Modulation of inflammation and oxidative stress in canine chondrocytes

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Objective—To determine whether oxidative stress could be induced in canine chondrocytes in vitro.

Sample—Chondrocytes obtained from healthy adult mixed-breed dogs.

Procedures—Harvested chondrocytes were maintained at 37°C with 5% CO2 for 24 hours. To assess induction of oxidative stress, 2 stimuli were used: hydrogen peroxide and a combination of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). To determine the effect of hydrogen peroxide, a set of chondrocyte-seeded plates was incubated with control medium alone or hydrogen peroxide (100, 200, or 300 µM) for 24 hours. For inhibition of oxidative stress, cells were incubated for 24 hours with N-acetylcysteine (NAC; 10mM) before exposure to hydrogen peroxide. Another set of chondrocyte-seeded plates was incubated with control medium alone or with IL-1β (10 ng/mL) and TNF-α (1 ng/mL) for 24 hours. Supernatants were obtained for measurement of prostaglandin E2 production, and cell lysates were used for measurement of superoxide dismutase (SOD) activity and reduced-glutathione (GSH) concentration.

Results—Chondrocytes responded to the oxidative stressor hydrogen peroxide with a decrease in SOD activity and GSH concentration. Exposure to the antioxidant NAC caused an increase in SOD activity in hydrogen peroxide–stressed chondrocytes to a degree comparable with that in chondrocytes not exposed to hydrogen peroxide. Similarly, NAC exposure induced significant increases in GSH concentration. Activation with IL-1β and TNF-α also led to a decrease in SOD activity and increase in prostaglandin E2 production.

Conclusions and Clinical Relevance—Canine chondrocytes responded to the oxidative stress caused by exposure to hydrogen peroxide and cytokines. Exposure to oxidative stress inducers could result in perturbation of chondrocyte and cartilage homeostasis and could contribute to the pathophysiology of osteoarthritis. Use of antioxidants, on the other hand, may be helpful in the treatment of arthritic dogs. (Am J Vet Res 2013;74:983–989)
Oxide, by chondrocytes in vitro. Excessive generation of ROS such as nitric oxide, nitrite, hydroxyl radicals, superoxide anions, and hydrogen peroxide is believed to result in attack of fatty acids, phospholipids, DNA, and RNA, causing cell damage. These ROS cause damage to DNA by altering nucleic acids by base modifications, double base lesions, and strand breaks. Indeed, oxidative stress can induce chondrocyte telomere instability, leading to chondrocyte senescence and a shortened cellular replicative life span.

The ability of oxygen to modulate chondrocyte activity is not fully understood. As an avascular tissue, articular cartilage relies on the diffusion of oxygen from the surrounding synovial fluid, where the oxygen tension is only approximately 1% to 7%. Several studies have found that in vivo oxygen tension in cartilage tissue is < 8%. Thus, chondrocytes have to be able to survive in a low-oxygen environment. An oxygen tension of < 1% leads to the inhibition of glucose uptake, lactate production, and RNA synthesis, which indicates that some oxygen is needed for chondrocyte metabolic activity. An optimal oxygen concentration is believed to be needed for physiologic cellular function. Certain low concentrations of ROS can act as different secondary messengers in the expression of various gene products. However, high concentrations can lead to cell destruction and death.

Because osteoarthritis is a chronic and progressive disease, new treatments are sought to help decrease the severity of clinical signs. A multimodal approach is currently used to help decrease inflammation and reduce pain associated with the disease, involving NSAIDs, intra-articular corticosteroid injection, oral opioid administration, omega-3 fatty acids, polyunsaturated glycosaminoglycans, dietary restriction, and chondroprotectants. Research has yielded limited information regarding therapeutic modalities that specifically minimize oxidative stress in osteoarthrosis. For example, omega-3 fatty acids have some antioxidant qualities but are commonly recommended for their anti-inflammatory properties in managing osteoarthrosis.

In vitro research in humans has revealed that oxidative stress and inflammation work together in the induction of extracellular matrix destruction, with matrix metalloproteinases and ROS as the 2 key participants in matrix component degradation.

The objectives of the study reported here were to investigate in vitro the potential influence of oxidative stress in the development of osteoarthrosis in dogs, evaluate whether inflammatory mediators would cause an inflammatory and oxidative stress response in canine chondrocytes, and assess the ability of an antioxidant to counteract the influence of oxidative stress. We hypothesized that oxidative stress could be induced in canine chondrocytes by exposure to hydrogen peroxide or a cytokine combination of IL-1β and TNF-α and that this induction could be inhibited through exposure to the antioxidant NAC. We also hypothesized that an inflammatory response could be induced in the cells by exposure to the combination of IL-1β and TNF-α.

**Materials and Methods**

**Cell culture**—Cartilage specimens were obtained from the stifles of previously healthy adult mixed-breed dogs that had been euthanized for reasons unrelated to this study and aseptically diced into pieces < 5 mm in size. The diced cartilage was digested with type II collagenase (110 U/mL) for 12 to 18 hours at 37°C in 9% CO₂. Chondrocytes were filtered through a wire mesh screen to remove debris and rinsed 4 times with Hank balanced salt solution. Cells were counted and assessed for viability with the trypan-blue exclusion method and propagated in monolayer culture until approximately 80% confluency was attained in canine chondrocyte medium.

**Experimental design**—All experimental tests were conducted in triplicate. Chondrocytes (5 × 10⁵) were seeded onto 6-well plates and maintained at 37°C with 5% CO₂ for 24 hours prior to treatment. To assess induction of oxidative stress, cells were incubated for an additional 24 hours with canine chondrocyte medium and hydrogen peroxide (100, 200, or 300 µM) or a cytokine combination of IL-1β (10 ng/mL) and TNF-α (1 ng/mL). The concentration of hydrogen peroxide was determined after running dose titrations to identify the lowest concentration that would induce oxidative stress as measured by SOD and the highest concentration that would induce oxidative stress without killing the chondrocytes. To determine whether any induction achieved could be inhibited, selected wells were incubated for 24 hours with NAC at 10 mM before exposure to hydrogen peroxide. The effect of cytokines on chondrocyte inflammation was tested by incubating cells with control medium alone or with IL-1β (10 ng/mL) and TNF-α (1 ng/mL) for 24 or 48 hours. Supernatants and lysates were frozen at −80°C for further assessment of PGE₂ production and measurement of SOD enzyme activity and GSH concentration. All cellular lysates were collected with the use of a nonionic detergent.

**PGE₂ high-sensitivity immunoassay**—A commercially available PGE₂ immunoassay kit was used to quantify secreted PGE₂ concentrations in the cellular supernatant in accordance with the manufacturer’s instructions. A PGE₂ standard was assayed in parallel to each of the supernatant samples run in triplicate. Immediately following cell incubation, optical density was measured at 450 nm by use of a plate reader with wavelength correction set at 540 nm.

**SOD activity assay**—A commercial SOD kit was used to quantify the activity of secreted SOD (percentage inhibition rate) in the cell lysate, in accordance with the manufacturer’s instructions. An SOD activity standard was assayed in parallel to the lysate samples with 20 µL of each standard, and samples were plated onto a 96-well plate in triplicates. The plates were then incubated at 37°C for 20 minutes. Optical density at 450 nm was measured immediately with the plate reader.

**GSH assay**—The GSH assay technique used was a modification of the method of Prieto-Sagredo et al. Sodium phosphate (0.1 M)–EDTA (0.005 M) buffer (pH 8), o-phthalaldehyde (0.1% [wt/vol]), and glutathione stock (200 µg/mL) were all prepared as described. Reduced glutathione standards were analyzed in parallel to the lysate samples. Optical density was measured
immediately with a plate reader$^7$ set at an excitation of 350 nm and emission of 420 nm.

**Chondrocyte structure immunohistochemical analysis**—Chondrocytes were plated on microscope slides, fixed with 10% (vol/vol) paraformaldehyde, and immunostained as described elsewhere.$^{29,30}$ Briefly, slides were incubated with goat anti–type I collagen, anti–type II collagen, or anti-aggrecan antibody.$^1$ The slides were next washed 3 times with PBS solution$^4$ and incubated with fluorescein isothiocyanate–labeled anti-goat antibodies. Immunostaining of cells was detected with an epifluorescent microscope.$^1$

**Statistical analysis**—Data are summarized as mean ± SD. Pairwise multiple comparisons were performed among chondrocyte treatment groups by means of a 1-way ANOVA with Tukey post hoc testing with the aid of statistical software.$^6$ Data were confirmed to be compatible with the assumptions of Gaussian distribution and homogenous variance. Values of $P < 0.05$ were considered significant.

**Results**

**Canine chondrocyte phenotype**—More than 80% of cultured chondrocytes obtained from stifle joint cartilage specimens from canine cadavers had positive immunostaining results for type II collagen. Approximately 20% of the cells had positive results for both type II collagen and type I collagen, and a negligible number (< 1%) had positive results for type I collagen only (Figure 1). All cells had positive immunostaining results for aggrecan.

**Effect of oxidative stress on SOD expression**—The chondrocytes exposed to 100, 200, and 300 µM hydrogen peroxide had nearly complete depletion of SOD activity, compared with findings in chondrocytes cultured in control medium alone ($P < 0.001$ for all 3 concentrations; Figure 2). Exposure to all 3 concentrations of hydrogen peroxide yielded the same response. Chondrocyte treatment with 10mM NAC followed by incubation with 100, 200, or 300µM hydrogen peroxide significantly ($P < 0.001$, $P = 0.001$, and $P = 0.003$, respectively) resulted in an increase in SOD activity, compared with activity in nonpretreated, hydrogen peroxide–activated chondrocytes. No significant difference in the SOD activity was identified between the chondrocytes in the control medium and the cells pretreated with NAC followed by activation with any concentration of hydrogen peroxide.

**Effect of oxidative stress on GSH concentration**—Chondrocyte activation with hydrogen peroxide (100, 200, or 300µM) resulted in a decrease in GSH concentration; however, the decrease was not significant, compared with the GSH concentration of chondrocytes cultured in control medium alone (Figure 3). Treatment with 10mM NAC followed by activation with hydrogen peroxide (100, 200, or 300µM) significantly increased the GSH concentration, compared with hydrogen peroxide activation ($P < 0.001$ for all 3 hydrogen peroxide concentrations) and incubation in control medium alone ($P < 0.001$ for all 3 hydrogen peroxide concentrations).

![Figure 1](image1.png)  
**Figure 1**—Photomicrograph of immunostained chondrocytes from the stifle joint of a canine cadaver showing few cells with positive staining for collagen type I (red) and a preponderance of staining for collagen type II (green). Bar = 20 µm.

![Figure 2](image2.png)  
**Figure 2**—Mean ± SD SOD activity (percentage inhibition) in cellular lysates in response to exposure of canine chondrocytes (3 tests/treatment) to hydrogen peroxide (100, 200, and 300µM) with (yellow bars) or without (red bars) 10mM NAC or to culture medium alone (control treatment; blue bars). *Value is significantly ($P < 0.001$) different from that of the control treatment. †Value is significantly ($P ≤ 0.003$) higher than the corresponding value for hydrogen peroxide alone.

![Figure 3](image3.png)  
**Figure 3**—Mean ± SD intracellular GSH concentrations in response to exposure of canine chondrocytes (3 tests/treatment) to hydrogen peroxide (100, 200, and 300µM) with (yellow bars) or without (red bars) 10mM NAC or to culture medium alone (control treatment; blue bars). *Value is significantly ($P < 0.001$) different from that for hydrogen peroxide alone or control medium alone.
Effect of cytokines on SOD activity—Chondrocytes incubated in the control medium alone had higher SOD activity than did cytokine-activated chondrocytes (Figure 4). Chondrocytes activated with a combination of IL-1β (10 ng/mL) and TNF-α (1 ng/mL; red bars) or to culture medium alone (control treatment; blue bars) at 24 and 48 hours of incubation. *Indicated values are significantly different from control values at 24 hours ($P < 0.001$) and 48 hours ($P = 0.026$).

Effect of cytokines on PGE$_2$ production—Chondrocytes incubated in the control medium alone had higher PGE$_2$ production than did cytokine-activated chondrocytes (Figure 5). Chondrocytes activated with a combination of IL-1β (10 ng/mL) and TNF-α (1 ng/mL) had significantly lower SOD activity at 24 hours ($P < 0.001$) and 48 hours ($P = 0.026$) after activation, compared with that in the chondrocytes in control medium alone. The response was time dependent, with a significantly greater decrease identified 48 hours after chondrocyte activation.

Effect of cytokines on PGE$_2$ production—Chondrocytes activated with a combination of IL-1β (10 ng/mL) and TNF-α (1 ng/mL), which are both proinflammatory mediators, for 24 hours ($P < 0.001$) and 48 hours ($P = 0.010$) yielded significantly greater PGE$_2$ concentrations than did chondrocytes incubated in control medium alone (Figure 5). No significant difference was identified between the same lines or passages of chondrocytes at the 24- and 48-hour activation points, but PGE$_2$ concentrations were slightly lower at 48 hours. Although PGE$_2$ production was expressed as a percentage of PGE$_2$ production in control chondrocytes, production was found to vary among the various chondrocyte treatment or culture groups, and, as a whole, the changes within groups were similar (Figure 6).

**Discussion**

The principal finding of the present study was that oxidative stress could be induced in chondrocytes isolated from stifle joint cartilage specimens from cadavers of previously healthy dogs through incubation of the cells with hydrogen peroxide or cytokines. Most of the chondrocyte cultures used had intensely positive immunostaining results for type II collagen, indicating that the cells had maintained features typical of chondrocytes within healthy cartilage. We then showed that in vitro exposure of canine chondrocytes to hydrogen peroxide or cytokines could induce an oxidative stress response as demonstrated by a decrease in intracellular SOD and GSH activity. The chondrocytes also responded to cytokine activation, as reflected by a marked increase in PGE$_2$ production following cytokine exposure. These findings are consistent with published findings in humans, in whom oxidative stress has been implicated in the progression and development of osteoarthritis.

An effect was identified in the canine chondrocytes of mediators known to be involved in the development of osteoarthritis. The proinflammatory mediators IL-1β and TNF-α appeared to have an important influence on inflammation and oxidative stress in the chondrocyte
model, leading to the production of PGE₃. Production of PGE₃ is reportedly greater than typical in the joints of humans with osteoarthritis.³⁴ Prostaglandin E₃ also has catabolic effects through induction of degradative enzymes such as matrix metalloproteinases.³⁵ The finding that exposure of canine chondrocytes to IL-1β and TNF-α led to an increase in PGE₃ production is in agreement with findings in humans.³⁴,³⁶,³⁷

Chondrocytes are believed to have a well-coordinated antioxidant enzyme system consisting of SOD, catalase, and glutathione peroxidase, which helps prevent cytotoxic effects and cell death by ROS. We chose to use SOD activity as a biomarker of oxidative stress because SOD is the most abundant antioxidant available intracellularly and extracellularly. During inflammation or oxidative stress in humans, expression of oxidative defense genes such as that for SOD is reportedly downregulated in multiple tissues, including chondrocytes.³²,³⁸,³⁹

The present study showed that a decrease in chondrocyte SOD activity can occur with cell exposure to 3 concentrations of hydrogen peroxide. A decrease in physiologic SOD stores secondary to an increase in ROS exposure may help explain this observation. Over time, SOD activity could possibly become 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 investigated in clinical studies such as that of Scott et al,⁴⁰ who reported a decrease in cartilage SOD activity during end-stage osteoarthritis in humans. Those investigators found that production of all 3 members of the SOD family is downregulated in diseased cartilage.⁴¹ Our results are consistent with reports of a decrease in SOD activity in conditions of increased oxidative stress and inflammation.

Although the finding was not significant, GSH concentrations in chondrocytes appeared to decrease upon exposure to hydrogen peroxide (Figure 3). Little is known about GSH production in response to oxidant exposure by chondrocytes in dogs. Glutathione is an abundant cellular antioxidant,⁴² which serves as a substrate for enzymes such as glutathione peroxidase, which in turn neutralizes hydrogen peroxide and other peroxides during conditions of oxidative stress.⁴³ The glutathione system is comprised in part of glutathione reductase and its 2 forms (GSH and oxidized glutathione), which play a primary role in the detoxification of hydrogen peroxide and other peroxides to water and oxygen.⁴⁴ When oxidized glutathione leaves cells, it can reportedly create a GSH depletion environment, which can favor apoptosis.⁴⁵,⁴⁶

Carlo et al⁴⁷ revealed that cell death significantly increases in conditions of GSH depletion and oxidative stress. In conditions where GSH was abundant, the chondrocytes were protected from the same oxidative stressors.⁴⁸ This protection could occur because an increase in GSH production serves as a substrate for the glutathione peroxidase pathway, thus eliminating hydrogen peroxide and other toxic peroxides.⁴⁹ Apoptosis occurs in chondrocytes in articular cartilage of humans with osteoarthritis and rheumatoid arthritis,⁵⁰ and it has been proposed that chondrocytes in such patients may become deprived of GSH and thus become prone to oxidative stress. These cells may be unable to detoxify or scavenge various ROS.⁵¹ Because of GSH depletion, dysregulated production of ROS leads to a decrease in the production of proteoglycan and hyaluronic acid.⁵² Research is needed into both isoforms of glutathione (oxidized and reduced) and their expression in relation to short- and long-term oxidative stress in canine chondrocytes.

The present study also revealed that exposure of chondrocyte cultures to NAC inhibited oxidative stress, which is similar to findings in another study in which chondrocyte treatment with NAC not only restored GSH production but also increased the physiologic reserve of glutathione. The exact mechanism underlying this observation is unknown but may involve a rapid decrease in and inability to rapidly increase chondrocyte SOD activity. N-acetylcysteine may help prevent complete depletion of SOD. In contrast, physiologic GSH concentrations are not readily depleted; therefore, NAC not only maintains GSH concentrations to prevent apoptosis but also yields a protective effect by increasing GSH stores. Another possible explanation is that NAC could serve as a substrate to help replenish or maintain physiologic GSH concentrations. Only a few antioxidants have been explored for the management of oxidative stress in response to chondrocyte damage. N-acetylcysteine exposure can protect temporomandibular chondrocytes from oxidative stress by fully restoring viability and function of chondrocytes and increasing glutathione reserves in the presence of hydrogen peroxide.⁵³,⁵⁴

One limitation to the present study was that the chondrocytes were obtained from adult healthy mixed-breed dogs that had been euthanized for reasons unrelated to the study. Each joint appeared free of gross lesions, but the presence of microscopic lesions could not be ruled out. Another major limitation was the study's in vitro nature; however, use of the canine chondrocyte cultures showed that oxidative stress could be induced with hydrogen peroxide or the combination of IL-1β and TNF-α. The oxidative stress response appeared to be counteracted by chondrocyte exposure to NAC. The in vitro model used may therefore be useful for additional research to identify agents that inhibit oxidative stress or inflammation, with the ultimate goal of improving the clinical management of osteoarthritis in dogs.

a. Gibco Invitrogen, Carlsbad, Calif.
b. ATCC, Manassas, Va.
d. Triton-100X, Sigma-Aldrich Corp, St Louis, Mo.
e. R&D Systems Inc, Minneapolis, Minn.
f. SpectraMAX 340 microplate reader, Molecular Devices, Sunnyvale, Calif.
g. Sigma-Aldrich Corp, St Louis, Mo.
h. Corning Costar, Corning, NY.
i. SpectraMAX Gemini XS microplate reader, Molecular Devices Corp, Sunnyvale, Calif.
j. Southern Biotechnology Associates Inc, Birmingham, Ala.
k. PBS solution, Gibco Invitrogen, Carlsbad, Calif.
l. Eclipse epifluorescent microscope TE200, Nikon, Tokyo, Japan.
m. SigmaStat, version 3.11 for Windows, Systat Software Inc, Chicago, Ill.
References


