Effect of a Single Intra-Articular Injection of Bupivacaine on Synovial PGE$_2$

Concentrations in Normal Canine Stifles

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Jenna Elizabeth Giangarra

ABSTRACT (ACADEMIC)

Intra-articular bupivacaine is a common analgesic used in dogs with orthopedic disease. Bupivacaine has been linked to chondrotoxicity. The mechanism for bupivacaine’s chondrotoxicity is unknown, but may involve inflammation. Prostaglandin E₂ (PGE₂) is an inflammatory mediator and a marker of joint inflammation. The aim of this study was to compare synovial fluid PGE₂ concentrations after a single intra-articular injection of bupivacaine with a saline control in normal canine stifles. We hypothesized that bupivacaine stifles would have a significantly elevated PGE₂ concentration compared to controls. Stifles from eight healthy, adult Beagles were randomly selected as the treated stifle and infused with bupivacaine. The contralateral stifle was injected with saline. Synovial fluid was collected before and after injection. PGE₂ was quantified using a commercial ELISA. Data were transformed and mixed model ANOVA was performed with significance set at p<0.05. There were no significant differences in PGE₂ concentration between treatment groups or times. Samples acquired with one or two aspiration attempts had significantly lower PGE₂ concentrations than samples with ≥3 aspiration attempts (p=0.001). When adjusted for number of attempts, PGE₂ concentrations were significantly higher 24 (p=0.003) and 48 (p=0.041) hours after injection compared to baseline in the bupivacaine group, but not in the saline group. Intra-articular bupivacaine injection did not result in increased synovial fluid PGE₂ concentrations compared to controls; however, multiple aspiration attempts did, suggesting that synovial fluid PGE₂ concentration is sensitive to multiple fluid collection attempts. Future studies investigating synovial fluid inflammatory mediators should consider methods to minimize aspiration attempts.
Effect of a Single Intra-Articular Injection of Bupivacaine on Synovial PGE2 Concentrations in Normal Canine Stifles

Jenna Elizabeth Giangarra

ABSTRACT (PUBLIC)

Intra-articular bupivacaine is a popular pain relief medication commonly used in joint surgery. Despite its historically wide use, bupivacaine has been scrutinized due to its potentially toxic effects on joint cartilage. Currently, the mechanism of this toxicity has not been identified, though it may be associated with inflammation. Prostaglandin E2 (PGE2) is considered an indicator of joint inflammation. The purpose of this study was to quantify the concentration of PGE2 within the joint fluid following a single injection of bupivacaine in normal canine stifles as compared to a saline control. Eight healthy, adult Beagles were used for this study. Stifles were randomized into treatment (bupivacaine) or control (saline) groups such that each dog had one stifle infused with bupivacaine and the opposite stifle with saline. Joint fluid was collected at the following time points: before injection (T0), 30 minutes, 60 minutes, 24 hours and 48 hours. Samples were analyzed in duplicate for PGE2 concentration. There was no significant effect of treatment group (bupivacaine vs. saline) or time on joint fluid PGE2 concentration. The number of sampling attempts did have an effect. Samples acquired with only one or two attempts had significantly lower PGE2 concentrations than samples that required 3 or more sampling attempts. When adjusted for number of attempts, PGE2 concentrations were significantly higher 24 and 48 hours after injection compared to baseline within the bupivacaine group, but not the saline group. Intra-articular bupivacaine injection did not result in increased joint fluid PGE2 concentration compared to saline control. The data indicates that joint fluid PGE2 concentration is highly sensitive to fluid collection attempts.
DEDICATION

This work is dedicated to my family, specifically my husband and son, who have personally sacrificed in witness to the realization of my professional goals. I could not have done it without your love and support.
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It is important to note the colleagues intimately associated in the research and composition of Chapter 2: Effect of a single intra-articular injection of bupivacaine on synovial fluid PGE\textsubscript{2} concentrations in normal canine stifles. All contributors listed are co-authors and their contributions are acknowledged below.

- Sabrina L. Barry, DVM, DACVS-SA, is an assistant professor of Small Animal Clinical Sciences at the Virginia-Maryland College of Veterinary Medicine. Dr. Barry assisted directly in study design, sample collection, and manuscript preparation.

- Linda A. Dahlgren, DVM, PhD, DACVS, is an associate professor of Large Animal Clinical Sciences at the Virginia-Maryland College of Veterinary Medicine. Dr. Dahlgren assisted in study design and contributed to data analysis and interpretation as well as manuscript preparation.

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- Stephen R. Werre, PhD, is a Research Assistant Professor of Veterinary Medicine Experimental Statistics at the Virginia-Maryland College of Veterinary Medicine. Dr. Werre performed statistical analysis on all study data and was essential to data interpretation.
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<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>EP</td>
<td>E prostanoid G protein-coupled receptor</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL1β</td>
<td>Interleukin 1 beta</td>
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<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<td>MRP</td>
<td>Multidrug resistant protein</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
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<tr>
<td>NO</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>PAGCL</td>
<td>Post-arthroscopic glenohumeral chondrolysis</td>
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<tr>
<td>PG</td>
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<td>Prostaglandin E synthase</td>
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THEESIS ORGANIZATION

The thesis presented here contains an extended version of a journal publication as the primary focus of the document. The publication is entitled “Effect of a single intra-articular injection of bupivacaine on synovial fluid PGE$_2$ concentrations in normal canine stifles” and contains its own introduction, materials and methods, results, discussion, and references. The discussion found in Chapter 1 provides an overview and literature review of the research topic and is intended to be an extension of the introduction to the manuscript.
CHAPTER 1 – BACKGROUND AND LITERATURE REVIEW

1. NORMAL JOINT ANATOMY AND PHYSIOLOGY

A synovial joint consists of two or more articulating bone surfaces confined by the joint capsule. The potential space this creates, or synovial space, contains the articular cartilage surface of the articulating bones bathed in synovial fluid. The volume of synovial fluid can vary between 0.2 and 2 mL in the adult dog, depending on the size of the dog and the specific joints (1). These components work in concert to provide near frictionless motion and even force transmission during weight bearing (1, 2).

The joint capsule is confluent with the peristium of the adjacent bones and consists of two layers. The fibrous outer layer predominately consists of type 1 collagen and elastin. In most joints, ligaments are extensions of this fibrous layer. In the case of the stifle, the menisci are intimately associated with the fibrous joint capsule. The inner layer, or synovium, is further divided into two layers: the intimal and subintimal layers. The subintimal layer contains loosely arranged fibrous and fatty tissue, as well as the rich synovial neurovascular supply (3-5). The intimal layer is innermost and consists of multiple layers of synoviocytes lacking a basement membrane. Three types of synoviocytes are described: macrophages (Type A synoviocytes), fibroblasts (Type B synoviocytes) and dendritic cells (6, 7). Type A synoviocytes are phagocytic. Type B synoviocytes produce collagens, hyaluronan and other proteins for the formation of healthy synovial fluid. In addition, synoviocytes contribute to the production of cytokines and inflammatory mediators (5).
The lack of a basement membrane and the vast vascular supply of the subintimal layer facilitate the passage of plasma from the blood into the synovial space. Only proteins <10kDa can pass through the endothelium of the subintimal layer. This rapid exchange across the synovium allows for the efficient supply of nutrients and removal of waste products from the joint space (1, 5). Thus, the synovial fluid is an ultrafiltrate of blood formed by the passage of plasma through the synovium. Synovial fluid is formed in part by the passive processes of hydrostatic pressure and colloid oncotic pressure, which favor filtration into the joint. However, a large excretory component is provided by the synoviocytes. In particular, Type B synoviocytes supply hyaluronan and lubricin that provide the viscosity and contribute to the lubrication functions of synovial fluid (5, 8).

The articular cartilage, also termed hyaline cartilage, lines the articulating bone surfaces and is produced by chondrocytes that are surrounded by an extracellular matrix (ECM). Chondrocytes typically make up 1-12% of normal cartilage dry weight. While contributing to only a small percentage of cartilage tissue, chondrocytes are responsible for the turnover of the remaining cartilage matrix. While the water content can vary with age, water contributes the majority of the weight of cartilage tissue, estimated at 70-80%. Collagen and other substances such as proteoglycans, glycoproteins, and lipids account for the remaining 8-29% (5).

Classically, four histologic zones of articular cartilage have been described. The most superficial is termed the tangential zone followed, in order, by the transitional, radiate and calcified zones (9, 10). Each of these zones are differentiated histologically based on chondrocyte and collagen morphology and orientation. The tangential zone consists of densely packed, flattened
chondrocytes parallel to the joint surface with similarly arranged collagen fibers. The transitional zone contains ovoid chondrocytes with randomly arranged collagen. The largest chondrocytes are found within the radiate zone with the long axis of the cells and collagen fibers arranged perpendicular to the joint surface. The calcified zone contains rounded chondrocytes as well as the tidemark, the demarcation between calcified and uncalcified cartilage. Ninety to 95% of collagen in articular cartilage is type II collagen, which is produced by chondrocytes. The remaining 5-10% of collagen is a combination of types VI, IX, and XI collagen, which help with the stability of type II collagen (10, 11).

Aggrecan accounts for 85% of the proteoglycans found in cartilage. Each aggrecan monomer consists of glycosaminoglycan side chains bonded to a core protein. The glycosaminoglycans are hydrophilic with a high negative charge so that they repel each other, contributing to cartilage swelling pressure (12). The core protein contains an amino terminal side and a carboxyl terminal side with three domains, G1, G2, and G3, in between the two terminal ends. The amino terminal G1 domain is often connected to hyaluronan via a link protein (13). Between G2 and G3 domains are upwards of 30 keratin sulfate and 100 chondroitin sulfate side chains. Multiple aggrecan monomers are bound to the same hyaluronan creating large aggregates (14, 15). Therefore, proteoglycans are important in the maintenance of cartilage volume and buoyancy. Turnover of proteoglycans can take upwards of 300 days in the canine, though it may decrease in the presence of osteoarthritis.

Subchondral bone is the layer of bone immediately deep to the layer of calcified cartilage. Deeper to the subchondral bone layer, often 10 µm thick in most locations is the trabecular bone.
Together, the subchondral and trabecular bone support the articular cartilage, allowing the bone to adapt to the loading it undergoes. The subchondral bone generally supplies firm, rigid support, though it remains ten times more plastic compared to cortical diaphyseal bone. The trabecular bone provides elastic support (16). The bone will remodel in the face of abnormal loading, resulting in thickened sclerotic subchondral bone. The changes that occur deep to the articular surface may alter the mechanical loading of the articular cartilage and can be the source of joint pain. The subchondral bone is rich in vasculature and innervation allowing the tissue to respond to physiologic and pathologic conditions. The subchondral bone supplies cytokines, inflammatory mediators as well as pain perception in the presence of joint disease (17).

The neurovascular supply to the joint is not provided by a singular vessel or nerve. Circulus articularis vasculosus are anastomosing loops from surrounding arteries and veins, which form a network to supply the epiphysis and synovial membrane. Cutaneous and muscular branches of nearby nerves provide the innervation of the synovial joint including the subchondral bone, synovium, and ligaments and are the source of nociception. Interestingly, cartilage is aneural and, therefore, does not contribute to nociception (18). The innervation provides not only pain perception in the event of injury or disease, but also proprioceptive input (1). Several sensory receptors are present in the dog and include Pacinian-like and Ruffini-like receptors found within the synovial membrane, Golgi tendon organs found in ligaments, and free nerve endings (19).

Other intra-articular structures, such as the meniscus, contribute to pain sensation. Joint nociception and the pain pathway will be further described in detail with respect to the pelvic limb in Section 6, Sensory Innervation of the Joint and the Pain Pathway.
2. ARTHRITIS

Arthritis is inflammation of the joint as a whole organ. Historically, within human medicine in particular, arthritis is considered a “wear and tear” disease due to abnormal mechanical loading of normal joints or normal loading on abnormal cartilage (20). While direct damage to the cartilage certainly occurs, it is currently also accepted that inflammatory mediators can lead to cartilage degeneration even in the absence of mechanical trauma. As previously described, the joint organ contains a number of tissues: ligaments, cartilage, subchondral bone, synovium, and fibrous joint capsule. Each can be damaged individually resulting in the progression of the inflammatory cascade and arthritis.

A balance of anabolic and catabolic metabolism is necessary for the maintenance of cartilage (21). The “wear and tear” theory of osteoarthritis was supported by knowledge of the generally low metabolic activity of chondrocytes and little ability to repair itself. With increased awareness of the role of inflammatory mediators in the joint, a new “inflammatory” theory has been postulated. It has become clear that considering arthritis a cartilage-only disease is narrow sighted and while synovial inflammation may not always be a precursor, it is intricately involved in cartilage destruction (22, 23). It is accepted that various tissues including cartilage, subchondral bone, and synovium can release inflammatory mediators found in elevated concentrations in the presence of osteoarthritis (24). For the purposes of this review, synovitis will be used to illustrate the progressive nature of joint inflammation, which can result in cartilage degeneration.
Inflamed synovium produces inflammatory mediators that lead to excess proteolytic enzymes promoting cartilage catabolism. The cartilage breakdown products are then phagocytosed by synovial cells amplifying the inflammatory cascade (25). Synovitis in human osteoarthritis was found only at sites adjacent to degenerate cartilage but degenerate cartilage was not always connected to synovitis. This suggests that the cartilage degradation products caused the synovial inflammation (6, 26). However, Fernandez-Madrid et al 1994, challenged that assertion in human patients with early osteoarthritis, as 73% of patients had thickening of the synovial membrane suggesting that synovial changes are more likely a primary event (6, 27). Regardless of the initiating event, synovial inflammation and cartilage breakdown create a positive feedback loop and a self-perpetuating cycle.

Synovial inflammation is characterized by infiltration of white blood cells including neutrophils, T lymphocytes, and monocytes, as well as neo-vascularization, synovial fluid accumulation, and synovial hyperplasia (6, 28-31). The increased intra-articular pressure, due to increased fluid volume, decreases lymphatic drainage of the joint. Additionally, a decrease in the quality and quantity of hyaluronan results in decreased viscosity of the synovial fluid (32). Increased volume within the joint contributes to loss of function and decreased viscosity results in increased friction. Both contribute simultaneously to cartilage wear due to the derangement of normal joint biomechanics.

According to the inflammatory theory of osteoarthritis, synovitis contributes to the development of cartilage degeneration and eventual end result of osteoarthritis through the continuous supply of damaging inflammatory cytokines. Cytokines are small, signaling proteins with both paracrine
and autocrine functions and have been associated with synovitis and osteoarthritis severity and predicted progression (22, 33-35). Cytokines are frequently linked to chondrocytes, though chondrocytes are not the only source. Non-cartilaginous sources, such as synovial macrophages and fibroblasts, are important contributors of these inflammatory mediators. Initially, much of the cytokine expression is predominantly from synovial macrophages, promoting inflammatory and destructive responses in osteoarthritis (6, 35). Catabolic processes are governed by the main pro-inflammatory cytokines thought to be involved in the pathogenesis of osteoarthritis: interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) (24, 36, 37). IL1β and TNFα are synthesized by synoviocytes, chondrocytes, and leukocytes (6, 38, 39). These cytokines act on synoviocytes’ and chondrocytes’ IL1R type and TNF-R55 cell surface receptors, respectively (6). Expression of these receptors is up regulated in the presence of joint inflammation (40, 41). The cytokines stimulate other chondrocytes and synoviocytes to synthesize more cytokines and degradative proteases, which lead to degradation of cartilage matrix by way of chondrocyte release of matrix metalloproteinases (MMP)-1, -3, and -13 (6, 42-47). Other active substances that contribute to the pathogenesis of inflammation and osteoarthritis, such as PGE₂, nitrous oxide, and others are induced by pro-inflammatory cytokine expression (6, 48, 49).

Experimentally, inflammatory cytokines (IL1β and TNFα) peak and then decrease between 48 and 72 hours (50). The immune response that is triggered following release of these cytokines suggests that synovitis stimulates cytokine-driven cartilage changes with the additional production of MMP, aggrecanases, prostaglandins (PG) and free radicals (6, 38). Inflammatory cytokines accelerate the degradation of cartilage when it is subjected to damage from abnormal mechanical forces (34, 51). For example, cytokines peak 24 hours from injury and remain
elevated due to altered biomechanics in people with an acute anterior cruciate ligament tear (52, 53).

In dogs, the available research regarding synovitis pertains to its relationship to naturally occurring cranial cruciate ligament disease. The fact that dogs with naturally occurring cranial cruciate ligament disease have lymphoplasmacytic synovitis may support the theory that inflammation has a role in ligament rupture in dogs (54, 55). The majority of dogs with cranial cruciate ligament disease with contralateral clinically stable canine stifles have ligamentous fiber disruption of the cranial cruciate ligament, contributing to the development of synovitis and preceding complete ligament rupture (56-58). Approximately 50% of dogs with naturally occurring disease have evidence of lymphoplasmacytic synovitis (54). Lymphoplasmacytic synovitis can also be found in the contralateral stable stifle in dogs (54, 58-60). Evidence of synovitis in the contralateral limb of dogs presenting for cranial cruciate ligament injuries suggests ligamentous instability may be secondary to the onset of synovitis (54, 58, 61). It has been reported that persistent synovitis induces substantial deterioration of the biomechanical integrity of the cranial cruciate ligament (56, 62).
3. PROSTAGLANDIN E₂ FORMATION AND METABOLISM

PGE₂ is the primary eicosanoid end product of the arachidonic acid pathway. Free arachidonic acid, a 20-carbon chain fatty acid, is produced from plasma membrane phospholipids in the presence of phospholipase A₂ in response to cytosolic increases in calcium (63). Eicosanoids are paracrine hormones derived from this free arachidonic acid and are further divided into two groups: leukotrienes and prostanoids. Prostanoids are formed within the cytoplasm following the transformation of arachidonate to the precursor PGH₂ by prostaglandin synthase (PGS). This conversion occurs in two steps. PGS converts free arachidonic acid to an unstable intermediate PGG₂ using its cyclooxygenase activity (COX), which is then immediately converted to PGH₂ via PGS hydroperoxidase activity on the luminal side of nuclear and endoplasmic reticulum membranes (64). From PGH₂, prostanoids including prostaglandins, thromboxanes and prostacyclins are formed. There are four common forms of prostaglandins: PGE₂, PGI₂, PGD₂ and PGF₂α. These are important to normal homeostasis, including kidney function, platelet aggregation, reproduction, and others (65).

For many years it was believed that only one gene and protein existed for the production of prostaglandin synthase, commonly referred to as COX enzymes; however the identification of glucocorticoid inhibition of prostanoid production lead to the discovery of two “pools” of COX. The first pool is constitutive. Production is not increased by inductive triggers or decreased in the presence of steroids (COX1). The second is a ligand-inducible or glucocorticoid-inhibited pool (COX2) (66). Various cells can produce these enzymes, including fibroblasts, monocytes/macrophages, endothelial cells, as well as synoviocytes, chondrocytes and osteoblasts.
in the presence of inflammation. Elevated levels of COX enzymes are found within the synovial lining, synovial fibroblasts, and chondrocytes (67-69).

Prostaglandin E synthase (PGES) is the terminal enzyme that results in PGE\textsubscript{2} production. Multiple isoforms of this enzyme exist including a cytosolic PGES and microsomal PGES1 and 2. In particular, microsomal PGES1 is upregulated by pro-inflammatory stimuli and functionally paired with COX2, resulting in delayed PGE\textsubscript{2} synthesis and additional PGE\textsubscript{2} to perpetuate the inflammatory cascade (70, 71). PGE\textsubscript{2} leaves the cytoplasm via either active transport by the ATP-dependent multidrug resistance protein-4 (MRP4) or passive diffusion across the plasma membrane (64). PGE\textsubscript{2} then can act at or nearby the site of excretion by binding to one of four E prostanoid G protein-coupled receptors (EP). EP2 and EP4 receptors activate adenylyl cyclase, increasing intracellular cAMP. EP1 activates phosphatidylinositol metabolism forming inositol triphosphate with mobilization of intracellular free calcium. EP3 can also contribute to elevation of intracellular calcium as well as inhibition of cAMP generation (72, 73). Due to this latter activity, EP3 is therefore considered in part an inhibitory receptor (63).

PGE\textsubscript{2} is then rapidly metabolized often within minutes due to cytosolic enzymes 15-ketoprostaglandin delta 13-reductase and 15-hydroxyprostaglandin dehydrogenase (74). The lack of a cellular reservoir necessitates this rapid metabolism (75, 76). It is believed that PGE\textsubscript{2} is released from cells nearly exclusively in response to inflammation. The response has been described as quick and short lived following an experimental inflammatory insult (77) with a half-life of approximately 30 seconds in human circulation (78). The rise of PGE\textsubscript{2} is rapid and peaks 2-9 hours after experimental acute unsustained inflammatory insult in horses (79-81). In
contrast, PGE$_2$ also plays a predominant role in sustained inflammation peaking 14 days after an acute sustained injury via experimental transection of the cranial cruciate ligament in dogs (82).
4. PROSTAGLANDIN E\textsubscript{2} FUNCTIONS AND ROLE IN JOINT INFLAMMATION

PGE\textsubscript{2} is produced by virtually any nucleated cell in the body\cite{83}. It is an important mediator for various homeostatic processes. PGE\textsubscript{2} contributes to thermoregulation, circadian cycle, hyperalgesia, bone formation and healing, vasodilation, contraction and relaxation of smooth muscle for the purposes of blood pressure, parturition, as well as the peristaltic contractions of the gastrointestinal tract, and gastric mucosal barrier functions. However, the role PGE\textsubscript{2} plays in inflammation is the most extensively studied, as it is associated with the classical signs of inflammation: redness, heat, pain, and edema\cite{83}. It is described as the most important inflammatory mediator in experimental inflammatory exudates\cite{82,84}. PGE\textsubscript{2} increases blood flow through arterial dilation and increased microvascular permeability, resulting in redness and edema. Hyperalgesia is mediated through EP1 receptors acting on peripheral sensory neurons contributing to nociception as well as central neuronal sites\cite{63}.

\textit{In vivo}, PGE\textsubscript{2} is found in very low concentrations in normal equine joints. However, there is evidence that supports a PGE\textsubscript{2} inflammatory positive feedback loop maintaining increased concentrations within the synovial fluid following an inflammatory insult. IL1\textbeta and TNF\alpha are important pro-inflammatory cytokines enhancing PGE\textsubscript{2} production through the up regulation of COX2\cite{85-88}. The autocrine function allows PGE\textsubscript{2} to drive the arachidonic acid pathway by maintaining calcium elevations within the cell via EP1 receptor activation. The paracrine function allows PGE\textsubscript{2} to induce the release of PGE\textsubscript{2} from other cells from similar receptor activity and in the presence of IL1\textbeta, which may be important in sustaining chronic synovitis\cite{89,90}. 


Under inflammatory conditions within the joint, PGE$_2$ is capable of vasodilation, angiogenesis, and increased vascular permeability of the synovium, while also sensitizing peripheral nociceptors (82, 91-96). Higher concentrations in the synovial fluid are also directly correlated with clinical assessments of pain based on force plate analysis in canines (82). PGE$_2$ elevations have been shown to correlate with degree of pain in both dogs and humans (82, 97). PGE$_2$ contribution to pain sensation was once thought to be limited to peripheral sites, though it is now understood that PGE$_2$ plays a role in pain transmission within the spinal cord contributing to hyperalgesia and central sensitization (98).

Within the joint, the presence of increased levels of PGE$_2$ can contribute to osteoarthritis and cartilage degeneration in multiple ways. Cyclooxygenase activation enhances production of MMP3 along with PGE$_2$. Additionally, PGE$_2$ can induce MMP expression alone (82, 91-96). Increased PGE$_2$ concentrations also contribute to the production of cartilage-degrading proteases to induce extracellular matrix degradation and osteoarthritis pain pathways. PGE$_2$ causes further cartilage degradation by the inhibition of proteoglycan synthesis and direct induction of apoptosis of articular chondrocytes (99, 100). Additionally, it is a potent stimulator of osteoclast activity, resulting in subchondral bone resorption impacting the mechanical support of the articular cartilage (101). In one study, elevated PGE$_2$ concentrations within the synovial fluid was consistent with the presence of joint disease in horses (102). This finding was further supported by a study by de Grauw et al 2006, which also identified elevated PGE$_2$ concentrations in the presence of joint pathology in equines (103).
Different components of the joint contribute to the release of PGE$_2$ into the synovial space and the overall inflammatory process. These structures include the synovial membrane, cartilage, subchondral bone as well as menisci (104). Studies report that PGE$_2$ is found in higher concentrations in the synovial membrane rather than in cartilage in horses, supporting it as a specific indicator of synovial inflammation (48, 105). Similarly, PGE$_2$ is also found in greater concentrations in the synovial fluid from joints with osteoarthritis in horses compared to normal joints (84, 105). Given its elevation in the disease process, it is a proposed indicator of an existing inflammatory state within the joint (105).
5. PGE₂ COMPETETIVE ELISA

Given the sensitive nature of the protein, handling and processing of the cytokine is important when attempting to quantify its concentration. A commercially available competitive enzyme linked immunosorbent assay (ELISA) has been employed in other studies for PGE₂ quantification (50, 82, 97, 106). The competitive ELISA plate wells are coated with an anti-IgG to which a PGE₂ antibody binds. Free PGE₂ versus AChE linked PGE₂ (tracer) then compete for binding sites on the antibody. Once the colorimetric reagent containing the substrate for AChE is added, the ELISA plate is incubated until it can be read by spectrophotometer. The intensity of the color produced is proportional to the amount of bound tracer and, therefore, inversely proportional to the amount of free PGE₂ in the well.

The use of this method allows for reliable quantification of PGE₂ in the substrate tested. Cayman Chemical Inc. provides a PGE₂ canine specific competitive ELISA. This particular ELISA is supplied in a kit with all necessary reagents. It has been validated for urine, plasma, and tissue culture media. Though it has not been validated for synovial fluid, studies have reported its use with reliable data (50, 97).

As previously mentioned, PGE₂ is a sensitive protein, as a result proper handling and storage is necessary for successful quantification. If samples cannot be analyzed immediately, storage in a -80°C freezer is recommended to maintain PGE₂ stability. However, it is stated that samples can maintain their reliability for quantification if stored in such a manner for up to 2 years according to the manufacturer.
6. SENSORY INNERVATION OF THE JOINT AND THE PAIN PATHWAY

For the purposes of this discussion, the canine stifle will be used as an example to describe the sensory innervation and pain pathway of joints. The stifle has the vastest innervation of all joints considering the presence of multiple ligaments as well as menisci (107-109). The stifle is described as a complex condylar synovial joint attributed to the articulation of the femoral and tibial condyles as well as the interaction of the patella and femoral trochlea. The joint capsule of the stifle is the largest in the body with three interconnected sacs. The stifle also contains two fibrocartilaginous discs, or menisci, that develop from the fibrous layer of the joint capsule, with the medial meniscus retaining its attachment to the joint capsule (110). Several ligaments are associated with the stifle including ligaments that secure the menisci, two cruciate ligaments that lie intra-articularly though are considered extra-synovial, and collateral ligaments that provide medial and lateral stability and arise from the fibrous layer of the joint capsule similar to the menisci.

As previously stated, pain receptors are located in non-cartilaginous tissues of the joint and are supplied by the articular branches of nearby nerves (18). The articular branches of peripheral nerves contain myelinated and unmyelinated afferent fibers, as well as unmyelinated sympathetic efferent fibers (92). Nociceptive input travels along thinly myelinated, A δ fibers, as well as unmyelinated C fibers. Mechanoreceptor input travels along myelinated afferent fibers, A β fibers. Post-ganglionic sympathetic efferent nerve fibers are often found near blood vessels within the synovium, and are implicated in the maintenance of vascular tone as seen with inflammation (92). Neural transmission from afferent fibers ascend to the dorsal horn of the spinal cord before initiating a reflex arc or projecting the impulse to the brain resulting in pain.
perception (111). The region of the dorsal horn that is activated can be done so by either mechanical, nociceptive stimuli, or both. This is considered regional activation and further emphasizes the mechanoreceptor role in nociception and pain perception (111). The initial activation of a receptor lowers the threshold for subsequent stimulation, termed hyperalgesia. The subsequent reactivation of sensory receptor organs is often correlated with inflammatory mediators that directly excite nociceptors, which include, but are not limited to bradykinin, histamine, and PGE₂ (112).

Mechanoreceptors contribute to pain sensitization of the joint both directly and indirectly. Mechanoreceptors are encapsulated sensory end organs involved in proprioceptive function. These end organs innervated by nerve fibers that transform mechanical stimulus into neural excitation, allowing for control of joint position during movement (113, 114). These mechanoreceptors are classified as Ruffini corpuscles or Type I mechanoreceptors, Pacini corpuscles or Type II mechanoreceptors, and Golgi-like corpuscles or Type III mechanoreceptors. Type I and II mechanoreceptors generally respond to pressure and tension and are often activated during joint movement, participating directly in proprioception. These mechanoreceptors are not likely to have a role in nociception (115). Type III receptors activate large myelinated afferent fibers, Aα fibers. They have a high threshold for activation and are generally activated when a strong mechanical stimulus approaches the level that may result in tissue damage, such as hyperextension of the stifle. Therefore, Type III mechanoreceptors are considered nociceptors (116).
Type IV receptors are actually free nerve endings rather than a receptor organ activating myelinated A $\delta$ and unmyelinated C fibers (116). They are found in all tissues of the joint save for articular cartilage and are considered polymodal given the broad spectrum of stimuli that activate these receptors, including not only mechanical and thermal but also chemical as with the inflammatory cascade. C afferent fibers are unmyelinated resulting in slower transduction often perceived as a slow, burning pain. A $\delta$ afferent fibers are myelinated resulting in a faster, acute pain perception (117). A subcategory of Type IV receptors are mechano-insensitive under normal circumstances, as with normal range of motion, however, they demonstrate mechano-sensitivity under inflammatory conditions. These receptors are termed silent nociceptors (92, 115). Chemical stimuli including bradykinin, histamine, PGE$_2$ as well as others can activate these receptors (112).

Human literature has demonstrated the nociceptive innervation of the abaxial aspect of the meniscus (118). This neuroanatomy was then explored in the equine species to ascertain any similarities between species. The saphenous nerve is described as the major innervation to the medial meniscus contributing to its nociceptive and sympathetic innervation (119). The cranial pole of the medial meniscus contains nociceptive fibers coursing along and through connective tissue, or associated with blood vessels, similar to humans. It is suggested that the presence of nociceptive fibers near blood vessels may have a role in inflammation by local vasodilation and plasma extravasation stimulated by substance P release (119). Ruffini corpuscles are the most numerous in the equine medial meniscus (114). It is postulated that the changes to the joint seen after partial meniscectomy may be due to both biomechanical disadvantages and deterioration of
the proprioceptive functions contributing to joint inflammation further perpetuating nociception and pain perception (120).

The phases of the pain pathway include transduction, transmission, modulation, projection, and perception (121). Nociception initiates the activation of the pain pathway in the processing noxious stimuli by the nervous system resulting in pain perception. This stimulus may be thermal, chemical, or mechanical in nature stimulating the reciprocal nociceptor. Once stimulated, nociceptors begin transduction or the conversion of the activated stimulus into an electrical impulse or action potential traveling towards the spinal cord (122). The next step requires the action potential to move from the peripheral nervous system to the central nervous system. The action potential travels to the nerve cell bodies in the dorsal root ganglion synapsing with a neuron in the dorsal horn completing transmission. Modulation is the third step and arguably the most influential. During modulation the brain interacts with spinal nerves in response to the nociceptive stimulus which occurs within the dorsal horn of the spinal cord. The dorsal horn contains interneurons and ascending neurons. It is at this level that peripheral sensory impulses are modulated, either amplified or suppressed. This makes the dorsal horn a central terminal receiving both inputs from the brain and outputs to the brain and brain stem. The majority of the nociceptive input is received in the rhesis lamina 1 and 2 of the dorsal horn of the spinal cord which are the most superficial portions of the grey matter.

Interestingly, the peripheral sympathetic nervous system is composed of postganglionic noradrenergic nerve fibers which via a direct action excite nociceptors during acute inflammation or via the immune system (123). This activation of the immune system acts in two ways. First, a
pro-inflammatory role to clear the lesion and an anti-inflammatory function to restore homeostasis (124). In chronic conditions, the dysregulation of the sympathetic nervous system favors amplification over suppression promoting inflammation, which in turn perpetuates dorsal horn activation leading to the local and systemic damaging effects of inflammation (124).

Projection begins in the dorsal horn with excitatory neurotransmitters. Presynaptic excitatory terminals release glutamate, the primary excitatory neurotransmitter for acute pain, thereby activating AMPA or kainite subtypes of the glutamate receptor. Conversely, NMDA receptors are the primary receptor for chronic pain. However, chronic nociceptive stimuli allow magnesium to be released from the NMDA channels during depolarization. This allows the NMDA receptor to become the primary channel for glutamate with a glycine co-agonist. Persistent NMDA receptor activation contributes to central sensitization commonly referred to as “wind-up” pain.

The final stage is perception, which occurs within the brain indicated by the conscious experience of discomfort within the cortex. The stimulation within the cortex allows the patient to consciously identify the pain. However, evidence suggests the rhesis lamina 1 contains a nociceptive pathway that also projects to the amygdala resulting in the emotional component of pain perception (125). The amygdala also participates in endogenous pain control and opioid analgesia mediated through the periaqueductal grey (126). Approximately 65% of neurons from the central nucleus of the amygdala neurons receive input from the knee with excitation as the predominant effect (127).
Joint pain has different characteristics to other types of pain in that it produces a diffuse, dull ache that is difficult to adequately localize owning, in part, to the regional activation of the spinal cord. In fact, there are demonstrated differences between primary afferents of skin, knee, and muscle suggesting that the perception of joint pain is different from that of other sites (128-131). Arthritis of the knee results in pain sensation during weight bearing or when mechanically manipulated, such as during a physical examination. This phenomenon is termed mechanical hyperalgesia (132). The hypersensitization of nociceptors and spinal cord neurons elevates pain sensitivity sustaining inflammation and promoting a hyperalgesic state. The source of joint pain can originate from any joint tissue that is innervated including the synovium, joint capsule, ligaments, and subchondral bone. It is important to remember that cartilage is not, in fact, the source of arthritic pain due to its lack of innervation.
7. PAIN MANAGEMENT

Analgesia is provided any mammalian species by interfering at any point along the pain pathway. A multimodal approach can be utilized to maximize analgesia thereby preventing the perception of pain. Common medications used to provide multimodal analgesia include non-steroidal anti-inflammatory drugs, opioids, local anesthetics, as well as others. Just as there are many medications to consider, there are equally numerous ways to administer these medications, including systemically, such as per os or intravenously, regionally, such as epidurally or femoral-sciatic blockade, or locally as with intra-articular administration. Further, the timing of medication administration is also considered. The ideal analgesic is site specific, long lasting, and easily administered with a high safety index.

Opioids are the cornerstone of analgesia in the small animal veterinary species (133). Prior to the application of regional analgesia and anesthesia, systemic administration was the route of choice. Parenteral routes were favored due to the rapid onset of action and distribution as well as the relative ease of administration and re-administration during the post-operative period. Unfortunately, given the diffuse nature of this modality, it is not without the potential for adverse effects, including central nervous system depression (sedation), hypothermia, nausea and vomiting, respiratory depression, vasodilation and hypotension secondary to histamine release, and ileus (133). As a result, regional techniques were explored in an effort to reduce systemic side effects.

Epidural regional analgesia is the prototypical form of adjunctive analgesia for surgery of the pelvic limb in small animals given its reliability and relative technical ease. Epidural morphine
provides analgesia for up to 24 hours with a single injection and an onset of action of 60 minutes (134). Often, epidural morphine is combined with a local anesthetic to bridge the gap of the onset of action. These local anesthetics likely act directly at the rhesis lamina 1 and 2 of the dorsal horn following diffusion(135). Reported side effects of epidurals include nausea, vomiting, respiratory depression, urinary retention, and pruritus (136, 137). Additional disadvantages of epidurals include bilateral motor blockade with certain medication combinations and the potential for iatrogenic complications such as injury to the cauda equina in cats (138, 139).

Femoral-sciatic neural blockade is a technique that has been evaluated in recent years often compared to the more traditional regional blockade of epidural anesthesia (140). Evidence supports its use in providing adequate perioperative analgesia in various species (141-145). Femoral sciatic blockade provides less systemic hemodynamic effects and urinary retention when compared to epidural (141, 145). Additionally, it is thought to provide better analgesia via blockade of the peripheral pain pathway, while some studies also support the reduction of maintenance anesthesia requirements when this technique is employed (141, 145). An additional benefit of the femoral-sciatic technique is the lateralization it provides, while there is motor blockade to the operated limb the contralateral limb is unaffected resulting in improved patient recovery evidenced by reduced post-operative stress biomarkers (143). Unfortunately, this technique necessitates additional equipment such as electrical nerve stimulators or ultrasound in order to facilitate administration directly to the intended site.

Systemic administration allows for rapid distribution to sites of action whereas regional blockades interrupt the pain pathway more peripherally with the intention of preventing pain
perception. Analgesia is best achieved the more peripheral the intervention site. For example, systemic administration of opioids acts to modulate synaptic neurotransmission of nociception after it has reached the dorsal horn of the spinal cord, whereas local anesthetics block afferent neural impulses peripherally before nociceptors are even stimulated by an insult (146). It is for this reason and the resultant blockage of afferent articular nerves, that lends itself to the clinical appeal of the intra-articular approach (147).

Commonly, local anesthetic agents are used intra-articularly in conjunction with stifle surgery. One example agent is bupivacaine, an amino-amide, lipophilic local anesthetic that works via reversible blockade of neuronal cell membrane voltage gated sodium channels. These medications act at the site of injury, preventing transduction of nerve fibers with minimal systemic effects. With the interruption of transduction, the dorsal horn remains unstimulated, ultimately preventing projection and pain perception. Intra-articular administration is technically straightforward and can provide reliable analgesia without the use of controlled pharmaceuticals. Intra-articular administration have been compared for efficacy against epidurals in various species requiring orthopedic surgery (148-150). Substantial intra-operative analgesia was provided by intra-articular administration of bupivacaine although short lived and required a second intra-articular injection as well as systemic rescue analgesia in one study using goats (148). It is reported that intra-articular bupivacaine can provide 4-6 hours of analgesia in dogs after stifle surgery, but there are reports of analgesia lasting for up to 24 hours in dogs and people (147, 151). Another study found no significant differences in the need for rescue analgesia between epidural morphine with bupivacaine, and intra-articular bupivacaine, advocating for the use of bupivacaine in dogs (149).
In addition to bupivacaine, other medications have also been explored with intra-articular applications. Peripheral opioid receptors are found outside the central nervous system and are associated with primary afferent neurons (152). These peripheral opioid receptors are activated in the presence of inflammation such that opioid agonists under inflammatory conditions, as with synovitis or osteoarthritis, can provide anti-nociception. Intra-articular morphine provides reliable and effective analgesia for up to 6-7 hours with one study reporting a duration of up to 48 hours (150, 153, 154). Interestingly, systemic administration of the same opioid does not provide the same degree of analgesia when compared to intra-articular administration (152). While this application provides a similar duration of analgesia it is important to note that intra-articular morphine does not outperform bupivacaine. As a result, the combination of the two medications have been advocated for superior analgesia (153).

Other combinations of intra-articular medications aim to provide a synergistic effect such as the use of dexmedetomidine with morphine in the stifle. This combination produced a longer duration of analgesia compared to the same medications used separately (154). The mechanism by which dexmedetomidine provided this effect is considered two-fold. First, an indirect effect of vasoconstriction reducing the absorption thereby retaining morphine in the joint space and, second, a direct effect acting at peripheral α2 receptors to provide direct analgesia (154). Other studies explore the use of non-steroidal anti-inflammatory medications intra-articularly with the intention to reduce joint inflammation as well as the resultant inflammatory nociception at the site of inflammation with mixed results. The addition of tenoxicam to bupivacaine provided
decreased need for analgesia in humans (155). While intra-articular meloxicam was found to have no perceived benefit when used alone in dogs (156).

As stated previously, the goal of analgesia is to prevent or mitigate pain perception reducing stress and overall discomfort of the patient. Given the ease of use and accessibility of intra-articular local anesthetics, one may question why this technique is not used ubiquitously. The answer is associated with the use of intra-articular bupivacaine infusion pumps following arthroscopic surgery in humans. This technique of using pumps was employed in an effort to lengthen the duration of action and prevent multiple intra-articular injections, but was associated with the later development of chondrolysis in those joints. The next section discusses the impact of this technique in both human and veterinary medicine.
8. BUPIVACAINE AND CHONDROTOXICITY

Although widely available and efficacious, reports of chondrolysis following intra-articular administration of local anesthetics such as bupivacaine caution their use in the clinical setting. Chondrolysis is described as the diffuse degeneration of articular cartilage. Various risk factors have been proposed, including the gentian-violet color-test in the shoulder, joint infection, slipped capital physeal fractures in the hip, as well as other surgical interventions such as implants, suture material, meniscectomies, or thermal injury from intra-articular cautery use (9, 157-161). In 2004, Petty, et al. was the first to describe three cases of young athletes who developed chondrolysis following arthroscopic surgery of the shoulder. A clear cause was not identified in that report (162). Later, in 2007, Hansen, et al. coined the term post-arthroscopy glenohumeral chondrolysis (PAGCL) and reported a 63% incidence of PAGCL at a mean of 4.3 months post-operatively in patients that received a 48-hour infusion of 0.25% bupivacaine and epinephrine through a post-operative intra-articular pain pump (163). His group concluded that the local anesthetic was the most likely cause of the chondrolysis and cautioned its continued use in human orthopedics (163). This concern inspired further research into the mechanism by which the rapid destruction of articular cartilage may be the result.

Recent systematic reviews summarize the findings of available clinical cases and experimental literature (164, 165). In 2015, Gulihar, et al. reported on 41 clinical cases and laboratory studies evaluating the effects of local anesthetics on articular cartilage. Among the clinical case reports, they identified a 40.6% incidence of chondrolysis in patients following the use of post-operative intra-articular pain pumps. Risk factors associated with chondrolysis included not only the pain pump itself, but also high flow rates (166). Laboratory studies have employed various local
anesthetic agents, concentrations, and methods to assess cellular viability. Among these studies, the terminology is shifted to reflect cellular viability by using the term chondrotoxicity rather than chondrolysis, as a mechanism for chondrolysis still had yet to be elucidated. Gulihar, et al. summarized that bupivacaine, lidocaine, ropivacaine, and levobupivacaine were all toxic to chondrocytes and there was a time- and dose-dependent relationship. Additionally, the effects of other features of the infusion on chondrotoxicity, such as pH, combination of epinephrine, and preservatives, was unclear and, at times controversial among the available literature. In 2017, Kreuz, et al. reported on 12 laboratory studies that evaluated the effects of single doses of bupivacaine on chondrocytes and cartilage and discussed the findings individually by type and dose of the local anesthetic agent used. Briefly, they concluded that there is significant evidence that supports a type- and dose-dependent effect of local anesthetics, even after a single administration. They recommended avoiding the use of lidocaine and bupivacaine in favor of very low concentrations of mepivacaine and ropivacaine. However, given the variability among study methods, a threshold dose for chondrotoxicity could not be identified.

Over the last 30 years, these studies utilized different local anesthetic agents, at different concentrations, for variable lengths of time against cell cultures from variable species that either were grown or harvested (167-172). Regardless of the study methods, local anesthetics have resulted in immediate necrosis followed by increasing cellular apoptosis after several days. The pathophysiology remains unclear (173-176). Unfortunately, much of the bench top research available fails to consider expected physiologic influences in vivo, such as how quickly the medication elutes out of the joint space, host metabolism of the medication, and how the presence of osteoarthritis may affect the chondrocytes’ ability to respond to an insult.
Historical literature regarding the kinetics of local anesthetics following a single intra-articular injection explain the continued concern regarding this method of administration. To review, the initial concern for the development of chondrolysis was the association with intra-articular infusion pumps given the large volume of local anesthetics chondrocytes are exposed to over time reaching a toxic threshold. However, the dose given with a single injection is much higher when compared to a constant infusion over an extended period of time. (165). Thus, it is thought to be more likely to reach the chondrotoxicity threshold (177). Evidence, in direct support of this assertion, reports peak plasma concentrations of bupivacaine occurring in 10 minutes in normal dogs following intra-articular administration (178). This is slower than reported for intravenous administration, suggesting sequestration within the joint space during absorption. Therefore, the presumption is that the theoretical toxic effects on the joint would occur within an hour of intra-articular administration. However, more recent literature challenges this theory. Within 30 minutes following intra-articular injection, bupivacaine concentrations decreased to 25% and 11% of the original concentration in osteoarthritic and normal canine stifles, respectively, in direct contrast to previous theory proposed by Saltzman et al. (179). The rapid clearance of bupivacaine following intra-articular administration supports its continued use clinically.

Given the potential for catastrophic chondrolysis, many studies utilized in vitro or ex vivo methods, however, there are several studies evaluating the in vivo response to intra-articular administration of single injections in various species. While the true chondrotoxic mechanism remains to be elucidated, the inflammatory effects of local anesthetics are consistently documented. In equine joints, single intra-articular injections of lidocaine and mepivacaine
resulted in increased total nucleated cell counts for up to 24 hours post-injection (180). In bovine joints, single intra-articular injections of lidocaine and procaine resulted in increased total nucleated cell counts and total protein within the synovial fluid for up to 24 hours, with procaine having a greater effect over lidocaine. This study also noted that the acute inflammation was short lived and within 72 hours cytologic changes returned to baseline values (181). These studies identify an inflammatory response, however brief, in species other than the canine. This begs the question can we assume the same to be true for the dog.

*In vivo* studies have also used histopathology to further document the dangers or safety of local anesthetics. While pain pumps are largely implicated in the development of cartilage degeneration, a single intra-articular injection of bupivacaine resulted in histopathologic changes to the articular cartilage that are similar to osteoarthritis (182). Dogan et al, was the first evaluating inflammatory changes histopathologically up to 10 days post injection following a single injection of 0.5% bupivacaine in rabbit stifles. Histopathologic changes, such as synovium hypertrophy and hyperplasia, inflammation of the articular cartilage, and white blood cell infiltration, did not get worse over time but remained until 10 days post injection (182). The use of histopathology identified that while nucleated cell counts may diminish in 72 hours, the implications of the injection on the synovial membrane lasts far longer. Chu, et al in 2010 further classified the changes that occur to the cartilage following a single injection of 0.5% bupivacaine, saline, or 0.6% monoiodoacetate (used as positive control) in rat stifles for up to 6 months. The authors reported a 50% reduction of chondrocyte density without cartilage tissue loss up to six months following injection, supporting the concern for chondrotoxicity. This study in particular also provides evidence that subsurface pathologic changes may not be evident on
gross inspection making the potential toxic effects of bupivacaine difficult to assess clinically with typical modalities such as arthroscopy and radiography (174).

The use of an in vivo design provides evidence that most closely resembles the clinical patient. However, even with the results of these studies it remains difficult to draw definitive conclusions on the safety of the intra-articular use of local anesthetics. In 2012, Piat et al used horses for the evaluation of 0.5% bupivacaine, 2% lidocaine, and saline following a single intra-articular injection with synovial fluid collected up to 14 days post injection. The study concluded that a single intra-articular injection of bupivacaine caused changes in both synthesis and degradation markers, specifically a rise in markers for proteoglycan synthesis with the absence of an expected rise in collagen degradation, which was an unexpected observation. Given the minimal effects on degradation, the authors affirmed a single injection was likely safe and suggested a possible secondary process to explain this phenomenon (183).

In 2015, Sherman et al, provided a fresh perspective by taking a step back from the literature that was available, which primarily focused on the effects of local anesthetics on chondrocytes. His group evaluated the effects of local anesthetics on synoviocyte viability in dog shoulders in vivo. The shoulders were injected with various local anesthetic and steroid combinations. All groups had lower cartilage viable cell density compared to saline injection, without significant differences among the treatment groups 24 hours post injection. After 7 days of tissue culture, all groups had lower viable cell density compared to day 1. However, there was no difference between bupivacaine and control synoviocyte subjective viability scores. The authors concluded that 0.0625% bupivacaine with triamcinolone was not associated with deleterious effects on
chondrocytes or synoviocytes (184). It is important to note that this study, although performed in canines, utilized the lowest concentration of bupivacaine. Perhaps the results would reflect significant changes at the higher concentrations more commonly used in practice. This study also proposes an alternative mechanism to chondrocyte death by way of synovitis.

Some of the major shortcomings of in vitro studies are the inability to approximate the host metabolism of the medication and the lack of a clinically representative population. Di Salvo et al in 2015 evaluated the pharmacokinetic profile and toxicity of intra-articular lidocaine in dogs. Elbows were infused with either 2% lidocaine and epinephrine or saline. Blood samples were collected before infusion and at 10 different time intervals beginning 5 minutes post injection up to 6 hours post injection. Time to maximum concentration (Cmax) was reached at a mean of 70.0 minutes for lidocaine. The active metabolite of lidocaine was detected as early as 5 minutes post injection but the Cmax for the metabolite was reached at a mean of 130 minutes post injection. A significant decrease in chondrocyte viability was found in all treatment groups with respect to controls (185). Unfortunately, this study failed to utilized bupivacaine and it would be inappropriate to presume a similar pharmacokinetic profile for the two different medications (bupivacaine and lidocaine). Iwasaki, et al. performed weekly injections of saline of 0.5% bupivacaine for 5 weeks intro normal and osteoarthritic rat stifles. Osteoarthritis was created experimentally by transecting the cranial cruciate ligament 4 weeks prior to the first injection. There were no differences in chondrocytes viability between saline and bupivacaine, or normal and osteoarthritic joints. However, the osteoarthritic stifles had evidence of erosion of articular cartilage that extended into the middle and deep zones. The Osteoarthritis Research Society International score was insignificantly higher in the bupivacaine group compared to the saline
group. This study concluded that bupivacaine may not have a harmful effect on normal joints and while there is concern regarding the abnormal articular surface in diseased joints, this study does not support any increase in vulnerability of the articular cartilage even with repeated injections (186). The mixed results from the research available continue to raise the question if extrapolation from other species is appropriate in this context.

Available research using a canine in vivo model is sparse. Nole et al was the first to evaluate the effect of intra-articular administration of bupivacaine. However, the authors did not recommend the discontinuation of intra-articular bupivacaine use at that time given that proteoglycan synthesis returned to normal within 3 days of administration and no evidence of direct chondrocyte damage was noted on electron microscopy (167). An ex vivo study by Hennig et al in 2010, attempted to mimic the arthritic joint response to bupivacaine exposure. After exposure to 0.5% bupivacaine for up to 30 minutes, 53.8% of superficial zone chondrocyte death was identified, which was significantly increased from 5-minute exposure with 23.6% chondrocyte death. However, even with damaged articular cartilage, percentage of chondrocyte death did not differ over time in the superficial zone or in the middle and deep zones (170). This study fails to approximate the host metabolism of the medication following administration, which the authors adequately acknowledge in their limitations. We know now that 11-25% of initial bupivacaine concentration is expected in the stifle joint 30 minutes following injection, allowing for further scrutiny of this study (179). Still the majority of research available, even in canines, focuses on the chondrocyte response to local anesthetic administration. Sherman, et al, performed a study that evaluated local anesthetic effects on other cells within the joint, specifically synoviocytes. Various concentrations of common local anesthetics were used, which indicated decreased viable
cell densities of cartilage and subjective viability scores of synovial explants compared to negative controls with the one exception of 0.0625% bupivacaine with triamcinolone. Additionally, no significant differences were found in the level of tissue metabolism with this concentration. This study established that various commonly used local anesthetics at common concentrations are not only chondrotoxic but also synoviotoxic substantiating an alternative mechanism for cartilage degeneration (172). Early evidence combined with more recent research raise the question whether the available studies reliably quantify the joint response to intra-articular administration of bupivacaine.
9. CONCLUSION

The joint is a complex organ with various tissues working in concert to maintain homeostasis. Disturbance of this homeostasis affects all of the organ’s tissues as a whole. Arthritis and subsequent cartilage degeneration is a cascade, which bupivacaine may initiate, contribute to, or accelerate by the promotion of inflammation. Studies fail to adequately describe the inflammatory response \textit{in vivo} after a single intra-articular injection of bupivacaine in the canine patient. This creates a difficult atmosphere for the practicing clinician who must weigh the benefits of adequate analgesia against possible consequences when making treatment decisions. PGE$_2$ is an inflammatory biomarker that can be used to identify joint inflammation. The objective of the \textit{in vivo} study reported here was to identify if there is an inflammatory response in the normal canine stifle following a single intra-articular dose of bupivacaine.
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CHAPTER 2 – Effect of a Single Intra-Articular Injection of Bupivacaine on Synovial PGE\textsubscript{2} Concentrations in Normal Canine Stifles


Introduction

Bupivacaine is a local anesthetic that works directly on the neuronal cell membrane, reversibly binding sodium channels and blocking the impulse conduction of nerve fibers. When used intra-articularly, as part of a multimodal analgesic protocol, bupivacaine provides long-lasting local pain relief (1-3). The ease of administration, wide availability, inexpensive nature, familiarity and reliable clinical efficacy of intra-articular bupivacaine make it an attractive option for the management of postoperative pain in the clinical setting.

In recent years, the safety of intra-articular use of bupivacaine has come into question, despite decades of clinical use without immediate side effects (4-8). A retrospective case series along with several systematic reviews of the literature concluded an association between continuous infusion of intra-articular bupivacaine and glenohumeral chondrolysis in people (4, 9-11). Chondrolysis is characterized by severe, diffuse degeneration of the articular cartilage from the bone surface (4, 8) and when associated with bupivacaine is thought to be the results of direct toxicity leading to increased chondrocyte apoptosis and necrosis (4-8, 12, 13) and is time and dose dependent (4, 7, 11, 13-15). The delay of several months between bupivacaine infusion and clinically evident chondrolysis suggests that chondrolysis may be a sequela to an acute process.
that indirectly leads to chondrocyte toxicity or makes chondrocytes more vulnerable to subsequent insults (16).

In humans and equids, sustained synovitis can indirectly lead to cartilage destruction via the activation and release of enzymes, inflammatory mediators, and cytokines (17-19). It is well established that an insult to the joint induces release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) from synoviocytes (20-23). These cytokines increase the synthesis of other inflammatory mediators from the joint such as prostaglandin (PG) E₂ (20, 21). PGE₂ is considered a marker of joint inflammation and synovitis (17) and stimulates expression of matrix metalloproteinases that play a major role in articular cartilage degeneration (24). PGE₂ synovial fluid concentrations are increased and sustained in dogs with osteoarthritis induced by experimental transection of the cranial cruciate ligament (25). In addition, studies also suggest that synovitis may precede cranial cruciate ligament degeneration, stifle instability and subsequent osteoarthritis (26-28).

The idea that bupivacaine may cause acute synovial joint inflammation is supported by studies involving other local anesthetics in multiple species (29-31). Increased nucleated cell counts and protein levels within synovial fluid has been reported 12-24 hours after injection of lidocaine and mepivacaine in equine joints (29), and lidocaine and procaine in bovine joints (30). In rabbit stifles harvested 24 hours, 48 hours and 10 days after bupivacaine injection, scored histologic changes were consistent with synovitis and were significantly worse than histologic scores in the saline controls (31).

There is reason to question whether ex vivo studies reporting bupivacaine’s chondrocyte toxicity accurately model its actual effects in the clinical patient. Isolation of chondrocytes from the surrounding extracellular matrix and the concentration and duration of bupivacaine to which
chondrocytes are exposed in these in vitro and ex vivo studies far exceed synovial fluid concentrations found after a single joint injection in vivo (32). Therefore, it is clinically important to know whether a single intra-articular bupivacaine injection, when given at the dose for perioperative analgesia, causes synovial joint inflammation that could lead to cartilage damage in dogs. The objective of this in vivo study was to identify if there is a synovial joint response in the normal canine stifle following a single intra-articular dose of bupivacaine. We hypothesized that a single intra-articular bupivacaine injection would result in a detectable synovial joint response as indicated by a significantly increased synovial fluid PGE$_2$ concentration, compared to intra-articular saline injection in normal canine stifles.
Materials and Methods

Study Population

Eight healthy adult male intact purpose bred Beagle dogs were used. The study was approved by the Institutional Animal Care and Use Committee. Dogs were housed in pairs in approved 1.5x2.1 meter runs and hand walked daily. The mean age of the dogs was 1.59 years (range: 1.57-1.62 years) and the mean weight was 12.1 kg (range: 10.1-13.4 kg). Dogs were included if they were deemed healthy based on normal physical and orthopedic examinations (i.e. no evidence of lameness at a walk, and absence of cranial drawer test or tibial thrust). Exclusion criteria consisted of evidence of orthopedic disease either at the time of orthopedic examination or on radiographic examination of the stifle. Dogs were heavily sedated with hydromorphone (0.05 mg/kg) and dexmedetomidine (5 mcg/kg) intravenously and eyes were lubricated with a sterile petrolatum ophthalmic ointment. Bilateral orthogonal stifle radiographs were obtained and a single board certified radiologist reviewed all radiographic images.

Sample Collection

Stifles were randomized using a random number generator\(^1\) such that each dog had one stifle allocated to bupivacaine and the contralateral stifle allocated to saline treatments. Immediately prior to sampling, dogs received a subcutaneous injection of a long acting cephalosporin, cefovecin sodium (8 mg/kg), to protect against bacterial contamination as a result of repeated arthrocentesis during this study. Synovial fluid was sampled at five time points: immediately prior to intra-articular injection (T0), thirty minutes following injection (T30), sixty minutes following injection (T60), twenty-four hours following injection (T24) and forty-eight hours following injection (T48).

\(^1\) Excel 2016, Microsoft Corporation, Redmond, WA, USA
Following radiographs, under the same sedation, stifles were clipped and aseptically prepared using 70% alcohol and povidone iodine scrub. When necessary, dogs were administered additional sedation (dexmedetomidine 5 mcg/kg, intravenously). The first three time points (T0, T30 and T60) were collected on the first day (Day 0). A standard medial or lateral para-patellar arthrocentesis technique was utilized as described previously, based on dog positioning (i.e. medial arthrocentesis of the right stifle and lateral arthrocentesis of the left stifle) (33). Following collection of the first sample, the syringe (1 mL) and needle (22 gauge) were removed and a new syringe (3 mL) and needle (22 gauge) were used to administer either 0.5% preservative-free bupivacaine\(^2\) (0.2 mL/kg) or an equal volume of 0.9% saline. On Day 0, following intra-articular injection, a digital timer was set for 30 minutes and ten passive range of motion cycles were performed. Following the last sample collection, dogs were reversed with atipamezole (equal volume to dexmedetomidine) intra-muscularly. The remaining time points (T24, T48) were collected on Days 1 and 2, respectively. Dogs were sedated; samples collected and then reversed using the above protocol. Following collection, all synovial fluid was transferred into 0.5 mL microfuge tubes and stored at -80°C until batch analysis could be performed.

**PGE\(_2\) Quantification**

Samples were thawed on ice and PGE\(_2\) quantified by competitive ELISA\(^3\) (25) and read by spectrophotometer\(^4\). All samples were processed within 6 months of collection. The manufacturer reports that samples can maintain reliability for quantification for up to 2 years with appropriate storage. Each sample was quantified in duplicate according to the

\(^2\) Marcaine 0.5% (preservative free), Hospira, Lake Forest, IL, USA

\(^3\) Cayman Chemical Company, Ann Arbor, MI, USA

\(^4\) Spectramax 5, Molecular Device, Sunnyvale, CA, USA
manufacturer’s instructions using the standard curves generated for each ELISA plate (7.8-1,000 pg/mL). Samples were initially diluted 1:2 with ELISA buffer. When necessary additional dilutions were performed to ensure results were within the standard curve of the plate (32/80 samples).

**Statistical Analysis**

Prior to the study, a power analysis was performed using data from an unpublished pilot study that measured the increase in PGE$_2$ concentration 30 minutes following intra-articular injection of bupivacaine. A sample size of 6 stifles per group was estimated to achieve 83% power to detect a mean of paired differences of 480 pg/ml between groups with a significance level (alpha) of 0.05.

Data were analyzed using commercial software$^5$. T0 concentrations were corrected to account for the inherent dilution created by infusion of either bupivacaine or saline into the joint using the following formula (T0c): $T0c = \frac{T0 \times Vi}{Vf}$. Where initial volume (Vi) equals 0.08 mL/kg (32) and final volume (Vf) equals (Vi – Vaspirated) + Vtreatment.

Normal probability plots showed that PGE$_2$ concentrations were skewed and are therefore summarized as median (range). A logarithmic transformation (base e) was applied to the concentrations before any downstream data analysis. Effects of treatment and time were assessed using mixed-model repeated-measures ANOVA followed by Tukey’s procedure for multiple comparisons. The linear model specified treatment and the interaction between treatment and time as fixed effects with Kenward-Roger approximation as the denominator degrees of freedom. G-side variation in the data was modeled by specifying dog identification as a random effect while the R-side variation in the data was modeled by specifying a first order, autoregressive, 

$^5$ SAS version 9.4, SAS Institute Inc., Cary, NC, USA
covariance matrix. Residuals were inspected to verify that the errors followed a normal distribution with constant variance. A post hoc analysis (mixed-model ANOVA) was performed to assess any association between number of aspiration attempts required to collect an adequate sample and PGE$_2$ concentration. Based on the post-hoc analysis revealing that $\geq$3 attempts resulted in significantly elevated PGE$_2$ concentrations, a sensitivity re-assessment of the effects of treatment and time using data obtained after 1 or 2 aspiration attempts was performed. Statistical significance was set at $p < 0.05$. 
Results

All dogs were included in the study. No radiographic evidence of osteoarthritis was identified in any stifle in any dog. Most dogs (7/8) required additional sedation administration for Day 0 sample collections. No other complications were noted before, during or after sample collection. No lameness following injection or joint aspiration was observed at any time throughout the study period.

There was no significant difference in PGE\textsubscript{2} concentration between the bupivacaine and saline groups (p=0.229-0.898) or over time within each group (p=0.152 for bupivacaine; p=0.343 for saline) when all data were included in the analysis without consideration for number of aspiration attempts (Table 1). The number of aspiration attempts required to collect an adequate sample was 1 in the majority of cases (62/80), but was as high as 6 attempts in a single dog (Table 2) and PGE\textsubscript{2} concentrations varied significantly based on number of aspiration attempts (Table 3; p = 0.007). PGE\textsubscript{2} concentrations for 1 attempt was not significantly different than for 2 attempts (p=0.984); however, PGE\textsubscript{2} concentrations in samples requiring either 1 (p = 0.005) or 2 (p = 0.041) attempts were significantly lower than those requiring 3 attempts.

Based on the analysis of number of attempts, samples requiring ≥3 aspiration attempts were omitted and the data re-analyzed. A total of 11 of 80 samples were omitted (Table 4). Following re-analysis, PGE\textsubscript{2} concentrations in the bupivacaine group increased significantly over time (p = 0.008). PGE\textsubscript{2} concentrations in samples collected at T24 (p = 0.003) and T48 (p = 0.041) were significantly higher compared to T0 (Table 4). There were no changes in PGE\textsubscript{2} concentrations in saline-treated joints over time (p = 0.207). There were no significant differences between saline- and bupivacaine-injected joints at any time point (p=0.261-0.949).
Finally, a second post hoc analysis was performed to evaluate the effect of \( \geq 3 \) attempts on all subsequent synovial fluid samples. Using a mixed model ANOVA, all samples collected before \( \geq 3 \) attempt-samples were compared to samples collected after \( \geq 3 \) attempt-samples, excluding samples that required \( \geq 3 \) attempts. No evidence was found to suggest that \( \geq 3 \) aspiration attempts influenced the PGE\(_2\) concentration in subsequent samples, regardless of whether all stifles were analyzed together (p=0.221) or whether bupivacaine and saline stifles were analyzed separately (p=0.092 and 0.582, respectively).
Discussion

In the normal canine stifles in our study, a single intra-articular injection of bupivacaine did not result in increased synovial fluid PGE$_2$ concentrations compared to saline-injected controls. Measurement of synovial fluid PGE$_2$ concentrations was complicated by changes in synovial fluid volume following injection, as well as the variable number of aspiration attempts for each joint. Our data indicate that synovial fluid PGE$_2$ concentration is highly sensitive to number of aspiration attempts. This is an important consideration when performing arthrocentesis in clinical cases and in designing future studies.

There is conflicting evidence of the expected inflammatory effects of local anesthetics on the synovium (29, 31, 34, 35). In an *in vitro* canine co-culture model of the synovial joint, the presence of 0.05% bupivacaine inhibited an expected rise in PGE$_2$ following addition of IL-1β to the culture medium (5). In this same study, a decrease in chondrocyte viability was also noted, but a mechanism was not identified (5). Neurogenic inflammation was reduced following a 30-minute continuous infusion (200 uL/min) of low concentrations of either lidocaine (0.02%) or bupivacaine (0.05%) in inflamed rat stifles; however, these same effects were not identified at higher concentrations (35). In our study, higher, clinically relevant concentrations did not achieve the anti-inflammatory properties of local anesthetics noted at low concentrations. Our results, in fact, identified a significant increase in PGE$_2$ from baseline within the bupivacaine group, suggesting an inflammatory response following injection. However, PGE$_2$ concentrations did not differ significantly from those in saline-injected joints. Whether these differences in PGE$_2$ response are solely due to the differences in local anesthetic concentration or other details of study design and/or species is not known.
An unexpected observation in our study was the significant increase in synovial fluid PGE$_2$ concentrations following repeated arthrocentesis (≥3 attempts in a single setting). Frequent needle penetration of the synovium is the most likely source of this inflammation rather than the treatment agent because it occurred in both treatment groups. Increased PGE$_2$ concentrations in synovial fluid following arthrocentesis is also documented in horses and as a result, the authors suggest separating repeated aspirations by 7 days to account for the inflammation created by arthrocentesis (36). Delaying arthrocentesis was not possible in our study. The small size of the joint and resulting small synovial fluid volume available for sampling likely contributed to the necessity for repeated arthrocentesis attempts in our study.

We anticipated that the normal Beagle stifle would contain a small synovial fluid volume (approximately 1 mL for a 12 kg Beagle). As a result, following aspiration at T0 and the infusion of the treatment volume (approximately 2 mL), subsequent PGE$_2$ concentrations would be lower due to the larger relative fluid volume having a dilutional effect. In a previous study evaluating how quickly bupivacaine eluted from the stifle following intra-articular administration, a similar dilutional effect was noted following treatment injection, and the pre-injection fluid volume approximation was calculated based on those methods from this previous study (32). Therefore, a 0.08 mL/kg synovial fluid volume approximation was made for each stifle defined as initial volume (32).

The effect of aspiration attempts influencing our data resulted in 11/80 (13.75%) samples being excluded from the final analysis. Of those excluded, 72.7% were collected from Dogs 1 and 5 (4 of 11 samples each). Dog 1 required 4 aspiration attempts on the bupivacaine stifle at T0, T30 and T60 and six aspiration attempts on the saline stifle at T0. In Dog 5, the bupivacaine stifle required 3 aspiration attempts for T0, T30 and T60 as well as 4 attempts for the T48
sample. The difficulty with Dog 1 may simply have been due to the learning curve associated with the procedures. However, individual dogs may be more difficult to aspirate than others, such as with Dog 5, and this variable should be expected in future studies.

As with any study, there are limitations to our study design. First, the results of our study may not accurately portray the response within a diseased joint (i.e. osteoarthritis), when bupivacaine is most likely to be used. Elevated PGE$_2$ concentrations are a common feature of osteoarthritis and synovitis (25, 26). These changes may prime the joint for further insult, resulting in increased release of PGE$_2$ in response to bupivacaine injection. However, a recent study suggests that the presence of osteoarthritis may not result in cartilage degeneration following multiple intra-articular bupivacaine injections compared to normal rat stifles (37). As a result, we cannot speculate how these data might translate to diseased joints. A second limitation is the small available synovial fluid volume at T0. This presented a particular challenge for aspiration, resulting in additional aspiration attempts that affected our data, as described. A lavage technique to expand the synovial fluid volume could be considered, but would require correction of the dilutional factor.

Liposomal bupivacaine formulations are available for use in both people and animals. This alternative formulation provides the convenience of a single peri-incisional injection with the duration of a continuous infusion (up to 72 hours) and a similar complication profile (38, 39). Liposomal bupivacaine consists of multi-vesicular liposomes encapsulating aqueous bupivacaine that release bupivacaine gradually over 96 hours. A recent study evaluating the chondrotoxicity of the liposomal bupivacaine formulation found that the liposomal formulation resulted in less chondrotoxicity over a 1 hour exposure compared to traditional ropivacaine and bupivacaine using an in vitro model of bovine chondrocytes (40). The veterinary liposomal bupivacaine
formulation is not yet currently labeled for intra-articular applications and was not used in our study. Further research evaluating peri-incisional and intra-articular use of liposomal bupivacaine compared to traditional intra-articular bupivacaine in the dog is indicated.

There is a time- and dose-dependent effect of bupivacaine on chondrolysis in vitro (4, 7, 11, 14, 15). However, to our knowledge, chondrolysis has not been reported in the veterinary literature following a single intra-articular dose of bupivacaine in vivo. A pharmacologic study of a single intra-articular dose of bupivacaine identified a rapid decrease in bupivacaine concentrations (11-25% of the original bupivacaine concentration) 30 minutes after injection in dogs (32). Given the rapid clearance of a single intra-articular dose of bupivacaine and the results of our study, a single injection of bupivacaine is unlikely to cause sufficient synovial joint inflammation to be the sole cause of chondrolysis. Our study establishes that repeated arthrocentesis results in elevated synovial fluid PGE2 concentrations. Further evaluation of additional markers of synovial joint inflammation (i.e. IL-1β, TNF-α), while minimizing the number of arthrocentesis attempts, may be useful in continuing to investigate the synovial response to bupivacaine in dogs. Additionally, the response of a clinically representative population of dogs (i.e. those with cranial cruciate rupture) may differ from that of dogs with normal stifles.
References


Table 1: PGE$_2$ concentration over time in synovial fluid collected from canine stifles following injection with either bupivacaine or saline regardless of number of aspiration attempts

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Sample Size</th>
<th>Median (pg/mL)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>Bupivacaine</td>
<td>8</td>
<td>287.1</td>
<td>58.3-734.9</td>
</tr>
<tr>
<td>T0</td>
<td>Saline</td>
<td>8</td>
<td>208.3</td>
<td>96.2-954.9</td>
</tr>
<tr>
<td>T30</td>
<td>Bupivacaine</td>
<td>8</td>
<td>244.2</td>
<td>128.9-2,026.0</td>
</tr>
<tr>
<td>T30</td>
<td>Saline</td>
<td>8</td>
<td>269.9</td>
<td>91.7-430.1</td>
</tr>
<tr>
<td>T60</td>
<td>Bupivacaine</td>
<td>8</td>
<td>512.3</td>
<td>142.3-3,669.5</td>
</tr>
<tr>
<td>T60</td>
<td>Saline</td>
<td>8</td>
<td>468.3</td>
<td>172.3-1,074.2</td>
</tr>
<tr>
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<td>Bupivacaine</td>
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<td>726.2</td>
<td>197.8-1,675.8</td>
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<tr>
<td>T24</td>
<td>Saline</td>
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<td>446.4</td>
<td>159.8-910.3</td>
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<tr>
<td>T48</td>
<td>Bupivacaine</td>
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<td>391.6</td>
<td>210.0-1,722.4</td>
</tr>
<tr>
<td>T48</td>
<td>Saline</td>
<td>8</td>
<td>390.2</td>
<td>118.5-5,032.2</td>
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</tbody>
</table>
Table 2: Number of aspiration attempts for each stifle at each collection time point following T0 injection with either bupivacaine or saline

<table>
<thead>
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<th>DOG</th>
<th>TIME</th>
<th># ATTEMPTS</th>
<th>Bupivacaine</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>4</td>
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<td></td>
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<td>1</td>
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<tr>
<td></td>
<td>T24</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>T48</td>
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<td></td>
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<tr>
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<td></td>
<td>T24</td>
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<td>T48</td>
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<th>Bupivacaine</th>
<th>Saline</th>
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<td>T48</td>
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<tr>
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<td>T0</td>
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<tr>
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### Table 3: Effect of number of aspiration attempts on synovial fluid PGE\(_2\) concentration

<table>
<thead>
<tr>
<th>Attempts</th>
<th>Sample Size</th>
<th>Median (pg/mL)</th>
<th>Range</th>
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</thead>
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<tr>
<td>1</td>
<td>62</td>
<td>304.3(^a)</td>
<td>58.3-1,722.4</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>244.5(^a)</td>
<td>110.9-2,425.9</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>734.9(^b)</td>
<td>306.7-5,032.2</td>
</tr>
</tbody>
</table>

Superscript letters indicate significant differences between groups.

\(^a\)One attempt was not significantly different from two attempts (p = 0.984).

\(^b\)Two attempts were significantly different from three attempts (p = 0.041).

\(^b\)One attempt was significantly different from three attempts (p = 0.005).
Table 4: PGE$_2$ concentration over time in synovial fluid collected from canine stifles following injection with either bupivacaine or saline and requiring ≤2 aspiration attempts

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Sample Size</th>
<th>Median (pg/mL)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Bupivacaine*</td>
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<td>110.9#§</td>
<td>58.3-382.1</td>
</tr>
<tr>
<td>T0</td>
<td>Saline</td>
<td>7</td>
<td>184.8</td>
<td>96.2-434.8</td>
</tr>
<tr>
<td>T30</td>
<td>Bupivacaine</td>
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</tr>
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<td>T30</td>
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<td>6</td>
<td>362.4</td>
<td>142.3-669.1</td>
</tr>
<tr>
<td>T60</td>
<td>Saline</td>
<td>8</td>
<td>468.3</td>
<td>172.3-1,074.2</td>
</tr>
<tr>
<td>T24</td>
<td>Bupivacaine</td>
<td>8</td>
<td>726.2#</td>
<td>197.8-1,675.8</td>
</tr>
<tr>
<td>T24</td>
<td>Saline</td>
<td>8</td>
<td>446.4</td>
<td>159.8-910.3</td>
</tr>
<tr>
<td>T48</td>
<td>Bupivacaine</td>
<td>6</td>
<td>391.6§</td>
<td>210.0-1,722.4</td>
</tr>
<tr>
<td>T48</td>
<td>Saline</td>
<td>7</td>
<td>274.3</td>
<td>118.5-2,425.9</td>
</tr>
</tbody>
</table>

*Significant difference over time within the bupivacaine group (p=0.008)

# T24 is significantly different from T0 (p=0.003)

§ T48 is significantly different from T0 (p=0.041)