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GLUCOCORTICOID-INDUCED CHONDROCYTE CYTOTOXICITY AT DOSES RECOMMENDED FOR INTRA-ARTICULAR THERAPY IN HORSES

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GLUCOCORTICOID-INDUCED CHONDROCYTE CYTOTOXICITY AT DOSES RECOMMENDED FOR INTRA-ARTICULAR THERAPY IN HORSES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food, and Environment at the University of Kentucky

By
Wenying Zhu

Lexington, Kentucky

Director: Dr. James N. MacLeod, Professor of Veterinary Science
Lexington, Kentucky
2015

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Intra-articular glucocorticoid injections are commonly used to treat synovitis and osteoarthritis in horses. These agents are highly effective at relieving pain, swelling, and other symptoms of joint inflammation. The drugs also have therapeutic benefits by down regulating the expression of cytokines and protease enzymes that participate in the degradation of articular cartilage. However, detrimental effects on chondrocyte function and cell viability that is independent of osteoarthritis pathogenesis have been described and linked to glucocorticoid use. These side effects are both drug- and dose-dependent. This study tested the hypothesis that manufacturer recommended dosage levels of methylprednisolone, betamethasone, and triamcinolone that are widely used in equine clinical practice are cytotoxic to articular chondrocytes. Drug-induced chondrocyte cytotoxicity was evaluated in monolayer cultures, cartilage explants, and equine fetlock joints. Total RNA was isolated from control and IL-1β stimulated primary chondrocytes and synoviocytes in culture. Changes in steady state mRNA for targeted gene transcripts related to inflammation and normal cell function were measured using reverse transcription and quantitative PCR. Inducible nitric oxide synthase activity was evaluated using nitrite production. Drug-induced chondrocyte cytotoxicity occurred at drug dosage levels frequently used in equine clinical practice. Both drug- and dose-dependent effects on chondrocyte and synoviocyte gene expression were observed. Maximum anti-inflammatory activities for the glucocorticoids were observed at in vitro concentrations below manufacturer-recommended levels. Results from this study suggest that lower glucocorticoid dose ranges for intra-articular therapy in horses should be validated to maximize the ratio of their therapeutically beneficial anti-inflammatory efficacy against detrimental effects on cell function and viability.

Keywords: Equine, Articular Cartilage, Corticosteroid, Chondrocyte Cytotoxicity, Anti-Inflammation
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8/21/15
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Chapter I Background and Literature Review

I. Importance of Osteoarthritis: both in horses and humans

Osteoarthritis (OA), a degenerative joint disease, is the most common musculoskeletal disease both in humans and horses. Lameness due to joint injury and joint disease is the most prevalent cause of diminished function and poor performance in racehorses. Several epidemiologic studies have documented the substantial impact of joint disease. Thirty-six to fifty-two percent of racehorses up to four years old have clinical sign of lameness (Jeffcott et al., 1982, Rossdale et al., 1985). According to a study done at Cornell University between 1983 and 1990 on equine patient admissions, 42% of racehorses older than 24 months with lameness had clinical sign of OA (Fubini et al., 1999). Likewise, OA not only affects racehorses, but also non-racing horses. A survey suggested that 60% of non-racing horses older than 24 months with lameness had clinical sign of OA (Caron et al., 2003). Osteoarthritis is also recognized as a major concern in human health, because of a large number of people involved and its severe impact on quality of life. It affects about 60% of men and 70% of women over the age of 65 (Sarzi-Puttini et al., 2005). It is a common cause of long-term disability in many populations of middle aged and older people (Praemer et al., 1999; Felson et al., 1995). Approximately 40% of adults over 70 suffer from OA of the knee, 80% of people with OA have limitations in movement, and about 25% cannot perform daily living activities (World Health Organization and bone and joint decade, 2001). Osteoarthritis has significant health
and quality of life implications for individuals, societies, and economies around the world – problems that are expected to get even worse as with aging populations (Brooks et al., 2002).

Osteoarthritis is first and foremost characterized by the ongoing destruction of articular cartilage in diarthrodial joints. Although it may develop in any joint, areas most commonly affected are weight bearing joints of the legs, including knees, and hips in humans, or stifle, carpus, and fetlock joints in horses. Osteoarthritis may result from injury, an abnormal growth pattern, or inherited factors. Mechanical impact is recognized as an important parameter for all of these issues (Brandt et al., 2009), and may also limit intrinsic joint repair mechanisms attempting to restore joint function. Due to years of mechanical stress, cartilage extracellular matrix components, which are responsible for the biomechanical properties of the tissue, are broken down and the normal balance between their synthesis and degradation is lost. Over time, the structural integrity of articular cartilage fails and joint movement becomes increasingly painful and restricted.

II. Joint Inflammation in Osteoarthritis

Osteoarthritis is characterized by changes in composition, structure and function of the articular cartilage. Although articular cartilage degeneration followed by erosion is the main identifying characteristic of OA, recent evidence supports a newer perspective that OA is a whole joint disorder affecting all joint tissues
including all connective tissues within and around the joints. OA results from joint degeneration, a process that includes degradation of the articular cartilage accompanied by attempted repair of articular cartilage, thickening of the subchondral bone, formation of subchondral bone cysts and osteophytes, and variable degrees of inflammation of the synovium (Buckwalter et al., 1995, 1997, 2000, Gerwin et al., 2004) (Figure 1). These changes usually limit joint movement and typically cause pain. Pain is the most common appearance of OA and the major cause of lameness associated with the disease. It can be generated by two types of factors: mechanical stimuli and inflammation. Mechanical stimulation may elicit inflammation response through tissue injury (Rene et al., 2010). OA is a process of changes of joint tissues responding to inflammatory mediators within the affected joint. It is frequently associated with signs and symptoms of inflammation, such as joint pain and swelling, leading to significant functional impairment and disability (Felson et al., 2006). Inflammation, that occurs in response to mechanical irritation and injury is central to the pathogenesis of OA and involves not only chondrocytes, but also cells in the synovial membrane and subchondral bone. Inflammatory cytokines, chemokines, and other inflammatory mediators, produced by the synoviocytes and chondrocytes, can be measured in the synovial fluids of osteoarthritis patients (Rainbow et al., 2012).
The role of synovial membrane in joint inflammation

Osteoarthritis research is traditionally focused on the understanding of changes on articular cartilage integrity and chondrocyte pathobiology. The synovial membrane changes are largely neglected. However, synovial membrane is an important portion of the joint and synovial inflammation is an important component of OA, contributing to the imbalance of chondrocyte catabolic and anabolic activities (Loeser et al., 2006).

The synovial membrane is a vascular connective tissue that lines the noncartilaginous surfaces within a joint cavity (Blom et al., 2007, Radin et al., 2001). The synovial lining cells consist of two different cell types: type A and type B synoviocytes. The two cell types execute different functions. Type A synoviocytes are macrophage-like cells that can eliminate excess materials and potential pathogens from the joint (Iwanaga et al., 2000; Blom et al., 2007). Type B
synoviocytes are fibroblast–like cells that are responsible for producing hyaluronan (HA) which is the main component of synovial fluid. In a normal joint, these two type of synoviocytes function together to maintain a healthy environment. However, in pathologic conditions, type A synoviocytes can stimulate type B synoviocytes to produce inflammatory mediators and matrix degrading enzymes.

Articular cartilage relies on adjacent tissues, such as subchondral bone and synovial membrane, to help maintain the health of the chondrocytes. The synovial membrane regulates molecules moving into and out of the joint space and maintains the composition of synovial fluid (Scanzello et al., 2012). Synovial fluid not only is important for reducing friction of joint movement, but also functions to transport nutrients and oxygen to the cartilage (Blom et al., 2007; Hui et al., 2011). However, the permeability of the synovial membrane can be altered when inflammation occurs. This likely contributes to the decreased concentration of HA and lubricin in synovial fluid. The level of HA has been reported to be elevated in concentration during inflammation, and serum HA concentrations have been used as a marker of synovitis (Goldberg et al., 1991).

Synovial inflammation is common in both early- and late-stage OA, although they are generally of lower grade than those observed in rheumatoid arthritis (RA) (Sellam et al., 2010). Moderate or marked inflammatory synovitis is present in nearly 50% of the synovial membranes from patients with OA (Goldenberg et al., 1982; Pearle et a. 2007). This condition is marked by dramatically increased secretion of pro-inflammatory cytokines and proteolytic enzymes from synoviocytes.
Pro-inflammatory cytokines, including interleukin 1β (IL-1β), interleukin 6, and tumor necrosis factor-α (TNF-α), are reported to mediate the cartilage degeneration and joint pain associated with OA (Sellam et al., 2010; Wassilew et al., 2010). The most extensively studied cytokines are IL-1β and TNF-α, which can induce expression of matrix degrading proteases, suppress matrix synthesis and promote cartilage catabolism (Aigner et al., 2006; Goldrin et al. 2009). Matrix metalloproteinases (MMPs), including MMP-1, MMP-3, and MMP-13, can be detected in OA synovial fluid samples, although at significantly lower levels than in RA joints (Pozgan et al., 2010).

Articular cartilage in osteoarthritis

Articular cartilage is a highly specialized, avascular, and aneural connective tissue that forms the smooth gliding surface of the diarthrodial joints (Figure 2). Cartilage allows the frictionless motion of the joint, in which it absorbs and dissipates loading stress. It is largely an extracellular matrix (ECM), which is synthesized by sparsely distributed resident cells, named chondrocytes. ECM is composed mainly of a high concentration of proteoglycans (aggrecan), collagen fibers, non-collagenous non-proteoglycanous proteins, and a large amount of water (Heinegard et al., 1992). The integrity of articular cartilage is altered to some degree in all joints with OA (Figure 3). Cartilage matrix components are broken down and normal balance of synthesis and degradation is lost. A net loss of proteoglycan content is one of the hallmarks of all stages of osteoarthritic cartilage degeneration (Figure 3) (Mankin et al., 1971).
Figure 2. Diarthrodial joint (A, adopted from www.studyblue.com) and structure of articular cartilage (B, adopted from php.med.unsw.edu.au).

Figure 3. Cartilage in healthy and osteoarthritic joints. The normal cartilage has smooth articular surface (A) and OA cartilage shows fissuring of articular surface (B). Proteoglycans abundant in cartilage matrix was stained with red color using Safranin-o. Histological appearance of healthy articular cartilage showed a good Safranin-o staining. Safranin-o stain was decreased in OA cartilage.
Chondrocytes, the only cell type in articular cartilage, are responsible for the balanced turnover of the ECM and maintenance of structural integrity in cartilaginous tissues (Aigner et al., 2006). During the osteoarthritic disease process, cellular reaction patterns of chondrocytes are changed. It can respond to joint injury or biomechanical perturbation by undergoing cell death, proliferation, or phenotypic modulation. Compared to normal cartilage, chondrocytes alter their gene expression profile in OA (Aigner et al., 2001). It is largely reported that chondrocytes have the capacity to up-regulate synthetic activity or increase the production of inflammatory mediators and matrix degradation enzymes, which are also produced by other joint tissues (Goldring et al., 2009). Chondrocytes obtained from OA tissues actively express MMPs, IL-1β, TNF-α, IL-6, IL-8, and a number of other genes that enhance or modulate inflammatory and catabolic responses, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) (Loester et al., 2012; Pelletier et al., 2001). These molecules act within cartilage in an autocrine or paracrine manner to promote a catabolic state, which leads to progressive cartilage degradation in OA joints (Attur et al., 1998). Chondrocytes also produce a large amount of NO when stimulated by IL-1β and TNF-α. NO inhibits the synthesis of extracellular matrix components such as type II collagen and proteoglycans, but increases activity of MMPs (Aigner et al., 2002).
Pro-inflammatory cytokines and matrix metalloprotease

It is believed that different inflammatory molecules in the development of OA play an important role. IL-1β and TNF-α produced by activated synoviocytes, mononuclear cells or by chondrocytes are the 2 major cytokines in the pathogenesis of OA (Fernandes et al., 2002). IL-1β is primarily synthesized as a precursor and released in the active form (Mosley et al., 1987). The catabolic effects of IL-1β are multiple. It not only can stimulate its own production, induce the expression of MMPs and other catabolic genes, but also inhibit the synthesis of matrix constituents such as collagens and proteoglycans (Pelletier et al., 2008). This cytokine also plays important roles in normal joints, including stimulation of the turnover of extracellular matrix (Pelletier et al., 2008). Although TNF-α has only been detected in OA articular tissue at low levels, it also appears to be an important mediator of cartilage matrix degradation and a pivotal cytokine in inducing synovial membrane inflammation (Fernandes et al., 2002). IL-1 and TNF-α can stimulate the mitogen-activated protein kinase (MAPK) pathways and nuclear factor κB (NF-κB) pathway which are central inflammatory pathways in OA (Saklatvala et al., 2007). These kinase cascades lead to the expression of many inflammatory genes and MMP genes which facilitate the degradation of several ECM proteins, such as collagens, proteoglycans, fibronectin, link protein and other matrix proteins which are critical for normal cartilage function and integrity (Malemud et al., 2004).
The matrix degrading enzymes found in the OA joint include aggrecanases and collagenases, which are members of the MMP family. These include stromelysin-1 (MMP-3), collagenase -1(MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13), and aggrecanases (Clark et al., 1998; Woessner et al., 1991). These proteinases are very much involved in the cartilage matrix degradation in OA (Poole et al., 1995). Cartilage matrix degradation in early OA may be due to aggrecanase and MMP-3, which degrade aggrecan. Then increased activity of MMP-13 is highly efficient at degrading type II collagen (Dahlberg et al., 2000). Once the collagen network is degraded, the damage to the cartilage structure cannot be reversed.

The main objectives in the management of OA are to reduce symptoms, minimize functional disability, and limit progression of the structural changes. Therefore, controlling joint inflammation and immediate damage to cartilage, as well as minimizing long-term degenerative structure changes, are important objectives of OA therapy.

III. Anti-inflammatory therapies commonly used for synovitis and osteoarthritis

There are a number of treatments available for synovitis and osteoarthritis. Aims of these treatments are to relieve the pain, prevent the cytokines and matrix degrading enzymes from compromising the articular cartilage, and finally return the joint to normal as quickly as possible. Non-pharmacological therapies and medical treatments are discussed in this section.
Non-pharmacological therapies to reduce inflammation

Non-pharmacological therapies can be used as the first step, both in humans and horses, for joint injury and disease treatment (Roos et al., 2012). These treatments can be used alone or in combination with medical and surgical treatments (Figure 4).

![Figure 4. The osteoarthritis treatment pyramid. Non-pharmacological treatments can be used for all patients. Pharmacological therapies are useful for some of the patients. Few patients need to have the surgery to maintain the joint function.](image)

Physical therapy and rehabilitation are commonly used non-pharmacological treatments for horse synovitis and osteoarthritis. Cold hydrotherapy might be extremely useful as a primary treatment immediately after an acute joint injury. It is indicated to retard the inflammatory processes and reduce edema (Hickman J, 1964). After 48 hours, hot hydrotherapy might help to relieve pain and reduce tension in inflamed tissues (Adams OR, 1974; Lehmann JF et al., 1982; Michlovitz SL
et al., 1996; Prentice WF et al., 1994). Swimming is the closest treatment to non-weight bearing motion, which is practiced in human sports medicine and in horse sport medicine (McIlwraith et al., 2001). It is used in the convalescent period with joint injury to maintain the horse’s condition while relieving joint trauma.

Recently, there also has been considerable use of treatments such as electromagnetic therapy, electrostimulation, and low level laser for various musculoskeletal conditions including traumatic joint disease. The value of extracorporeal shock wave therapy (ESWT) has been demonstrated with experimental osteoarthritis in the horse (Frisbie et al., 2009). It significantly reduced the degree of lameness in horses, although no disease-modifying effects were evident (Frisbie et al., 2009). The effects of non-surgical defocalized carbon dioxide laser therapy (DLT) on acute synovitis and capsulitis was evaluated (Lindholm et al., 2002). Although no control was established, observer-blind and prospective studies still observed beneficial effects of DLT on reducing the degree of lameness, which was comparable with conventional intra-articular medical treatments (Lindholm et al., 2002).

Current commonly used anti-inflammatory drug therapy

Drug therapy is one of the most utilized treatments for OA in equine practice. Evaluating therapeutic efficacy has largely focused on improvements in pain management and joint function. The mainstay of medical treatment has included systemic non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular (IA) corticosteroids, viscosupplementation, and chondroprotectants (Goodrich et al.,
The following section discusses systemic medical treatment of OA, followed by IA and other topical therapies.

**Systemic treatment of OA**

NSAIDs are by far the most commonly used drugs in this category. The effect of NSAID is primarily the inhibition of cyclooxygenase (COX) enzymes in the arachidonic acid cascade, thus inhibiting prostaglandins production (Higgins et al., 1984). There are two forms of cyclooxygenase: constitutive COX 1 and inducible COX 2. It is reported that COX 2 enzyme may be primarily responsible for inflammatory response and COX 1 is responsible for producing prostaglandins involved in regulating normal cellular processes, such as protection of mucosal barriers in the gastrointestinal tract (Meade et al., 1993). The identification of COX 1 and COX 2 may explain, the variability of efficacy, as well as toxicity, of different NSAIDs. Most of the older NSAIDs have been shown to inhibit both COX 1 and COX 2. Moreover, some of them, such as aspirin, indomethacin and piroxicam, are reported to be more potent inhibitors of COX 1 than COX 2, which means that they more negatively affect normal physiological function than beneficially inhibit inflammation (May et al., 1996). Selective COX 2 inhibitors have been developed and demonstrated a superior gastrointestinal safety profile in humans, while providing comparable anti-inflammatory potency (Chen et al., 2008). However, selective COX 2 inhibitors are known to cause cardiovascular (CV) events (McGettigan et al., 2006), and its gastrointestinal protective benefits may be negated if the patient is also taking aspirin (Chan et al., 2007). Selective COX 2 inhibitors have also become available for the treatment of OA in horses, but traditional nonselective NSAIDs are
still routinely used. Table 1 summarizes dosages and ways of administration for the most commonly used NSAIDs in horses.

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Route of Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylbutazone</td>
<td>Oral</td>
</tr>
<tr>
<td>Flunixin</td>
<td>Oral or IV</td>
</tr>
<tr>
<td>Carprofen</td>
<td>Oral or IV</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>IV or IM</td>
</tr>
<tr>
<td>Vedaprofen</td>
<td>Oral</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>Oral</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Oral or IV</td>
</tr>
</tbody>
</table>

Table 1. Overview of the most commonly used NSAIDs with their route of administration.

Other systemic treatments for OA in horses include intramuscular (IM) polysulfated glycosaminoglycans (PSGAG), IV or oral sodium hyaluronate (HA), and neutraceuticals. PSGAGs alter OA progression by sustaining or promoting chondrocyte metabolic activity and inhibiting the detrimental effects of cytokines or prostaglandins on cartilage. HA has anti-inflammatory effects in the joint. Horses treated with HA have reduced lameness, better synovial membrane scores, and a reduction in prostaglandins in joints compared to joints of horses receiving saline...
Neutraceuticals are used to treat horses suffering from OA or other chronic joint disorders and in most cases contain mixtures of chondroitin sulfate and glucosamine. These compounds are supposed to have a disease-modifying effect. There has been controversy about the potential usefulness of these compounds in horses since these products are not FDA controlled (Goodrich et al., 2006).

**Local treatment of OA**

Intra-articular glucocorticoids are commonly used and can be extremely effective for the therapeutic management of OA symptoms. Glucocorticoids are powerful anti-inflammatory agents that strongly suppress the immune response. However, glucocorticoid use became controversial because of side effects, particularly on steroid-induced deterioration of articular cartilage, which also known as steroid arthropathy. There now seems to be common agreement that if used judiciously, intra-articular glucocorticoid therapy can be extremely beneficial. Mechanism of glucocorticoids, side effects, and type of corticosteroid used are discussed in detail in the following section.

Hyaluronic acid (HA), also called hyaluronan, is a large nonsulphated glucosaminoglycan and synthesized by fibroblast-like synoviocytes of the synovial membrane (Howard et al., 1996). It is an important component of the joint fluid and serves various functions, such as viscoelasticity of the synovial fluid and boundary lubrication of the articular cartilage (Scanzello et al., 2012). HA is also an important component in articular cartilage matrix and can be locally produced by
chondrocytes. It has been extensively used as intra-articular treatment in horses as viscosupplementation and anti-inflammatory agent (Goodrich et al., 2006). The combination of intra-articular glucocorticoids and HA is popular in equine practice, as it may permit the reduction of the dose of glucocorticoids and may counteract the possible deleterious effects of these drugs on the cartilage (Caron et al. 2005).

IV. Intra-articular glucocorticoids: mechanisms of action and side effects

As noted above, glucocorticoids are potent anti-inflammatory agents widely used in therapeutic applications for osteoarthritis (Todhunter et al. 1998, Goodrich et al. 2006 and Stitik et al. 2006). Glucocorticoids exert their effects by binding to glucocorticoid receptors (GR) localized in the cytoplasm of target cells. Central to the action of glucocorticoids is the interaction of glucocorticoid molecules with the cytoplasmic glucocorticoid receptor, in response to which a vast number of intracellular events are entrained. The efficacy of glucocorticoids in alleviating inflammatory disorders results from the pleiotropic effects of the glucocorticoid receptor on multiple signaling pathways (Adcock et al. 2000, Smoak et al. 2004, and De Bosscher et al. 2003). Within the cell, glucocorticoid acts in three ways: direct and indirect genomic effects and nongenomic mechanisms (Buttgereit et al 1998 and Rhen et al. 2005). First, the glucocorticoid receptor complex moves to the nucleus, and binds as a homodimer to DNA sequences called glucocorticoid-responsive elements (GRE) or negative glucocorticoid-responsive elements (nGRE) (Smoak et al, 2004 and Rhen et al. 2005), thereby facilitating or inhibiting
transcription (Figure 5). This process is highly dynamic and has demonstrated to increase the synthesis of certain anti-inflammatory proteins, including lilpocortin-1, IL-1 receptor antagonist, and IL-10, but is also related to glucocorticoid-induced side effects (Smoak et al, 2004 and Rhen et al. 2005). Second, glucocorticoid receptor sequesters and interacts with other transcription factors, such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1), to suppress transcription (Figure 5). The third mechanism is called non-genomic actions, which are mediated through membrane-associated receptors and second messengers (Buttgereit et al 1998).

Although these medications effectively decrease inflammation, side effects independent of OA pathogenesis have been identified (Fubini et al. 2001). Cartilage collected from joints treated with glucocorticoids have pathological changes that include chondrocyte cell death, reduced proteoglycan content and rate of synthesis, decreased collagen synthesis, and an increase in the percentage of water (Chunekamrai et al. 1989, Richardson et al. 2003). Over time, these changes compromise the structural integrity and biomechanical properties of articular cartilage. In regards to bone metabolism, glucocorticoids have been found to inhibit osteoblasts which function in bone production and increase osteoclast activity which aid in bone resorption, thereby creating a bone that is more susceptible to the development of osteoporosis or fractures. Another side effect of glucocorticoids includes avascular necrosis and altered bone repair (Bitto et al, 2009). The effects of glucocorticoids on bone metabolism and structure has been extensively studied in humans and lab animals, but not in horses. The therapeutic
use of glucocorticoids for OA is limited by these side effects, especially during long-term treatment.

Methylprednisolone acetate (MPA), betamethasone, and triamcinolone are three commonly used intra-articular corticosteroids in horses (Caron et al. 2005). Although these drugs have strong anti-inflammatory activities, some detrimental effects have been reported. Many studies have been conducted to observe the beneficial and side effects of MPA on cartilage, subchondral bone, and synovium. Side effects were reported both in vivo and in vitro studies (Frisbie et al. 1998, MacLeod et al. 1998, Shoemaker et al. 1992). It appears that lower doses inhibit inflammation while maintaining the normal joint metabolism (Todhunter et al. 1996). Further studies are needed to accurately identify the best MPA dose in the horse. A study looked at one dose of MPA (100 mg administered in the middle carpal joint) and found no significant side effects on subchondral bone, but indicated further research with multiple drug compounds at varying doses need to be examined to draw conclusions regarding glucocorticoid effects on bone structure and metabolism in the equine (Murray et al, 2002). There is no significant detrimental effect observed in the cartilage following administration of betamethasone in horse joints (Foland et al. 1994). However, side effects like suppression of proteoglycan synthesis are detected with in vitro work (Frean et al. 2002). Triamcinolone was reported to inhibit the inflammatory molecules without side effects on cartilage extracellular matrix (Celeste et al. 2005, Dechant et al. 2003, Frisbie et al. 1997, Kawcak et al. 1998). According to the above finding, the specific
type of glucocorticoid used and the concentration achieved following administration plays an important role on side effects of these drugs on cartilage.

Figure 5. Mechanisms of transactivation and transrepression actions of glucocorticoids and glucocorticoid receptor in the cell.

V. New anti-inflammatory pharmacologic agents: dissociative glucocorticoids

As previously mentioned, the genomic action of glucocorticoids can be divided into two processes: transactivation and transrepression. It is widely accepted that the numerous desirable anti-inflammatory and immunomodulating effects of
glucocorticoids are mediated by their transrepression properties (Lin et al., 2002), whereas most of the adverse side effects are mediated through transactivation mechanisms (Schacke et al., 2002). Significant side effects of glucocorticoids lead to the development of several new compounds. Dissociative glucocorticoids, a novel group of glucocorticoid ligands, bind selectively to the glucocorticoid receptor and differentially recruit downstream cofactors (Catley et al. 2007). These compounds cause a receptor conformation that prefers monomer glucocorticoid receptor and protein interaction rather than homodimer glucocorticoid receptor DNA binding (Stahn et al., 2007). As a consequence, they can inhibit the activity of MAPK and NF-κB pathways by protein-protein interaction like glucocorticoids, while having fewer side effects than glucocorticoids (Cogylan et al. 2003, Humphrey et al. 2006, and Owen et al. 2007). Some dissociative glucocorticoids have a similar level of anti-inflammatory activity compared to prednisolone, both in vitro and in vivo (Cogylan et al. 2003, Humphrey et al. 2006, and Owen et al. 2007). This list of potential alternative compounds tested in various in vitro and in vivo studies are provided in Table 2.
<table>
<thead>
<tr>
<th>Drug</th>
<th>In vitro testing</th>
<th>In vivo animal model testing</th>
<th>Human clinical trial</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL-438</td>
<td>✓</td>
<td>✓</td>
<td>NT</td>
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<tr>
<td>LGD-5552</td>
<td>✓</td>
<td>✓</td>
<td>NT</td>
</tr>
<tr>
<td>ZK 216348</td>
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<td>✓</td>
<td>Phase II (Atopic Dermatitis)</td>
</tr>
<tr>
<td>Compound A</td>
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<td>✓</td>
<td>NT</td>
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<tr>
<td>PF-803</td>
<td>✓</td>
<td>✓</td>
<td>Phase II (rheumatoid arthritis)</td>
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<tr>
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<td>✓</td>
<td>NT</td>
</tr>
<tr>
<td>VBP1</td>
<td>✓</td>
<td>✓</td>
<td>NT</td>
</tr>
<tr>
<td>VBP15</td>
<td>✓</td>
<td>✓</td>
<td>Phase I (Duchenne muscular dystrophy)</td>
</tr>
</tbody>
</table>

Table 2. Potential alternative therapeutic dissociative compounds for treating inflammatory diseases. “✓” means has already been completed. “NT” stands for not tested.

*Dissociative non-steroidal compounds*

AL-438 (Coghlan et al., 2003; Elmore et al., 2001) and LGD-5552 (Miner et al., 2007; Lopez et al., 2008) are nonsteroidal glucocorticoid receptor ligands which bind the glucocorticoid receptor with specificity and relatively high affinity. *In vitro* experiments indicate that AL-438 and LGD-5552 both effectively repress the
production of TNF-α or IL-1β induced IL-6 and E-selectin levels similar to steroids (Coghlan et al., 2003; Elmore et al., 2001; Miner et al., 2007; Lopez et al., 2008). These two compounds were also tested in rat models showing desirable anti-inflammatory effects and a promising lack of adverse effects, such as without affecting glucose or bone metabolism (Coghlan et al., 2003; Elmore et al., 2001; Miner et al., 2007; Lopez et al., 2008).

Compound A (Bosscher et al., 2005; Dewint et al., 2008) and ZK216348 (Schacke et al., 2004) are also non-steroidal glucocorticoid receptor ligands which demonstrated anti-inflammatory effects with reduced detrimental side effects both in vitro and in vivo.

**Dissociative steroidal compounds**

The first dissociative steroidal compounds were discovered by Roussel-Uclaf (RU), including a series of RU-steroidal compounds. These RU compounds were determined to retain transrepression action with weakly transactivation properties in vitro but not in vivo (Belvisi et al., 2001; Schacke et al., 2007; Chen 2008).

In recent years, an increasing number of dissociative glucocorticoids have been described. A very interesting substance is, for example, VBP1 and VBP15 (anecortave desacetate) (Baudy et al., 2012). VBP's are Lazaroid Δ-9,11 analogs, which were developed originally as nonglucocorticoid steroids with effects on cell membranes and tested clinically for neuroprotection by the inhibition of lipid peroxidation (Taylor et al., 1996; Bracken et al., 1997; Kavanagh and Kam, 2001). Lazaroids have been reported to inhibit acute inflammation through the
suppression of inducible nitric oxide synthase (iNOS) (Altavilla et al., 1999), TNF-α (Altavilla et al., 1998), and NF-κB (Fukuma et al., 1999). In the recent study by Baudy et al (2012), VBP1 and VBP15 were tested for anti-inflammatory efficacy and side effects both in vitro and in vivo. The results showed potent inhibition of TNF-α induced NF-κB signaling in cell reporter assays, and a reduction of muscle inflammation and improvements in multiple muscle function assays in two mouse models of muscular dystrophy. Meanwhile, VBP15 exhibited lower affinity for mineralcorticoid receptor than the other VBPs and maintained comparable anti-inflammatory efficacy to prednisolone (Heier et al. 2013). Accordingly, VBP15 shows promise as a potentially safer therapeutic agent for chronic inflammatory disorders. Currently, VBP15 is undergoing the Phase I clinical trial.

VI. Articular chondrocyte culture

*Primary articular chondrocytes and cell lines*

Chondrocyte culture experiments are widely used for investigating the intracellular and molecular events associated with chondrocyte biological function and activation. Several culture models have been proved useful for studying chondrocyte function, such as primary cultures of animal or human chondrocytes, and cell lines from chondrosarcoma. However, chondrocyte cell lines cannot be entirely substituted for primary cultures, as major cell biology differences have been shown between normal and transformed chondrocytes (Mallein-Gerin et al. 1993, Stokes et al. 2001).
Primary articular chondrocyte culture methods

Many in vitro culture methods have been developed for the investigation of chondrocyte and cartilage biological properties, including monolayer cultures, several forms of three-dimensional (3D) aggregate cultures systems, and cartilage explants models (Adolphe et al., 1992; Tew et al., 2008). Monolayer culture is a commonly used in vitro method to grow chondrocytes and generate sufficient cell numbers for experimental analyses. However, chondrocytes cultured using this approach may undergo a characteristic process of cellular dedifferentiation, marked by a decrease in type II collagen synthesis and aggrecan core protein expression and the induction of type I collagen expression (Darling et al., 2005; Goessler et al., 2004; Goessler et al., 2005). This phenomenon is influenced to some extent by seeding density and is accelerated by growth in medium supplemented with serum and by passage (Hering et al., 1994; Ronziere et al., 1997; Matmati et al., 2012). Three-dimensional aggregate cultures systems, which include nonadherent aggregates, pellets, and cells embedded in an artificial material made of alginate, agarose, or collagens (Thirion et al., 2004), maintain their differentiated phenotype better than that of monolayer cultures. However, cell proliferation is limited so that large numbers of primary cells are needed for each experiment. In addition, extracting the cells from the artificial matrix can be technically challenging (Thirion et al., 2004). Experiments designed using a combination of chondrocyte culture methods, supported by in vivo studies where possible, facilitates biological discovery.
VII. Knowledge gaps, hypothesis, and specific aims.

Many important knowledge gaps remain, both with regard to OA pathogenesis and treatment. For equine patients, basic questions such as the optimal use of established medical therapies continue to be debated. Even with the extensive use of intraarticular corticosteroids on equine patients, objective data on dose-dependent relationships between anti-inflammatory efficacy and chondrocyte toxicity are limited. The objective of this dissertation was to evaluate these relationships for three commercial glucocorticoids that are widely used in clinical settings for the management of equine synovial joint inflammation. The hypothesis tested was that methylprednisolone, betamethasone, and triamcinolone that are widely used in equine clinical practice are cytotoxic to articular chondrocytes. Dose-dependent anti-inflammatory profiles of methylprednisolone, betamethasone, and triamcinolone in articular chondrocytes and synoviocytes were defined and compared. Glucocorticoid-induced chondrocyte cytotoxicity in monolayer culture, articular cartilage explants, and in vivo was evaluated.

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Chapter II Materials and Methods

I. Cell isolation and culture

Equine articular chondrocytes and explants

1. Articular cartilage collection and chondrocyte cell isolation

Full-thickness articular cartilage was harvested immediately postmortem from normal synovial joints of adult horses. For each horse, chondrocytes were enzymatically isolated from the cartilage matrix and cryopreserved as described previously (Nixon et al., 1992; Stewart et al., 1998). Articular cartilage was minced with a scalpel and digested overnight in basal digestion medium [OPTI-MEM, 5% fetal bovine serum, 1% L-glutamine, 25 μg/mL L-ascorbic acid phosphate (Wako Chemicals USA, Richmond, VA), 50 U/mL penicillin, and 50 μg/mL streptomycin], and 0.75% collagenase II (Worthington Biochemicals, Freehold, NJ). After digestion, the cell suspension was filtered through a sterile funnel containing a base layer of 44μm mesh (Nylon membrane, Millipore, NY) and 4 layers of cheesecloth into 50 ml conical tubes. The isolated chondrocytes were rinsed with PBS solution and counted. Cell viability was assessed using trypan blue staining with an automated (ViCell, Beckman Coulter) analyzer. An experiment was continued only when the proportion of viable cells was > 95%.

2. Chondrocyte monolayer culture

Monolayer chondrocyte cultures were established at a seeding density of 0.4 x 10^6 cells/well in 6-well plates and cultured for 5 days in Opti-MEM (Gibco, 11058-
021) with 5% fetal bovine serum, 1% L-glutamine, 25 µg/ml L-ascorbic acid phosphate, 50 U/ml penicillin and 50 µg/ml streptomycin. On day 6, the confluent cultures were washed twice with serum-free Opti-MEM medium, followed by culturing in Opti-MEM medium without serum for another 5 days. Various experiments were started on day 11. Chondrocytes used to assess glucocorticoids-induced cytotoxicity were seeded in 96 well plates at a density of 1 x 10⁴ cells/well and cultured in the same Opti-MEM with 5% fetal bovine serum for 24 hours. Medium was changed every 2-3 days.

3. Chondrocyte aggregate culture

Nonadherent aggregate cultures were maintained in hydrogel-coated 6-well plates to prevent cell adhesion and maintain cartilage phenotype. Three million cells were placed in each well in 5 ml of medium and cultured at 37°C with 5% carbon dioxide at saturated humidity in Opti-MEM serum free medium, supplemented with 1% L-glutamine, 25 µg/ml L-ascorbic acid phosphate, 50 U/ml penicillin and 50 µg/ml streptomycin. During the first 72 h of culture, the floating cells spontaneously came together to form clearly visible aggregates. At each medium change, spent medium was aspirated and centrifuged at 300 rcf for 5 minutes. Aspirated cells were resuspended in 5 ml of fresh medium and returned to the appropriate wells. Various experiments were started on day 11.

4. Cartilage explants culture

Articular cartilage was aseptically collected as full-thickness explants from normal synovial joints of adult horses between the age of 3 and 4 years. A 6-mm
diameter biopsy punch instrument was used to maintain approximate uniformity in size and weight of each sample. The explants were washed in sterile PBS solution with 1% penicillin-streptomycin, 2.5µg/ml amphotericin B, and placed in 6-well plates within 2 hours of collection, then incubated in Opti-MEM medium supplemented with 5% (v/v) FBS, L-ascorbic acid (25 µg/ml), L-glutamine (300 µg/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in 5% CO2 and 95% humidity. Explants were adapted to culture conditions for 24 hours before experimental variables were applied.

*Equine synovial fibroblast isolation and culture*

Synovial membrane tissue was collected from normal synovial joints of three horses. Synovial fibroblasts were prepared by a method described in Kojima et al (2002). Briefly, tissues were dissociated by mincing and then digested overnight with 1 mg/ml type I collagenase (Sigma, St.Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM) in a humidified 5% CO2 incubator at 37°C. Dissociated cells were cultured in 75 cm² culture flasks in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mM), 50 U/ml penicillin and 50 µg/ml streptomycin. At greater than 95% confluency, the adherent synovial fibroblasts were passaged by digestion with 0.05% trypsin/EDTA. For all experiments, passage 4 cells were plated into 6-well plates (0.4x10⁶ cells/well) in DMEM containing 10% FBS for 24 h. After seeding, the synovial cells were cultured for an additional 24 h in DMEM with 2% FBS and then incubated with or without various experimental stimuli.
II. Experimental variables in tissue culture

Pro-inflammatory stimuli with IL-1β and LPS

To stabilize baseline cell function and levels of gene expression, chondrocyte monolayers were cultured for 10 days and synovial fibroblasts for 24 hours prior to stimulation. After the adaptation period, cells were treated with medium containing equine recombinant IL-1β (R&D system) (1 ng/ml or 10 ng/ml), or Escherichia coli lipopolysaccharide (LPS) (1 ng/ml, 10 ng/ml, or 100 ng/ml) for either 24 hours or 72 hours. At the end of the treatment period, the culture medium was collected to analyze nitric oxide levels and the cells were lysed for total RNA isolation.

Glucocorticoids

1. Molecular structures

Methylprednisolone sodium succinate (MPS) was purchased from Pharmacia-Upjohn (Kalamazoo, MI). Betamethasone 21-phosphate disodium (BPD) and triamcinolone acetonide (TA) were purchased from Sigma (St. Louis, MO). VBP1 was synthesized by Bridge Organics (Kalamazoo, MI) (Figure 6).
2. Concentrations and solubility

MPS and BPD were prepared at the initial concentration levels of $10^{-2}$ M in Opti-MEM. Serial dilution was performed to reach the target concentrations. TA and VBP1 are both water insoluble compounds. Stock solutions at a concentration of 10 mM were prepared for these two compounds using dimethyl sulfoxide (DMSO; Sigma, St.Louis, MO) as a diluent. Serial dilutions were used to reach target experimental concentrations. The final volume of DMSO in culture medium was below 1% (v:v).
3. Evaluation of glucocorticoids administration protocols

a) Timing of methylprednisolone treatment relative to IL-1β stimulation

Chondrocyte aggregates and synovial fibroblasts were treated with a dose of 10^{-4} M methylprednisolone (Pfizer) with or without 1 ng/ml equine recombinant IL-1β (R&D system). Three methylprednisolone (MPS) treatment models: pre-treated with MPS (IL-1β 24h; MPS 36h); post-treated with MPS (IL-1β 24h; MPS 12h); treated together with MPS and IL-1β (IL-1β 24h; MPS 24h) were tested. Chondrocyte aggregates were pre-cultured in Opti-MEM medium without any stimulus for 10 days and synovial fibroblasts were pre-cultured in DMEM medium without any stimulus for 24 hours to adapt to culture conditions. After pre-culturing, cells were re-fed with fresh medium with 1 ng/ml IL-1β or 10^{-4} M MPS alone, 1 ng/ml IL-1β and 10^{-4} M MPS, or fresh medium without any treatment for 12 hours. Cell culture medium was changed and cells were re-fed with medium containing MPS alone, IL-1β and MPS, or without any treatment for other 12 h. Cells in the MPS pre-treatment group were continued culturing in the medium with IL-1β and MPS for 12 more hours. Cells and medium in the other two groups were collected for the following study. Cells stimulated with IL-1β alone were used as positive controls, and cells without any stimulation were used as negative controls for this study.

b) Anti-inflammatory efficacy

Methylprednisolone acetate (MPS) from Pfizer, betamethasone 21-phosphate disodium (BPD) from Sigma, and triamcinolone acetonide (TA) from Sigma were used for this study. VBP1 was donated by Bridge Organics. The following treatment
groups were investigated: control treatment with no corticosteroid treatment and free of recombinant equine interleukin-1β (IL-1β); control treatment with DMSO without corticosteroid treatment and free of IL-1β; IL-1β stimulation containing only 1 ng/ml IL-1β without corticosteroid addition; MPS (10^{-4} or 10^{-6} M), BPD (10^{-4} or 10^{-6} M), TA (10^{-5} or 10^{-6} M) or VBP1 (10^{-5} or 10^{-6} M) only without IL-1β stimulation; MPS (10^{-4} or 10^{-6} M), BPD (10^{-4} or 10^{-6} M), TA (10^{-5} or 10^{-6} M) or VBP1 (10^{-5} or 10^{-6} M) each with 1 ng/ml IL-1β added to the medium (Figure 7). The drug concentrations were extrapolated from dosages used clinically and the previous cytotoxicity study. After incubation with treatment, chondrocytes and spent medium were harvested. All medium were stored at -70 °C until analyzed. Before collection, chondrocytes were rinsed twice using PBS, scraped from the culture plate, and stored at -70 °C.
Figure 7. Timing and sequence of drug treatment and IL-1β stimulation for evaluating the anti-inflammatory efficacy of methylprednisolone, betamethasone, triamcinolone, and VBP1 treatment on articular chondrocytes.
III. RNA isolation

Total RNA was isolated from chondrocytes and synovial fibroblasts using the QIAGEN RNeasy Mini Kit (QIAGEN). After cells were rinsed with D-PBC, total RNA was isolated according to manufacturer’s protocol. Total RNA quantity and purity were assessed with a NanoDrop ND-1000. RNA integrity assessments were performed with a BioAnalyzer 2100 (Agilent, Eukaryotic Total RNA Nano Series II) and were deemed suitable for downstream applications based upon RNA integrity number >8, 28S:18S rRNA ratio >1.8, UV 260:280 ratio >1.8, and UV 260:230 ratio >1.7.

IV. Reverse transcription and Real-time qPCR

Differential expression of nine genes, interleukin-1 β (IL-1β), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 9 (MMP9), matrix metalloproteinase 13 (MMP13), tissue inhibitor of metalloproteinases 2 (TIMP2), type II collagen (COL2A1) and hyaluronic acid synthase 2 (HAS2), was analyzed using quantitative polymerase chain reactions (RT-qPCR) (Table 3). Briefly, total RNA was reverse-transcribed into cDNA using an oligo-dT primer with the Promega Reverse Transcription System (Promega, cat. no. A3500). Total RNA (0.5 µg) was diluted in 39 µl nuclease-free water, and combined with 41 µl reverse transcription master mix: 3 µl (20 U/ µl) avian myeloblastosis virus (AMV) reverse transcriptase, 4 µl oligo dT primer (0.5 µg/ µl), 2 µl RNAsin (40 U/ µl), 8 µl dNTP (10 mM), 8 µl AMV buffer, and 16 µl MgCl₂ (25 mM) (Promega, Madison, WI), for each reverse transcription reaction. Samples of cDNA were then stored at -20 °C until further analyzed.
Quantitative “real-time” PCR (7900HT Fast Real-Time PCR Systems, Applied Biosystems, Foster City, CA) was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) and intron-spanning primer/probe sets (Assays-by-Design, Applied Biosystems) designed from equine genomic sequence data (Ensembl – http://www.ensembl.org/Equus_caballus/index.html; UCSC Genome Browser – http://genome.ucsc.edu). Equine-specific RPLPO, EIF2B2, TMF1, IL-1α, IL-6, iNOS, MMP3, MMP9, MMP13, TIMP2, COL2A1, and HAS2 primer/probe sets were designed for this purpose. Given thought to the gene’s stability, efficiency, CT range, and CT values compared to target genes, endogenous control genes were used on the target gene plates. Large ribosomal protein (RPLPO), eukaryotic translation initiation factor 2B (EIF2B2), TATA element modulatory factor (TMF1) and hypoxanthine phosphoribosyltransferase (HPRT) were selected as endogenous control genes because they showed the greatest stability for the sample set as defined by the geNorm reference gene application (data not shown). Amplification efficiencies were measured by the default fit option of LinRegPCR (LinRegPCR 7.0, J. M. Ruijter and C. Ramakers, Academic Medical Center, Amsterdam, the Netherlands) while maintaining the cycle threshold as a data point within the measured regression line. Mean efficiencies for each gene across the whole experimental groups were utilized for data analysis. Relative expression levels of target genes were normalized to the relative quantities of endogenous control genes using geometric mean with the geNorm VBA applet.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
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<td>Eukaryotic translation initiation factor 2B</td>
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<td>TTCTTCAAGGCACCCACAAAACTTTG</td>
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Table 3. Primer nucleotide sequences used in RT-qPCR assays for genes described in the study
V. Nitric oxide production

For measurement of nitrite in the cell supernatants, chondrocytes and synovial fibroblasts were plated into the wells of a 6-well plate at a density of 0.4 x 10^6 cells/well in phenol red free Opti-MEM containing 5% FBS or phenol red free DMEM containing 2% FBS and incubated for 24 h in the presence or absence of IL-1β, with or without treatment. Nitrite concentrations were measured in cell supernatants as an indicator of NO production using a commercially available Griess reaction kit (Cayman, Ann Arbor, MI, USA). Assay was performed according to the manufacturer’s recommendation.

VI. Glucocorticoid-induced chondrocyte cytotoxicity

In vitro analysis

1. Culture methods

Primary articular chondrocytes were seeded in 96-well plates at a density of 1 x 10^4 cells/cm². The cultures were maintained in Opti-MEM medium supplemented with 5% (v/v) FBS, L-ascorbic acid (25 µg/ml), L-glutamine (300 µg/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml), and incubated at 37°C in 5% CO₂ and 95% humidity for 24 hours to adapt to culture conditions.
2. Dose-and time-dependent effects of glucocorticoids

Once adapted to culture conditions, chondrocytes were washed 3 times in D-PBS solution and placed in fresh Opti-MEM medium (control) or medium containing MPS, BPD, or TA in varying concentrations in 96-well plates with 4 replicates. Triamcinolone acetonide powder was dissolved in 2-hydroxypropal-β-cyclodextrin (HC) (Sigma-Aldrich, St Louis, MO). The percentage of HC in culture media was kept at levels no higher than 2%, according to the cytotoxicity assay for HC solution in chondrocytes. The concentrations of MPS, BPD and TA included levels routinely used in clinical practice (Table 4). Chondrocytes were treated with MPS, BPD, or TA for 3, 6, 12, 24 or 72 hours. Catilage explants were also treated with previously mentioned drug at the same concentration levels for 72 hours.
Table 4. Concentrations of methylprednisolone, betamethasone, and triamcinolone used to evaluate the glucocorticoid-induced chondrocyte cytotoxicity in chondrocyte cell culture and cartilage explants.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS</td>
<td>2x10^{-8} 2x10^{-7} 2x10^{-6} 2x10^{-5} 2x10^{-4} 4x10^{-4} 6x10^{-4} 1.6x10^{-3} 2x10^{-3} 2x10^{-2} 1x10^{-1}</td>
</tr>
<tr>
<td>BPD</td>
<td>2x10^{-7} 2x10^{-6} 2x10^{-5} 2x10^{-4} 2x10^{-3} 4x10^{-3} 6x10^{-3} 1.2x10^{-2} 4x10^{-2} 1x10^{-1}</td>
</tr>
<tr>
<td>TA</td>
<td>5x10^{-9} 5x10^{-8} 5x10^{-7} 5x10^{-6} 1x10^{-5} 2.3x10^{-5} 5x10^{-5} 9x10^{-5} 2x10^{-4}</td>
</tr>
</tbody>
</table>
3. Assessing cell viability

a) Chondrocyte monolayer cell culture

Chondrocytes cytotoxicity assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method using a commercially available kit (CellTiter 96 Aqueous one Solution Cell Proliferation Assay; Promega, Madison, WI, USA) according to the manufacturer’s instruction. This method has shown to be an accurate indicator of cell viability by measuring mitochondrial NADPH or NADH-dependent dehydrogenase activity. The MTS tetrazolium compound is directly reduced by cellular NADH and NADPH to generate a colorimetric reaction. Briefly, chondrocyte cells were seeded in 96 well plates at a density of 1 x 10^4 cells/well and cultured in the Opti-MEM with 5% fetal bovine serum.

Chondrocyte cell viability was assessed after 3h, 6h, 12h, 24h and 72h culturing with methylprednisolone, betamethasone, or triamcinolone respectively. The cells were incubated with 20 µl of CellTiter 96 Aqueous one Solution for 3h at 37°C in a humidified atmosphere of 5% CO₂. Plates were read on a microplate spectrophotometer at 490 nm. The cells were evaluated regularly for morphological changes. Data are expressed as the percentage of viable cells compared to control samples.
b) Cartilage tissue

After 24 hours pre-culture, explants were washed 3 times with D-PBS and re-fed with fresh media containing different concentrations of glucocorticoid drugs. After 72 hours treatment, the cell culture medium was removed and explants were washed 3 times in D-PBS solution.

The LIVE/DEAD viability/cytotoxicity kit for mammalian cell from Molecular Probe was used to simultaneously detect live and dead cells. It achieves this by utilizing two fluorescent labels; calcein-AM is metabolised by living cells to produce a green fluorescent substrate, ethidium homodimer enters cells whose membranes have been compromised and emits a red fluorescence. Glucocorticoid treated explants were incubated individually with 1 ml PBS containing 10 μM calcein-AM and 2 μM ethidium homodimer at 37°C for 30 min, and washed again in PBS, mounted onto slides and viewed by confocal microscopy. Data are expressed as the percentage of viable cells compared to control samples.

In vivo analysis

1. Animals

Nine horses at 3-4 years of age were studied (Table 5). All horses had a thorough physical, lameness and radiographic examination of their metacarpal-phalangeal and metatarsal-phalangeal (fetlock) joints before entry into the study. All horses included in the study were free of both clinical and radiographic evidence of
osteoarthritis and severe joint lesions. Horses were stabled on treatment days and were allowed free exercise in a paddock on non-treatment days. All procedures for this study were approved by the Animal Care and Use Committee at University of Kentucky.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Horse ID</th>
<th>Gender</th>
<th>Age (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylprednisolone</td>
<td>I-4</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>N-124</td>
<td>F</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>I-5</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>J-4</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>J-7</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>J-11</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>J-14</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>J-16</td>
<td>M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>J-17</td>
<td>M</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5. Horse ID, gender and age for nine horses in each treatment group.

The nine horses were randomly assigned into the three treatment groups: methylprednisolone, betamethasone, or triamcinolone treatment. Each treatment group had 3 horses. The same drug was administered to the left front and left hind fetlock joint of each horse, with the contra-lateral fetlock (right front, right hind) receiving saline. This design results in a total of 6 replicates for each medication with a further division of 3 applications per drug in the front fetlock and 3
applications of the drug in the rear fetlock (Figure 8). The same treatment was administered a total of two times, separated by an interval of one month.

Figure 8. Experimental design for intra-articular corticosteroid drug treatment in nine horses. Each experimental group had 3 horses and each horse received two injections with the same drug at 4 weeks interval. The left front and hind fetlock joints were received corticosteroid injections, with the contra-lateral fetlocks receiving saline.

2. Medicating joints

Fetlock joints at the time of injection were aseptically prepared by cleaning the area with 70% EthOH and 2% Chlorhexidine using standard aseptic procedures to minimize the potential of contaminating bacteria at the injection site. After preparation of the injection site, the appropriate volume of drug or saline was injected into the joints. Intra-articular injections of methylprednisolone,
triamcinolone, and betamethasone were administered in the fetlock joint by a 
licensed veterinarian at the upper limit of the treatment range as recommended by 
the manufacturer. Drug-treated fetlock joints were received either 80 mg/joint of 
methylprednisolone (Depo-Medrol), 12 mg/joint of betamethasone (Celeston), or 9 
mg/joint of triamcinolone (Kenalog) individually (Table 6). The contra-lateral 
paired joints receiving the same amount of saline were used as controls.
<table>
<thead>
<tr>
<th>Drug (Generic name, Trade name)</th>
<th>Commercial product concentration</th>
<th>Intra-articular dose for proposed experiment</th>
<th>Volume of drug (ml/joint)</th>
<th>Volume of amikacin (ml/joint)</th>
<th>Total volume of solution injected into joint (ml/joint)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triamcinolone acetonide Kenalog</td>
<td>10 mg/ml</td>
<td>9 mg/joint</td>
<td>0.9 ml</td>
<td>0.25</td>
<td>0.9 +0.25 + 1.1 ml saline = 2.25 ml</td>
</tr>
<tr>
<td>Betamethasone Celeston, Soluspan</td>
<td>3 mg betamethasone acetate, 3 mg betamethasone sodium phosphate, total 6 mg/ml</td>
<td>12 mg/joint</td>
<td>2 ml</td>
<td>0.25</td>
<td>2.25 ml</td>
</tr>
<tr>
<td>Methylprednisolone acetate Depo-Medrol</td>
<td>40 mg/ml</td>
<td>80 mg/joint</td>
<td>2 ml</td>
<td>0.25</td>
<td>2.25 ml</td>
</tr>
</tbody>
</table>

Table 6. Volumes and concentrations of steroid drug solutions injected into horse fetlock joints.
3. Sample collections

Three days after the second injection, all horses were humanely euthanized using commercial sodium pentobarbital euthanasia solution (Beuthanasia D, Intervet). All tissue samples were collected postmortem. The fetlock joint was dissected free from surrounding skin and subcutaneous tissues and in its entirety isolated by transecting the proximal phalangeal and third metatarsal/metacarpal bone with a band saw within 1 h after euthanasia. After opening, the joints were inspected visually to exclude macroscopic pathology. Subsequently, articular cartilage plugs were collected perpendicular to the articular surface from 2 predefined locations on the proximal articular surface of the first phalanx using a 6-mm diameter biopsy punch to maintain approximated size and weight uniformity of each sample (Brama et al. 2009). One cartilage plug was collected from the dorsal edge of the articular surface (site 1, Figure 9a, b). This site is exposed to dynamic, intermittent peak-type loading other than the weight bearing during standing or moving at slow speed (Figure 9c, Brama et al. 2001). A second cartilage plug was harvested from the mid-region of the joint cavity (site 2, Figure 9a, b). This site is constantly loaded whenever the limb is weight bearing, but it is not exposed to the peak loads and shear forces to which site 1 is exposed (Figure 9c, Brama et al. 2001).
4. Analysis of cell death

Cell viability within treated and control samples was evaluated immediately after sample collections. Two section vertical slices 1 mm thick were cut through each articular cartilage sample with a scalpel and incubated in 1 ml PBS containing 2 μM calcein-AM and 5 μM ethidium homodimer at 37°C for 30 min. Confocal fluorescence imaging (Leica TCS SP5) using 495 nm excitation and separation of green (515 nm) and red (635 nm) fluorescence was used to visualize live and dead...
cells, respectively. A 10 x objective was used and six images (1.5 x 1.5 mm²) were combined to obtain one of the entire slice. To quantify cell viability, the images were analyzed using Image J (National Institute of Health, Maryland) as described previously (Natoli et al. 2008). Percent viability was measured over a square area defined by the full thickness of the explants (Figure 10 and 11).

Figure 10. Articular cartilage sample collection, dissection and staining. The articular cartilage plugs (diameter 4 mm) were taken perpendicular to the articular surface and down to the calcified cartilage from 2 predefined locations on the joint surface of the proximal phalanx using 4 mm diameter biopsy punch. Samples were trimmed to 1 mm thick slice and incubated in 1 ml PBS containing 2 µM Calcein-AM and 5 µM EthD-1. Confocal microscope was used to visualize the live and dead cells. Cartilage schematic image was taken from Geistlich Surgery.
Figure 11. Visualization with Confocal Microscope. The imaging area was 1.5 x 1.5 mm². A 10 x objective was used to observe the samples and six images (1.5 x 1.5 mm²) were combined to obtain one sample. Live cells were stained with green color and dead cells were stained with red color.

VII. Statistical analysis

The data are expressed as mean +/- sem. Statistical analysis was done using one-way analysis of variance followed by Turkey's multiple comparison procedure. 

\( P < 0.05 \) was considered statistically significant.

Data for in vivo study were analyzed using the General Linear Model procedure (GLM procedure) of the SAS software. Each treated or untreated fetlock joint of horse was considered as one block. The model included horse, block and their interactions as covariates, and a treatment as a primary variable of interest. Data were analyzed by use of ANOVA. Significance was defined as values of p-value<0.05.
Chapter III Results

I. Establish an *in vitro* glucocorticoid treatment model

In order to develop a cell culture model for studying the effects of glucocorticoids on primary equine articular chondrocytes, length of pre-culture, pro-inflammatory stimuli, and timing of treatments were compared.

*Establish a chondrocyte culture model with stable gene expression pattern.*

Three genes including the housekeeping gene RPLPO were investigated by quantitative RT-PCR. Following normalization to steady state levels RPLPO mRNA, time-dependent changes in the expression of MMP3 and MMP9 were evaluated over 14 days in culture. The relative mean expression levels of each gene were compared between chondrocytes prior to seeding (defined as 1.00) and cultured chondrocytes at seven time points (days 1, 2, 3, 5, 7, 10, 14). The expression levels genes exhibited dramatic changes during the culture period. As shown in Figure 12, MMP3 and MMP9 exhibited higher expression levels in the early culture period than those in the late culture period. The relatively stable expression of MMP3 was observed after 5 days culturing (Figure 12). MMP9 had relatively stable expression after 10 days culturing (Figure 12). The similar gene expression patterns were observed in both aggregate and monolayer cultures for two genes.
Figure 12. Changes over time of MMP3 and MMP9 mRNA levels in monolayer and aggregate cultures of primary equine articular chondrocytes. Steady state mRNA levels for MMP3 and MMP9 genes were measured using quantitative RT-PCR, and normalized to RPLPO. Each value represents the mean±SEM with three replicates.

Comparing the effects of IL-1β and LPS stimulation on pro-inflammatory gene expression in primary articular chondrocytes and synovial fibroblasts.

Both IL-1β and LPS have been applied to stimulate the inflammatory response in joint tissues in published studies. In order to establish an appropriate model of joint inflammation, the expression levels of IL-6 and MMP13 were evaluated in primary articular chondrocytes and synovial fibroblasts following IL-1β and LPS stimulation (Figure 13). This experiment was used to compare the efficacy of IL-1β and LPS on stimulating the inflammatory response in these two cell types. IL-1β and LPS both significantly (p<0.05) stimulated these two gene expressions in chondrocytes and synovial fibroblasts after 24 or 72 hours stimulation. IL-1β was the more potent of stimulus than LPS, especially for synovial fibroblasts. More than 100-fold differences were observed between IL-1β and LPS stimulated gene
expression levels of IL-6 and MMP13 in synovial fibroblasts. Comparing two different concentrations (1ng/ml or 10ng/ml) of IL-1β, no significant difference was identified (p>0.05).
Figure 13. Changes of IL-6 and MMP13 gene expression in primary articular chondrocytes (A) and synovial fibroblasts (B) exposed to different concentrations of IL-1β and LPS. Cells were stimulated with recombinant equine IL-1β (1ng/ml or 10ng/ml) or LPS (1ng/ml, 10ng/ml, or 100ng/ml) for 24 or 72 hours. Steady-state mRNA levels of IL-6 and MMP13 were measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. IL-1β was the more potent of stimulus, but both significantly (*p<0.05, *) stimulated inflammatory responses in two cell types. No significant difference was identified between two different concentrations of IL-1β. Data are expressed as mean+/- SEM of three biological replicates.
Comparing differential expression of six genes in chondrocyte aggregate and synovial fibroblasts culture with three different sequences of methylprednisolone treatment relative to IL-1β stimulation.

In order to study the anti-inflammatory effects of glucocorticoids on joint tissues, chondrocyte aggregate and synovial fibroblast inflammatory culture models were established. Three methylprednisolone (MPS) treatment models: pre-treated with MPS (IL-1β 24h; MPS 36h); post-treated with MPS (IL-1β 24h; MPS 12h); treated together with MPS and IL-1β (IL-1β 24h; MPS 24h), were tested in chondrocytes and synovial fibroblasts. The relative mean expression levels of each gene were compared between cells with methylprednisolone treatment and control cells with IL-1β stimulation alone. The three treatment models all significantly (p<0.05) inhibited IL-1β, IL-6, iNOS, MMP3, MMP13 gene transcriptions stimulated by IL-1β (2-5-fold, Figure 14). The transcription levels of IL-1β, IL-6, iNOS, MMP3, MMP13, COL2A1 (chondrocytes), HAS2(synovial fibroblasts) were also compared among these three different treatment models. Significant differences were not identified among three models (p>0.05).
Figure 14. Effects of timing of glucocorticoid treatment on the expression of marker genes related to inflammation and normal cell function in primary articular chondrocytes (A) and synovial fibroblasts (B). Three methylprednisolone (MPS) treatment models: pre-treated with MPS (IL-1β 24h; MPS 36h); post-treated with MPS (IL-1β 24h; MPS 12h); treated together with MPS and IL-1β (IL-1β 24h; MPS 24h), were tested in chondrocytes (A) and synovial fibroblasts (B). Steady-state mRNA levels were measured for each gene by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. The three treatment models all significantly (p<0.05, *) inhibited pro-inflammatory gene transcriptions stimulated by IL-1β. Significant differences were not identified between the treatment groups. Data are expressed as mean+/− SEM of three biological replicates, with IL-1β positive control cells defined as 1.00.
II. Dose-dependent effects of glucocorticoids on the expression of pro-inflammatory genes for synoviocytes and chondrocytes

Inflammation is central to the pathogenesis of osteoarthritis, responsible for both the clinical symptoms and progressive destruction of articular cartilage through increased production of cytokines and degradative enzymes such as matrix metalloproteinases. Controlling inflammation, therefore, is an important therapeutic goal and a major beneficial effect of glucocorticoid administration.

Pro-inflammatory gene expressions in primary articular chondrocytes and synovial fibroblasts with glucocorticoid treatment.

To study the effects of glucocorticoids, steady-state mRNA levels of 5 pro-inflammatory genes were tested using quantitative RT-PCR. As previously reported (Richardson et al., 2000), IL-1β increased steady-state mRNA of IL-6, IL-1β, MMP3, MMP13 and iNOS dramatically in primary articular chondrocytes and synovial fibroblasts. Methylprednisolone (MPS) (10^{-4}M or 10^{-6}M) significantly (p<0.05) inhibited IL-6, IL-1β, MMP3, and MMP13 gene expression in both cell types, as well as iNOS in chondrocytes, when compared to control cells with IL-1β stimulation alone (Figure 15). These decreases ranged over the tested two doses range from 30% to 80% (Figure 15). Comparing the effects of two different doses of MPS on pro-inflammatory gene transcriptions, no significant difference was observed between these two concentrations (p>0.05).
The similar results were observed in betamethasone (BPD) and triamcinolone (TA). BPD (10^{-4}M or 10^{-6}M) significantly suppressed IL-6, IL-1β, MMP3, MMP13 and iNOS gene transcriptions in chondrocytes (Figure 16A), IL-1β and MMP13 in synovial fibroblasts as well (Figure 16B). However, no significant difference was observed between the two BPD concentrations (p>0.05). TA (10^{-5}M or 10^{-6}M) also significantly suppressed IL-6, MMP3, MMP13 and iNOS gene transcriptions in chondrocytes (Figure 17A), IL-6, IL-1β and iNOS in synovial fibroblasts as well (Figure 17B). The same as MPS and BPD, no significant difference was observed between the two TA concentrations (p>0.05).
**Figure 15.** Effect of two different concentrations of methylprednisolone (MPS) on expression of pro-inflammatory marker genes in primary articular chondrocytes (A) and synovial fibroblasts (B) following IL-1β stimulation. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were stimulated by IL-1β 12 hours prior to MPS treatment. The cells were then refed with medium containing both IL-1β and MPS for another 12 hours. Steady-state mRNA levels for IL-6, IL-1β, MMP3, MMP13, and iNOS were measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. Anti-inflammatory activities of MPS were evaluated at concentration levels of $10^{-4}$ M and $10^{-6}$ M. Cells treated with MPS showed significant (p<0.05, *) changes in gene expression compared to control cells with IL-1β stimulation alone. No significant difference was observed between the two different MPS concentrations. Data are expressed as mean +/- SEM of three biological replicates, with IL-1β positive control cells defined as 1.00.
Figure 16. Effect of two different concentrations of betamethasone (BPD) on expression of pro-inflammatory marker genes in primary articular chondrocytes (A) and synovial fibroblasts (B) following IL-1β stimulation. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were stimulated by IL-1β 12 hours prior to BPD treatment. The cells were then refed with medium containing both IL-1β and BPD for another 12 hours. Steady-state mRNA levels for IL-6, IL-1β, MMP3, MMP13, and iNOS were measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. Anti-inflammatory activities of BPD were evaluated at concentration levels of 10^{-4} M and 10^{-6} M. Cells treated with BPD showed significant (p<0.05, *) changes in gene expression compared to control cells with IL-1β stimulation alone. No significant difference was observed between the two different BPD concentrations. Data are expressed as mean+/- SEM of three biological replicates, with IL-1β positive control cells defined as 1.00.
Figure 17. Effect of two different concentrations of triamcinolone (TA) on expression of pro-inflammatory biomarker genes in primary articular chondrocytes (A) and synovial fibroblasts (B) following IL-1β stimulation. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were stimulated by IL-1β 12 hours prior to TA treatment. The cells were then refed with medium containing both IL-1β and TA for another 12 hours. Steady-state mRNA levels for IL-6, IL-1β, MMP3, MMP13, and iNOS were measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. Anti-inflammatory activities of TA were evaluated at concentration levels of $10^{-5} \text{M}$ and $10^{-6} \text{M}$. Cells treated with TA showed significant ($p<0.05$, *) changes in gene expression compared to control cells with IL-1β stimulation alone. No significant difference was observed between the two different TA concentrations. Data are expressed as mean+/- SEM of three biological replicates, with IL-1β positive control cells defined as 1.00.
Effects of glucocorticoids and VBP1 on five pro-inflammatory gene expressions in primary articular chondrocytes and synovial fibroblasts.

VBP1, a dissociative glucocorticoid, was reported to maintain comparable anti-inflammatory efficacy to prednisolone with reduced side effects in muscle cells. Accordingly, VBP1 shows promise as a potentially safer therapeutic agent for joint inflammation. Steady-state mRNA levels of 5 pro-inflammatory genes (IL-6, IL-1β, MMP3, MMP13 and iNOS) were tested using quantitative RT-PCR. MPS, BPD, and TA significantly (p<0.05) inhibited mRNA of pro-inflammatory genes in both cell types relative to control cells with IL-1β stimulation alone. There was a significant (p<0.05) association between different type of glucocorticoid treatment and expression of IL-6, IL-1β, MMP3, and MMP13. Significant differences were observed among MPS, BPD, and TA for IL-6, IL-1β, and MMP3 in chondrocytes (Figure 18A), and IL-6, MMP3, and MMP13 in synovial fibroblasts (Figure 18B). Comparing VBP1 to three glucocorticoids, anti-inflammatory efficacies of VBP1 were similar to those of TA, however, significantly lower than MPS and BPD.
Figure 18. Effects of glucocorticoids and VBP1 on expression of pro-inflammatory marker genes in primary articular chondrocytes (A) and synovial fibroblasts (B) following IL-1β stimulation. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were stimulated by IL-1β 12 hours prior to drug treatment. The cells were then refed with medium containing both IL-1β and a drug for another 12 hours. Steady-state mRNA levels for IL-6, IL-1β, MMP3, MMP13, and iNOS were measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2 for chondrocytes, and HPRT and TMF1 for synovial fibroblasts. Methylprednisolone (MPS, 10^-6 M), betamethasone (BPD, 10^-6 M), and triamcinolone (TA, 10^-6 M) significantly (p<0.05, *) inhibited pro-inflammatory gene expression in both cell types relative to control cells with IL-1β stimulation alone. Significant differences were observed among different drug treatment groups for IL-6 and MMP3 in both cell types, IL-1β in chondrocytes, and MMP13 in synovial fibroblasts. Comparing VBP1 to three glucocorticoids, anti-inflammatory efficacies of VBP1 were similar to those of TA, however, significantly lower than MPS and BPD. Data are expressed as mean+/- SEM of three biological replicates, with IL-1β positive control cells defined as 1.00.
iNOS gene expression and inducible nitric oxide synthase activity

Steady-state mRNA level of iNOS was significantly increased by IL-1β stimulation (more than 100-fold) in primary articular chondrocytes. The effect of IL-1β on iNOS gene transcription was significantly (p<0.05) reversed by glucocorticoids (MPS, BPD, and TA) or VBP1 (Figure 19A). VBP1 at the concentrations of $10^{-5}$M or $10^{-6}$M both inhibited iNOS gene transcription, however, significant difference was only observed at $10^{-5}$M.

Nitric oxide (NO) production was significantly increased by IL-1β stimulation (more than 100-fold). The effect was reversed by increasing doses of glucocorticoids and VBP1. Glucocorticoids and VBP1 reduced IL-1β-induced NO production in a dose-dependent manner (Figure 19B). BPD and TA significantly (p<0.05) reduced NO production more than 30% when concentrations increased above $10^{-8}$M. MPS at the concentration levels above $10^{-6}$M significantly decreased NO production more 30%. VBP1 also significantly (p<0.05) inhibited NO production when concentration was increased to $10^{-5}$M.
Figure 19. Effects of glucocorticoids and VBP1 on iNOS gene expression and nitric oxide production in primary articular chondrocytes. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were stimulated by IL-1β 12 hours prior to drug treatment. The cells were then refed with medium containing both IL-1β and a drug for another 12 hours. Steady-state mRNA for iNOS was measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2. Nitrite in drug treated chondrocytes was assessed at concentration levels of methylprednisolone (MPS: 10⁻⁴, 10⁻⁶, 10⁻⁸, 10⁻⁹, or 10⁻¹⁰ M), betamethasone (BPD: 10⁻⁴, 10⁻⁶, 10⁻⁸, 10⁻⁹, or 10⁻¹⁰ M), triamcinolone (TA: 10⁻⁵, 10⁻⁶, 10⁻⁸, 10⁻⁹, or 10⁻¹⁰ M) or VBP1 (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, or 10⁻⁹ M) by Griess reagents. (A) Steady state mRNA of iNOS was increased by IL-1β stimulation and the effect was significantly (p<0.05, *) reversed by glucocorticoids or VBP1 treatment. Data are expressed as mean+/- SEM of three biological replicates, with IL-1β positive control cells defined as 1.00. (B) Nitric oxide production was increased by IL-1β stimulation. The effect was reversed by increasing doses of glucocorticoids and VBP1. Significant differences were observed. Data are expressed as mean+/- SEM of three biological replicates.
Type II collagen gene expression in primary articular chondrocytes with glucocorticoids treatments.

Methylprednisolone, betamethasone, triamcinolone are three commonly used intra-articular glucocorticoids in horse, yet drug-induced suppression of type II collagen synthesis by chondrocytes has been documented. VBP1 as a potential therapeutic agent for joint inflammation may have a reduced side effect on type II collagen gene transcription. Dose-dependent effects of glucocorticoids and VBP1 on the steady-state mRNA level of type II collagen in chondrocytes were tested. Methylprednisolone, betamethasone, triamcinolone, and VBP1 all displayed numerically lower levels of steady-state COL2A1 mRNA, but not to an extent that was significantly different from the control samples (Figure 20).
Figure 20. COL2A1 gene expression in primary articular chondrocytes with glucocorticoids or VBP1 treatment. Cells were exposed to four drugs individually at a concentration level of methylprednisolone (MPS, 10^{-4} or 10^{-6} M), betamethasone (BPD, 10^{-4} or 10^{-6} M), triamcinolone (TA, 10^{-5} or 10^{-6} M) or VBP1 (10^{-5} or 10^{-6} M). Steady state mRNA for COL2A1 was measured by RT-qPCR, and normalized to the expression of reference genes HPRT and EIF2B2. Data are expressed as mean±/ SD of three biological replicates, with IL-1β control cells defined as 1.00.
HAS2 gene expression in synovial fibroblasts following equine recombinant IL-1β stimulation and treatment with glucocorticoids.

Hyaluronan (HA), which is produced by synovial fibroblasts, is an important component of synovial fluid contributing to properties of viscosity and boundary lubrication. In order to evaluate the inhibition of glucocorticoids and VBP1 on HA, HA transcription in synovial fibroblasts was analyzed. Hyaluronic acid synthase 2 (HAS2) mRNA levels were significantly (p<0.05) upregulated by stimulation with 1 ng/ml IL-1β, as compared with untreated controls (Figure 21). Methylprednisolone, triamcinolone, and VBP1 all repressed the HAS2 mRNA levels which were induced by IL-1β stimulation, and change levels were from 0.7 fold to 0.89 fold (Figure 21). However, significant difference between these three drugs treated samples and samples with only IL-1β stimulation was not observed. Betamethasone at concentration levels of 10^-4 M and 10^-6 M significantly (p<0.05) down-regulated the HAS2 mRNA levels (Figure 21).
Figure 21. HAS2 gene expression in synovial fibroblasts following recombinant IL-1β stimulation and glucocorticoids or VBP1 treatment. Recombinant equine IL-1β (1ng/ml) was used as a pro-inflammatory stimulus. Cells were exposed to 4 drugs following IL-1β stimulation at a concentration level of methylprednisolone (MPS, 10^{-4} or 10^{-6} M), betamethasone (BPD, 10^{-4} or 10^{-6} M), triamcinolone (TA, 10^{-5} or 10^{-6} M) or VBP1 (10^{-5} or 10^{-6} M). Steady state mRNA for HAS2 was measured by RT-qPCR, and normalized to the expression of reference genes HPRT and TMF1. Data are expressed as mean+/- SD of three biological replicates, with IL-1β positive control cells defined as 1.00.
III. Glucocorticoid-induced chondrocyte cytotoxicity

As a potent anti-inflammatory drug for osteoarthritis, glucocorticoids effectively reduce expression of matrix metalloproteinases and help in relieving the symptoms of joint inflammation. However, several side effects of glucocorticoids including potential cytotoxicity on chondrocytes have been reported. The purpose of this study is to evaluate the chondrocyte cytotoxicity of different doses of the three most commonly used corticosteroids with different length of exposure on equine primary articular cartilage in vitro. Subsequently, chondrotoxicities of manufacture recommended dosage levels of methylprednisolone, betamethasone, and triamcinolone are evaluated on normal equine fetlock joints in order to assist the clinician when using an evidence-based approach to the use of intra-articular corticosteroid medications.

*Methylprednisolone, betamethasone, and triamcinolone induced chondrocyte cytotoxicity in monolayer and explants culture.*

Several in vitro studies have demonstrated conflicting results for the effects of corticosteroid on chondrocyte cytotoxicity. In these studies, different experimental models and corticosteroids were used, and different endpoints were chosen. In order to compare the cytotoxic effects of three commonly used glucocorticoids on equine articular chondrocytes, the same experimental models were used in our study, and several different time points and drug concentrations were evaluated. In chondrocyte monolayer cultures, cell viability decreased below 90% in the presence of MPS which concentration levels were equal to or higher than 0.8 mg/ml (1.6x10⁻³...
M) at three hour time point (Figure 22A). After six hours of incubation, cell viability fell below 90% when MPS concentrations were equal to or higher than 0.2 mg/ml (4x10^{-4} M) (Figure 22A). At the highest concentration of 40 mg/ml (8x10^{-2} M), almost all of the chondrocytes were dead after 3 hours incubation (Figure 22A). After 12 hours of incubation, almost all of the cells were dead when MPS concentrations were equal to or higher than 10 mg/ml (2x10^{-2} M) (Figure 22A). Cell death and viability in response to corticosteroid treatment in explants was assessed by uptake of ethidium homodimer (red) and calcein-AM (green) fluorescent label respectively (Figure 23). The cytotoxicity curve for MPS treated chondrocyte explants is quite similar to the curve observed for chondrocytes in monolayer culture (Figure 22A). According to these two experiments, MPS concentrations higher than 0.1 mg/ml (2x10^{-4} M) are cytotoxic to chondrocytes in both monolayer and explant culture.

Similar cytotoxicity curves were observed for betamethasone and triamcinolone. At the 3 hour time point, cell viability decreased below 90% when BPD concentrations were equal to or higher than 6 mg/ml (1.2x10^{-2} M) (Figure 22B). After 6 hours of treatment, cell viability fell below 90% when BPD concentrations were equal to or higher than 2 mg/ml (4x10^{-3} M) (Figure 22B). At the highest concentration of 50 mg/ml (1x10^{-1} M), almost all of the chondrocytes were dead after 3 hours incubation (Figure 22B). After 24 hours of incubation, almost all of the cells were dead when BPD concentrations were equal to or higher than 20 mg/ml (4x10^{-2} M) (Figure 22B). Chondrocyte cell viability dropped below 90% when TA concentration increased to 0.08 mg/ml (1.8x10^{-4} M) after 24 hours treatment.
(Figure 22C). The same to the MPS cytotoxicity experiments, the cytotoxicity curves for BPD or TA treated chondrocyte explants and monolayer cultures are very similar (Figure 22B, C). According to these experiments, BPD concentrations higher than 1 mg/ml (2x10^{-3} M) and TA concentrations higher than 0.04 mg/ml (9x10^{-5} M), are cytotoxic to chondrocytes in both monolayer and explants culture.
A

Concentration of MPS (M)

Cell viability (%)

3 h 6 h 12 h 24 h 72 h Explants

B

Concentration of BPD (M)

Cell viability (%)

3 h 6 h 12 h 24 h 72 h Explants

C

Concentration of TA (M)

Cell viability (%)

3 h 6 h 12 h 24 h 72 h Explants
Methylprednisolone, betamethasone, and triamcinolone induced chondrocyte cytotoxicity in equine fetlock joints.

Some limitations of in vitro study, however, must be considered. First, chondrocytes cultures may not reflect the complex and highly differentiated cartilage tissue. Second, the chondrocytes are stored in the media in a static manner and diffusion characteristics likely differ in a moving joint. Another limitation is the use of constant concentration of drug in vitro as a model of the clinical injection, which is not equivalent to the in vivo joint environment and metabolism. Effect of glucocorticoids on chondrocyte cytotoxicity therefore need to be evaluated in vivo to see if drugs at the manufacture recommended dosage level would cause cell death in equine joints.

All experimental procedures were well tolerated by all of the horses and no evidence of joint inflammation and lameness was observed following the administration of corticosteroids.

To assess chondrocyte viability, 2µM of Calcein AM and 10 µM of EthD-1 were used to stain dead cells (Red) and live cells (green), respectively. To quantify cell viability, the outer contour of the articular cartilage section and the area containing viable cells were identified manually. Typical confocal microscopy images of cell
viability within the articular cartilage layer which were intra-corticosteroid treated (Figure 23 A) and untreated control (Figure 23B), are shown in Figure 23.

![Figure 23](image)

Figure 23. LIVE/DEAD labeling of articular cartilage from equine fetlock joints treated with 80 mg/joint methylprednisolone (MPS) or saline. Live cells were stained green by calcein-AM, whereas dead cells were stained red by ethidium homodimer. A. Equine articular cartilage from a left front fetlock joint of a horse treated with 80 mg/joint MPS. B. Equine articular cartilage from a right hind fetlock joint of the same horse treated with saline.

Methylprednisolone acetate (MPA) caused a significant (P<0.05) reduction in chondrocyte viability compared with the control saline injections (Figure 24). The average cell death was increased from 12% in controls to 17.4% with 80 mg/joint of MPA injections. Biological variation was observed when cell death of MPA treated samples was compared to control samples for each horse. Significant differences were observed for two horses. The cell death was increased from 11.4% in controls to 20.9% with MPA injections in one horse, and from 9.6% in controls to 17.7% with MPA injections in another horse. There, however, is no significant (P>0.05) difference was observed for the third horse.
Betamethasone containing betamethasone sodium phosphate and betamethasone acetate caused a significant (P<0.05) reduction in chondrocyte viability compared with the controls with saline injections (Figure 24). The average cell death was increased from 13.2% in controls to 18.5% with 12 mg/joint of betamethasone injections. Biological variation was observed when cell death of drug treated samples was compared to control samples for each horse. Significant differences were observed for two horses. The cell death was increased from 15.5% in controls to 22.6% with betamethasone injections in one horse, and from 10.3% in controls to 19.5% with betamethasone injections in another horse. There, however, is no significant (P>0.05) difference was observed for the third horse.

Triamcinolone acetonide (TA) caused a significant (P<0.05) reduction in chondrocyte viability compared with the controls with saline injections (Figure 24). The average cell death was increased from 13.1% in controls to 17.1% with 9 mg/joint of TA injections. Biological variation was observed when cell death of TA treated samples was compared to control samples for each horse. Significant differences were only observed for one horse. The cell death was increased from 9.4% in controls to 18.3% with TA injections in one horse. There, however, is no significant (P>0.05) difference was observed for the other two horses.
Figure 24. Methylprednisolone (MPA), betamethasone (BPD) and triamcinolone (TA) induced chondrocyte cytotoxicity in articular cartilage from equine fetlock joints. Intra-articular MPA (80 mg/joint), BPD (12 mg/joint) and TA (9 mg/joint) were injected into left front and hind fetlock joints for 3 horses, respectively. The contra-lateral fetlocks (right front, right hind) were received saline injection. Each horse was received 2 injections with the same drug. Effect of MPA, BPD and TA on cell viability was assessed 3 days after the second injection by LIVE/DEAD kit. Significant differences were observed between treated groups and control groups. Data are expressed as means ± S.E.M (n= 6). The percentages of cell death in each treatment group are expressed as mean ± S.D in the table.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MPS</th>
<th>BPD</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death (%)</td>
<td>12.4 ± 3.0</td>
<td>17.4 ± 3.9</td>
<td>18.5 ± 4.9</td>
<td>17.3 ± 4.0</td>
</tr>
</tbody>
</table>
Chapter IV Discussion

I. Establish an *in vitro* glucocorticoid treatment model

Tissue culture parameters and protocols were compared to develop a model system for studying the effects of glucocorticoids on primary equine articular chondrocytes and synovial fibroblasts. Several cell culture methods have been developed to investigate chondrocyte and articular cartilage biological properties (Adolphe et al., 1992; Tew et al., 2008). Although nonadherent aggregates and pellet culture improve the maintenance of chondrocyte phenotype, they slow the cell growth, yielding lower amounts of cells and RNA for analysis. Alternatively, articular cartilage can be studied as explant cultures, in which chondrocytes remain embedded within their own extracellular matrix (Poole et al., 1989). However, many experimental manipulations are done more easily using isolated chondrocytes maintained as adherent monolayer cultures (Otero et al., 2012). After considering these factors and culturing logistics, monolayer cultures were selected for subsequent experiments in this study.

Length of culture, pro-inflammatory stimuli, and timing of treatments were compared. The expression patterns of matrix proteases, protease inhibitors, and cytokines in equine articular chondrocytes were evaluated during a 14 day culture period to find out when steady state levels of mRNA for target genes of interest stabilized. This initial culture period prior to introducing an experimental variable was used to minimize error introduced by the effects of chondrocyte isolation and cell plating. PCR analysis showed that the expression of all genes of interest was markedly and differentially affected by tissue digestion, with differences up to 10 to
15 fold (Martin et al., 2001). Consistent with previous reports (Lin et al., 2008), the expression of matrix proteinases MMP3 and MMP9 decreased with increasing culturing period (Figure 12). Our results also indicated that the expression of MMP3 became relatively stable after 5 days in culture, and the expression of MMP9 became relatively stable after 10 days in culture. Based on these preliminary data, the pre-culture period for chondrocytes was determined to be 10 days before starting the treatment or stimulation, to allow cells to adapt to the culture conditions and eliminate the effects of tissue collection and cell isolation.

Inflammatory cytokines such as Interleukin-1 (IL-1) β and tumor necrosis factor-α (TNF-α) play pre-eminent roles in joint destruction. In joint diseases, IL-1β is synthesized by synoviocytes and cartilage chondrocytes (Martel-Pelletier et al., 1999; Tiku et al., 1992). IL-1β has been commonly used in articular cartilage and synovial membranes for creation of a joint inflammation model. It has been shown to be increased in inflammatory conditions and stimulate the production of matrix degradation enzymes such as matrix metalloproteinase (MMP) (Pelletier et al., 1993). Lipopolysaccharide (LPS) has similar effects to IL-1β on chondrocyte-mediated matrix metabolism and is, therefore, a useful experimental inducer of chondrocyte-generated pathology (Jasin et al., 1983; MacDonald et al., 1994). In order to establish a joint inflammation model in vitro, our experiments investigated the changes in pro-inflammatory gene expressions in response to LPS or IL-1β in chondrocytes and synovial fibroblasts. Similar to previous studies (Jasin et al., 1983; MacDonald et al., 1994; Pelletier et al., 1993), both IL-1β and LPS were able to significantly stimulate the inflammatory response in articular chondrocytes and
IL-1β was more potent than LPS, especially in synovial fibroblasts. However, no significant difference was identified between 1 ng/ml and 10 ng/ml of IL-1β. IL-1β with 1 ng/ml was used to stimulate the inflammatory response in chondrocytes and synovial fibroblasts for subsequent studies.

Several different in vitro glucocorticoid treatment models were established in previous studies: pre-treated with glucocorticoids, then started the inflammatory stimulation (Baundy et al., 2009; 2012; Crofford et al., 2004); stimulated the inflammatory response first, then post-treated with glucocorticoids (Bertone et al., 2008; Lu et al., 2011); treated together with glucocorticoids and pro-inflammatory mediators (Frisbie et al., 2001; Busscher et al., 2010; Richardson and Dodge, 2003). Three different glucocorticoid treatment models were compared in our studies. The length of IL-1β stimulation was controlled to 24 hours. The expression of cytokines, matrix proteinases and matrix proteins in chondrocytes and synovial fibroblasts was determined by quantitative real-time PCR. All three models repressed pro-inflammatory gene expressions induced by IL-1β stimulation, and the expression of COL2A1 was inhibited in pre-treatment and simultaneous treatment models, not in post-treatment model. However, significant differences were not identified among these three models. Previous studies by Lin et al. 2011, also demonstrated that treating cartilage with glucocorticoids either before or after inflammatory stimulation significantly reduced glycosaminoglycans (GAG) loss and increased proteoglycan biosynthesis. It suggested that even when catabolic processes have already begun in cartilage, glucocorticoid treatment could still suppress GAG loss and increase biosynthesis. In clinical cases, patients experience joint pain and joint
swelling, and then clinicians treat the inflammatory joints using anti-inflammatory medicine. The chondrocytes and synovial fibroblasts used in this study were isolated from the normal joints without any sign of joint disease. Therefore, these cells may have different biological properties from cells from inflamed joints in which inflammatory response has already been initiated.

Based on these preliminary data and clinical cases, the glucocorticoid treatment models in primary equine chondrocytes and synovial fibroblasts were established. Primary equine chondrocytes were pre-cultured in cell culturing medium without passaging for 10 days. After that, IL-1β at 1 ng/ml was used to initiate the inflammatory response in both cell types. Twelve hours later, cells were treated with glucocorticoids in addition to continued stimulation with IL-1β for another 12 hours. The following experiments were performed using this drug treatment model.
II. Dose-dependent effects of glucocorticoids on the expression of pro-inflammatory genes in chondrocytes and synoviocytes.

A major finding of this study was that glucocorticoids can maximally inhibit pro-inflammatory gene transcriptions in both equine articular chondrocytes and synovial fibroblasts at lower doses than widely used clinical dosages.

The hallmark of osteoarthritis is loss of articular cartilage structure integrity and function. In the early stage of disease, multiple structural proteins of the matrix are actually increased (MacLeod et al., 1998), however, the net outcome is progressive loss of matrix integrity. The elements which play the most important role in the degradation of the cartilage matrix are the matrix metalloproteinases (MMP). Although there are obvious similarities among the MMPs, each MMP has a preferred substrate that presumably is related to its role in development, normal tissue remodeling, as well as disease processes. MMP3 (stromelysin 1) is well characterized in horse cartilage and believed to have the proteoglycans as preferred substrates (Morris et al., 1994; Richardson et al., 1998; Balkman et al., 1998). MMP13 (collagenase 3) has type II collagen as a preferred substrate and has been studied in equine chondrocytes (Caron et al. 1996; Richardson et al., 2000). It is generally accepted that MMP increases in arthritis and that it specifically increases in response to many cytokines, which is clear from our results (Figure 15) and those of others (Richardson and Dodge, 2003; Pelletier et al., 1993, 1994).

While the role of NO in the physiology and pathobiology of equine vascular reactivity has been the subject of recent research, its role in equine OA pathogenesis
has received little study. Results of subsequent short-term experiments using IL-1β stimulated cultures complement previous reports describing NO synthesis by equine chondrocytes in response to stimulation with proinflammatory mediators (Frean et al., 1997; Bird et al., 1997). Our results indicated that methylprednisolone, betamethasone, and triamcinolone attenuated IL-1β induced NO release by primary equine articular chondrocytes in a dose-dependent manner. It was also confirmed by our results that IL-1β induced NO release by equine chondrocytes correlates with a direct stimulation of iNOS gene expression (Figure 19). Real-time qPCR results indicated that expression of iNOS in chondrocyte was inhibited by all of three glucocorticoids at $10^{-6}$M. In other species, a number of isoforms of NOS have been described and vary in their sensitivity to glucocorticoids (Caron et al., 1993; Tung et al., 2002). Many iNOS isolated from mammalian chondrocytes are insensitive to corticosteroids at modest doses, however, paralleling our findings, equine iNOS expression is inhibited with high doses of methylprednisolone, betamethasone, and triamcinolone. These findings may suggest a limited influence of endogenous glucocorticoids on iNOS expression but that pharmacologic doses are inhibitory.

Glucocorticoids clearly have complex effects on many genes which are important in the synthesis and maintenance of articular cartilage. The mechanism of corticosteroid inhibition of matrix metalloproteinase and cytokines synthesis is probably related to the inhibition of NF-κB-regulated pro-inflammatory transcripts (Richardson and Dodge, 2003). Because the effects of glucocorticoids on matrix-related genes are dose-dependent, specific to certain genes and occur through various mechanisms, it appears that beneficial effects of steroids may be obtained at
doses that do not adversely affect matrix protein synthesis. The independent mechanisms also offer the future possibility of independently regulating these genes.

Although it is impossible to directly relate synovial fluid concentrations of glucocorticoids with those actually affecting the chondrocytes within articular cartilage, the molar concentrations were approximately $10^{-3}$ M to $10^{-5}$ M following intra-articular injection of 100 mg of the steroid. This corresponds to the range of concentrations studied in our experiments. Although concentrations of glucocorticoids that we studied in vitro were close to the range of expected tissue concentrations seen in vivo, the actual concentrations exposed to the chondrocytes are not known. It also must be acknowledged that the complexity of glucocorticoid effects on other cells in the joint may be important in the net therapeutic result.

Dose-dependent effects of glucocorticoids and VBP1 on the steady-state mRNA level of COL2A1 in chondrocytes and HAS2 in synovial fibroblasts were tested. The same as the previous studies (Tanimoto et al., 2001; 2010), HAS2 gene expression was significantly up-regulated by IL-1β stimulation. From the Figure 20, it can be seen that glucocorticoid treatments suppressed the gene expression of type II collagen, however, significant differences were not detected (P<0.05). Suppression of steady-state gene expression of type II collagen wasn’t expected as we hypothesized. The data was consistent with our previous studies (Figure 14A). In our drug treatment model comparison experiments, steady-state mRNA level of type II collagen was only significantly decreased in models with 24h or 36h methylprednisolone treatment, not in the model with 12h drug treatment (Figure
One of the reasons is that cells were not treated with glucocorticoids long enough to see the inhibition of steady-state level of type II collagen mRNA. Degradation of mRNA is one of the key processes that control the steady-state level of gene expression. In general, mRNA species with a short half-life were enriched among genes with regulatory functions, whereas mRNA species with a long half-life were enriched among genes related to metabolism and structure (extracellular matrix, cytoskeleton) (Sharova et al., 2009). The mRNA half-life for type II collagen is about 17 hours in rabbit articular chondrocytes and 18 hours in human costal chondrocytes (Galera et al., 1992; Goldring et al., 1994; Mark et al., 2004). In this study, chondrocytes were treated with glucocorticoids for 12 hours, therefore, the level of type II collagen mRNA was still elevated upon withdrawal of drug treatment. Our results verified the former studies that type II collagen mRNA, as an important extracellular matrix protein gene in chondrocytes, has a relatively long half-life which is longer than 12 hours in equine articular chondrocytes. In this study, steady-state mRNA of type II collagen was evaluated, however, significant differences might be detected between control cells and glucocorticoid treated cells if only newly synthesized type II collagen mRNA were evaluated.

From what we observed, some further experiments could be planned for future studies. One of the experiments which could be done is using the same drug treatment model to analyze newly synthesized type II collagen mRNA to see if glucocorticoids would suppress type II collagen mRNA synthesis or not. The effect of glucocorticoids on gene expression levels of some other biomarker genes (aggrecan, type I collagen, link protein) for articular cartilage also could be
evaluated to see if glucocorticoids alter the differentiated phenotype of articular chondrocytes or stimulate dedifferentiation into fibroblasts. The other drug treatment model also could be tested in the future studies. In the previous reports, chondrocytes were treated with medium containing glucocorticoids for at least 24 h (Fubini et al., 2001; Richardson and Dodge, 2003; Busschers et al., 2010) prior to harvesting cells. The length of drug treatment could be extended. Chondrocytes could be cultured in medium containing glucocorticoids for 24, 48, or 72 hours. After that, cells could be collected and the effect of glucocorticoids on steady-state type II collagen mRNA could be evaluated. Then results also could be compared to 12 hour results to see if there is any difference between these two drug treatment models.
III. Glucocorticoid-induced chondrocyte cytotoxicity

The comparison of effects of methylprednisolone, betamethasone, and triamcinolone on equine articular cartilage was conducted in this study. The drug concentrations used in this study were at the high end of the manufacturer recommended doses for equine joint injection, which were commonly used by veterinarians according to a survey paper published by Ferris et al. 2011. Taken together, all these three corticosteroids have dose-dependent cytotoxic effects on equine articular chondrocytes in vitro. Manufacturer recommended concentration levels of these three commonly used intra-articular corticosteroids in equine practice have cytotoxic effects on equine articular chondrocyte in vivo. The results of both our in vitro and in vivo studies are consistent (Table 7).

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Clinical dose (mg)</th>
<th>In vivo Cytotoxicity conc. (mg/joint)</th>
<th>In vitro Cytotoxicity conc. (mg/ml)</th>
<th>Duration of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylprednisolone</td>
<td>40-120</td>
<td>80</td>
<td>0.2</td>
<td>Medium</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>3-18</td>
<td>12</td>
<td>2</td>
<td>Medium to long</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>6-18</td>
<td>9</td>
<td>0.08</td>
<td>Long</td>
</tr>
</tbody>
</table>

Table 7. Concentrations of glucocorticoids which have cytotoxicity in vitro and in vivo, as well as commonly used corticosteroid concentrations for horse OA treatment.

Corticosteroid medications are often injected into the joint space to decrease joint inflammation. Even though there is evidence that these medications effectively reduce patient symptoms (Blyth et al., 1994; Cole et al., 2005; Pelletier et al., 1994),
little is known with regard to their potential cytotoxicity on chondrocytes. Concentration-dependent cytotoxicity of methylprednisolone on chondrocytes monolayer cultures was observed by Fubini et al. 2009 and Seshadri et al. 2009, and these results were consistent with our studies. A recent investigation by Dragoo et al. 2012, examined the effect of a single dose of betamethasone, methylprednisolone, and triamcinolone on monolayer cultures of human chondrocytes. The authors found betamethasone to be significantly cytotoxic to chondrocytes when compared with controls (Dragoo et al., 2012). The same results were also observed in both our in vitro and in vivo studies. The cytotoxic effects of methylprednisolone and triamcinolone on chondrocytes were not reported in Dragoo’s study. However, several studies, including our investigations, have also shown detrimental effects of triamcinolone on chondrocyte viability (Celeste et al., 2005; Choi et al., 2005; Syed et al., 2011). It is reported by Syed et al. 2011 that triamcinolone alone or combination with bupivacaine was cytotoxic to chondrocytes when compared with controls. These results partially corroborate our findings, however, some in vivo studies demonstrated that triamcinolone may not have detrimental effects in patients with osteoarthritis (Pelletier et al. 1995) and may actually contribute to histopathological improvements in a canine model of osteoarthritis (Raynauld et al., 2003). The conflicting results observed in this study coupled with the published reports indicate that a cautious clinical approach may be best when evaluating indications for the use of triamcinolone and methylprednisolone for intra-articular injections.

Our study represents a direct assessment of chondrocyte viability after defined exposure to three corticosteroids individually using quantitative evaluations. The
major advantage of this study was that chondrocyte viability was evaluated in both *in vitro* and *in vivo*. Multiple drug concentrations and different drug treatment points can be tested in chondrocyte cell cultures. Animal models precisely maintain the *in vivo* condition of chondrocytes within articular cartilage bathed in synovial fluid, physical pH, and drug diffusion in joint spaces. Some other advantages of this study protocol include contra-lateral control and consistency over experimental conditions and direct quantitative measurements of chondrocyte viability using fluorescent staining. Cytotoxicity of methylprednisolone on equine articular cartilage *in vivo* was reported by Chunekamrai et al. according to the histological staining results (Chunekamrai et al. 1989). The concentration of methylprednisolone (120 mg/joint) used in that study, however, was higher than the commonly used clinical dosage, and the appearance of empty lacunae at the histological level may not be directly related to the effect of the corticosteroid. In our study, two fluorescent dyes were used to visualize the dead and live cells (Figure 11), and the percentages of cell death were quantified using Image J.

Variability was observed in our study. Chondrocyte cell death was also observed in our control samples with only saline and antibiotic injections. The average chondrocyte viability of controls in all treatment groups was about 87% which is lower than that in the explants without any injections (Table 8). Since same tissue collection, dissection, and staining processes were performed for explants and equine fetlock cartilage samples, the increased cell death in equine fetlock cartilage without any corticosteroid treatment may have been caused by the injection process and/or combined antibiotic injections. However, chondrocyte
viability at 84% for control samples cultured in saline solution was reported in a published study (Seshadri et al., 2009). This level is lower than the chondrocyte viability in our control samples. In future studies, chondrocyte viability needs to be tested in joints without any injection and with only saline treatment.

<table>
<thead>
<tr>
<th>Treatment arms</th>
<th>Experimental group</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline injection</td>
<td>Control samples in methylprednisolone group</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td>Control samples in betamethasone group</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td>Control samples in triamcinolone group</td>
<td>87%</td>
</tr>
<tr>
<td>Non-injection</td>
<td>Explants plug</td>
<td>93%</td>
</tr>
</tbody>
</table>

Table 8. Percentage of live chondrocytes in parallel control joints with normal saline injection and in methylprednisolone, betamethasone, or triamcinolone treatment groups, and in explants control sample without any treatment.

The potential for artifacts introduced during tissue processing also need to be considered. Cut edges were avoided during the imaging of stained articular cartilage samples (Figure 4). Dead cells were observed along the cut surface of the deep zone of cartilage, and they were consistent in control and treated sample. Chondrocyte viability for tissues from the superfacial layer down to the calcified cartilage was compared to a subsection of each sample limited to 75% of the cartilage that eliminated the cut surface of the deep zone. Correlation analysis was conducted for each treatment group. Dead cells in full thickness samples were well correlated to those in 75% of cartilage tissues. The correlation coefficients for methylprednisolone, betamethasone, and triamcinolone are 0.85, 0.90 and 0.96.
respectively (Figure 25). Statistical analysis was also performed using student t-test, demonstrating no significant difference (P>0.05).

Figure 25. Correlation analysis of dead cells in full thickness equine cartilage samples and those in 75% of cartilage samples. A. Methylprednisolone treated equine fetlock joints. B. Betamethasone treated equine fetlock joints. C. Triamcinolone treated equine fetlock joints.

Limitations of this study include the small number of biological replicates. Three biological replicates with paired comparisons were used for in vivo experiments in this study. Obviously, biological variation was observed for all of the treatment groups (Figure 24). By including more biological replicates for each treatment group, more statistical power would be achieved. Another limitation is that the cytotoxicity of corticosteroids on chondrocyte was tested in healthy equine fetlock joints rather than inflamed joints that would be selected for treatment in clinical situations. Effusion and changes in drug pharmokinetics in inflamed joints...
might alter the results. Further studies, therefore, may need to be conducted to evaluate the cytotoxicity of corticosteroid on inflamed chondrocytes.

Taken together, this study clearly shows a decrease in chondrocyte viability after exposure to corticosteroid treatments both in vitro and in vivo. The critical finding from this study is that the manufacture’s recommended dose of methylprednisolone, betamethasone, and triamcinolone can cause cell death and are cytotoxic to chondrocytes. As such, the data support using the lowest concentration of corticosteroids possible to achieve desired clinical goals.

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Chapter V Conclusion

I. Reflections

Research efforts in this dissertation thesis were aimed at examining important questions related to the chondrocyte cytotoxicity and anti-inflammatory efficacy of glucocorticoids used in the treatment of equine synovial joint inflammation. The primary hypothesis of this work was that manufacture's recommended dose of glucocorticoids currently used in equine clinical practice exceed the amount necessary to achieve maximal anti-inflammatory efficacy. In addition, these levels are cytotoxic to articular chondrocytes. To test this hypothesis, experiments were designed to characterize changes in steady state mRNA for targeted gene transcripts in chondrocytes with glucocorticoid treatments and to evaluate drug-induced cytotoxicity both in vitro and in vivo. Within this dissertation results have been reported for experiments that have been performed to meet the following two objectives: 1) compare the dose-dependent anti-inflammatory profiles of MPS, BPD, and TA in synoviocytes and chondrocytes, 2) evaluate glucocorticoid-induced chondrocyte cytotoxicity in chondrocytes, articular cartilage explants and equine fetlock joints. By focusing on these objectives, the goal is to generate scientific data to provide a reference for administrating intra-articular glucocorticoid injections in equine practice.

Intra-articular glucocorticoid injections are commonly used to treat synovitis and OA in horses. Since side effects of glucocorticoid on articular cartilage and underlying bone have been reported, detrimental side effects remain a major concern among the equine practitioners. These side effects are drug- and dose-
dependent, so research is needed to define whether the manufacture’s recommended dose of glucocorticoids are above a therapeutic optimum in terms of relative anti-inflammatory efficacy and chondrocyte cytotoxicity. We described the first study to examine the three glucocorticoids (MPS, BPD, and TA) induced equine articular chondrocytes cytotoxicity both in vitro and in vivo. Live/Dead assay was used to evaluate the chondrocyte cytotoxicity. The results from in vitro and in vivo studies were consistent. Dose-dependent effects of glucocorticoids on chondrocyte cell death were observed in chondrocyte monolayer culture and cartilage explants. With the increase of concentration of glucocorticoids at ranges commonly used in equine practice, the percentage of chondrocyte cell death increased. This study supported the hypothesis that manufacture recommended dosage of MPS, BPD, and TA can cause chondrocyte cell death. According to the results from our study testing the anti-inflammatory efficacy of glucocorticoids, glucocorticoids were able to maintain the full functional efficacy at the low concentration levels. Since the survival of articular chondrocytes is essential to maintain cartilage function, the data support using the lowest concentration of corticosteroids possible to achieve desired clinical goals.

II. Looking ahead to future studies

Follow-up studies to those described in the dissertation should focus on two areas: 1) determination of effects of glucocorticoids on the expression of biomarkers for articular chondrocytes. 2) evaluation of effects of dissociative glucocorticoids on pro-inflammatory gene and type II collagen gene expression in articular chondrocytes.
Effects of glucocorticoids on the expression of biomarkers for articular chondrocyte

Dose-dependent effects of glucocorticoids on the steady-state mRNA level of type II collagen in chondrocytes were tested. It has shown that glucocorticoid treatments suppressed the gene expression of type II collagen, however, significant differences were not detected (P<0.05).

From what has been observed, further research on chondrocyte cell biology is needed. Since both detrimental (Dahlberg et al., 2000; Dechant et al., 2003; Sandler et al., 2004) and chondroprotective (Pelletier et al., 1989; Williams et al., 1985) roles of glucocorticoids for cartilage have been reported, it would be interesting to detect the effects of glucocorticoids on articular cartilage by measuring newly synthesized mRNA of cartilage biomarkers. Type II collagen is the principle collagen of articular cartilage and plays an essential role in maintaining cartilage architecture and biomechanical performance. Aggrecan is the major proteoglycan in the articular cartilage and interacts with hyaluronan and link protein to endow the cartilage with load-bearing properties. Link protein is also essential for normal biomechanical function of articular cartilage. It can attach the aggrecan to hyaluronan thus forming the supermolecular aggregate. In the light of their important role in articular cartilage, it is important to understand how glucocorticoids affect transcription of cartilage biomarkers, such as with nuclear run-on assay.

Nuclear run-on is used to measure the transcriptional activity of specific genes in intact nuclei (Bates et al., 2002; Laufs et al., 1999). It provides information on the synthesis of a specific gene, other than a change in mRNA degradation, transport
from the nucleus to the cytoplasm, or steady state mRNA (Figure 26). The same drug treatment model in this study, therefore, can be used to analyze effects of three glucocorticoids on newly synthesized mRNA of type II collagen, aggregan core protein and link protein. The first step of the nuclear run-on assay is the isolation of intact nuclei of treated cells. And then the nuclei are incubated with four ribonucleotide triphosphates including radiolabelled UTP. This allows the transcription of a labeled total mRNA over a defined time \textit{in vitro}. After that, the radiolabeled RNA is isolated and hybridized with appropriate DNA sequences immobilized on a membrane (Figure 26). The DNAs usually include the sequences of target genes and of an endogenous control gene as a standard. The amount of specifically hybridized RNA proportional to the standard and therefore reflects the transcriptional activity of genes in the intact cell.

Figure 26. Schematic diagram of the process of nuclear run-on.
Effects of dissociative glucocorticoids on pro-inflammatory gene and type II collagen gene expression in articular chondrocytes

One of the dissociative glucocorticoids, VBP1, has been tested in this study. VBP1 significantly inhibited the iNOS gene transcription and NO production. However, the suppression effects of VBP1 on several pro-inflammatory gene transcriptions in articular chondrocytes were not significant. There remain some questions to the mechanism of action of the VBP compounds on chondrocytes.

VBP1 is one of Lazaroid Δ-9,11 analogs and is a water-insoluble compound with poor solubility in organic solvent. It is also reported recently that VBP1 can bind to both glucocorticoid receptor and mineralocorticoid receptor with relatively high affinity (Reeves et al. 2013). Another dissociative glucocorticoid, VBP15 which is a lead compound of the Δ-9,11 steroids, has been reported to inhibit the production of NFκB-regulated pro-inflammatory transcripts in human macrophages (Dillingham et al. 2015), bronchial epithelial cells (Damsker et al. 2013), and skeletal muscle cells (Heier et al. 2013). VBP15 showed greater affinity for the glucocorticoid receptor and lower affinity for mineralocorticoid receptor than other Δ-9,11 steroids (Heier et al. 2013), indicating that VBP15 has the possibility to be a potential candidate for OA therapy. Although VBP15 is also a water-insoluble compound, it has better solubility than VBP1 and can be solved in 8% DMSO + 8% Ethanol + 50% PEG400 + 34% HP-β-CD (20% W/V) (Reeves et al. 2013), which makes VBP15 as a better candidate that can be tested than VBP1.
Since anti-inflammatory efficacy of VBP15 has been reported in several cell types, it would be interesting to address the effect of VBP15 on NF-κB-regulated pro-inflammatory transcripts in chondrocytes. Chondrocytes can be transfected with NF-κB luciferase reporter plasmids (Rockel et al. 2008, Feng et al. 2003). Cells expressing NF-κB luciferase reporter will be cultured for the luciferase assay. Drug treatment model in the published study (Baudy et al. 2009) will be adopted. Cells will be treated with VBP15 at the concentration level of $10^{-5}$ M for 24 hours. After that, TNF-α at the concentration level of 10ng/ml will be used to induce the NF-κB transcription and cells will be treated with TNF-α for 24 hours. Commercially available C2C12 cells that expressing NF-κB response elements coupled to luciferase in which VBP15 inhibit the NF-κB luciferase activity (Baudy et al. 2009) will be used as a positive control. Cells with only TNF-α treatment will be used as a negative control. Luciferase values will be normalized as a percentage of TNF-α treated only group. This study could be used to determine if VBP15 can inhibit the NF-κB transcription in chondrocytes and chondrocytes are refractory to VBP15.

Additionally, effects of VBP15 on pro-inflammatory and chondrocyte biomark genes transcription can be evaluated as well. To confirm effects of VBP15 on NF-κB target genes in chondrocytes, several inflammatory transcripts such as IL-1β, IL-6, Cox2, iNOS, MMP3, and MMP13 can be assayed by Real-time qPCR in VBP15 and MPS treated chondrocytes. One of the reported detrimental effects of glucocorticoids on articular cartilage is that glucocorticoids suppressed the transcription of type II collage. In order to evaluate the detrimental effects of VBP15 on articular cartilage, type II collage transcription can be analyzed in VBP15
treated chondrocytes. Inhibition effects of VBP15 on type II collage transcription in chondrocytes will be compared to those of MPS treated groups.

Glucocorticoids are important drugs in joint inflammation therapy. Although they have strong anti-inflammatory efficacy, several detrimental side effects are identified. Most of anti-inflammatory effects are mediated by transrepression, while many side effects are due to transactivation. Dissociative glucocorticoids, such as VBP15, can induce transrepression with little or no transactivation activity. Accordingly, dissociative glucocorticoids may be potential agents for joint inflammation therapy. This study may be able to provide molecular insight into the molecular mode of dissociative glucocorticoids activities in joint tissues.

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REFERENCES


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**Publications in Preparation**

**Abstracts**
