with CCCM (43.3%) than in SI treated with CCCM (25.3%). In the middle zone, chondrocyte death was significantly higher in the SR cartilage treated with CCCM (19.3%) at 5 minutes than in SI cartilage treated with CCCM (8.4%). In the deep zone, chondrocyte death was significantly higher in the SR cartilage treated with B (17.0%) at 30 minutes than in SI cartilage treated with B (6.4%).

3.2.4 pH

The pH significantly increased during the treatment period for all treatments with exception to CCCM at 5 minutes in both the SI and SR osteochondral cores (Tables 3.4, 3.5 & 3.6). The pH was always significantly lower for B (SI 5.81, SR 6.52) and BP (SI 5.36, SR 6.36), than CCCM (SI 7.51, SR 7.85). The ph of BP at the end of treatment (SI 6.38, SR 6.35) was significantly lower than B at 5 minutes (SI 6.34, SR 6.32) and 15 minutes (SI 6.45, SR 6.48), but by 30 minutes (SI 6.52, SR 6.51) it was the same. When comparing the SI and SR cores, there was no difference for any treatment solution at any time.

Table 3.1 Mean (standard deviation) methylene blue stain penetration measured in microns from the cut sides of the cores following either 15 or 30 minute exposure.

Surface	Time	Side penetration (μm)	Width stain-free core (μm)
Surface Intact	**15 minutes**	39 (10.7)	5921 (21)
	30 minutes	49 (11.7)	5901 (23)
Surface Removed	**15 minutes**	37 (12.5)	5925 (25)
	30 minutes	47 (9.3)	5905 (19)

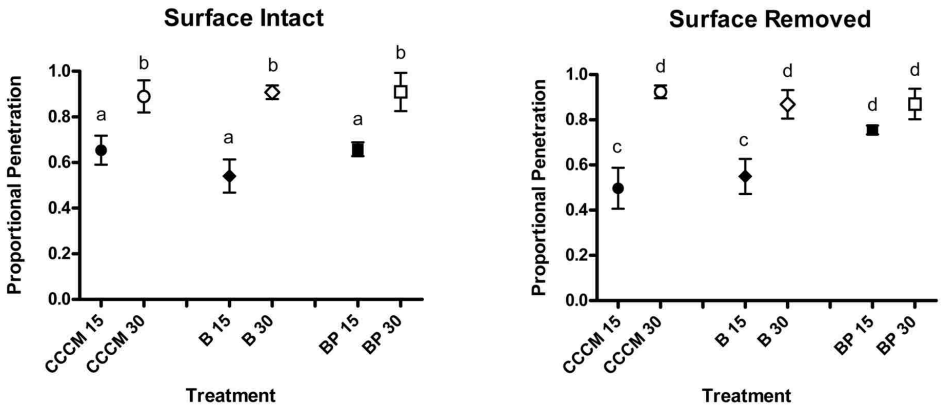

Figure 3.1 Mean (standard deviation) proportional penetration of methylene blue stain from the articular surface of surface intact and surface removed osteochondral cores following either 15 or 30 minute exposure to canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP). For surface intact and surface removed, means with like superscripts are not significantly different.

Table 3.2 Mean (standard deviation) proportional penetration of methylene blue stain from the articular surface of surface intact and surface removed osteochondral cores following either 15 or 30 minute exposure to canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP). For surface intact and surface removed, means with like superscripts are not significantly different.

Surface Intact	**15 Minutes**	**30 Minutes**
CCCM	0.7 (0.16) [a]	0.9 (0.17) [b]
B	0.5 (0.18) [a]	0.9 (0.07) [b]
BP	0.7 (0.08) [a]	0.9 (0.21) [b]
Surface Removed	**15 Minutes**	**30 Minutes**
CCCM	0.5 (0.22) [c]	0.9 (0.07) [d]
B	0.6 (0.19) [c]	0.9 (0.15) [d]
BP	0.8 (0.05) [d]	0.9 (0.16) [d]

Table 3.3 Mean (standard deviation) of chondrocyte death following 5 minute, 15 minute or 30 minute exposure to canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP) for surface intact (SI) and surface removed (SR) osteochondral cores. For each zone, superscripts indicate that means with like superscripts (a,b,c) across each row are not significantly different. For each zone, subscripts indicate that means with like subscripts (x,y,z) down each column are not significantly different. Bold numbers indicate surface intact means are significantly lower compared to corresponding surface removed means.

Surface	Treatment	5 minutes	15 minutes	30 minutes
SUPERFICIAL ZONE				
SI	B	**23.6 (14.67)** $^{a}_{x}$	49.9 (21.53) $^{b}_{x}$	53.8 (14.96) $^{b}_{x}$
	BP	47.7 (16.54) $^{ab}_{y}$	37.6 (23.0) $^{a}_{xy}$	62.5 (19.42) $^{b}_{x}$
	CCCM	**25.4 (16.42)** $^{a}_{x}$	**25.3 (18.9)** $^{a}_{y}$	20.0 (23.29) $^{a}_{y}$
SR	B	48.2 (27.06) $^{a}_{x}$	38.8 (28.29) $^{a}_{x}$	47.2 (19.9) $^{a}_{xy}$
	BP	37.7 (20.72) $^{a}_{x}$	48.6 (20.92) $^{ab}_{x}$	59.0 (19.24) $^{b}_{y}$
	CCCM	42.7 (25.94) $^{a}_{x}$	43.3 (23.08) $^{a}_{x}$	28.9 (19.76) $^{a}_{x}$
MIDDLE ZONE				
SI	B	8.6 (6.84) $^{a}_{x}$	24.0 (24.42) $^{a}_{x}$	18.0 (15.24) $^{a}_{xy}$
	BP	16.7 (20.66) $^{a}_{x}$	28.8 (20.62) $^{a}_{x}$	27.6 (18.22) $^{a}_{x}$
	CCCM	**8.4 (9.35)** $^{a}_{x}$	11.9 (10.68) $^{a}_{x}$	7.6 (8.10) $^{a}_{y}$
SR	B	9.2 (16.13) $^{a}_{x}$	12.1 (11.45) $^{a}_{x}$	19.8 (18.89) $^{a}_{x}$
	BP	19.6 (19.28) $^{a}_{x}$	17.1 (18.75) $^{a}_{x}$	29.5 (18.99) $^{a}_{x}$
	CCCM	19.3 (16.67) $^{a}_{x}$	13.3 (16.92) $^{a}_{x}$	14.8 (11.30) $^{a}_{x}$
DEEP ZONE				
SI	B	5.0 (4.64) $^{a}_{x}$	10.7 (19.92) $^{a}_{x}$	**6.4 (12.58)** $^{a}_{x}$
	BP	13.0 (24.60) $^{a}_{x}$	12.2 (16.98) $^{a}_{x}$	14.1 (11.15) $^{a}_{y}$
	CCCM	5.2 (6.42) $^{a}_{x}$	10.38 (8.06) $^{a}_{x}$	7.2 (7.06) $^{a}_{xy}$
SR	B	7.8 (12.70) $^{a}_{x}$	15.5 (14.28) $^{a}_{x}$	17.0 (13.68) $^{a}_{x}$
	BP	12.2 (16.49) $^{a}_{x}$	10.5 (7.29) $^{a}_{x}$	16.1 (17.86) $^{a}_{x}$
	CCCM	8.9 (9.03) $^{a}_{x}$	5.9 (6.50) $^{a}_{x}$	11.0 (17.53) $^{a}_{x}$

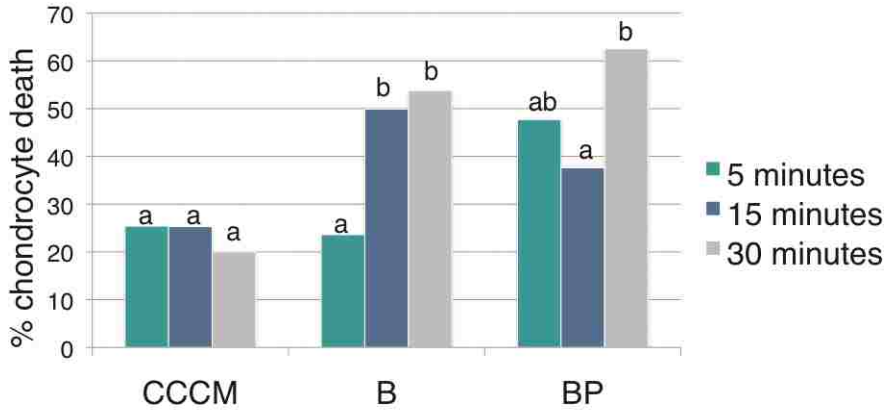

Figure 3.2 Mean % chondrocyte death comparisons within treatment solutions across time in the superficial zone of surface intact osteochondral cores. Means with like superscripts are not significantly different.
SI (Surface Intact); SR (Surface Removed);
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative

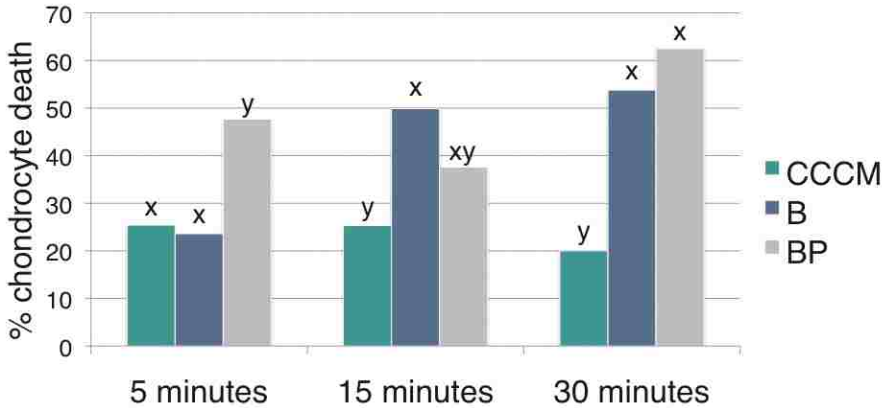

Figure 3.3 Mean % chondrocyte death comparisons across time in the superficial zone of surface intact osteochondral cores. Means with like superscripts are not significantly different.
SI (Surface Intact); SR (Surface Removed);
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

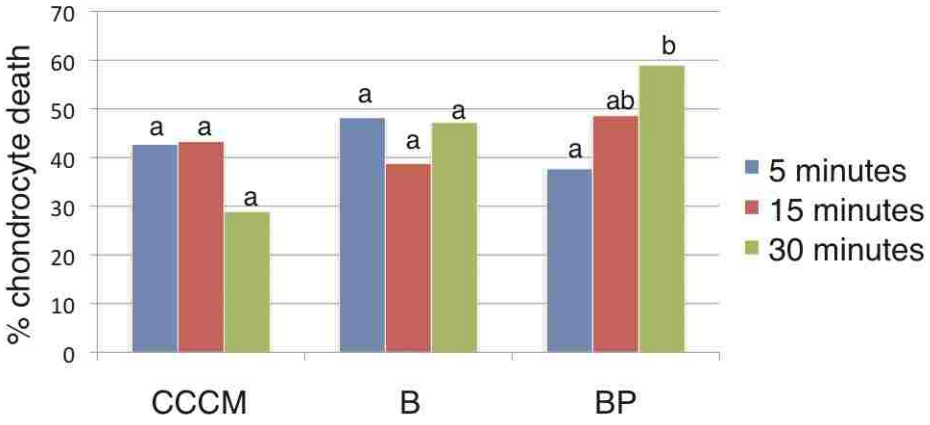

Figure 3.4 Mean % chondrocyte death comparisons within treatment solutions across time in the superficial zone of surface removed osteochondral cores. Means with like superscripts are not significantly different.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

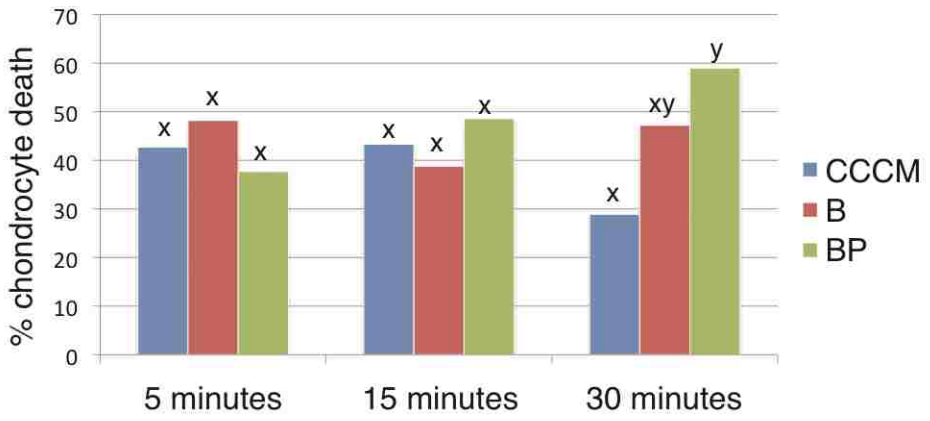

Figure 3.5 Mean % chondrocyte death comparisons across time in the superficial zone of surface removed osteochondral cores. Means with like superscripts are not significantly different.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

Table 3.4 Mean (standard deviation) pH of canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP) for surface intact (SI) and surface removed (SR) osteochondral cores before and after 5 minute treatment. Means with like subscripts (x,y,z) across each row (within surface and treatment solution) are not significantly different. Asterisks (*) indicate that the pH is significantly higher than initial pH.

Surface	Treatment	5 minutes		
		Before	After	Difference
SI	B	5.8 $_{x}$ (0.42)	*6.3 $_{x}$ (0.26)	-0.5 $_{x}$ (0.33)
	BP	5.6 $_{x}$ (0.22)	*6.1 $_{y}$ (0.17)	-0.4 $_{x}$ (0.20)
	CCCM	7.5 $_{y}$ (0.26)	7.8 $_{z}$ (0.28)	-0.2 $_{y}$ (0.30)
SR	B	5.8 $_{x}$ (0.36)	*6.3 $_{x}$ (0.24)	-0.6 $_{x}$ (0.31)
	BP	5.6 $_{y}$ (0.23)	*6.1 $_{y}$ (0.16)	-0.4 $_{x}$ (0.21)
	CCCM	7.6 $_{z}$ (0.28)	7.7 $_{z}$ (0.28)	-0.2 $_{y}$ (0.31)

Table 3.5 Mean (standard deviation) pH of canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP) for surface intact (SI) and surface removed (SR) osteochondral cores before and after 15 minute treatment. Means with like subscripts (x,y,z) across each row (within surface and treatment solution) are not significantly different. Asterisks (*) indicate that the pH is significantly higher than initial pH.

Surface	Treatment	15 minutes		
		Before	After	Difference
SI	B	5.8 $_{x}$ (0.42)	*6.5 $_{x}$ (0.20)	-0.7 $_{x}$ (0.34)
	BP	5.6 $_{x}$ (0.22)	*6.3 $_{y}$ (0.11)	-0.6 $_{x}$ (0.17)
	CCCM	7.5 $_{y}$ (0.42)	*7.8 $_{z}$ (0.26)	-0.7 $_{y}$ (0.45)
SR	B	5.8 $_{x}$ (0.36)	*6.5 $_{x}$ (0.21)	-0.7 $_{x}$ (0.27)
	BP	5.6 $_{x}$ (0.23)	*6.3 $_{y}$ (0.11)	-0.6 $_{x}$ (0.16)
	CCCM	7.6 $_{y}$ (0.28)	*7.8 $_{z}$ (0.27)	-0.3 $_{y}$ (0.30)

Table 3.6 Mean (standard deviation) pH of canine chondrocyte culture medium (CCCM), 0.5% bupivacaine (B), or 0.5% bupivacaine with preservative (BP) for surface intact (SI) and surface removed (SR) osteochondral cores before and after 30 minute treatment. Means with like subscripts (x,y,z) across each row (within surface and treatment solution) are not significantly different. Asterisks (*) indicate that the pH is significantly higher than initial pH.

Surface	Treatment	30 minutes		
		Before	After	Difference
SI	B	5.8 $_{x}$ (0.42)	*6.5 $_{x}$ (0.22)	-0.7 $_{x}$ (0.45)
	BP	5.6 $_{x}$ (0.22)	*6.4 $_{x}$ (0.10)	-0.7 $_{x}$ (0.15)
	CCCM	7.5 $_{y}$ (0.26)	*7.9 $_{y}$ (0.23)	-0.3 $_{y}$ (0.28)
SR	B	5.8 $_{x}$ (0.36)	*6.5 $_{x}$ (0.22)	-0.8 $_{x}$ (0.41)
	BP	5.6 $_{x}$ (0.23)	*6.4 $_{x}$ (0.09)	-0.7 $_{x}$ (0.17)
	CCCM	7.6 $_{y}$ (0.28)	*7.9 $_{y}$ (0.22)	-0.3 $_{y}$ (0.29)

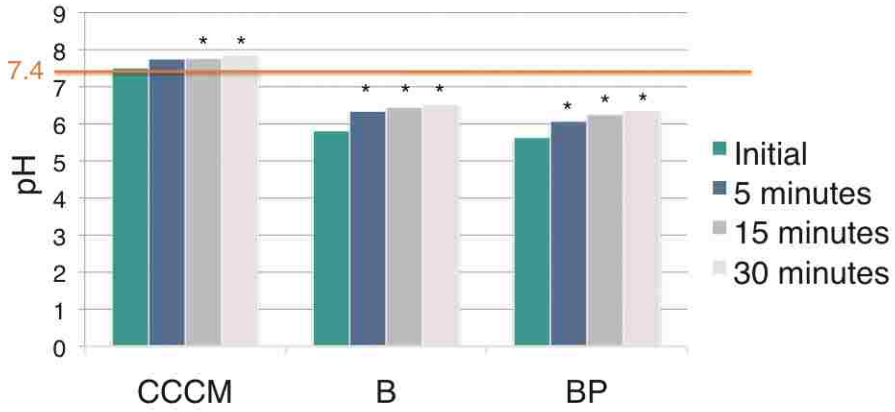

Figure 3.6 Mean pH within treatment solutions across time of surface intact osteochondral cores. Asterisks indicate significant increases compared to initial pH.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

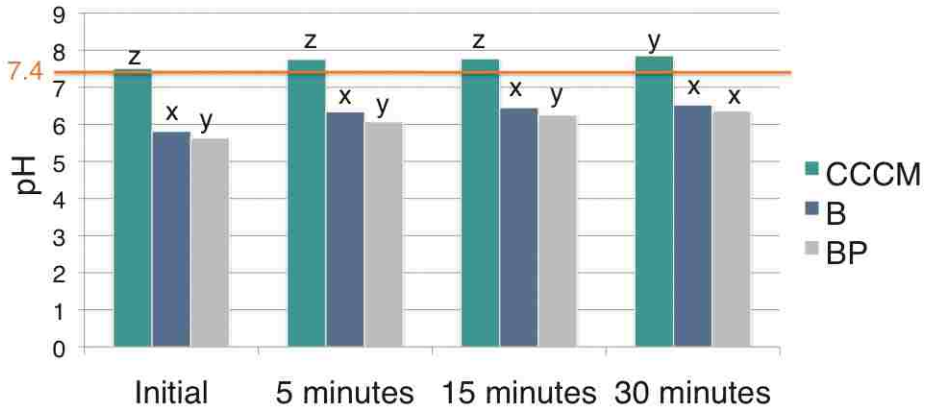

Figure 3.7 Mean pH across time of surface intact osteochondral cores. Means with like superscripts are not significantly different.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

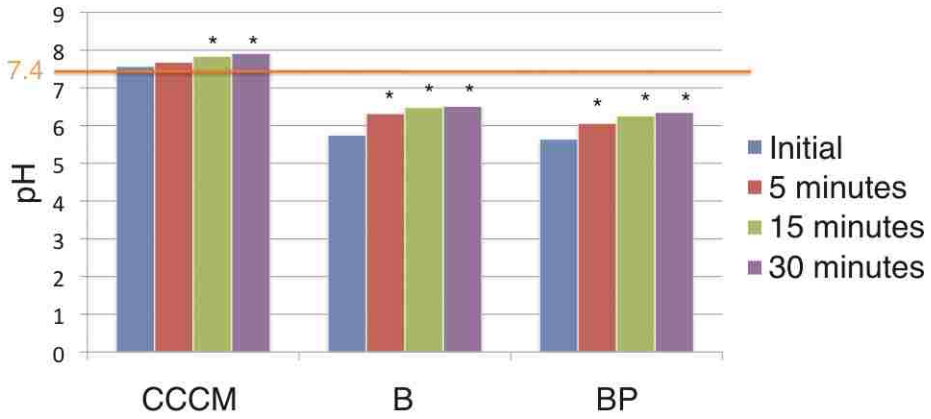

Figure 3.8 Mean pH within treatment solutions across time of surface removed osteochondral cores. Asterisks indicate significant increases compared to initial pH.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

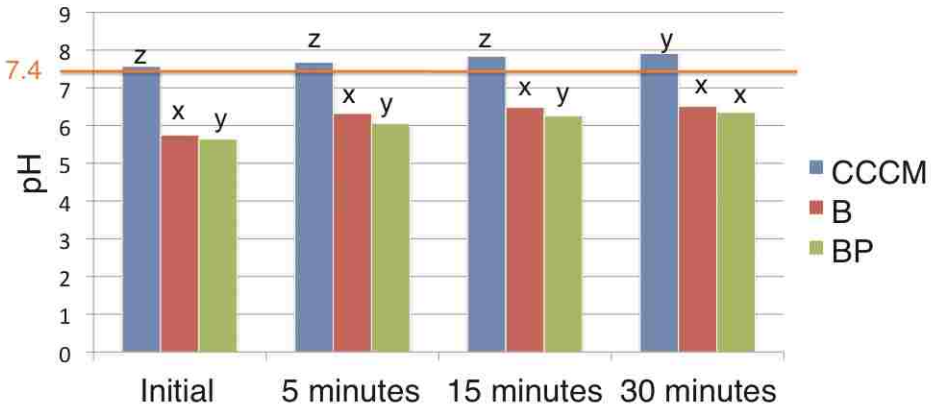

Figure 3.9 Mean pH across time of surface intact osteochondral cores. Means with like superscripts are not significantly different.
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

CHAPTER 4: DISCUSSION

Phase 1 was performed in part to confirm that osteochondral cores used for Phase 2 of the study were of adequate dimensions. The results of Phase I demonstrated that there was no penetration from the deep cut surface at the level of the subchondral bone, and that side penetration was minimal and did not approach the central core. The remaining central core was of more than adequate dimensions to evaluate chondrocyte death attributed to surface penetration.

Phase 1 results also showed an increasing mean proportional penetration of MB from the articular surface in a time dependent manner from 15 to 30 minutes in all treatment solutions and in both SI and SR cores. Our finding of time-dependent surface penetration from the articular surface of cartilage consistent with previous research[111]. The increased penetration of BP with MB in the SR cores suggested that when the articular surface was mechanically debrided, BP with MB penetrated significantly faster compared to B with MB or CCCM with MB. When comparing the Phase 2 chondrocyte death results to the Phase 1 MB penetration results, a direct causal relationship between BP rate of penetration and BP chondrocyte death could not be proven. In the superficial zone of SI cores, cores treated with BP for 30 minutes had significantly higher chondrocyte death compared to 15 minutes. This pattern is consistent with phase 1 results, where SI cores treated with BP and MB for 30 minutes had significantly more penetration compared to 15 minutes. In the superficial zone of SI cores, cores treated with B for 15 and 30 minutes had significantly higher chondrocyte death compared to 5 minutes. This pattern is not consistent with the results of Phase 1, where SI cores treated with B and MB for 30 minutes had significantly more penetration compared to 15 minutes. In the superficial zone of SR cores, cores treated with BP for 30 minutes had significantly higher chondrocyte death compared to 5 minutes. These results are not consistent with the results of Phase 1, where SR cores treated with BP and MB for 30 minutes did not have more penetration compared to 15 minutes. Both bupivacaine solutions caused significantly greater chondrocyte death over time compared to CCCM, but the rapid penetration of BP treated cores in Phase 1 and the chondrocyte death in BP treated cores of Phase 2 can not be considered a causal relationship. The increased chondrocyte death in the superficial zone and the increasing chondrocyte death over time are more likely a reflection of a time-dependent toxic effect on the exposed chondrocytes.

Methylene blue was mixed with each of the tested solutions to act as a visual marker to quantify the depth of surface penetration over time. Methylene blue has been shown to penetrate the surface of articular cartilage in a time-dependant manner[111]. An assumption of this study was that the depth of methylene blue penetration was dependent upon and consistent with the penetration of the treatment solution in which it was added. It is not known how the different treatment solutions interact with MB or if MB penetration is independent of treatment solution penetration. If the MB and the treatment solution penetrate independently and since Phase 1 results are a reflection of only MB penetration, then Phase 1 and Phase 2 results would have to be considered independently.

In this study, both B and BP treated cores exhibited time-dependent chondrocyte death and the greatest chondrocyte death occurred in the superficial zone of both the SI and SR cores. This is a consistent finding in other studies examining bupivacaine-associated chondrocyte death[14-16]. This could be at least partially due to the lower proteoglycan content in the superficial zone of the articular cartilage. The concentration of proteoglycans increases with increasing depth from the articular surface[108]. Chondrocytes in the histologic superficial zone degrade proteoglycans more rapidly and synthesize less collagen and proteoglycans than do cells in the deeper zones[103]. Also, higher concentrations of fibronectin and water are present in the superficial zone[103]. Any insult that decreases chondrocyte proteoglycan production will result in extracellular matrix changes that could no longer sustain chondrocytes. It has been shown that 0.5% bupivacaine causes a decline in proteoglycan synthesis[6,13] and loss of 50% of the proteoglycans from articular cartilage results in irreversible degeneration of the joint[183]. This decreased metabolism could leave chondrocytes vulnerable to the toxic effect of bupivacaine. Methylene blue penetration and chondrocyte death were attributed to time-dependent solution penetration from only the articular surface. Therefore, the superficial zone is going to experience the most exposure to bupivacaine for the most time. It follows that the superficial zone would experience the most chondrocyte death.

When the SI and SR cores were compared, both had the greatest chondrocyte death in the superficial zone. In the middle and deep zones of SI cores, there were no statistical differences in chondrocyte death across time within treatments, but there were significant differences between treatment solutions at 30 minutes. Interestingly, in the middle and deep zones of SR cores, there were no statistical differences in chondrocyte death across time within treatments or between treatment solutions at any given time. Experimentally, removal of the superficial zone increases

the permeability of cartilage[103]. We hypothesized that SR cores exposed to bupivacaine would have greater chondrocyte death than SI cores. This was true for several of the time points but not a consistent finding as reported by other studies[14-16].

The hypothesis that BP would cause greater chondrocyte death compared to B was based on the report of a crystalline precipitate when BP was mixed with human osteoarthritic synovial fluid[6]. Complications associated with methylparaben are reported to be hypersensitivity reactions following parenteral administration in people[68]. Investigations comparing chondrocyte death following B and BP exposure have not been published[15]. Our results support our hypothesis that cartilage exposed to BP had greater chondrocyte death compared to B or CCCM. We did not mix B or BP with synovial fluid to see if crystalline precipitates developed as previously described[6]. This mechanism is not suspected to be the cause of increased chondrocyte death since there was no synovial fluid associated with the project design. The pH was always significantly lower for B and BP, than CCCM. The ph of BP at the end of treatment was significantly lower than B at 5 minutes and 15 minutes, however, by 30 minutes it was the same. Based on these results, the low pH of BP could potentially be responsible for the increased chondrocyte death compared to the other treatment solutions.

Our study evaluated the pH of the treatment solutions to determine if pH might contribute to differences in chondrocyte death. The pH increased significantly for all treatments during the treatment period (with exception to the CCCM at 5 minutes) suggesting that ongoing metabolism offers some buffering. Both B and BP had a consistently significantly lower pH than CCCM. Although a causal relationship has not been established, the lower pH of both bupivacaine solutions may play a role in canine chondrocyte death. Bovine osteochondral explants were treated with saline at pH 7.4, pH 7.0 and pH 5.0 for 15 to 60 minutes[176]. There were no differences in chondrocyte death of the pH of 5.0 and pH 7.0 treated chondrocytes compared to the pH 7.4 control group of at any time point, suggesting that saline with a pH as low as 5.0 did not cause chondrocyte death. Due to species variation, the effect of pH on canine cartilage remains unknown. The critical pH for causing canine chondrocyte death has not been determined. Furthermore, the relationship between pH and bupivacaine-associated canine chondrocyte death also remains unknown. Given that the pH of both B and BP were significantly lower than CCCM, lower than physiologic pH, and that B and BP treated cores had greater chondrocyte death compared to CCCM treated cores, it is possible that a low pH could contribute to, accentuate, or cause bupivacaine-associated chondrocyte death. Ideally a physiologic pH

bupivacaine would be compared to differentiate the effect of pH and the effect of bupivacaine itself. However, buffering bupivacaine solutions to a pH of 7.4 would alter the pKa and chemical effect of the solutions, in turn potentially negating the analgesic effect.

In this in vitro study, canine osteochondral cores exposed to both B and BP treatment solutions showed a time-dependant chondrocyte death compared to CCCM, however, the mechanisms responsible for bupivacaine-associated death of canine chondrocytes are unknown. It has been shown that there is a relationship between local anesthetic exposure and cartilage inflammation. A single intra-articular dose of 0.5% bupivacaine in rabbits caused increased articular cartilage inflammation by 24 hours after administration[9]. Bupivacaine has also been shown to cause inflammation through potentiating nitric oxide synthase-2 activity in rat glial cells and astrocytes[174]. Nitric oxide synthase-2 activity can be induced in normal cells through immunostimulation and bupivacaine has been shown to exacerbate ongoing inflammation through the production of nitric oxide[174,175]. Lidocaine has been shown to selectively up-regulate pro-inflammatory proteins and down-regulate anti-inflammatory and some pro-resolution peptides and proteins thereby inhibiting resolution of ongoing inflammation[184]. It is possible that bupivacaine shares this characteristic of inflammation inhibition with lidocaine. However, an inflammatory mechanism causing chondrocyte death in this study is unlikely because the joints were considered free of overt inflammation based on gross examination and histopathology of each joint.

Bupivacaine caused a time and dose-dependent apoptosis in Schwann cell cultures by stimulating the production of reactive oxygen species (ROS)[177]. When the ROS was blocked with anti-oxidants, bupivacaine induced apoptosis was significantly inhibited. Apoptosis of human articular chondrocytes has also been observed and proposed to have at least a partial role in chondrolysis following exposure to 0.25% bupivacaine[15]. In our study, B and BP had greater chondrocyte death in the superficial zones of both the SI and SR cores compared to CCCM. It has been shown that a greater proportion of apoptotic chondrocytes are within the histologic superficial zone of articular cartilage, especially in the face of osteoarthritis[185,186]. These chondrocytes undergo early apoptosis and have been detected with techniques such as TUNEL[186-189]. Theoretically, cartilage in this study exposed to bupivacaine could undergo decreased proteoglycan synthesis, leading to early chondrocyte apoptosis and the increased chondrocyte death that was observed. The combination of low proteoglycan content in the superficial aspect of cartilage, proteoglycan synthesis inhibition caused by bupivacaine, and the

propensity for chondrocytes in the histologic superficial zone to undergo apoptosis could result in the chondrocyte death that was observed in the superficial zones following exposure to bupivacaine.

Bupivacaine has also been shown to disrupt mitochondrial homeostatic mechanisms and could therefore contribute to bupivacaine-associated chondrocyte death. Local anesthetics with a high lipid-solubility, such as bupivacaine, penetrate the cell and reach the mitochondria, disrupt the mitochondrial transmembrane potential and subsequently lead to cellular apoptosis[65,190]. If the local anesthetic is removed before apoptosis is initiated, the effect is reversible and the mitochondria recover. Bupivacaine has been shown to cause mitochondrial depolarization and opening of permeability transition pores which play a key role in many forms of cell death[191]. The uncoupling effect on the mitochondria was noticed 6 minutes after incubating chondrocytes with bupivacaine[65] and our studied exposed chondrocytes for up to 30 minutes. The time to initiation of apoptosis was not defined so the critical exposure time is unknown. This mitochondrial mechanism could be at least partially responsible for chondrocyte death in our study.

With the recent in vitro evidence that bupivacaine causes chondrocyte death in bovine and human articular cartilage, the effects of other local anesthetics on chondrocytes were studied. Lidocaine[176] and ropivacaine[16] have both been shown in vitro to cause increased chondrocyte death compared to saline controls. Similar to bupivacaine, they caused greater chondrocyte death in cell cultures than intact tissues[16,155]. Lidocaine and ropivacaine are useful as intra-articular analgesics[50,158,159,171] as they reportedly have minimal systemic side effects and are therefore considered safer than bupivacaine when administered intra-articularly[156,157,172]. These local anesthetics have been shown to cause chondrocyte death, but not to the same degree as bupivacaine, which suggests that an inherent behavioral characteristic of bupivacaine is responsible for chondrocyte death. Mepivacaine has also been shown to cause increased cell death in equine articular cartilage explants challenged with lipopolysaccharide[173]. Chu et al[14,15] and Piper et al[16] compared the effect of cartilage surface integrity and found that cell cultures were much more sensitive to the effects of local anesthetics than intact tissues with the surface debrided. This suggests that not only an intact articular surface may have a protective effect but also that the cartilage matrix itself may have an integral role in chondrocyte protection.

The phase 2 viability assessment methodology provided objective assessment of cell viability. Confocal microscopy with vital staining is recognized as an accurate and sensitive

method of determining cell viability[155,188,192-195]. Living cells with intact plasma membranes and active cytoplasm metabolize calcein and exhibit green fluorescence and cell membranes of dead, damaged or dying cells are penetrated by ethidium and their nuclei will exhibit red fluorescence. This methodology easily discriminates between the live or dead/dying state of a given cell. However, the methodology of actually counting the cells was potentially associated with variability. Due to the number of cells in a given sample tissue, manual cell counts were impractical from a time and labor perspective. An average pixel count for cells of each zone of each image was used due to the layered structure of cartilage. The cells in the superficial zone of articular cartilage are flatter and would appear to be smaller in a 2-dimensional microscopic image than more spherical cells in the deeper zones. The average pixel count was done for both live and dead cells due to the structure of cartilage and the size discrepancy between green and red staining cells. Also, by nature of the staining properties, red cells will always have fewer pixels than a green staining cells since it is the nucleus that stains red and the cytoplasm that stains green. This methodology does have some shortcomings, especially when counting the SR cores. The surface debridement process caused debris to collect on the articular surface that could not be completely rinsed off the surface. When stained and imaged with the laser confocal microscope, this debris stained red and was then subtracted from the pixel counts. This added variability to the assessment that could have affected the results of Phase 2.

To the author's knowledge, the tissue collection methodology used in this study has not been previously described. We investigated the possibility of using similar collection methodology described by Chu et al for their research on bovine chondrolysis but were restricted by the size of the canine humeral head[14,15]. We found that we could collect osteochondral explants of similar dimensions of 6mm x 6mm x 6mm cubes. In vivo models of canine osteoarthritis have been reported in which disruption of the superficial zone occurs as a consequence[169,170]; however, to the authors' knowledge, an in vitro osteochondral surface disruption protocol has not been established. Chu et al removed the superficial 1mm of cartilage of their bovine osteochondral explants[14,15], however, the thickness of canine humeral head cartilage is considerably thinner than that of bovine stifles so this methodology could not be duplicated with consistency in this study. Our surface debridement methodology may have contributed to increased chondrocyte death in the SR cores. Preliminary histopathology confirmed disruption of the superficial layers of the articular surface and that the collection of these tissues did not cause overt pathology along the cut edges of the cartilage. The debridement

methodology was not standardized therefore disruption may have not been uniform from core to core. Any variation in pressure could easily result in variation of surface debridement. When the chondrocyte death was compared between SI and SR, significantly more chondrocyte death occurred in the superficial zone of the SR cores treated with CCCM for 5 and 15 minutes compared to the SI cores. In the middle zone, SR cores treated with CCCM for 5 minutes had significantly greater chondrocyte death than SI cores. This is probably a reflection of the severe trauma inflicted by the surface debridement, which set in motion a terminal process for chondrocytes no matter to which treatment solution these cores were exposed. Therefore, our study does not clearly evaluate the effect of cartilage disruption, but it does suggest a potential protective effect of an intact articular cartilage surface.

Osteochondral core collection and storage prior to treatment may have affected the chondrocyte viability, especially of the superficial zone chondrocytes. Articular cartilage undergoes primarily anaerobic metabolism and exposing the articular cartilage to air may have detrimental effects[195]. Collection of the cores occurred immediately following euthanasia to minimize any post-mortem autolysis. The osteochondral cores were collected as quickly as possible, rinsed with 37°C PBS during collection to maintain moisture and minimize trauma, and then immediately placed in CCCM at 37°C in a 5% CO_2/95% air atmosphere. The CCCM is manufactured specifically for use with canine chondrocytes. All tissues were treated and imaged within 24 hours of euthanasia and collection to minimize in vitro chondrocyte death. Every joint was collected with this same protocol to minimize variability that may have been due to collection or storage artifact. However, this methodology likely inserted some degree of variation to the evaluation and analysis.

This in vitro study showed that 0.5% bupivacaine with and without methylparaben preservative are associated with a time-dependant penetration of canine articular cartilage and increased chondrocyte death compared to controls, with both an intact and mechanically debrided surface. Significantly higher chondrocyte death occurred in the superficial zones of both SI and SR cores at every time period. These results suggest that an intact articular cartilage surface is susceptible to chondrocyte death when exposed to 0.5% bupivacaine and that when the surface is mechanically debrided chondrocyte death increases.

CHAPTER 5: SUMMARY AND CONCLUSIONS

With the popularity of day-case arthroscopy procedures in people, a need for safe and effective analgesia became a necessity. Bupivacaine has been thoroughly investigated as an intra-articular analgesic following arthroscopic procedures and has been widely accepted as an effective perioperative analgesic in people and animals. As an amide local anesthetic, it has an immediate onset of action and a long duration of activity, so when combined with decreased amounts of systemic analgesics, post-operative pain and systemic drug complications are minimized so these patients can be discharged from the hospital the same day as the surgical procedure. A variety of side effects associated with the use of intra-articular bupivacaine, however, when accepted protocols are adhered to these side effects can be minimized.

While the use of intra-articular bupivacaine was gaining popularity among human arthroscopic surgeons, research was performed to evaluate the safety of intra-articular administration of irrigating solutions and bupivacaine on articular cartilage. Mature cartilage is virtually devoid of blood supply and lymphatics so nutrition is provided by synovial fluid[103]. However, due to this difference compared to other tissues, response to cartilage injury is poor and the risks of degenerative changes are increased[196]. Early animal studies showed that chondrocytes exposed to bupivacaine suffered from a transient proteoglycan synthesis inhibition however no structural alterations were noted[6]. Intra-articular bupivacaine was therefore considered non-toxic to chondrocytes and research continued to determine the optimal protocol to maximize efficacy and minimize associated side effects.

Years after the administration of intra-articular bupivacaine was considered non-toxic to cartilage, concerns arose that intra-articular bupivacaine may actually be associated with more delayed detrimental effects. Cartilage exposed to 0.5% bupivacaine showed evidence of worsening inflammation beyond the time frame of previous studies[9]. Also, human case reports of chondrolysis were emerging and speculation arose that intra-articular bupivacaine may be responsible[10-12,69,175]. In vivo research in rabbits supported this theory[13]. In vitro studies assessing chondrocyte death following bupivacaine exposure revealed a time-dependent toxic effect on bovine and human cartilage[14,15]. Lidocaine and ropivacaine have since been shown to cause chondrocyte death as well, but to a lesser degree than bupivacaine[16,176].

The primary objective of this in vitro study was to determine if canine osteochondral cores exposed to 0.5% bupivacaine would have increased chondrocyte death compared to

controls. We measured chondrocyte death in articular cartilage of dogs exposed to 0.5% bupivacaine with and without methylparaben preservative and compared chondrocyte death when the articular surface was intact to when it was mechanically debrided. We hypothesized that exposure of canine articular cartilage to 0.5% bupivacaine would increase chondrocyte death in a time-dependent manner over 5 to 30 minutes and that chondrocyte death would be increased to a lesser extent in intact cartilage cores compared to those with the surface mechanically debrided.

Results of this study showed that articular cartilage of dogs following in vitro exposure to 0.5% bupivacaine with and without methylparaben preservative has greater chondrocyte death than controls. Cartilage with an intact surface had less chondrocyte death following exposure to the bupivacaine solutions compared to cartilage with the surface mechanically debrided, however, our debridement methodology was so severe that results were potentially confounded. Also, the pH of bupivacaine with and without methylparaben preservative were recorded and found to be significantly lower than the control at pH 7.4.

The mechanisms responsible for bupivacaine-associated chondrocyte death are currently unknown. The role of the low pH of the bupivacaine solutions may be important but researchers have exposed cartilage to acidic saline solutions and found no increases in chondrocyte death compared to controls[176]. Chondrocytes have been shown to undergo early apoptosis when exposed to bupivacaine. Our results are consistent with other studies that found the greatest chondrocyte death in the superficial zones of the cartilage where chondrocytes appear susceptible to early apoptosis[15]. Cartilage inflammation has been implicated to exacerbate bupivacaine's toxic effect, however, in this study the cartilage was considered histologically free of inflammation. Long acting, highly lipid soluble local anesthetics including bupivacaine have been shown to disrupt mitochondrial membrane potentials, leading to cell death. This mechanism could theoretically been a mechanism of chondrocyte death in this study.

Aside from the given in vitro study limitations, several other limitations should be considered. There was a temporal delay from tissue collection to treatment and evaluation that could have introduced increased chondrocyte death. The Phase 1 penetration results were based on the assumption that methylene blue penetration was a direct reflection of treatment solution penetration. It is unknown if methylene blue penetrates articular cartilage independently from the treatment solutions. Also, chondrocytes were counted using digital image editing software and pixel counts to calculate chondrocyte numbers which could have been over- or underestimated

due to technical considerations. The surface debridement methodology resulted in increased chondrocyte death in controls so this methodology was potentially too aggressive.

Based on the results of this in vitro study, canine articular cartilage exposed to 0.5% bupivacaine has greater chondrocyte death compared to controls. Further in vitro and in vivo studies will be necessary to further evaluate time or dose dependent effects, single injections and continuous infusions, healthy and osteoarthritic cartilage, and the role of pH in bupivacaine-associated chondrolysis. Further research is indicated to determine if intact healthy cartilage is less susceptible to injury, or if only cartilage with pathology is affected. This is of clinical significance as many animals undergoing joint-related surgical procedures have concurrent chondropathy or suffer iatrogenic cartilage injury during surgery. Joint pathology characterized by loss of articular cartilage surface continuity occurs with conditions such as osteochondritis dissecans[197], cranial cruciate ligament rupture[198], articular fractures[199], and patellar luxations[200]. This pathology results in subsequent chondrosis and osteoarthritis. Until further research is conducted, the intra-articular administration of 0.5% bupivacaine with or without methylparaben preservative should be used with caution in dogs, especially those with concurrent chondropathy.

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APPENDIX I: PHASE 1 RAW METHYLENE BLUE PENETRATION DATA

						MB Penetration (um)			
Specimen	Dog	Weight (kg)	Surface	Solution	Time (min)	Top	Right	Left	Total Depth
A-33	A3	19.8	SI	CCCM	30	72	F	F	72
A-34	A3	19.8	SI	CCCM	30	65	F	F	65
A-37	A3	19.8	SI	B	30	58	50	44	69
A-38	A3	19.8	SI	B	30	57	55	56	64
A-39	A3	19.8	SI	BP	30	25	45	52	51
A-40	A3	19.8	SI	BP	30	56	65	55	58
A-41	A3	19.8	SR	CCCM	30	60	61	52	67
A-42	A3	19.8	SR	CCCM	30	64	48	55	78
A-45	A3	19.8	SR	B	30	35	30	27	55
A-46	A3	19.8	SR	B	30	56	F	F	56
A-47	A3	19.8	SR	BP	30	43	62	40	59
A-48	A3	19.8	SR	BP	30	48	F	F	48
A-49	A4	16.5	SI	CCCM	30	72	F	F	72
A-50	A4	16.5	SI	CCCM	30	56	F	F	56
A-53	A4	16.5	SI	B	30	42	F	F	42
A-54	A4	16.5	SI	B	30	34	40	40	40
A-55	A4	16.5	SI	BP	30	37	F	F	37
A-56	A4	16.5	SI	BP	30	36	F	F	36
A-57	A4	16.5	SR	CCCM	30	60	F	F	60
A-58	A4	16.5	SR	CCCM	30	55	F	F	55
A-61	A4	16.5	SR	B	30	46	F	F	46
A-62	A4	16.5	SR	B	30	35	F	F	35
A-63	A4	16.5	SR	BP	30	55	50	70	63
A-64	A4	16.5	SR	BP	30	34	F	F	34
A-65	A5	14.8	SI	CCCM	30	40	42	35	64
A-66	A5	14.8	SI	CCCM	30	50	50	25	70

A-69	A5	14.8	SI	B	30	43	F	F	43
A-70	A5	14.8	SI	B	30	52	45	40	60
A-71	A5	14.8	SI	BP	30	44	F	F	44
A-72	A5	14.8	SI	BP	30	46	F	F	46
A-73	A5	14.8	SR	CCCM	30	40	45	30	45
A-74	A5	14.8	SR	CCCM	30	45	52	55	48
A-77	A5	14.8	SR	B	30	40	42	35	52
A-78	A5	14.8	SR	B	30	45	45	50	56
A-79	A5	14.8	SR	BP	30	44	F	F	44
A-80	A5	14.8	SR	BP	30	40	45	50	65
A-81	A6	9.6	SI	CCCM	15	27	48	42	50
A-82	A6	9.6	SI	CCCM	15	28	26	28	52
A-85	A6	9.6	SI	B	15	33	38	38	45
A-86	A6	9.6	SI	B	15	30	27	37	57
A-87	A6	9.6	SI	BP	15	32	30	50	57
A-88	A6	9.6	SI	BP	15	30	42	40	42
A-89	A6	9.6	SR	CCCM	15	10	27	30	51
A-90	A6	9.6	SR	CCCM	15	21	34	30	40
A-93	A6	9.6	SR	B	15	34	40	30	51
A-94	A6	9.6	SR	B	15	22	30	22	62
A-95	A6	9.6	SR	BP	15	42	34	30	55
A-96	A6	9.6	SR	BP	15	33	28	30	43
A-97	A7	17.6	SI	CCCM	15	50	45	50	60
A-98	A7	17.6	SI	CCCM	15	32	44	42	46
A-101	A7	17.6	SI	B	15	43	52	52	56
A-102	A7	17.6	SI	B	15	21	24	33	46
A-103	A7	17.6	SI	BP	15	31	47	48	50
A-104	A7	17.6	SI	BP	15	37	70	48	48
A-105	A7	17.6	SR	CCCM	15	35	62	51	54
A-106	A7	17.6	SR	CCCM	15	18	34	34	56

A-109	A7	17.6	SR	B	15	35	51	27	58
A-110	A7	17.6	SR	B	15	44	24	26	52
A-111	A7	17.6	SR	BP	15	36	32	20	49
A-112	A7	17.6	SR	BP	15	48	55	55	58
A-113	A8	16.4	SI	CCCM	15	30	48	54	62
A-114	A8	16.4	SI	CCCM	15	45	44	44	54
A-117	A8	16.4	SI	B	15	19	24	25	62
A-118	A8	16.4	SI	B	15	25	20	18	55
A-119	A8	16.4	SI	BP	15	37	30	42	60
A-120	A8	16.4	SI	BP	15	42	25	35	63
A-121	A8	16.4	SR	CCCM	15	31	21	32	65
A-122	A8	16.4	SR	CCCM	15	49	49	44	60
A-125	A8	16.4	SR	B	15	27	30	49	64
A-126	A8	16.4	SR	B	15	27	33	22	67
A-127	A8	16.4	SR	BP	15	40	43	39	59
A-128	A8	16.4	SR	BP	15	44	89	50	58

SI (Surface Intact); SR (Surface Removed); CCCM (Canine Chondrocyte Culture Medium); B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)
F: indicates that there was full penetration across the width of the core

APPENDIX II: PHASE 2 RAW CHONDROCYTE DEATH DATA

Specimen	Image	Zone	Live	Dead	% Death
B001	B001_Series10_z000.tif	1	37	42	53.0
B001	B001_Series10_z000.tif	2	28	3	10.5
B001	B001_Series10_z000.tif	3	5	0	0.0
B002	B002_B002_z001.tif	1	162	87	35.1
B002	B002_B002_z001.tif	2	115	28	19.7
B002	B002_B002_z001.tif	3	35	3	8.6
B003	B003_B003_z001.tif	1	53	201	79.0
B003	B003_B003_z001.tif	2	180	61	25.3
B003	B003_B003_z001.tif	3	53	3	5.9
B004	B004_B004_z002.tif	1	143	38	21.0
B004	B004_B004_z002.tif	2	127	16	11.2
B004	B004_B004_z002.tif	3	106	16	13.1
B005	B005_B005_z001.tif	1	93	97	51.1
B005	B005_B005_z001.tif	2	111	50	31.1
B005	B005_B005_z001.tif	3	97	43	30.7
B006	B006_B006_z000.tif	1	21	29	58.0
B006	B006_B006_z000.tif	2	127	4	3.1
B006	B006_B006_z000.tif	3	67	0	0.0
B007	B007_B004_z002.tif	1	108	97	47.3
B007	B007_B004_z002.tif	2	153	25	14.0
B007	B007_B004_z002.tif	3	157	32	16.9
B008	B008_B008_z001.tif	1	33	50	60.2
B008	B008_B008_z001.tif	2	103	28	21.4
B008	B008_B008_z001.tif	3	86	3	3.4
B009	B009_B009_z003.tif	1	31	92	74.8
B009	B009_B009_z003.tif	2	126	50	28.4
B009	B009_B009_z003.tif	3	73	12	14.1
B010	B010_B010_z000.tif	1	98	89	47.6

B010	B010_B010_z000.tif	2	147	29	16.5
B010	B010_B010_z000.tif	3	79	24	23.3
B011	B011_Series004_z004.tif	1	40	72	64.3
B011	B011_Series004_z004.tif	2	49	17	25.8
B011	B011_Series004_z004.tif	3	46	28	37.8
B012	B012_B012_z000.tif	1	288	50	14.8
B012	B012_B012_z000.tif	2	318	12	3.6
B012	B012_B012_z000.tif	3	172	3	1.7
B013	B013_B013_z005.tif	1	104	115	52.5
B013	B013_B013_z005.tif	2	190	15	7.3
B013	B013_B013_z005.tif	3	196	9	4.4
B014	B014_B014_z003.tif	1	55	122	68.9
B014	B014_B014_z003.tif	2	150	62	29.2
B014	B014_B014_z003.tif	3	107	54	33.5
B015	B015_B015_z002.tif	1	65	198	75.3
B015	B015_B015_z002.tif	2	150	84	35.9
B015	B015_B015_z002.tif	3	251	1	0.4
B016	B016_B016_z003.tif	1	30	29	49.2
B016	B016_B016_z003.tif	2	117	32	21.5
B016	B016_B016_z003.tif	3	143	0	0.0
B017	B017_B017_z000.tif	1	312	41	11.6
B017	B017_B017_z000.tif	2	272	40	12.8
B017	B017_B017_z000.tif	3	258	27	9.5
B018	B018_B018_z004.tif	1	13	93	87.7
B018	B018_B018_z004.tif	2	86	53	38.1
B018	B018_B018_z004.tif	3	55	17	23.6
B019	B019_B019_z008.tif	1	915	19	2.0
B019	B019_B019_z008.tif	2	831	3	0.4
B019	B019_B019_z008.tif	3	413	0	0.0
B020	B020_B020_z014.tif	1	349	177	33.7

B020	B020_B020_z014.tif	2	677	19	2.7
B020	B020_B020_z014.tif	3	399	2	0.5
B021	B021_B021_z004.tif	1	337	28	7.7
B021	B021_B021_z004.tif	2	805	9	1.1
B021	B021_B021_z004.tif	3	249	1	0.4
B022	B022r1_B022_z011.tif	1	824	675	45.0
B022	B022r1_B022_z011.tif	2	802	116	12.6
B022	B022r1_B022_z011.tif	3	403	0	0.0
B023	B023_B023_z009.tif	1	393	576	59.4
B023	B023_B023_z009.tif	2	702	47	6.3
B023	B023_B023_z009.tif	3	355	1	0.3
B024	B024_B024_z011.tif	1	225	168	42.7
B024	B024_B024_z011.tif	2	427	43	9.1
B024	B024_B024_z011.tif	3	181	2	1.1
B025	B025_B025_z014.tif	1	947	331	25.9
B025	B025_B025_z014.tif	2	901	0	0.0
B025	B025_B025_z014.tif	3	349	0	0.0
B026	B026_B026_z002.tif	1	274	104	27.5
B026	B026_B026_z002.tif	2	475	19	3.8
B026	B026_B026_z002.tif	3	70	1	1.4
B027	B027_B027_z006.tif	1	426	348	45.0
B027	B027_B027_z006.tif	2	659	134	16.9
B027	B027_B027_z006.tif	3	395	81	17.0
B028	B028_B028_z008.tif	1	494	71	12.6
B028	B028_B028_z008.tif	2	653	5	0.8
B028	B028_B028_z008.tif	3	261	0	0.0
B029	B029_B029_z004.tif	1	317	89	21.9
B029	B029_B029_z004.tif	2	493	0	0.0
B029	B029_B029_z004.tif	3	319	0	0.0
B030	B030_B030_z005.tif	1	812	419	34.0

B030	B030_B030_z005.tif	2	780	14	1.8
B030	B030_B030_z005.tif	3	218	7	3.1
B031	B031_B031_z012.tif	1	787	747	48.7
B031	B031_B031_z012.tif	2	839	28	3.2
B031	B031_B031_z012.tif	3	276	40	12.7
B032	B032_B032_z002.tif	1	435	424	49.4
B032	B032_B032_z002.tif	2	927	14	1.5
B032	B032_B032_z002.tif	3	178	0	0.0
B033	B033_B033_z003.tif	1	93	70	42.9
B033	B033_B033_z003.tif	2	392	15	3.7
B033	B033_B033_z003.tif	3	282	46	14.0
B034	B034_B034_z005.tif	1	278	175	38.6
B034	B034_B034_z005.tif	2	386	1	0.3
B034	B034_B034_z005.tif	3	286	1	0.3
B035	B035_B035_z003.tif	1	184	98	34.8
B035	B035_B035_z003.tif	2	455	33	6.8
B035	B035_B035_z003.tif	3	251	0	0.0
B036	B036_B036_z007.tif	1	185	204	52.4
B036	B036_B036_z007.tif	2	323	55	14.6
B036	B036_B036_z007.tif	3	329	17	4.9
B037	B037_Series004_z002.tif	1	215	100	31.7
B037	B037_Series004_z002.tif	2	151	0	0.0
B037	B037_Series004_z002.tif	3	89	0	0.0
B038	B038_Series005_z002.tif	1	66	106	61.6
B038	B038_Series005_z002.tif	2	224	3	1.3
B038	B038_Series005_z002.tif	3	37	0	0.0
B039	B039_Series007_z004.tif	1	551	69	11.1
B039	B039_Series007_z004.tif	2	382	13	3.3
B039	B039_Series007_z004.tif	3	184	5	2.6
B040	B040_Series005_z003.tif	1	557	62	10.0

B040	B040_Series005_z003.tif	2	398	10	2.5
B040	B040_Series005_z003.tif	3	407	8	1.9
B041	B041_Series004_z000.tif	1	125	107	46.1
B041	B041_Series004_z000.tif	2	221	6	2.6
B041	B041_Series004_z000.tif	3	72	0	0.0
B042	B042_Series005_z002.tif	1	25	65	72.2
B042	B042_Series005_z002.tif	2	27	9	25.0
B042	B042_Series005_z002.tif	3	35	1	2.8
B043	B043_Series004_z003.tif	1	16	14	46.7
B043	B043_Series004_z003.tif	2	38	10	20.8
B043	B043_Series004_z003.tif	3	4	0	0.0
B044	B044_Series004_z002	1	16	33	67.3
B044	B044_Series004_z002	2	70	29	29.3
B044	B044_Series004_z002	3	50	8	13.8
B045	B045_Series004_z003.tif	1	49	512	91.3
B045	B045_Series004_z003.tif	2	84	91	52.0
B045	B045_Series004_z003.tif	3	19	5	20.8
B046	B046_Series004_z002.tif	1	17	217	92.7
B046	B046_Series004_z002.tif	2	119	144	54.8
B046	B046_Series004_z002.tif	3	96	9	8.6
B047	B047_Series004_z006.tif	1	88	202	69.7
B047	B047_Series004_z006.tif	2	193	179	48.1
B047	B047_Series004_z006.tif	3	111	16	12.6
B048	B048_Series004_z000.tif	1	48	11	18.6
B048	B048_Series004_z000.tif	2	58	7	10.8
B048	B048_Series004_z000.tif	3	16	1	5.9
B049	B049_Series004_z003.tif	1	287	170	37.2
B049	B049_Series004_z003.tif	2	542	1	0.2
B049	B049_Series004_z003.tif	3	140	1	0.7
B050	B050_Series004_z001.tif	1	441	6	1.3

B050	B050_Series004_z001.tif	2	146	8	5.2
B050	B050_Series004_z001.tif	3	62	36	36.7
B051	B051_Series005_z006.tif	1	65	73	52.9
B051	B051_Series005_z006.tif	2	109	90	45.2
B051	B051_Series005_z006.tif	3	68	38	35.8
B052	B052_Series003_z003	1	131	106	44.7
B052	B052_Series003_z003	2	114	149	56.7
B052	B052_Series003_z003	3	101	110	52.1
B053	B053_Series004_z000.tif	1	97	155	61.5
B053	B053_Series004_z000.tif	2	265	34	11.4
B053	B053_Series004_z000.tif	3	224	1	0.4
B054	B054_Series004_z001.tif	1	137	324	70.3
B054	B054_Series004_z001.tif	2	209	66	24.0
B054	B054_Series004_z001.tif	3	111	0	0.0
B055	B055_Series005_z006.tif	1	419	107	20.3
B055	B055_Series005_z006.tif	2	266	10	3.6
B055	B055_Series005_z006.tif	3	134	20	13.0
B056	B056_Series004_z003.tif	1	99	63	38.9
B056	B056_Series004_z003.tif	2	481	20	4.0
B056	B056_Series004_z003.tif	3	191	9	4.5
B057	B057_Series004_z004.tif	1	498	40	7.4
B057	B057_Series004_z004.tif	2	491	22	4.3
B057	B057_Series004_z004.tif	3	298	31	9.4
B058	B058_Series005_z001	1	348	37	9.6
B058	B058_Series005_z001	2	154	21	12.0
B058	B058_Series005_z001	3	269	15	5.3
B059	B059_Series004_z002.tif	1	13	6	31.6
B059	B059_Series004_z002.tif	2	66	12	15.4
B059	B059_Series004_z002.tif	3	76	2	2.6
B060	B060_Series004_z003.tif	1	83	167	66.8

B060	B060_Series004_z003.tif	2	216	28	11.5
B060	B060_Series004_z003.tif	3	174	6	3.3
B061	B061_Series004_z003.tif	1	62	74	54.4
B061	B061_Series004_z003.tif	2	124	0	0.0
B061	B061_Series004_z003.tif	3	27	0	0.0
B062	B062_Series003_z003.tif	1	75	34	31.2
B062	B062_Series003_z003.tif	2	58	17	22.7
B062	B062_Series003_z003.tif	3	39	0	0.0
B063	B063_Series003_z004.tif	1	33	55	62.5
B063	B063_Series003_z004.tif	2	74	20	21.3
B063	B063_Series003_z004.tif	3	30	0	0.0
B064	B064_Series003_z003.tif	1	640	267	29.4
B064	B064_Series003_z003.tif	2	76	76	50.0
B064	B064_Series003_z003.tif	3	131	130	49.8
B065	B065_Series003_z001.tif	1	103	52	33.5
B065	B065_Series003_z001.tif	2	190	8	4.0
B065	B065_Series003_z001.tif	3	79	5	6.0
B066	B066_Series006_z002.tif	1	60	9	13.0
B066	B066_Series006_z002.tif	2	149	22	12.9
B066	B066_Series006_z002.tif	3	119	0	0.0
B067	B067_Series003_z003.tif	1	370	44	10.6
B067	B067_Series003_z003.tif	2	464	1	0.2
B067	B067_Series003_z003.tif	3	33	0	0.0
B068	B068_Series003_z004.tif	1	251	40	13.7
B068	B068_Series003_z004.tif	2	408	15	3.5
B068	B068_Series003_z004.tif	3	247	4	1.6
B069	B069_Series004_z001.tif	1	73	60	45.1
B069	B069_Series004_z001.tif	2	245	17	6.5
B069	B069_Series004_z001.tif	3	109	0	0.0
B070	B070_Series003_z005.tif	1	516	52	9.2

B070	B070_Series003_z005.tif	2	413	36	8.0
B070	B070_Series003_z005.tif	3	144	12	7.7
B071	B071_Series003_z000.tif	1	176	103	36.9
B071	B071_Series003_z000.tif	2	215	43	16.7
B071	B071_Series003_z000.tif	3	36	6	14.3
B072	B072_Series003_z001.tif	1	292	115	28.3
B072	B072_Series003_z001.tif	2	368	55	13.0
B072	B072_Series003_z001.tif	3	289	24	7.7
B073	B073_Series012_z002.tif	1	257	103	28.6
B073	B073_Series012_z002.tif	2	235	34	12.6
B073	B073_Series012_z002.tif	3	156	17	9.8
B074	B074_Series005_z006.tif	1	385	41	9.6
B074	B074_Series005_z006.tif	2	315	40	11.3
B074	B074_Series005_z006.tif	3	151	39	20.5
B075	B075_Series004_z001.tif	1	312	201	39.2
B075	B075_Series004_z001.tif	2	664	29	4.2
B075	B075_Series004_z001.tif	3	401	2	0.5
B076	B076_Series005_z002.tif	1	573	111	16.2
B076	B076_Series005_z002.tif	2	476	16	3.3
B076	B076_Series005_z002.tif	3	181	18	9.0
B077	B077_Series005_z005.tif	1	158	68	30.1
B077	B077_Series005_z005.tif	2	172	42	19.6
B077	B077_Series005_z005.tif	3	102	1	1.0
B078	B078_Series006_z000.tif	1	132	85	39.2
B078	B078_Series006_z000.tif	2	143	0	0.0
B078	B078_Series006_z000.tif	3	124	0	0.0
B079	B079_Series005_z001.tif	1	208	134	39.2
B079	B079_Series005_z001.tif	2	331	0	0.0
B079	B079_Series005_z001.tif	3	120	0	0.0
B080	B080_Series003_z004.tif	1	158	1	0.6

B080	B080_Series003_z004.tif	2	42	24	36.4
B080	B080_Series003_z004.tif	3	129	9	6.5
B081	B081_Series003_z003.tif	1	120	138	53.5
B081	B081_Series003_z003.tif	2	84	2	2.3
B081	B081_Series003_z003.tif	3	70	1	1.4
B082	B082_Series003_z001.tif	1	166	52	23.9
B082	B082_Series003_z001.tif	2	451	5	1.1
B082	B082_Series003_z001.tif	3	121	1	0.8
B083	B083_Series004_z000.tif	1	89	66	42.6
B083	B083_Series004_z000.tif	2	316	2	0.6
B083	B083_Series004_z000.tif	3	131	0	0.0
B084	B084_Series005_z006.tif	1	152	1	0.7
B084	B084_Series005_z006.tif	2	148	23	13.5
B084	B084_Series005_z006.tif	3	73	51	41.1
B085	B085_Series003_z002.tif	1	40	110	73.3
B085	B085_Series003_z002.tif	2	103	5	4.6
B085	B085_Series003_z002.tif	3	127	0	0.0
B086	B086_Series003_z006.tif	1	423	173	29.0
B086	B086_Series003_z006.tif	2	670	22	3.2
B086	B086_Series003_z006.tif	3	241	12	4.7
B087	B087_Series003_z003.tif	1	382	105	21.6
B087	B087_Series003_z003.tif	2	497	43	8.0
B087	B087_Series003_z003.tif	3	120	23	16.1
B088	B088_Series004_z006.tif	1	249	22	8.1
B088	B088_Series004_z006.tif	2	510	3	0.6
B088	B088_Series004_z006.tif	3	426	21	4.7
B089	B089_Series003_z000.tif	1	67	49	42.2
B089	B089_Series003_z000.tif	2	201	8	3.8
B089	B089_Series003_z000.tif	3	114	14	10.9
B090	B090_Series005_z005.tif	1	33	40	54.8

B090	B090_Series005_z005.tif	2	81	32	28.3
B090	B090_Series005_z005.tif	3	90	19	17.4
B091	B091_Series002_z003.tif	1	635	342	35.0
B091	B091_Series002_z003.tif	2	459	209	31.3
B091	B091_Series002_z003.tif	3	397	89	18.3
B092	B092_Series004_z003.tif	1	334	51	13.2
B092	B092_Series004_z003.tif	2	391	49	11.1
B092	B092_Series004_z003.tif	3	255	31	10.8
B093	B093_Series003_z001.tif	1	374	91	19.6
B093	B093_Series003_z001.tif	2	573	7	1.2
B093	B093_Series003_z001.tif	3	87	3	3.3
B094	B094_Series005_z003.tif	1	65	31	32.3
B094	B094_Series005_z003.tif	2	100	5	4.8
B094	B094_Series005_z003.tif	3	32	0	0.0
B095	B095_Series003_z000.tif	1	152	101	39.9
B095	B095_Series003_z000.tif	2	402	3	0.7
B095	B095_Series003_z000.tif	3	284	0	0.0
B096	B096_Series003_z002.tif	1	39	77	66.4
B096	B096_Series003_z002.tif	2	51	26	33.8
B096	B096_Series003_z002.tif	3	31	0	0.0
B097	B097_Series003_z001.tif	1	89	118	57.0
B097	B097_Series003_z001.tif	2	184	86	31.9
B097	B097_Series003_z001.tif	3	155	41	20.9
B098	B098_Series004_z004.tif	1	99	63	38.9
B098	B098_Series004_z004.tif	2	67	115	63.2
B098	B098_Series004_z004.tif	3	190	115	37.7
B099	B099_Series004_z002.tif	1	174	263	60.2
B099	B099_Series004_z002.tif	2	259	139	34.9
B099	B099_Series004_z002.tif	3	271	52	16.1
B100	B100_Series003_z002.tif	1	99	125	55.8

B100	B100_Series003_z002.tif	2	93	34	26.8
B100	B100_Series003_z002.tif	3	51	4	7.3
B101	B101_Series006_z002.tif	1	196	97	33.1
B101	B101_Series006_z002.tif	2	265	36	12.0
B101	B101_Series006_z002.tif	3	109	13	10.7
B102	B102_Series003_z003.tif	1	292	94	24.4
B102	B102_Series003_z003.tif	2	232	31	11.8
B102	B102_Series003_z003.tif	3	321	0	0.0
B103	B103_Series003_z002.tif	1	90	51	36.2
B103	B103_Series003_z002.tif	2	385	10	2.5
B103	B103_Series003_z002.tif	3	141	1	0.7
B104	B104_Series003_z003.tif	1	368	33	8.2
B104	B104_Series003_z003.tif	2	277	127	31.4
B104	B104_Series003_z003.tif	3	184	93	33.6
B105	B105_Series003_z003.tif	1	249	53	17.5
B105	B105_Series003_z003.tif	2	236	19	7.5
B105	B105_Series003_z003.tif	3	148	57	27.8
B106	B106_Series003_z004.tif	1	339	69	16.9
B106	B106_Series003_z004.tif	2	134	6	4.3
B106	B106_Series003_z004.tif	3	75	3	3.8
B107	B107_Series003_z002.tif	1	92	237	72.0
B107	B107_Series003_z002.tif	2	206	67	24.5
B107	B107_Series003_z002.tif	3	183	45	19.7
B108	B108_Series003_z000.tif	1	111	330	74.8
B108	B108_Series003_z000.tif	2	256	423	62.3
B108	B108_Series003_z000.tif	3	89	124	58.2
B109	B109_Series003_z001.tif	1	142	110	43.7
B109	B109_Series003_z001.tif	2	852	0	0.0
B109	B109_Series003_z001.tif	3	146	0	0.0
B110	B110_Series003_z002.tif	1	301	149	33.1

B110	B110_Series003_z002.tif	2	317	167	34.5
B110	B110_Series003_z002.tif	3	162	23	12.4
B111	B111_Series004_z004.tif	1	676	229	25.3
B111	B111_Series004_z004.tif	2	463	93	16.7
B111	B111_Series004_z004.tif	3	378	32	7.8
B112	B112_Series003_z003.tif	1	179	74	29.2
B112	B112_Series003_z003.tif	2	81	22	21.4
B112	B112_Series003_z003.tif	3	21	1	4.5
B113	B113_Series003_z002.tif	1	295	293	49.8
B113	B113_Series003_z002.tif	2	142	152	51.7
B113	B113_Series003_z002.tif	3	113	174	60.6
B114	B114_Series003_z001.tif	1	177	120	40.4
B114	B114_Series003_z001.tif	2	106	74	41.1
B114	B114_Series003_z001.tif	3	80	19	19.2
B115	B115_Series003_z000.tif	1	141	189	57.3
B115	B115_Series003_z000.tif	2	123	209	63.0
B115	B115_Series003_z000.tif	3	66	248	79.0
B116	B116_Series003_z001.tif	1	340	92	21.3
B116	B116_Series003_z001.tif	2	103	126	55.0
B116	B116_Series003_z001.tif	3	72	66	47.8
B117	B117_Series004_z002.tif	1	98	90	47.9
B117	B117_Series004_z002.tif	2	143	163	53.3
B117	B117_Series004_z002.tif	3	48	25	34.2
B118	B118_Series003_z006.tif	1	474	114	19.4
B118	B118_Series003_z006.tif	2	697	98	12.3
B118	B118_Series003_z006.tif	3	358	74	17.1
B119	B119_Series003_z003.tif	1	201	140	41.1
B119	B119_Series003_z003.tif	2	508	24	4.5
B119	B119_Series003_z003.tif	3	226	30	11.7
B120	B120_Series004_z001.tif	1	67	65	49.2

B120	B120_Series004_z001.tif	2	200	120	37.5
B120	B120_Series004_z001.tif	3	204	18	8.1
B121	B121_Series003_z000.tif	1	35	75	68.2
B121	B121_Series003_z000.tif	2	412	66	13.8
B121	B121_Series003_z000.tif	3	179	3	1.6
B122	B122_Series004_z002.tif	1	384	333	46.4
B122	B122_Series004_z002.tif	2	402	74	15.5
B122	B122_Series004_z002.tif	3	187	6	3.1
B123	B123_Series003_z001.tif	1	101	84	45.4
B123	B123_Series003_z001.tif	2	69	75	52.1
B123	B123_Series003_z001.tif	3	72	32	30.8
B124	B124_Series003_z003.tif	1	99	207	67.6
B124	B124_Series003_z003.tif	2	401	194	32.6
B124	B124_Series003_z003.tif	3	394	37	8.6
B125	B125_Series003_z002.tif	1	29	115	79.9
B125	B125_Series003_z002.tif	2	45	90	66.7
B125	B125_Series003_z002.tif	3	20	3	13.0
B126	B126_Series003_z000.tif	1	64	167	72.3
B126	B126_Series003_z000.tif	2	102	151	59.7
B126	B126_Series003_z000.tif	3	72	31	30.1
B127	B127_Series017_z006.tif	1	772	164	17.5
B127	B127_Series017_z006.tif	2	592	51	7.9
B127	B127_Series017_z006.tif	3	482	14	2.8
B128	B128_Series005_z000.tif	1	499	14	2.7
B128	B128_Series005_z000.tif	2	422	3	0.7
B128	B128_Series005_z000.tif	3	257	28	9.8
B129	B129_Series004_z002.tif	1	217	5	2.3
B129	B129_Series004_z002.tif	2	169	21	11.1
B129	B129_Series004_z002.tif	3	360	110	23.4
B130	B130_Series003_z002.tif	1	146	125	46.1

B130	B130_Series003_z002.tif	2	517	16	3.0
B130	B130_Series003_z002.tif	3	579	1	0.2
B131	B131_Series004_z001.tif	1	115	375	76.5
B131	B131_Series004_z001.tif	2	182	123	40.3
B131	B131_Series004_z001.tif	3	247	27	9.9
B132	B132_Series005_z002.tif	1	283	115	28.9
B132	B132_Series005_z002.tif	2	314	1	0.3
B132	B132_Series005_z002.tif	3	295	0	0.0
B133	B133_Series005_z000.tif	1	140	99	41.4
B133	B133_Series005_z000.tif	2	288	16	5.3
B133	B133_Series005_z000.tif	3	97	0	0.0
B134	B134_Series003_z001.tif	1	64	172	72.9
B134	B134_Series003_z001.tif	2	100	71	41.5
B134	B134_Series003_z001.tif	3	124	0	0.0
B135	B135_Series003_z002.tif	1	186	75	28.7
B135	B135_Series003_z002.tif	2	341	6	1.7
B135	B135_Series003_z002.tif	3	175	2	1.1
B136	B136_Series004_z000.tif	1	46	129	73.7
B136	B136_Series004_z000.tif	2	140	25	15.2
B136	B136_Series004_z000.tif	3	111	1	0.9
B137	B137_Series007_z003.tif	1	21	56	72.7
B137	B137_Series007_z003.tif	2	80	3	3.6
B137	B137_Series007_z003.tif	3	87	1	1.1
B138	B138_Series004_z000.tif	1	33	175	84.1
B138	B138_Series004_z000.tif	2	195	96	33.0
B138	B138_Series004_z000.tif	3	40	154	79.4
B139	B139_Series002_z000.tif	1	0	38	100.0
B139	B139_Series002_z000.tif	2	40	46	53.5
B139	B139_Series002_z000.tif	3	28	18	39.1
B140	B140_Series003_z001.tif	1	18	269	93.7

B140	B140_Series003_z001.tif	2	258	34	11.6
B140	B140_Series003_z001.tif	3	334	51	13.2
B141	B141_Series003_z002.tif	1	214	382	64.1
B141	B141_Series003_z002.tif	2	321	132	29.1
B141	B141_Series003_z002.tif	3	184	86	31.9
B142	B142_Series003_z003.tif	1	192	94	32.9
B142	B142_Series003_z003.tif	2	282	13	4.4
B142	B142_Series003_z003.tif	3	46	20	30.3
B143	B143_Series004_z002.tif	1	179	103	36.5
B143	B143_Series004_z002.tif	2	284	7	2.4
B143	B143_Series004_z002.tif	3	155	5	3.1
B144	B144_Series003_z001.tif	1	586	344	37.0
B144	B144_Series003_z001.tif	2	407	126	23.6
B144	B144_Series003_z001.tif	3	579	22	3.7
B145	B145_Series003_z002.tif	1	421	12	2.8
B145	B145_Series003_z002.tif	2	321	43	11.8
B145	B145_Series003_z002.tif	3	222	12	5.1
B146	B146_Series003_z002.tif	1	217	3	1.4
B146	B146_Series003_z002.tif	2	159	39	19.7
B146	B146_Series003_z002.tif	3	120	41	25.5
B147	B147_Series003_z000.tif	1	694	21	2.9
B147	B147_Series003_z000.tif	2	440	41	8.5
B147	B147_Series003_z000.tif	3	256	42	14.1
B148	B148_Series003_z001.tif	1	323	10	3.0
B148	B148_Series003_z001.tif	2	400	1	0.2
B148	B148_Series003_z001.tif	3	138	16	10.4
B149	B149_Series004_z002.tif	1	154	44	22.2
B149	B149_Series004_z002.tif	2	318	1	0.3
B149	B149_Series004_z002.tif	3	49	0	0.0
B150	B150_Series003_z003.tif	1	51	64	55.7

B150	B150_Series003_z003.tif	2	95	26	21.5
B150	B150_Series003_z003.tif	3	27	0	0.0
B151	B151_Series003_z000.tif	1	192	181	48.5
B151	B151_Series003_z000.tif	2	306	2	0.6
B151	B151_Series003_z000.tif	3	128	0	0.0
B152	B152_Series003_z001.tif	1	83	49	37.1
B152	B152_Series003_z001.tif	2	165	0	0.0
B152	B152_Series003_z001.tif	3	48	0	0.0
B153	B153_Series003_z002.tif	1	101	410	80.2
B153	B153_Series003_z002.tif	2	184	131	41.6
B153	B153_Series003_z002.tif	3	93	31	25.0
B154	B154_Series003_z000.tif	1	343	451	56.8
B154	B154_Series003_z000.tif	2	333	61	15.5
B154	B154_Series003_z000.tif	3	66	0	0.0
B155	B155_Series003_z003.tif	1	218	69	24.0
B155	B155_Series003_z003.tif	2	205	0	0.0
B155	B155_Series003_z003.tif	3	71	0	0.0
B156	B156_Series003_z001.tif	1	92	49	34.8
B156	B156_Series003_z001.tif	2	79	2	2.5
B156	B156_Series003_z001.tif	3	71	0	0.0
B157	B157_Series003_z000.tif	1	140	98	41.2
B157	B157_Series003_z000.tif	2	257	0	0.0
B157	B157_Series003_z000.tif	3	82	0	0.0
B158	B158_Series003_z001.tif	1	223	185	45.3
B158	B158_Series003_z001.tif	2	168	41	19.6
B158	B158_Series003_z001.tif	3	95	20	17.4
B159	B159_Series003_z002.tif	1	279	141	33.6
B159	B159_Series003_z002.tif	2	218	11	4.8
B159	B159_Series003_z002.tif	3	48	5	9.4
B160	B160_Series003_z000.tif	1	66	68	50.7

B160	B160_Series003_z000.tif	2	56	36	39.1
B160	B160_Series003_z000.tif	3	28	1	3.4
B161	B161_Series003_z001.tif	1	258	196	43.2
B161	B161_Series003_z001.tif	2	192	44	18.6
B161	B161_Series003_z001.tif	3	112	28	20.0
B162	B162_Series003_z000.tif	1	267	187	41.2
B162	B162_Series003_z000.tif	2	197	70	26.2
B162	B162_Series003_z000.tif	3	113	19	14.4
B163	B163_Series003_z001.tif	1	419	103	19.7
B163	B163_Series003_z001.tif	2	243	17	6.5
B163	B163_Series003_z001.tif	3	173	5	2.8
B164	B164_Series003_z001.tif	1	387	117	23.2
B164	B164_Series003_z001.tif	2	223	35	13.6
B164	B164_Series003_z001.tif	3	238	32	11.9
B165	B165_Series005_z002.tif	1	562	39	6.5
B165	B165_Series005_z002.tif	2	376	0	0.0
B165	B165_Series005_z002.tif	3	228	13	5.4
B166	B166_Series003_z003.tif	1	246	74	23.1
B166	B166_Series003_z003.tif	2	220	39	15.1
B166	B166_Series003_z003.tif	3	244	13	5.1
B167	B167_Series005_z002.tif	1	28	316	91.9
B167	B167_Series005_z002.tif	2	64	165	72.1
B167	B167_Series005_z002.tif	3	44	1	2.2
B168	B168_Series003_z004.tif	1	73	152	67.6
B168	B168_Series003_z004.tif	2	289	151	34.3
B168	B168_Series003_z004.tif	3	221	136	38.1
B169	B169_Series003_z000.tif	1	29	86	74.8
B169	B169_Series003_z000.tif	2	61	28	31.5
B169	B169_Series003_z000.tif	3	46	7	13.2
B170	B170_Series003_z004.tif	1	574	135	19.0

B170	B170_Series003_z004.tif	2	387	68	14.9
B170	B170_Series003_z004.tif	3	433	53	10.9
B171	B171_Series003_z002.tif	1	26	113	81.3
B171	B171_Series003_z002.tif	2	43	13	23.4
B171	B171_Series003_z002.tif	3	16	2	11.1
B172	B172_Series003_z002.tif	1	374	583	60.9
B172	B172_Series003_z002.tif	2	239	159	39.9
B172	B172_Series003_z002.tif	3	119	49	29.2
B173	B173_Series003_z003.tif	1	92	70	43.2
B173	B173_Series003_z003.tif	2	129	166	56.3
B173	B173_Series003_z003.tif	3	114	26	18.6
B174	B174_Series003_z002.tif	1	484	284	37.0
B174	B174_Series003_z002.tif	2	173	50	22.4
B174	B174_Series003_z002.tif	3	148	25	14.5
B175	B175_Series004_z001.tif	1	362	62	14.6
B175	B175_Series004_z001.tif	2	385	28	6.8
B175	B175_Series004_z001.tif	3	198	45	18.5
B176	B176_Series004_z003.tif	1	528	267	33.6
B176	B176_Series004_z003.tif	2	364	2	0.5
B176	B176_Series004_z003.tif	3	234	29	11.0
B177	B177_Series003_z003.tif	1	104	295	73.9
B177	B177_Series003_z003.tif	2	245	12	4.7
B177	B177_Series003_z003.tif	3	238	10	4.0
B178	B178_Series003_z000.tif	1	70	103	59.5
B178	B178_Series003_z000.tif	2	173	70	28.8
B178	B178_Series003_z000.tif	3	230	30	11.5
B179	B179_Series003_z000.tif	1	44	89	66.9
B179	B179_Series003_z000.tif	2	104	8	7.1
B179	B179_Series003_z000.tif	3	72	12	14.3
B180	B180_Series003_z000.tif	1	187	525	73.7

B180	B180_Series003_z000.tif	2	321	16	4.8
B180	B180_Series003_z000.tif	3	113	1	0.9

Zone 1 = Superficial Zone
Zone 2 = Middle Zone
Zone 3 = Deep Zone

APPENDIX III: PHASE 2 RAW pH DATA

Specimen	Dog	Surface	Treatment	pH: initial	pH: end
B001	1	SI	CCCM 5 min.	7.49	7.47
B002	1	SI	CCCM 15 min.	7.49	7.46
B003	1	SI	CCCM 30 min.	7.49	7.67
B004	1	SI	B 5 min.	5.49	6.14
B005	1	SI	B 15 min.	5.49	6.18
B006	1	SI	B 30 min.	5.49	6.48
B007	1	SI	BP 5 min.	5.45	5.96
B008	1	SI	BP 15 min.	5.45	6.10
B009	1	SI	BP 30 min.	5.45	6.23
B010	1	SR	CCCM 5 min.	7.49	7.47
B011	1	SR	CCCM 15 min.	7.49	7.46
B012	1	SR	CCCM 30 min.	7.49	7.67
B013	1	SR	B 5 min.	5.49	6.14
B014	1	SR	B 15 min.	5.49	6.18
B015	1	SR	B 30 min.	5.49	6.48
B016	1	SR	BP 5 min.	5.45	5.96
B017	1	SR	BP 15 min.	5.45	6.10
B018	1	SR	BP 30 min.	5.45	6.23
B019	2	SI	CCCM 5 min.	7.54	7.57
B020	2	SI	CCCM 15 min.	7.54	7.58
B021	2	SI	CCCM 30 min.	7.54	7.65
B022	2	SI	B 5 min.	5.41	6.28
B023	2	SI	B 15 min.	5.41	6.43
B024	2	SI	B 30 min.	5.41	6.60
B025	2	SI	BP 5 min.	5.39	6.13
B026	2	SI	BP 15 min.	5.39	6.24
B027	2	SI	BP 30 min.	5.39	6.35
B028	2	SR	CCCM 5 min.	7.54	7.57

B029	2	SR	CCCM 15 min.	7.54	7.58
B030	2	SR	CCCM 30 min.	7.54	7.65
B031	2	SR	B 5 min.	5.41	6.28
B032	2	SR	B 15 min.	5.41	6.43
B033	2	SR	B 30 min.	5.41	6.60
B034	2	SR	BP 5 min.	5.39	6.13
B035	2	SR	BP 15 min.	5.39	6.24
B036	2	SR	BP 30 min.	5.39	6.35
B037	3	SI	CCCM 5 min.	7.70	7.78
B038	3	SI	CCCM 15 min.	7.70	7.86
B039	3	SI	CCCM 30 min.	7.70	7.93
B040	3	SI	B 5 min.	5.80	6.31
B041	3	SI	B 15 min.	5.80	6.33
B042	3	SI	B 30 min.	5.80	6.36
B043	3	SI	BP 5 min.	5.37	6.21
B044	3	SI	BP 15 min.	5.37	6.16
B045	3	SI	BP 30 min.	5.37	6.31
B046	3	SR	CCCM 5 min.	7.70	7.78
B047	3	SR	CCCM 15 min.	7.70	7.86
B048	3	SR	CCCM 30 min.	7.70	7.93
B049	3	SR	B 5 min.	5.80	6.31
B050	3	SR	B 15 min.	5.80	6.33
B051	3	SR	B 30 min.	5.80	6.36
B052	3	SR	BP 5 min.	5.37	6.21
B053	3	SR	BP 15 min.	5.37	6.16
B054	3	SR	BP 30 min.	5.37	6.31
B055	4	SI	CCCM 5 min.	7.80	8.03
B056	4	SI	CCCM 15 min.	7.80	7.94
B057	4	SI	CCCM 30 min.	7.80	8.03
B058	4	SI	B 5 min.	5.48	6.31

B059	4	SI	B 15 min.	5.48	6.35
B060	4	SI	B 30 min.	5.48	6.14
B061	4	SI	BP 5 min.	5.81	6.05
B062	4	SI	BP 15 min.	5.81	6.48
B063	4	SI	BP 30 min.	5.81	6.50
B064	4	SR	CCCM 5 min.	7.80	8.03
B065	4	SR	CCCM 15 min.	7.80	7.94
B066	4	SR	CCCM 30 min.	7.80	8.03
B067	4	SR	B 5 min.	5.48	6.31
B068	4	SR	B 15 min.	5.48	6.35
B069	4	SR	B 30 min.	5.48	6.14
B070	4	SR	BP 5 min.	5.81	6.05
B071	4	SR	BP 15 min.	5.81	6.48
B072	4	SR	BP 30 min.	5.81	6.50
B073	5	SI	CCCM 5 min.	7.20	7.24
B074	5	SI	CCCM 15 min.	7.20	7.46
B075	5	SI	CCCM 30 min.	7.20	7.64
B076	5	SI	B 5 min.	5.20	5.83
B077	5	SI	B 15 min.	5.20	6.19
B078	5	SI	B 30 min.	5.20	6.53
B079	5	SI	BP 5 min.	5.36	5.68
B080	5	SI	BP 15 min.	5.36	6.14
B081	5	SI	BP 30 min.	5.36	6.21
B082	5	SR	CCCM 5 min.	7.20	7.24
B083	5	SR	CCCM 15 min.	7.20	7.46
B084	5	SR	CCCM 30 min.	7.20	7.64
B085	5	SR	B 5 min.	5.20	5.83
B086	5	SR	B 15 min.	5.20	6.19
B087	5	SR	B 30 min.	5.20	6.53
B088	5	SR	BP 5 min.	5.36	5.68

B089	5	SR	BP 15 min.	5.36	6.14
B090	5	SR	BP 30 min.	5.36	6.21
B091	6	SI	CCCM 5 min.	7.01	8.04
B092	6	SI	CCCM 15 min.	7.01	8.03
B093	6	SI	CCCM 30 min.	7.01	8.10
B094	6	SI	B 5 min.	5.67	6.73
B095	6	SI	B 15 min.	5.67	6.81
B096	6	SI	B 30 min.	5.67	6.95
B097	6	SI	BP 5 min.	5.66	6.01
B098	6	SI	BP 15 min.	5.66	6.25
B099	6	SI	BP 30 min.	5.66	6.32
B100	6	SR	CCCM 5 min.	7.01	8.04
B101	6	SR	CCCM 15 min.	7.01	8.03
B102	6	SR	CCCM 30 min.	7.01	8.10
B103	6	SR	B 5 min.	5.67	6.73
B104	6	SR	B 15 min.	5.67	6.81
B105	6	SR	B 30 min.	5.67	6.95
B106	6	SR	BP 5 min.	5.66	6.01
B107	6	SR	BP 15 min.	5.66	6.25
B108	6	SR	BP 30 min.	5.66	6.32
B109	7	SI	CCCM 5 min.	7.91	8.15
B110	7	SI	CCCM 15 min.	7.91	8.26
B111	7	SI	CCCM 30 min.	7.91	8.29
B112	7	SI	B 5 min.	6.11	6.17
B113	7	SI	B 15 min.	6.11	6.53
B114	7	SI	B 30 min.	6.11	6.35
B115	7	SI	BP 5 min.	5.66	6.04
B116	7	SI	BP 15 min.	5.66	6.31
B117	7	SI	BP 30 min.	5.66	6.33
B118	7	SR	CCCM 5 min.	7.91	8.15

B119	7	SR	CCCM 15 min.	7.91	8.26
B120	7	SR	CCCM 30 min.	7.91	8.29
B121	7	SR	B 5 min.	6.11	6.17
B122	7	SR	B 15 min.	6.11	6.53
B123	7	SR	B 30 min.	6.11	6.35
B124	7	SR	BP 5 min.	5.66	6.04
B125	7	SR	BP 15 min.	5.66	6.31
B126	7	SR	BP 30 min.	5.66	6.33
B127	8	SI	CCCM 5 min.	7.47	7.72
B128	8	SI	CCCM 15 min.	7.47	7.68
B129	8	SI	CCCM 30 min.	7.47	7.69
B130	8	SI	B 5 min.	6.30	6.58
B131	8	SI	B 15 min.	6.30	6.53
B132	8	SI	B 30 min.	6.30	6.56
B133	8	SI	BP 5 min.	5.85	6.18
B134	8	SI	BP 15 min.	5.85	6.22
B135	8	SI	BP 30 min.	5.85	6.46
B136	8	SR	CCCM 5 min.	7.68	7.77
B137	8	SR	CCCM 15 min.	7.68	7.81
B138	8	SR	CCCM 30 min.	7.68	7.78
B139	8	SR	B 5 min.	6.32	6.49
B140	8	SR	B 15 min.	6.32	6.65
B141	8	SR	B 30 min.	6.32	6.68
B142	8	SR	BP 5 min.	5.92	6.26
B143	8	SR	BP 15 min.	5.92	6.37
B144	8	SR	BP 30 min.	5.92	6.40
B145	9	SI	CCCM 5 min.	7.47	7.72
B146	9	SI	CCCM 15 min.	7.47	7.68
B147	9	SI	CCCM 30 min.	7.47	7.69
B148	9	SI	B 5 min.	6.30	6.58

B149	9	SI	B 15 min.	6.30	6.53
B150	9	SI	B 30 min.	6.30	6.56
B151	9	SI	BP 5 min.	5.85	6.18
B152	9	SI	BP 15 min.	5.85	6.22
B153	9	SI	BP 30 min.	5.85	6.46
B154	9	SR	CCCM 5 min.	7.60	7.87
B155	9	SR	CCCM 15 min.	7.60	8.02
B156	9	SR	CCCM 30 min.	7.60	8.00
B157	9	SR	B 5 min.	6.02	6.48
B158	9	SR	B 15 min.	6.02	6.65
B159	9	SR	B 30 min.	6.02	6.48
B160	9	SR	BP 5 min.	5.90	6.14
B161	9	SR	BP 15 min.	5.90	6.30
B162	9	SR	BP 30 min.	5.90	6.42
B163	10	SI	CCCM 5 min.	7.52	7.77
B164	10	SI	CCCM 15 min.	7.52	7.81
B165	10	SI	CCCM 30 min.	7.52	7.78
B166	10	SI	B 5 min.	6.32	6.49
B167	10	SI	B 15 min.	6.32	6.65
B168	10	SI	B 30 min.	6.32	6.68
B169	10	SI	BP 5 min.	5.92	6.26
B170	10	SI	BP 15 min.	5.92	6.37
B171	10	SI	BP 30 min.	5.92	6.40
B172	10	SR	CCCM 5 min.	7.83	7.87
B173	10	SR	CCCM 15 min.	7.83	8.02
B174	10	SR	CCCM 30 min.	7.83	8.00
B175	10	SR	B 5 min.	6.02	6.48
B176	10	SR	B 15 min.	6.02	6.65
B177	10	SR	B 30 min.	6.02	6.48
B178	10	SR	BP 5 min.	5.90	6.14

B179	10	SR	BP 15 min.	5.90	6.30
B180	10	SR	BP 30 min.	5.90	6.42

SI (Surface Intact); SR (Surface Removed)
CCCM (Canine Chondrocyte Culture Medium);
B (0.5% Bupivacaine); BP (0.5% Bupivacaine with Preservative)

VITA

Geoffrey Hennig was born and raised in Indianapolis, Indiana, where he graduated from Broad Ripple High School with honors in 1991. He then enrolled in the Biological Pre-Medical Illustration program at Iowa State University from the fall of 1991 through the spring of 1992. In the fall of 1992, he attended Indiana University where he graduated with distinction in May 1995, with a Bachelor of Arts in studio fine arts and a minor in art history.

Following graduation, he worked as a studio artist, graphic designer, medical illustrator, and taught anatomic illustration and functional ceramics. The fall of 2001 he was admitted to Ross University of Veterinary Medicine, on the island of St. Kitts in the West Indies. He completed his clinical training at the University of Georgia in January of 2005 and graduated with Highest Honors. He completed a rotating small animal medicine and surgery internship at the University of Georgia in June of 2006.

He started his residency in companion animal surgery at the Louisiana State University School of Veterinary Medicine in July of 2006. He entered the Louisiana State University Graduate School the fall of 2006 to pursue a master's in veterinary medical sciences. This thesis is the description of the research that was performed during his graduate studies and surgical residency. He will be awarded the degree of Master of Science in veterinary medical sciences in May of 2009 and will complete his companion animal surgery residency in July of 2009.