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Modulation of Inflammation and Oxidative Stress in Canine Chondrocytes

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Modulation of inflammation and oxidative stress in canine chondrocytes

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2012

Modulation of inflammation and oxidative stress in canine chondrocytes

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Pages in Study: 38

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Little research has focused on the involvement of oxidative stress as it relates to the pathophysiology of osteoarthritis (OA); while inflammation has been extensively studied. The present study evaluates the ability to modulate the response of canine chondrocytes to both inflammation and oxidative stress in an *in-vitro* model. Chondrocytes were incubated and then stimulated to under-go oxidative stress by using hydrogen peroxide or inflammation using interleukin-1 beta and tumor necrosis factor alpha. For inhibition of oxidative stress an antioxidant, N-acetyl-cysteine, was used prior to induction with hydrogen peroxide in a subset of chondrocytes. Measures of oxidative stress were superoxide dismutase and reduced glutathione. Prostaglandin E2 was used as a measurement of inflammation. Chondrocytes responded appropriately to both oxidative stress and inflammation. The antioxidant N-acetyl-cysteine provided adequate protection against oxidative stress. Oxidative stress and inflammation should be considered to play a role in the pathophysiology of canine OA.

DEDICATION

I would like to dedicate this research to my parents, Don and Vickie Dycus, as well as my wife and daughter Brooke and Kennedy Dycus for their faithful support of my pursuit of education.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Background

Diarthrodial joints of dogs are highly specialized organs that function to allow repetitive forces to be transmitted along the body in a pain free and frictionless environment (1). The anatomy of a normal diarthrodial joint is composed of a joint capsule, surrounding soft tissues, muscles, and tendons that contribute to the integrity and movement of the joint. Muscle mass further limits joint movement so that it does not exceed anatomical boundaries. Contained in the ligaments, tendons, and muscles are the mechanoreceptors that aid in providing proprioceptive input and fine motor control to prevent injury (2). Therefore, any disruption to the mechanoreceptors (i.e. trauma) will decrease fine motor control and predispose the joint to injury (3). The joint capsule is comprised of two layers, an outer fibrosis layer and an inner subsynovial layer both of which are vascular and innervated. The synovial lining covers every structure in the joint except the cartilage and menisci. Its function is to provide a low friction lining and produce synovial fluid. Synoviocytes make up the synovial lining. Type A synoviocytes are macrophage-like cells and function in phagocytosis. Type B synoviocytes are fibroblast-like and function to produce hyaluronan and enzymes to nourish the chondrocytes. Located throughout the synovium are cells that have immune function, and therefore the synovium contributes to the inflammation that occurs in joint disease (4).

Articular cartilage is a smooth compressible surface that transmits compressive forces across subchondral bone. It functions as a load-bearing cushion within the joint. Chondrocytes help to produce articular cartilage, specifically the extracellular matrix (ECM); they are the only metabolically active component of articular cartilage and have little capability to replicate. This is due to the lack of vascular, nervous, and lymphatic supply. It receives it nutrition from the synovial fluid via diffusion. It is composed of approximately 75-80% water, <5% chondrocytes, and an ECM.

The articular cartilage is composed of approximately 10% proteoglycan matrix, and 10% type II collagen fibers that make up the ECM. The proteoglycan matrix has a hyaluronic acid backbone. Aggrecan is a core protein with glycosaminoglycans attached (GAGS). The aggrecan attach to hyaluronic acid to form an aggrecan aggregate, which has a gel-like consistency with the ability to hold water. Aggrecan and water provide the compressive stiffness to the tissues whereas collagen provides tensile strength. The proteoglycan matrix is contained within a framework of mainly type II collagen. The collagen forms interlocking loops that provide added support to withstand mechanical forces (5, 6, 7).

Articular cartilage is divided into three zones (zones I to III), which is separated from the calcified cartilage (zone IV) by the tidemark. In adult animals, zone I has the highest cell density. In this zone the chondrocytes are small, flat, and oriented with the long axis parallel to the surface. Zone II is characterized by larger and more rounded cellular profiles while in zone III the chondrocytes are larger but arranged with their long axis approximately perpendicular to the surface (8). The organization of the collagen fibrils complements the cellular arrangement of articular cartilage. In the superficial zone

(zone I) the collagen fibrils are tangential to the articular surface. The middle zone (zone II) has fibrils arranged in an intricate three-dimensional network with many of the fibrils perpendicular to the surface and having branches that fan out. In the deep zone (zone III) and the calcified zone the fibrils are perpendicular and form a more rigid mesh. This organization allows each zone a specific function. In the superficial zone the layer forms a pre-stressed, wear-resistant protective area that can withstand tension in the plane of the articular surface. In contrast the fibrils in zones II and III are organized to provide increased resilience to compressive loading. The concentration of proteoglycan increases with increasing depth from the surface, which means that collagen fibrils are more concentrated at the surface (9).

Osteoarthritis

Osteoarthritis (OA) is a chronic progressive disease affecting diarthrodial joints. Estimates of 20% of middle aged and 90% of older aged dogs are affected by OA (10, 11). OA is a disease affecting the entire joint including: the articular cartilage, bone, and synovium. However, the relationship between the pathology of each of these tissues is poorly understood. Most attention has been focused on the articular cartilage in trying to understand the pathophysiology of OA. Undoubtedly biomechanics play an important role in disease initiation and progression in the articular cartilage, but biomechanical changes occur in all joint tissues. OA is characterized clinically by joint pain, limitation of movement, effusion, and variable degrees of inflammation. Biomechanically, a reduction in proteoglycan concentration, alterations in size of aggregation, increased

water content, collagen fibril disruption, and imbalance in the synthesis and degradation of matrix macromolecules characterize OA (12).

The etiology of OA is not clear. It is proposed that the onset can be the result of an abnormal stress on a normal joint (i.e. trauma, instability) that results from a single event or repetitive microtrauma, or normal stress on an abnormal joint (i.e. genetic (13), infection, inflammation) (1). Regardless of the cause, chondrocyte damage results in a vicious cycle of extracellular matrix destruction; which is the hallmark of OA. This degradation of the cartilage matrix results from an imbalance between chondrocyte catabolic and anabolic events (14, 15, 16).

Role of inflammation in OA

Inflammation of the synovial membrane (synovitis) leads to the production and release of inflammatory mediators from synoviocytes, which are mainly mononuclear cells. A well coordinated system of cytokines and chemokines acts as attractants when receptors have been activated. Chemokines are derived from chemotactic cytokines, which are small heparin binding proteins that were originally defined by their chemotactic activity. They are structurally related to cytokines, whose main function is to regulate cell trafficking (17, 18). They are secreted in response to signals such as proinflammatory cytokines where they have the important role of recruiting monocytes, neutrophils, and lymphocytes (17). Currently in the human literature more than 50 chemokines and 20 chemokine receptors have been identified (19). Chemokines are classified into four subfamilies based on the number and location of the cysteine residues at the N-terminus. The fours subfamilies are C-X-C, C-C, and C (20, 21). The C-C and

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C-X-C chemokines are further broken into 2 major subgroups. The C-X-C subgroup contains interleukin-8 (IL-8), which will be discussed in detail later. The C-C chemokine family comprises families of monocyte chemoattractant proteins (MCP-1, MCP-2, and MCP-3) as well as others. Chemokines exert their roles by binding to the corresponding G protein-linked cell surface receptors referred to as CXCR or CCR (18). In particular MCP-1 (also known as CCL2) is a potent chemotactic factor for monocytes. MCP-1 recruits monocytes into areas of high inflammation, but the exact signaling pathways still remain unclear. There is evidence to suggest the involvement of prostaglandin E2 (PGE_2) in the attraction of monocytes to the site of inflammation (22). It is produced by a variety of cell types either at the time of or after the induction by oxidative stress, inflammation (cytokines) growth factors (17), and tumor necrosis factor alpha (TNF-α), which have been shown to upregulate MCP-1 expression in sensory neurons (23-25). More recently MCP-1 expression has been found in chondrocytes (18). Seitz el al and Volin et al have demonstrated both *in-vivo* and *in-vitro* that synoviocytes from OA patients revealed production of IL-8, MCP-1, and macrophage inflammatory protein-1alpha (MIP-1α) (26, 27). Also, it was shown that chemokine receptors in human chondrocytes caused the induction of metalloproteinases (discussed later), which were found to be enhanced in OA chondrocytes (28). This particular study suggests a key role of the chemokine/cytokine receptor system in OA. Furthermore, Yuan et al found that MCP-1 and its receptor CCR2 are expressed in both OA and normal chondrocytes but the response to each was different with the stimulation of interleukin-1β (IL-1β) and TNF-α. This suggests that the signaling pathways between normal and OA chondrocytes might be different. They were also able to show that proteoglycan synthesis is inhibited by

chemokines (18). MCP-1 is one of the most studied chemokines and has been shown to be a potential intervention point for the treatment of multiple diseases. The idea is that once the signaling pathways of chemokines are more fully understood in OA then treatment can be aimed at blocking the binding of MCP-1 to its receptor site thus damping down the inflammatory response.

Inflammatory cells release a variety of inflammatory mediators such as prostaglandins, leukotrienes, metalloproteinases (14), proteases, free radicals, interleukins (17), tumor necrosis factor (17), and other cytokines. Of particular interest are the roles of interleukin-6, interleukin-8, and IL-1β. IL-6 is a pleiotrophic cytokine that has a wide range of roles from immunoregulation, mediation of acute-phase responses, and effects on bone metabolism (29). IL-6 has been suspected in the inflammatory response observed in patients with OA. This is further supported from studies showing that IL-6 knockout mice were resistant to inflammatory arthritis and had reduced levels of serum TNF-α (30). Elevated levels of IL-6 in both serum and synovial fluid of patients with OA seem to correlate with clinical and laboratory indices of disease activity (31). In multiple studies IL-6 is elevated in OA, which is associated with inflammation as well as cartilage and bone destruction (32, 33). Blocking IL-6 with its interactions with the IL-6 receptor (IL-6R) by using IL-6R antagonists reduces cartilage/bone destruction and invasion of cartilage by synovium in animal models and human patients (30, 34).

IL-8 is also produced in arthritic joints by activated synovial cells, and along with IL-6, is considered to be a potent catabolic factor in OA (29). IL-8 induces a massive accumulation of neutrophils, which produce neutrophil elastase. This leads to cartilage destruction. Furthermore, IL-8 injections have been shown to induce the expression of

IL-1β in the joint cavity (35). Several papers have reported that IL-1β is one of the most active catabolic cytokines in OA and plays a pivotal role in the pathogenesis of OA (36-39). As a result of Il-1β release, metalloproteinases such as collagenase-3 (MMP-13) and gelatinase A (MMP-2) are increased as well as production of nitric oxide (NO) which leads to collagen and chondrocyte destruction (15). It was discovered that the free radical, inducible nitric oxide (iNOS), expression in chondrocytes can be induced by a single stimulation of Il-1β or TNF-α while in other types of tissues multiple cytokines are required. The development of OA proinflammatory mediators such as IL-1β and TNF-α not only play a major role at inducing inflammation but also oxidative stress leading to the production of prostaglandins.

Prostaglandins are lipid mediators that have been shown to be locally increased in patients with OA (40). The origination of prostaglandins comes from the insult causing the release of arachidonic acid from the phospholipids of the chondrocytes. Once arachidonic acid is released, it causes the secretion cyclooxygenase and lipoxygenase. Lipoxygenase is responsible for causing the release of leukotrienes, while cyclooxygenase is responsible for the release of different prostaglandins such as PGE_2, which has catabolic effects on chondrocytes. These catabolic effects result in increased release of other inflammatory mediators such as metalloproteases (MMP's) and IL-1β (41). PGE_2 has also been shown to be involved in multiple conditions such as apoptosis, angiogenesis, structural changes, osteoclastic bone resorption (40), and symptoms of OA (42). It has been noted that cyclooxygenase production (especially COX-2) is up-regulated in both OA and rheumatoid arthritis, thus leading to increased levels of PGE_2 (41). Berenbaum et al discovered that TNF-α would induce cyclooxygenase expression

(especially COX-2) without concurrent release of IL-1β in rabbit chondrocytes; however, both TNF-α and IL-1β act synergistically to cause the release of PGE_2 (43).

Role of oxidative stress in OA

It is proposed that free radical are involved in the pathophysiology of OA. One theory is that oxidative stress and inflammation work synergistically together to cause extracellular matrix destruction. It was shown that MMP's and reactive oxygen species (ROS) are the two main mediators of matrix component degradation (41). However, more is known about inflammation while very little is known about the exact mechanisms oxidative stress plays on canine chondrocytes.

Oxidative stress can be defined as the imbalance of the oxidant-antioxidant ratio where the oxidant outweighs the antioxidant (44). This occurs by the free radicals indiscriminately reacting with molecular oxygen forming ROS (45). Excess ROS such as nitric oxide synthase (NOS), myeloperoxidase (MPO), nitrite, hydroxyl radical, superoxide anions, and hydrogen peroxide attack fatty acids, phospholipids, DNA, and RNA causing cell damage (41, 45). ROS causes damage to DNA by altering nucleic acids by base modification, double base lesions, and strand breaks (44, 45). Yudoh et al found that oxidative stress can induce chondrocyte telomere instability leading to chondrocyte senescence, and a decreased cellular replicative lifespan (46). Molecular oxygen is capable of accepting electrons becoming reduced to superoxide anion which functions as a strong oxidizing agent; but is not stable in aqueous solution and therefore can dismutate to hydrogen peroxide. Hydrogen peroxide is a weaker oxidizing agent than superoxide anion, and acts as an inducer of oxidative stress by interacting with metals such as

copper, Cu (I), and iron, Fe (I) which gives rise to reactive and toxic hydroxyl radicals through the Fenton reaction creating multiple free radicals and thus causing cellular destruction and/or apoptosis (45, 47-49). Multiple studies have proven that inflammation is a key secondary mediator involved with OA, however; only recently has the roles of oxidative stress been addressed in the development and progression of OA. It has been revealed that inflammatory mediators such as IL-1β can induce the production of ROS, along with macrophages, polymorphonuclear leukocytes (50), and chondrocytes (51) but the exact role ROS plays remains unclear. *In vitro* studies further showed that activation with either IL-1β or TNF-α increase iNOS expression in chondrocytes while in other types of tissues multiple cytokines are required (52).

As previously mentioned articular cartilage is an avascular tissue where its nutrients come from synovial fluid and the oxygen tension is only about 1-7% (52, 53). Several studies found that *in vivo* oxygen tension in cartilage is lower than 8%, therefore; chondrocytes have to be able to survive in a low oxygen environment (54, 55). Grimshaw et al found that oxygen tension below 1% will inhibit glucose uptake, lactate production and RNA synthesis, which indicate that some oxygen is needed for chondrocyte metabolic activity (56).

The ability of oxygen to modulate chondrocyte activity is still not fully understood, making it difficult to extrapolate data from other cell types regarding its oxidative response. For example, *in vitro* studies have evaluated the response of oxidative stress in the liver (57) which is a vascular organ receiving 70-80% of cardiac output and having an oxygen tension that is approximately 20-30%. An additional problem is that the self-healing ability of cartilage is severely limited due to its avascularity, lack of

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innervation and lymphatic circulation, as well as low cellularity (58). In certain

circumstances, oxygen may be transformed into ROS, which plays a role in cellular

functions such as cell proliferation, activation, and cell death. Low levels of ROS can act

as different secondary messengers in the expression of a wide variety of gene products;

however, high levels can lead to cell destruction and death (59, 60).

Management of OA: Pharmacologic and Non-Pharmacologic Treatment

Approaches

A major barrier in managing OA is the fact that it is not a single disease, but

rather a disease with different pathophysiological mechanisms that results in damage to

type II collagen (14). Multiple therapies have been developed to combat the

pathophysiology of OA as well as to relieve pain. Currently a multimodal approach to

help decrease inflammation and reduce pain with the use of NSAIDS, intra-articular

steroids, oral opioids, omega-3 fatty acids (61-63), polysulfated glycosaminoglycans

(64), dietary restriction (65), and chondroprotecants is utilized. Because OA is a chronic

and progressive disease new therapies are sought to help decrease the severity of clinical

signs. Research has not yet yielded information that identifies any therapeutic modalities

to combat oxidative stress in OA such as the use of antioxidants. In human *in vitro*

studies oxidative stress and inflammation are reported to work together in the induction

of extracellular matrix destruction. Previous studies demonstrated that MMPs and ROS

are the two main key participants in matrix component degradation (15).

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CHAPTER II

MODULATION OF INFLAMMATION AND OXIDATIVE STRESS IN CANINE

CHONDROCYTES

Objectives

The objectives of this study are to further investigate how oxidative stress

influences the pathophysiology of canine osteoarthritis and to evaluate if the same

inflammatory mediators will cause not only an inflammatory response but also an

oxidative stress response. Additionally, an insight into the ability of an antioxidant (N-

acetyl-cysteine) to counter-act the influence of oxidative stress was sought. Our

hypotheses are: (1) canine chondrocytes will respond to the oxidative stressor hydrogen

peroxide (H_2O_2), (2) canine chondrocytes will exhibit an oxidative stress and

inflammatory response to the inflammatory cocktail of interleukin-1 beta (IL-1β) and

tumor necrosis factor-alpha (TNF-α), and (3) the oxidative stress induction can be

inhibited with an antioxidant.

Materials and Methods

Cell Culture

Articular cartilage was harvested from canine stife joints and aseptically diced

into <5 mm pieces. The cartilage was digested with type II collagenase (110 U/ml)[a] for

12-18 hours at 37ºC in 5% CO_2. Chondrocytes were filtered through a wire mesh screen

to remove debris and rinsed four times with Hank's Balanced Salt Solution[b]. Cells were counted and assessed for viability using the Trypan-blue exclusion method, and propagated in monolayer culture until confluency in canine chondrocyte media[c].

Experimental Design

Chondrocytes (5×10^5) were seeded onto 6-well plates and maintained at 37°C with 5% CO_2 for 24 hrs. To assess induction of oxidative stress cells were incubated for 24 hours with: (1) canine chondrocyte media, (2) H_2O_2 at 100, 200, or 300 μM, or (3) a cytokine cocktail of IL-1β (10 ng/ml) and TNF-α (1 ng/ml). To determine if this induction could be inhibited, selected wells were pre-incubated for 24 hours with N-acetyl-cysteine (NAC) at 10 mM before exposure to H_2O_2. The effect of cytokines on chondrocyte inflammation was tested by incubating cells with control media alone or with IL-1β (10 ng/ml) and TNF-α (1 ng/ml) for 24 hours. Supernatants and lysates were frozen at -80ºC for further analysis of PGE_2 or measurement of SOD enzyme activity and GSH levels.

PGE_2 High Sensitivity Immunoassay

A commercially available PGE_2 immunoassay[d] kit was used to quantify secreted PGE_2 levels in the cellular supernatant, according to the manufacturer's instructions. A PGE_2 standard was run in parallel to the supernatant samples in parallel with triplicate samples. Optical density was measured immediately following incubation using the SpectraMAX 340 microplate reader[e] at 450 nm with wavelength correction set at 540 nm.

SOD Determination Assay

A commercial SOD kit[f] was used to quantify secreted SOD activity (% inhibition rate) in the cell lysate, according to the manufacturer's instructions. An SOD activity standard was run in parallel to the lysate samples using 20µL of each standard and samples were plated onto a 96 well plate[g] in triplicates. The plates were then incubated at 37°C for 20 minutes. Optical density was measured immediately using the SpectraMAX 340 microplate reader[e] at 450 nm.

GSH Assay

The GSH assay was modified from Prieto-Sagredo (1), et al using 0.1 M Sodium Phosphate-0.005 M EDTA Buffer: pH = 8, 0.1% w/v *o*-phthalaldehyde (OPT), and 200 µg/ml glutathione stock were all prepared according to authors description. GSH standards were run in parallel to the lysate samples. Optical density was measured immediately using the SpectraMAX Gemini XS microplate reader[e] set at excitation 350 nm and emission 420 nm.

Phenotype Analysis by Immunohistochemistry

Chondrocytes were plated on microscope slides and fixed with 10% v/v paraformaldehdye and immunostained as previously described (2,3). Briefly, slides were incubated with goat anti-type I collagen, anti-type II collagen, or anti-aggrecan antibodies[h]. The slides were next washed in phosphate buffered saline[i] three times and

incubated with FITC labeled anti-goat antibodies. Immunostaining was visualized using a Nikon Eclipse epifluorescent microscope TE200.

Statistical Analysis

Data is presented as the mean ± SD. Pair-wise multiple comparisons were carried out using one-way ANOVA, Tukey post-hoc using SigmaStat statistical software (Windows Version 3.11) where $p<0.05$ was considered statistically significant. The Sigma Stat program verified that our data is compatible with the assumptions of Normality- Gaussian distribution and homogenous variance.

Results

Characterization of canine chondrocyte phenotype

The majority of cultured canine chondrocytes immunostained for type II collagen. A few cells were shown to immunostain for both type II collagen and type I collagen while an insignificant number immunostained with type I collagen alone (Figure 1). All cells immunostained for aggrecan. This observation indicates that most of the chondrocytes studied maintained features of their original phenotype of articular cartilage in the joint.

The effect of oxidative stress on SOD expression in canine chondrocytes

The chondrocytes exposed to 100, 200, and 300 µM of H_2O_2 revealed depletion of SOD activity compared to the chondrocytes cultured in control media alone ($p<0.001$ for all 3 concentrations, Figure 2). Pre-treatment with 10mM NAC followed by incubation

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with 100, 200, or 300 μM H2O2 significantly increased SOD activity when compared to the activated chondrocytes alone (p<0.001, p=0.001, p=0.003, respectively, Figure 2). There was no significant difference in the SOD activity between the control media and the cells pre-treated with NAC followed by activated with any concentration of H_2O_2 (Figure 2).

The effect of oxidative stress on GSH levels in canine chondrocytes

Activation with 100, 200, or 300 μM of H_2O_2 decreased GSH levels; however, this was not significant when compared to the chondrocytes cultured in control media alone (Figure 3). Pre-treatment with 10mM NAC followed by activation with 100, 200, or 300 μM H_2O_2 significantly increased GSH levels when compared to the activated chondrocytes (p<0.001 for all three H_2O_2 concentrations, Figure 3) and control media (p<0.001 for all three H_2O_2 concentrations respectively, Figure 3).

The effect of cytokines on SOD activity in canine chondrocytes

Chondrocytes cultured with the control media alone showed higher levels of SOD activity compared to cytokine-activated chondrocytes (Figure 4). The chondrocytes activated with a cocktail of 10 ng/ml IL-1β and 1 ng/ml TNF-α revealed significantly decreased levels of SOD activity at 24 and 48 hour activation compared to the chondrocytes cultured in control media alone (p<0.001, and p=0.026, respectively, Figure 4). The response was time-dependent with a significantly greater decrease noted 48 hours post activation (Figure 4).
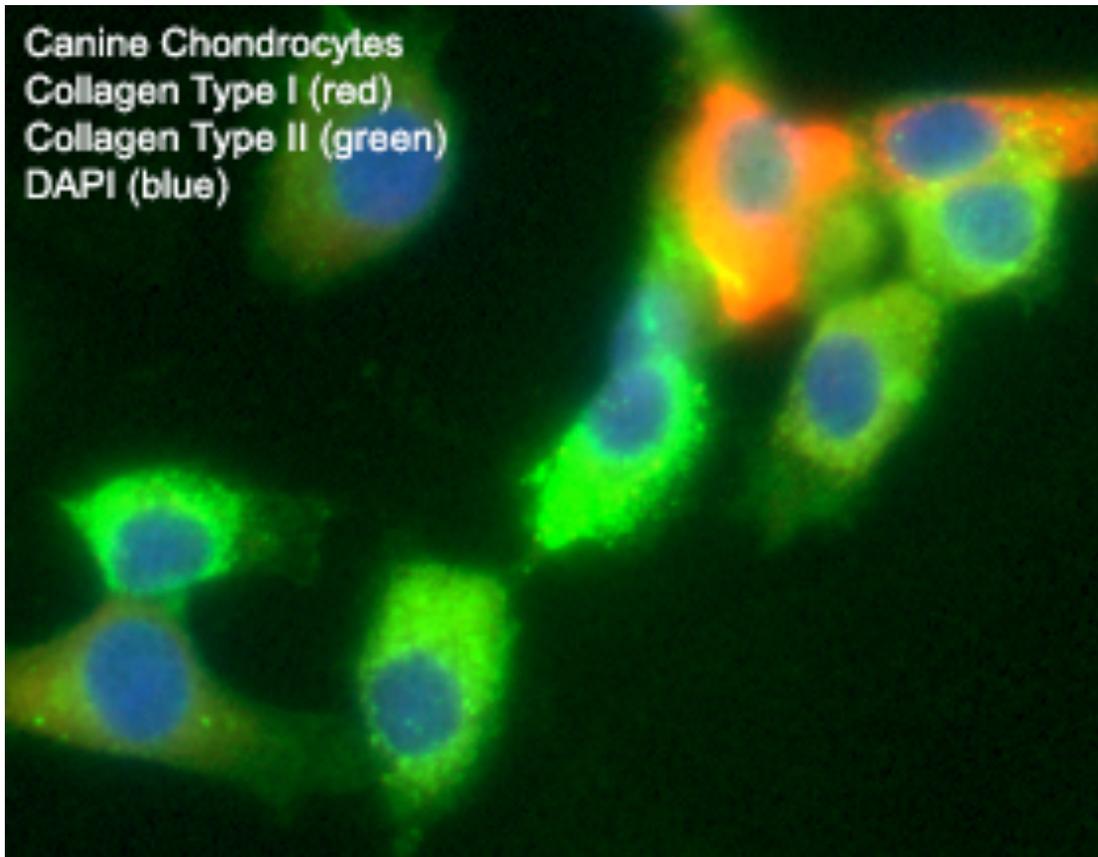
Figure 1

Immunostaining of canine chondrocytes

Immunostaining of canine chondrocytes showing few cells staining for collagen type I

(red), while the majority of cells stain for collagen type II (green)
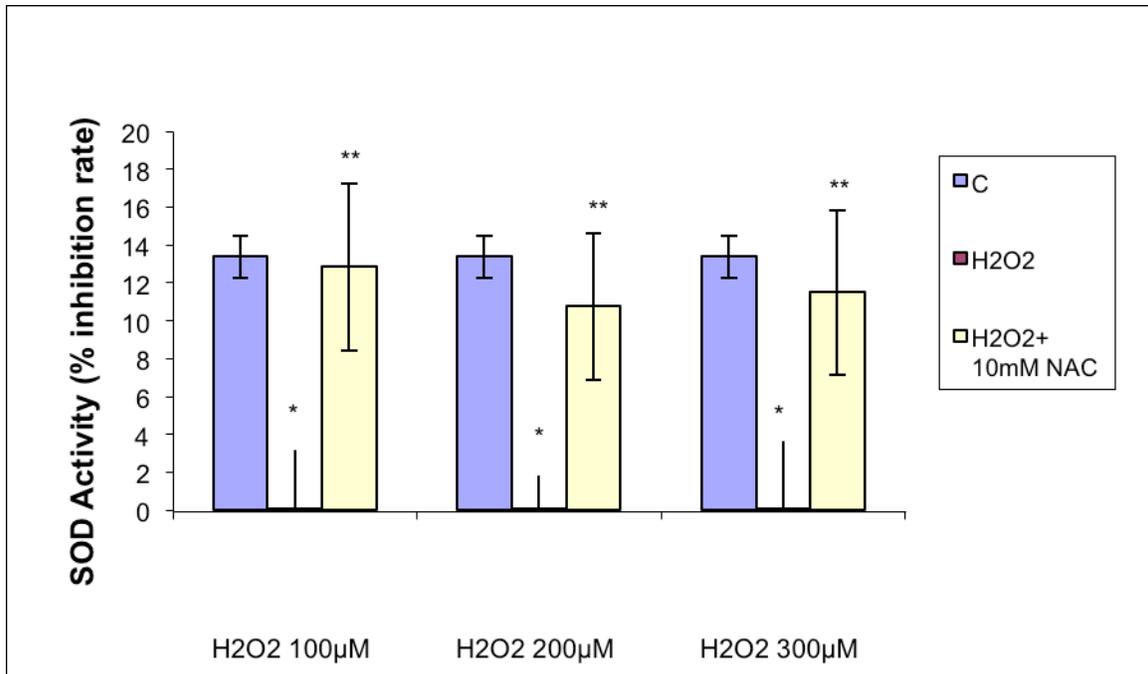
Figure 2

Effect of H_2O_2 on Chondrocyte SOD Activity

SOD activity in response to H_2O_2 concentrations (100, 200, 300 μM) and NAC (10 mM)

in canine chondrocytes lysates (mean $\pm$ SD). H_2O_2 completely depleted SOD activity.

*Mean SOD values were statistically significant when incubated with H_2O_2 ($p<0.001$ for

100, 200 and 300 μM H_2O_2) compared to control (C). **Treatment with NAC

significantly increased SOD values ($p<0.001$ for 100, 200 μM, $p=0.003$ for 300 μM

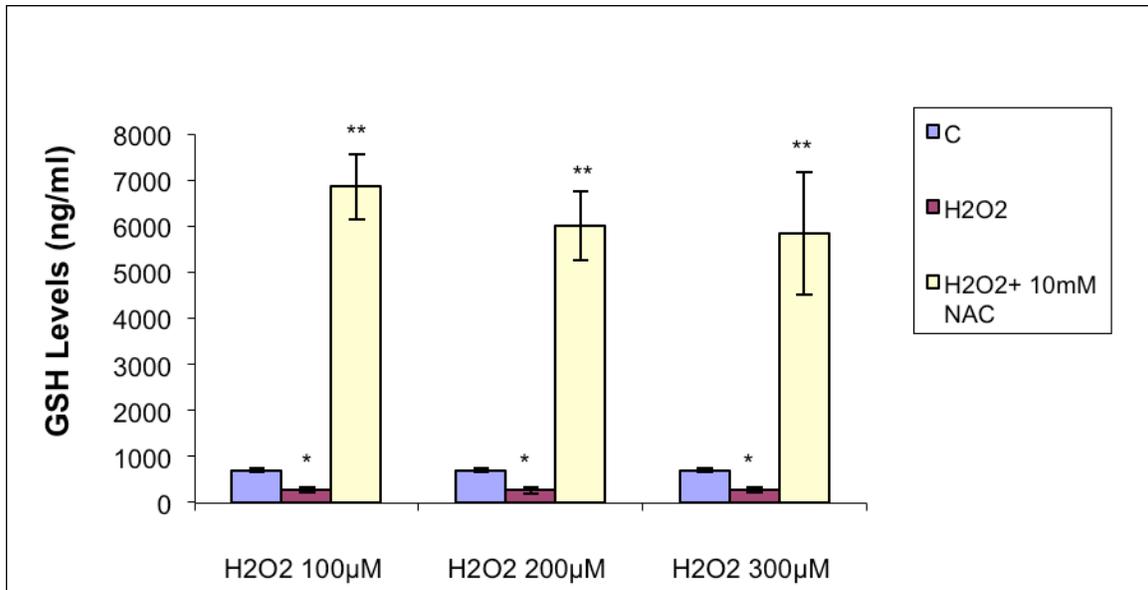H_2O_2) compared to cells activated with H_2O_2 alone

Figure 3

Effect of H_2O_2 on Chondrocyte GSH Levels

Intracellular GSH levels following exposure to H_2O_2 concentrations (100, 200, 300 μM)

and NAC (10 mM) (mean ± SD). *Mean GSH levels decreased when activated with all

concentrations of H_2O_2 compared to controls (C); however this response was not

significant. **Treatment with NAC significantly increased GSH values ($p<0.001$ for 100,

200, and 300 μM H_2O_2) compared to activated cells and compared to control ($p<0.001$
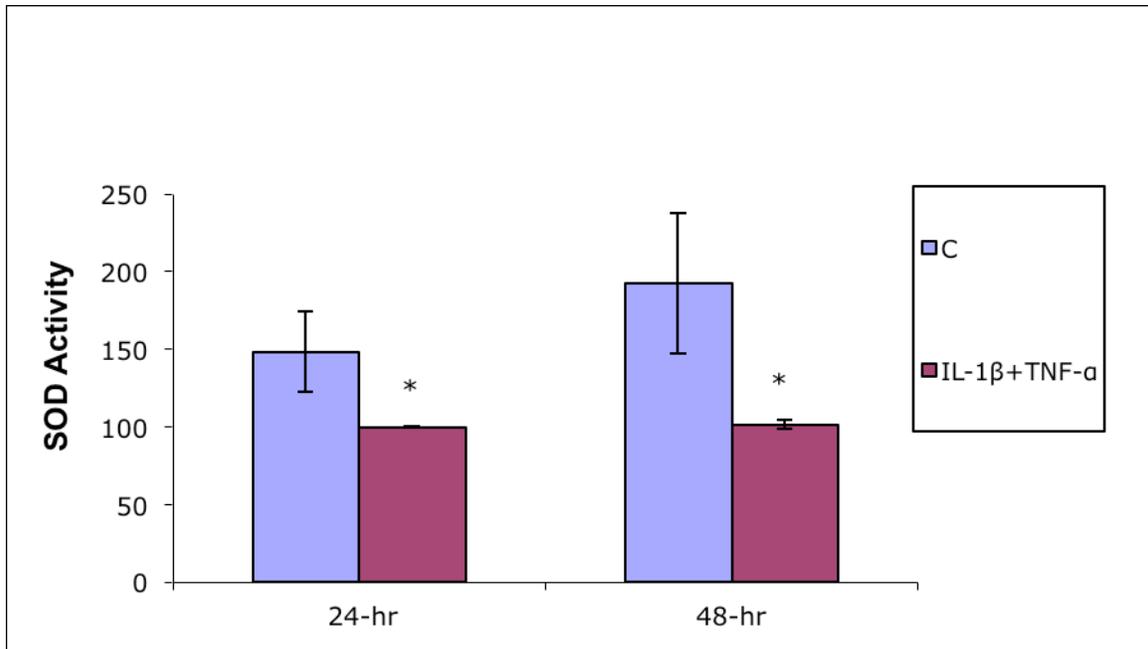
for 100, 200, and 300 μM H_2O_2)

Figure 4

Effect of Cytokine Induced Inflammation on Chondrocyte SOD Activity

SOD activity in canine chondrocyte cellular lysate (mean ± SD) following 24 and 48 hr

activation. Mean SOD activity levels are shown as percent of cytokine activated control.

*Mean values were statistically significant (p<=0.001 for 24 hr, p=0.026 for 48 hr)

compared to control values (C)

The effect of cytokine on PGE$_2$ production in canine chondrocytes

Cytokines IL-1β and TNF-α are well known pro-inflammatory mediators and

induced the production of PGE$_2$. Chondrocytes activated with a cocktail of 10 ng/ml IL-

1β and 1 ng/ml TNF-α for 24 and 48 hours exhibited significantly increased levels of

PGE$_2$ when compared to the chondrocytes cultured in control media alone (p<0.001 and

p=0.010 respectively, Figure 5). No statistically significant difference was noted between the chondrocytes at the 24 and 48-hour activation periods, but PGE_2 levels were slightly decreased at 48 hours (Figure 5). The PGE_2 production was expressed as a percentage of control due to the varying production levels amongst different sets. However, as a whole the trends were noted to be similar (Figures 6 and 7).

Discussion

The principal finding of the present study is that oxidative stress can be inducted with either hydrogen peroxide or cytokines in an *in vitro* canine chondrocyte culture model. Most of the chondrocyte cultures used in the present study immunostained intensely for type II collagen indicating that they maintained features of the cartilage phenotype (Figure 1). We then showed that H_2O_2 or cytokines induce an oxidative stress response as demonstrated by a decrease in intracellular SOD (Figures 2 and 4). Similar to earlier findings our results also show that chondrocytes respond to cytokine activation, reflected by a marked increase in PGE_2 production (Figure 5). These findings are consistent with other published data in humans where oxidative stress has been implicated in the progression and development of OA (4-7).
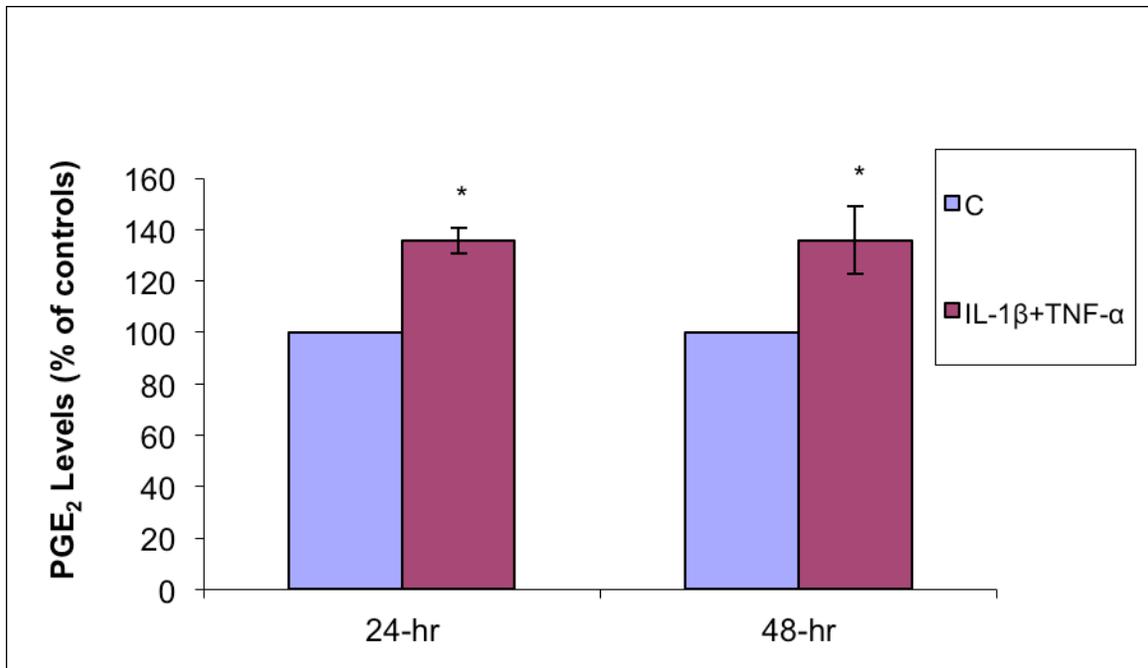
Figure 5

Effect of Cytokine Induced Inflammation on Chondrocyte PGE$_2$ Levels

PGE$_2$ levels (ng/ml) in canine chondrocyte supernatant (mean ± SD) following 24 and 48

hr cytokine activation expressed as percent of cytokine activated controls. Mean PGE$_2$

levels released into the cellular supernatant are shown as percent of activated control.

*Mean values were statistically significant (p<0.001 for 24 hr,

p<0.01 for 48 hr) compared to control values (C)

Figure 6

Effect of Cytokine Induced Inflammation on Chondrocyte PGE_2 Levels: 24 hr. activation

Representative PGE_2 levels in 3 separate runs to show differences in concentration

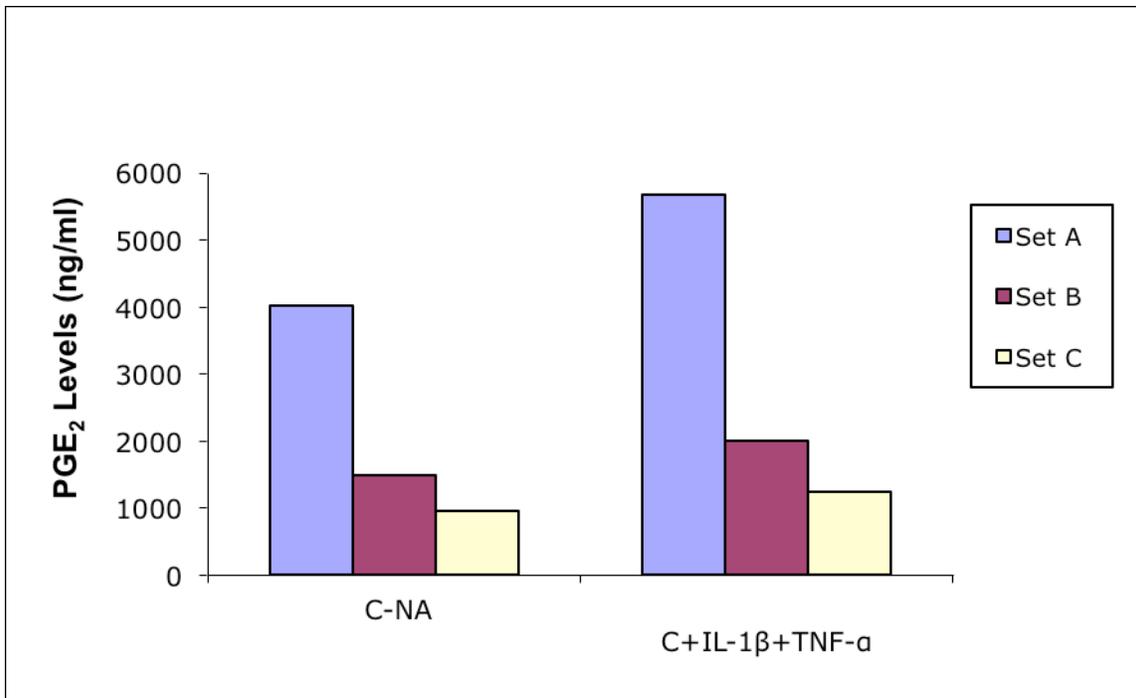between canine chondrocyte cell lines following 24 hr. cytokine activation

Figure 7

Effect of Cytokine Induced Inflammation on Chondrocyte PGE_2 Levels: 48 hr. activation

Representative PGE_2 levels in 3 separate runs to show differences in concentration

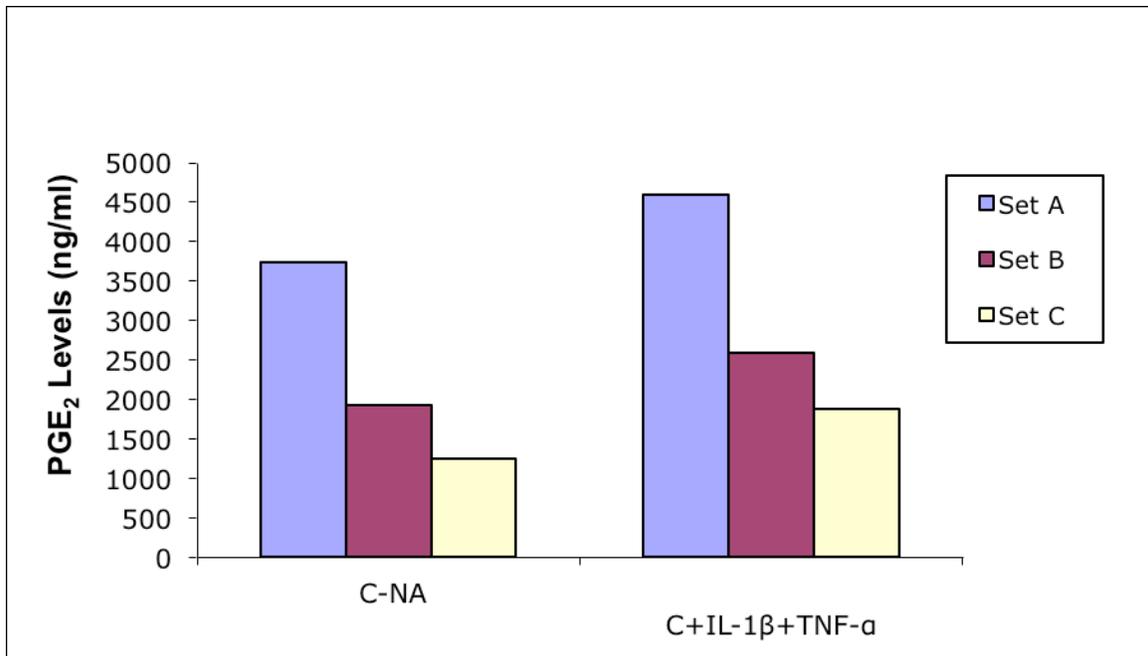between canine chondrocyte cell lines following 48 hr. cytokine activation

The observations in the present study identified mediators known to be involved

in the development of OA. Proinflammatory mediators such as IL-1β and TNF-α play a

major role at inducing both inflammation and oxidative stress leading to the production

of prostaglandins, particularly PGE_2. Production of this lipid mediator has been shown to

be increased in the joints of patients with OA (8). PGE_2 has catabolic effects on

chondrocytes, which result in increased release of other inflammatory mediators such as

MMPs and IL-1β (9). PGE_2 has also been shown to be involved in multiple conditions

such as apoptosis, angiogensis, structural changes, and osteoclastic bone resorption (8),

which are displayed in OA (10). Cyclooxygenase-2, the key enzyme that controls PGE2

production is upregulated by TNF-α (11). IL-1β and TNF-α induced inflammation in our chondrocyte cultures in agreement with other studies in showing that PGE$_2$ levels significantly increase with activation (Figure 5) (8, 12, 13).

The present study showed that SOD levels can be induced using three different concentrations of H$_2$O$_2$ (Figure 2). Depleted stores of SOD secondary to increased levels of ROS may help explain this observation. Overtime SOD could possibility be exhausted, thereby removing one of the first lines of defense against ROS, which may lead to cellular damage and apoptosis. The role of ROS and SOD has been implicated in clinical studies such as those of Scott et al who reported diminished SOD levels during end stage OA in human cartilage (14).

To prevent toxicity/cell death by ROS, chondrocytes are thought to have a well-coordinated antioxidant enzyme system consisting of SOD, catalase, and GPX. SOD transforms superoxide anion into H$_2$O$_2$ and oxygen. SOD plays an important role in managing ROS but is also very limited because H$_2$O$_2$ can still cause oxidative stress if other detoxification enzymes (i.e. GPX, catalase) are ineffective or depleted (15). We chose to use SOD as a biomarker of oxidative stress because it is the most abundant antioxidant available both intracellularly and extracellularly. During inflammation and/or oxidative stress, oxidative defense genes (i.e. SOD) are reported to be down-regulated in multiple tissues including astrocytes and chondrocytes (6, 16, 17). SOD has been discovered in three isoforms in humans. SOD1 (Cu/Zn-SOD) is found in the cytoplasm, nucleus, and intermembrane space of mitochondria. In the mouse model SOD1 mutations are associated with increased apoptosis and oxidative protein damage, and play a central role in cell survival (18). Overproduction of the inflammatory marker, TNF-α, has been

associated with increased oxidative stress consistent with decreases in SOD1 expression (19). TNF-α suppresses SOD1 production may explain the generation of superoxide anion. This also suggests its involvement in inflammatory and oxidative stress conditions as was shown in this study (18). SOD2 is found in the mitochondria and has been shown to provide a pivotal role against ROS generated by hyperoxia. Inflammatory cytokines such as IL-1β and TNF-α are well known activators of SOD2, which also contains sites that bind to NF-κB (20). ROS may induce transcription factors binding activity and can then act as secondary messengers of cytokines (21). SOD3 (EC-SOD) is found in the extracellular space; both isoforms (SOD1 and SOD3) use copper and zinc as cofactors. SOD3 has a strong affinity for proteoglycans in the ECM. Its main function is to protect cells and the ECM against the effects of superoxide anion (18). SOD2 has been shown to be down-regulated in OA while SOD3 is decreased in human OA and a mouse model of OA (16, 22, 23). Recently Scott et al found that all three SOD family members (SOD1, SOD2, and SOD3) were down regulated in diseased cartilage. It was shown in Hartley guinea pigs that down-regulation of SOD2 precedes the development of OA lesions raising the suspicion that SOD2 expression may be associated with the earliest stages of OA (14). Our results are consistent with these reports showing decreased levels of SOD in states of increased oxidative stress and inflammation (Figures 2 and 4).

Although not statistically significant, our results indicate that GSH levels tend to decrease upon exposure to H_2O_2 (Figure 3). It may be possible that SOD levels are exhausted prior to glutathione being activated. However, little is known about GSH in response to oxidants in chondrocytes. It would be informative if future research could focus on both isoforms of glutathione (oxidized and reduced), and evaluate their

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expression in relation to acute and chronic oxidative stress in canine chondrocytes. Glutathione is one of the most abundant cellular antioxidants (24), it serves as a substrate for enzymes such as glutathione peroxidase which neutralizes H_2O_2 and other peroxides during oxidative stress (25). Glutathione has been shown to be an effective $ONOO^-$ scavenger (26). A portion of the glutathione system is glutathione reductase, and its two forms: GSH, and GSSG, which play a primary role in detoxifying H_2O_2 and other peroxides to water and oxygen (27). During oxidative stress, glutathione reductase regenerates GSH from GSSG using NADPH. This activity was reported to prevent GSSG from leaving the cell and creating a GSH depletion environment, which can favor apoptosis (25, 28).

Under known oxidative stressors and glutathione depletion, cell death was significantly increased. In conditions where GSH was abundant, the chondrocytes were protected from the same oxidative stressors (25). This protection could occur as increased levels of GSH serve as a substrate for the glutathione peroxidase pathway thus eliminating H_2O_2 and other toxic peroxides (29). Studies have shown that apoptotic cell death is found in chondrocytes in articular cartilage of both OA and rheumatoid arthritis patients (25). It was proposed that chondrocytes may have been deprived of GSH, and are thus more prone to oxidative stress. These cells may be unable to detoxify or scavenge different ROS (28). Reduced levels of GSH can have damping effects on the production of cartilage extracellular matrix components. Because of GSH depletion, dysregulated production of ROS could reduce the production of proteoglycan and hyaluronic acid (29,30).

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The present study also showed that NAC inhibited oxidative stress in our stressed chondrocyte cultures (Figures 2 and 3). This is reminiscent of Ueno's study where treatment with NAC not only restored GSH, but also increased the glutathione reserves (31). The exact mechanism for this observation is unknown, but may be due to rapid depletion and inability to rapidly regenerate SOD stores. NAC may help keep the SOD expression from becoming completely depleted. In contrast, GSH expression is not readily exhausted, and therefore the NAC not only maintains GSH levels to prevent apoptosis, but also gives a protective effect by increasing GSH stores. Another possible explanation is that NAC could serve as a substrate to help replenish or maintain GSH levels. Currently, only a few antioxidants have been explored for the management of oxidative stress in response to chondrocyte damage. NAC has been shown to protect temporo-mandibular chondrocytes from oxidative stress by fully restoring viability and function of chondrocytes and increasing glutathione reserves in the presence of H_2O_2 (31.)

A major limitation to our study is that using an *in vitro* model does not recapitulate every condition *in vivo*. However, this canine chondrocyte culture model provided a useful tool to show that oxidative stress can be induced with H_2O_2 or the combination of IL-1β and TNF-α.

Footnotes

[a] Gibco Invitrogen, Carlsbad, CA, USA

[b] ATCC; Manassas, VA, USA

[c] Cell Applications, Inc; San Diego, CA, USA

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CHAPTER III

CONCLUSION

The principal finding of the present study is that oxidative stress can be inducted with either hydrogen peroxide or cytokines in an *in vitro* canine chondrocyte culture model. Most of the chondrocyte cultures used in the present study immunostained intensely for type II collagen indicating that they maintained features of the cartilage phenotype. We then showed that H_2O_2 or cytokines induce an oxidative stress response as demonstrated by a decrease in intracellular SOD. Similar to earlier findings our results also show that chondrocytes respond to cytokine activation, reflected by a marked increase in PGE_2 production. It was also shown that NAC inhibited oxidative stress in our stressed chondrocyte cultures. This *in vitro* model facilitated the study of cellular events involved with oxidative stress and inflammation. This model may be used for additional research to evaluate agents that inhibit oxidative stress and/or inflammation with the goal being to improve clinical management of canine OA.