DISSERTATION

EFFECTS OF DEXAMETHASONE AND OXIDATIVE ENVIRONMENT ON CHONDROGENESIS OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

Submitted by

Suwimol Tangtrongsup

Department of Clinical Sciences

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Doctoral Committee:

Advisor: John Kisiday

David Frisbie
Laurie Goodrich
Matt Kipper

ABSTRACT

EFFECTS OF DEXAMETHASONE AND OXIDATIVE ENVIRONMENT ON CHONDROGENESIS OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

Bone marrow-derived mesenchymal stem cell (MSCs) have received extensive consideration for applications to musculoskeletal tissue engineering based on their ability to differentiate into multiple skeletal lineage. For cartilage, MSCs-based therapies evaluated in vivo and in clinical studies have shown that MSCs can produce repair tissue that integrates with native tissue; however, defects remain partially cover, and the neotissue can contain fibrocartilage or evidence of hypertrophy. It is anticipated that a greater understanding of conditions that support MSCs chondrogenesis will lead to better results in cartilage tissue engineering.

In chapter 1, fundamental aspects of MSCs including chondrogenesis and uses in tissue engineering are reviewed. Further, information regarding reactive oxygen species, and their involvement in the functioning of MSCs and chondrogenesis are presented.

In chapter 2, MSC chondrogenesis was explored as a function of exposure to dexamethasone, anti-inflammatory glucocorticoid. Dexamethasone is known to support MSC chondrogenesis in vitro, although the effects of dose and timing of exposure are not well understood. Therefore, this study investigated these variables using a laboratory model of MSC chondrogenesis. In vitro MSCs chondrogenesis is conventionally induced in the presence of 100 nM dexamethasone; however, our result suggested that 1 nM dexamethasone was sufficient to supported robust cartilage-like ECM accumulation. By evaluating temporal exposure of MSCs to dexamethasone, we determined that exposure to dexamethasone during the first two days of culture was not critical,

and that sustained exposure of at least a week appears to be necessary to maximize ECM accumulation.

In chapter 3, we studied the oxidative environment associated with chondrogenic culture of MSCs. In conventional serum-free chondrogenic medium we noted that the concentration of intracellular reactive oxygen species (ROS) increased with time in culture. Previously, serum-free culture has been associated with increased ROS. Consistent with these reports, we found that supplementing chondrogenic cultures of MSCs with 5% fetal bovine serum reduced levels of intracellular ROS. Further, serum-supplementation increased the accumulation of collagen, a major component of cartilage extracellular matrix. Similar results were obtained using adult equine serum, which is as important as xenogeneic materials may be problematic for clinical applications. In summary, this study identified changes in the oxidative environment during MSC chondrogenesis, and suggested that lowering ROS may be an effective approach to increase collagen accumulation.

In chapter 4, the extent to which reducing intracellular ROS can improve chondrogenesis was evaluated in a more precise fashion using antioxidants. To do so, we tested the effects of N-acetylcysteine (NAC), glutathione ethyl ester (GSHEE), or ammonium pyrrolidine dithiocarbamate (PDTC). First, we evaluate the effect of each antioxidant on intracellular ROS using DCFDA staining. We found that NAC and GSHEE were not effective in reducing intracellular ROS over time in our MSCs chondrogenic cultures. In contrast, PDTC decreased intracellular ROS and evidence of oxidative damage, while modestly increasing GAG accumulation. However, PDTC also moderately decreased the compressive stiffness of the MSC-seeded hydrogels. In summary, this study indicated that lowering ROS with specific antioxidants could enhance MSCs chondrogenesis, although loss of mechanical integrity is a major concern.

The research described in this dissertation add to the knowledge of MSC chondrogenesis and the influences of dexamethasone and oxidative environment. We established that conventional doses of dexamethasone are at least a 100-fold higher than is necessary to support MSC chondrogenesis, which may be used to design dexamethasone delivery strategies to support MSCs chondrogenesis in vivo. During chondrogenesis, lowering levels of ROS that are encountered with conventional serum-free culture leads to higher levels of extracellular matrix accumulation. This information can be used to design in vitro or in vivo approaches to modulate the oxidative environment for optimal MSC chondrogenesis.

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CHAPTER 1: INTRODUCTION

1.1 Mesenchymal stem cell

1.1.1 Source and general characteristic

Mesenchymal stem cell (MSCs) are multipotent stromal cells that possess the ability to differentiate into musculoskeletal lineage including chondrocytes, osteoblasts, and myocytes.[1] MSCs are considered a promising cell type for tissue engineering and cell-based therapies based on the ease of tissue collection and cell isolation from different tissue sources, the self-renewal capacity with retention of multipotency with expansion in culture[2] that provides high cell numbers with weeks of culture. MSCs have been shown to differentiate in vitro and in vivo, while long-term observation has shown the safety of MSCs transplantation with no malignant tumor formation in vivo.[3-5] MSCs exhibit immunomodulatory properties through the release of bioactive agents that can inhibit autoimmune responses and escape immunerecognition,[6,7] which supports the potential for allogeneic treatments.

MSCs were first isolated from bone marrow in 1968, and have been studied extensively in the subsequent decades.[8] However, MSCS can be collected and isolated from many sources throughout the body such as adipose tissue, muscle, bone, umbilical cord vein, placenta, periosteum, synovial membrane, dental pulp, and others.[9-12] Since there is no unique cell surface marker distinguishing MSC from other stem cells, the International Society of Cell Therapy recommends the following three criteria to define human MSC.[13]

1) MSC must be plastic-adherent under standard culture conditions;

2) MSC must express CD105, CD73 and CD90, and lack expression of other marker, including CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules;

3) MSC must differentiate to osteoblasts, adipocytes and chondroblasts under established *in vitro* conditions.

Although these criteria have been adopted to identify MSC isolated from other animal species, murine MSC are known to express a different set of markers. Further, as cells are expanded in culture, the surface molecules and their level of expression may change as well.

1.1.2 MSC culture and chondrogenesis

The tri-lineage differentiation potential of bone marrow-derived MSCs[1] is the alternative candidate for cell-based therapy with musculoskeletal damage or disease, especially for articular cartilage. Since articular cartilage is absent of blood vessels, nerves, and lymphatic system, it has a limited capacity for intrinsic healing and repair when damage. Therefore, the goal of using MSCs in cartilage tissue engineering is to transplant cells that synthesize a cartilage-like extracellular matrix rich in proteoglycan and type II collagen.

The use of MSCs for cartilage tissue engineering is supported by in vitro experiments to study the biology of MSC chondrogenesis. In 1998, Johnstone et.al. published a method to induce robust chondrogenic differentiation.[14] One major aspect of this model is that MSCs need to be seeded into 3-D culture, such as in pellet or scaffolds, to resemble the physiological structure of chondrocytes in cartilage tissue.[14-16] Another major aspect is that MSC chondrogenesis is conducted in serum-free medium, since the cells resulted in unstable pellet formation in serum-supplement medium.[14] To compensate for the lack of serum, ITS+ premix was used as a baseline supplement. MSCs also need certain reagent and cytokine to induce and support chondrogenesis. Transforming growth factor-β (TGF-β) is required to induce chondrogenesis and generate the deposition of proteoglycans and type II collagen,[16,17] Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline.[18] Dexamethasone

is a glucocorticoid that has been used to support MSC chondrogenesis in vitro, although the specific role of dexamethasone is not known. This unknown is addressed in Chapter 2.

1.1.3 Problem of MSCs chondrogenesis in tissue engineering

Even though MSCs have been extensively researched for cartilage tissue engineering, in vivo studies have not proven that MSCs are capable of regenerating stable hyaline cartilage. The fact that MSCs under chondrogenic lineage progression can differentiate into auricular, articular or fibro-chondrocyte indicate that current methods for inducing chondrogenesis may not be suitable for articular cartilage repair (Figure 1.1). Although the outcome of bone marrow-derived MSCs chondrogenesis is the expression of hyaline ECM, type II collagen and proteoglycan, the content proportion of those biomolecules is not comparable to the weight-bearing hyaline cartilage. leading to inferior of tensile strength and load-carrying capabilities.[19-22]

The phenotype of differentiated MSCs in vitro suggests another potential obstacle to achieve cartilage regeneration. While hyaline cartilage is composed of type II collagen, chondrogenic MSCs have been reported to express type I collagen as well.[23-25] Fibrocartilage exhibits inferior mechanical and biochemical characteristics compared to normal hyaline articular cartilage and may affect their mechanical properties in the long term. In vitro and in vivo studies have demonstrated the propensity of chondrogenic MSCs to make fibrocartilage.[26-28] Also, chondrogenic MSCs have been reported to express hypertrophy-associated molecules such as type X collagen, alkaline phosphatase, matrix metalloproteinases-13, VEGF and RUNX2 on RNA and protein levels.[29-31] These data suggest endochondral ossification, rather than hyaline cartilage, might be the default lineage intrinsic of bone marrow-derived MSCs.[32-34]

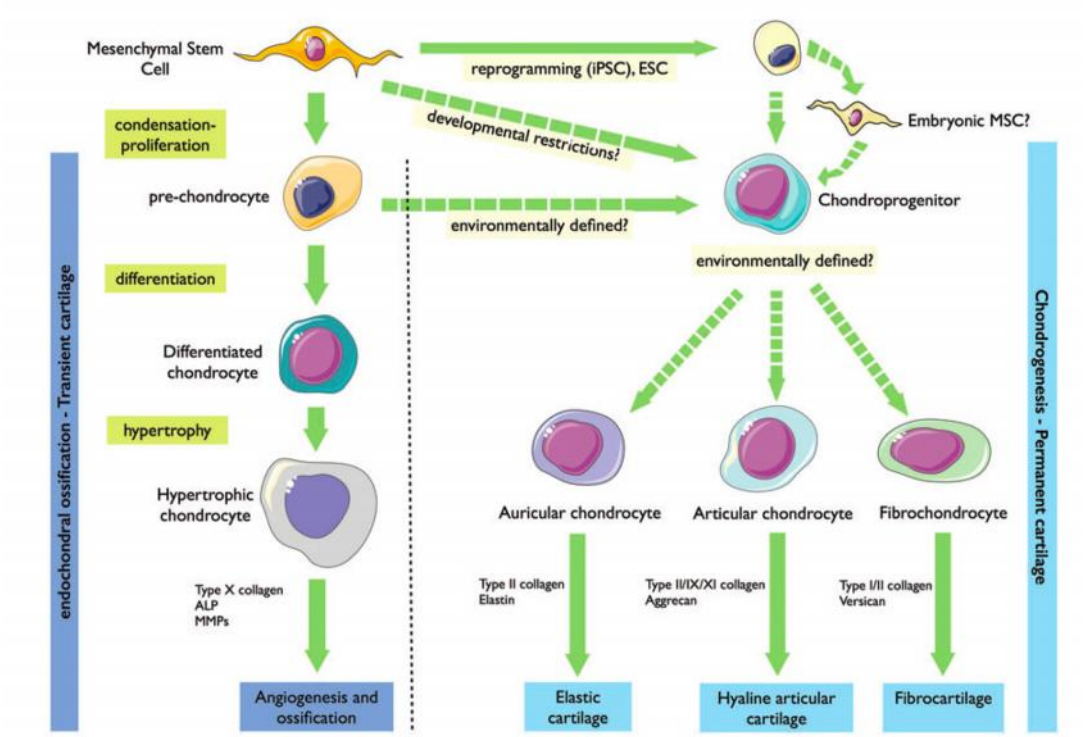

Figure 1.1 Chondrogenic lineage progression of mesenchymal stem cells (MSCs).[35] MSCs appear to be limited to follow an endochondral ossification program, which will end in vascular penetration, marrow deposition, and ossification of this cartilaginous tissue. Differentiation toward the stable hyaline cartilage phenotypes is theoretically restricted. However, it is possible that reproducing the complex environmental signals that are required for pathway switching has not been discovered. Bypassing these restrictions may be possible using reprogramming technology (induced pluripotent stem [iPS]) or embryonic stem cells (ESCs) as a new start state. Dashed arrows indicate possible routes, although evidence is lacking about the detailed differentiation pathway.

1.1.4 Current approaches to improving the chondrogenic potential of MSCs for cartilage tissue engineering

Several lines of research have focused on improving the chondrogenic potential of MSCs. Given that a preparation of MSCs contains significant heterogeneity among the cells, presorting cells with higher chondrogenic potential may be a useful for tissue engineering. For example, studies have demonstrated that $CD146^+$, $CD105^+$, and $CD29^+$ could serve as a sorting criterion to select cells with maximized chondrogenic potential.[36,37] Moreover, $CD271^+$ MSCs subpopulation

expressed highest level of type II collagen and aggrecan in chondrogenic induction over other MSCs subpopulation[38] and have greater chondrogenic potential both in vitro and in vivo[39]

A second approach has been coculture of MSCs with chondrocytes or cartilage explant as a source of bioactive factors such as growth factors. Coculture system provides the benefit of trophic effect and direct cell-to-cell contact that promotes MSCs chondrogenic differentiation.[40-43] Coculture has been reported to suppress hypertrophic markers such as type X collagen.[43-45]

Another way to improve MSCs chondrogenesis is through critical evaluation of the chondrogenic model to understand its biology and regulation. One aspect is redox status and reactive oxygen species in MSCs that has gained more attention during the past decade. Reactive oxygen species is critical to cartilage degradation,[46,47] and might associated in inhibition of cartilage repair in acute joint injury.[48] Therefore, understanding a role of reactive oxygen species on MSCs chondrogenesis will provide more insight and better strategy to manipulate MSCs in cartilage tissue engineering in vitro and in vivo.

1.2 Reactive oxygen species and antioxidant defense

1.2.1 Reactive oxygen species – production and function

Reactive oxygen species (ROS) are reactive molecules and free radicals derived from reduction of oxygen. ROS are categorized as radical containing one or more unpaired electrons in a single orbit, and non-radical derivatives of O_2. The production of oxygen-based radicals is an unavoidable consequence for all aerobic species. A major source of ROS within cells is mitochondria, which generated ROS as a by-product by the inevitable leakage of electrons from the electron transport activities during aerobic respiration. The electron leakage reacts with O_2 to form superoxide anion, a precursor of various types of ROS (Figure 1.2A). The dismutation of superoxide anion catalyzed by superoxide dismutases (SOD) generates hydrogen peroxide

(H_2O_2).[49] Superoxide anion can also combine with nitric oxide (NO) to form peroxynitrite,[50] another kind of pro-oxidant molecule that is classified as reactive nitrogen species. In Fenton's reaction, H_2O_2 can oxidize Fe^{2+} and form hydroxyl radical ($^{\cdot}OH$) and hydroxyl ion (OH^-).[51] Mainly, ROS are generated through complex I and III of electron transport chain in inner membrane of mitochondria (Figure 1.2B).[52,53] Another major source of ROS is from the membrane-bound NADPH oxidase (NOX) complex.[54,55] NOX consumes NADPH to generate superoxide anion and subsequently H_2O_2. While ROS are mainly generated from mitochondria and NOX complex, other cellular compartments such as endoplasmic reticulum or peroxisomes also take part in the production of pro-oxidant molecules.[56]

ROS are important in physiological and pathological processes in organisms. In immune cells, ROS participate in the defense mechanism against microorganism invasion by way of oxidative burst that kills the invading microorganism. Moreover, ROS function as important chemical mediator in the regulation of signal transduction, for which they are well-suited given that they are rapidly generated, highly diffusible, easily degraded and ubiquitously present in all cell types.[57] Physiological levels of ROS serve as a secondary messenger involving in processes of cell growth and differentiation in diversity of cell types,[58-60] including MSCs.[61-64]

The negative role of ROS involves damage of DNA or RNA,[65,66] oxidations of proteins,[67,68] lipids,[69,70] and cell apoptosis.[71-73] ROS also participates in variety of inflammation disease such as diabetes, atherosclerosis, neurodegenerative disease, and degenerative joint disease or osteoarthritis.[46,74]

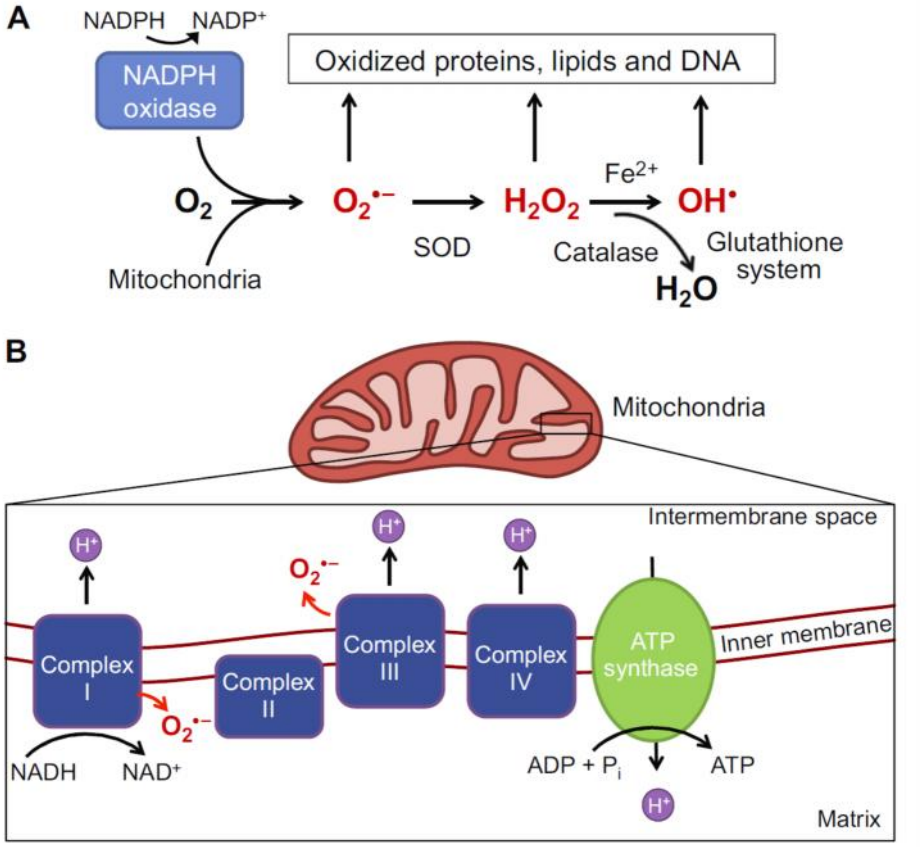

Figure 1.2 ROS generation and scavenging.[62] (A) Reactive oxygen species (ROS) include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\cdot}$) (shown in red). $O_2^{\cdot-}$ can be generated from complexes I and III (shown in B) or through the oxidation of NADPH by NADPH oxidases. Subsequent reduction to H_2O_2 is catalyzed by superoxide dismutase (SOD). H_2O_2 can be further reduced to water (H_2O) by catalase or can spontaneously oxidized iron (Fe^{2+}) to form the highly reactive $OH^{\cdot}$. Under conditions of oxidative stress, when ROS generation outpaces the ROS scavenging system, accumulating levels of ROS oxidize and damage various cellular components. (B) The electron transport chain complexes I-IV harness electrons from NADH in a series of redox reactions, which are coupled to pumping proton (H^+) into the mitochondria intermembrane space. The proton motive force, a combination of the membrane potential (charge) and the concentration gradient (pH), powers ATP synthase (complex V). Normally, O_2 acts as the final electron acceptor at complex IV, but aberrant reduction of O_2 can occur at complex I and III (red arrows), leading to the generation of $O_2^{\cdot-}$ (red).

1.2.2 Antioxidant defense

Living organisms are constantly producing ROS. Excessive ROS accumulation can lead to severely negative consequences such as oxidative stress, damage to biomolecules, and apoptosis. Therefore, cells possess a powerful antioxidant systems to scavenge ROS and regulate the redox homeostasis.

Antioxidant defense can be classified into 2 categories, enzymatic and non-enzymatic. Enzymatic antioxidants are a major component of antioxidant system that mobilize ROS into more stable and less reactive molecules. These important enzymes that play a role in cellular ROS defense include SOD, catalase, glutathione peroxidase and peroxiredoxin.

SOD catalyzes dismutation of superoxide anion to H_2O_2 and oxygen.[75] SOD are presented in almost all aerobic cells and in extracellular fluids. There are 3 isoforms of SOD in human and mammals. SOD1 is a homodimer contains copper and zinc (Cu/Zn-SOD) and located in the cytoplasm. SOD2 is a mitochondrial enzyme and exists as a tetramer containing manganese (Zn-SOD). SOD3 is a copper and zinc-containing tetramer and can be found in extracellular space.

Hydrogen peroxide, the product of dismutation of superoxide anion by SOD, can be further converted to water by several enzymes such as catalase, glutathione peroxidase and peroxiredoxins. Catalases are localized to peroxisomes in most eukaryotic cells, and use an iron or manganese cofactor to convert H_2O_2.[76] Glutathione peroxidase catalyzed H_2O_2 and other organic hydroperoxides by coupling to the oxidation of glutathione (GSH; reduced form) to glutathione disulfide (GSSG; oxidized form). To complete the cycle, GSH should be regenerated from GSSG by glutathione reductase using NADPH as the reducing agent. Glutathione peroxidase has several isozymes that vary in cellular location and substrate specificity, and some use selenium as a cofactor.[77,78] Glutathione peroxidase 1 is the most abundant, is found in cytoplasm, and is a very

efficient scavenger of H_2O_2. Glutathione peroxidase 4 is most active with lipid hydroperoxides, and can be found in nearly every mammalian cell. Peroxiredoxin in reduced form is another enzyme that plays a role in catalyzed H_2O_2, organic peroxides and peroxynitrite.[79] After H_2O_2 reduction, the oxidized form of peroxiredoxin is inactive and requires electron donation from thioredoxin to restore its catalytic activity.[80,81]

All cells contain non-enzymatic antioxidants including a variety of reducing substances such as glutathiones, thioredoxins, and vitamins C and E. They are efficiency scavengers that react with ROS to act as a redox buffer.

Glutathione is the most abundant non-protein thiol in mammalian cells. It plays a vital role in cellular defense against oxidative and nitrosative stress by its thiol group. Glutathione can also break the disulfide bridges formed within and between proteins by the action of oxidants. Glutathione is found in cells predominantly as reduced glutathione (GSH), which is a substrate of glutathione peroxidase,[82] with very low levels of oxidized glutathione (GSSG) present in cells under physiological conditions. Thioredoxin is also crucial antioxidant as it is used to recharge peroxiredoxin as mention above, and to oxidized cysteine residues and cleave disulfide bonds.[83] Glutathione and thioredoxin are very important from theirs abundance and versatility to counteract many kind of ROS such as H_2O_2, lipid hydroperoxides or xenobiotics by serving as a cofactor of ROS degrading.[84]

Vitamin C, or ascorbic acid, is a monosaccharide redox catalyst which can reduce ROS such as H_2O_2.[85] In cells, ascorbic acid is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins.[86,87] Although pathological doses of ascorbic acid may act as a pro-oxidant, physiological amounts have been demonstrated to be antioxidant even in the presence of metal ions.[88]

Vitamin E is a fat-soluble vitamin distributed in all cellular membranes, including mitochondria, and mainly prevents lipid peroxidation. The α-tocopherol form of vitamin E is thought to be the most important lipid-soluble antioxidant as it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction.[89,90] This removes the free radical intermediates and prevents the propagation reaction from continuing.

1.3 Reactive oxygen species in mesenchymal stem cells

1.3.1 ROS and MSCs stemness maintenance

After birth, adult stem cells continue to replenish and repair damaged or deteriorated tissues and organs through the generation of healthy, differentiated cells. An appropriate balance between self-renewal and differentiation is crucial for stem cell function during tissue homeostasis throughout life. Given that ROS works as a secondary mediator and may influence biological processes, ROS metabolism can be manipulated to generate stem cells and influence stem cell fate to improved differentiation and reprogramming.[62]

Bone marrow-derived mesenchymal stem cells are multipotent progenitor cells resided in a niche within the bone marrow, which maintained at low oxygen tension (2-8% oxygen).[91] Studies showed that hypoxia prolongs lifespan, increases proliferative capacity, and reduces differentiation of stem cell population, including MSCs.[91-93]

Within the bone marrow niche, undifferentiated quiescent MSCs have a high resistance to oxidative stress-induced death due to the low level of intracellular reactive species by effective ROS scavenging. In particular, high levels of total intracellular glutathione [94] catalyze the reactive species and correct oxidative damage of proteome and genome. Human MSCs constitutively express high levels of methionine sulfoxide reductase A, which is an enzyme crucial for the repair of oxidized proteins and for the recovery of methionine residues that act as scavengers of

oxidants.[95] In addition, undifferentiated MSC metabolism mainly relies on glycolysis instead of ROS-generating oxidative phosphorylation.[96] Such low levels of ROS are essential for maintaining self-renewal and stemness of MSCs to avoid senescence and apoptosis, while high levels of ROS effectively cease self-renewal and initiate stem cell differentiation.[97]

1.3.2 ROS and MSCS chondrogenesis

With differentiation, MSCs undergo a shift in redox status resulting in higher levels of ROS. Elevated ROS triggers signaling cascades that promote the proliferation, migration, survival and differentiation of stem cells.[62,98-100] With differentation, MSCs experience an increase in ROS production and a reduction in antioxidant defense. In general, mitochondrial biogenesis and metabolism shifts from glycolysis to oxidative phosphorylation [101,102]. For MSC chondrogenesis, increased concentrations of intracellular ROS[64] which coincide with decreases in protein and gene expression of the antioxidant enzyme superoxide dismutase-3,[103] and total glutathione capacity.[104] ROS produced by NOX-2 and NOX-4 promote cell survival during chondrogenesis, and enhanced expression of SOX-9, type II collagen and the accumulation of proteoglycans through AKT and ERK signaling [64]. Several aspects of the chondrogenic model presented in section 1.1.2 have been associated with increased levels of ROS. MSCs chondrogenesis is conducted in serum-free[105,106] and high glucose medium,[107,108] which can induce mitochondrial ROS generation. The presence of transforming growth factor-β (TGF-β) suppressed enzyme glutamate cysteine Ligase[109,110], which has been shown to lead to decreases in antioxidant glutathione (GSH) biosynthesis and increased levels of ROS. TGF-β has been shown to increase mitochondrial ROS production in lung epithelial cell line[111] and rat hepatocyte.[112] Taken together, it is clear that conventional methods for inducing MSC chondrogenesis in vitro have the potential to generate high levels of ROS. However, the

extent to which this model generates excessive ROS, leading to suboptimal differentiation as described in section 1.1.2, has not been evaluated.

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CHAPTER 2: EFFECTS OF DEXAMETHASONE CONCENTRATION AND TIMING OF EXPOSURE ON CHONDROGENESIS OF EQUINE BONE MARROW-DERIVED MESENCHYMAL STEM CELLS[1]

2.1 Summary

Objective. Dexamethasone is known to support mesenchymal stem cell (MSC) chondrogenesis, although the effects of dose and timing of exposure are not well understood. The objective of this study was to investigate these variables using a laboratory model of MSC chondrogenesis. Design. Equine MSCs were encapsulated in agarose and cultured in chondrogenic medium with 1 or 100 nM dexamethasone, or without dexamethasone, for 15 days. Samples were analyzed for extracellular matrix (ECM) accumulation, prostaglandin E2 and alkaline phosphatase secretion, and gene expression of selected collagens and catabolic enzymes. Timing of exposure was evaluated by ECM accumulation after dexamethasone was withdrawn over the first 6 days, or withheld for up to 3 or 6 days of culture. Results. Extracellular matrix accumulation was not significantly different between 1 and 100 nM dexamethasone, but was suppressed ~40% in dexamethasone-free cultures. Prostaglandin E2 secretion, and expression of catabolic enzymes including matrix metalloproteinase 13, and type X collagen was generally lowest in 100 nM dexamethasone and not significantly different between 1 nM and dexamethasone-free cultures. Dexamethasone could be withheld for at least 2 days without affecting ECM accumulation, while withdrawal studies suggested that dexamethasone supports ECM accumulation beyond day 6. Conclusion. One nM dexamethasone supported robust cartilage-like ECM accumulation despite

[1] Tangtrongsup S, Kisiday JD. Effect of Dexamethasone concentration and timing of exposure on chondrogenesis of bone marrow-derived mesenchymal stem cells. Cartilage. 2016 Jan;7(1):92-103. Doi: 10.1177/1947603515595263.

not having an effect on markers of inflammation, although higher concentrations of dexamethasone may be necessary to suppress undesirable hypertrophic differentiation. While early exposure to dexamethasone was not critical, sustained exposure of at least a week appears to be necessary to maximize ECM accumulation.

2.2 Introduction

The ability to undergo chondrogenesis is a hallmark of bone marrow mesenchymal stem cells (MSCs),[1] which has generated significant enthusiasm that they may be capable of repairing articular cartilage defects. Mesenchymal stem cells possess favorable properties for cartilage tissue engineering as they can be readily culture-expanded, and secrete robust quantities of cartilage-like extracellular matrix (ECM) after seeding into scaffolds and culture in the appropriate biochemical environment in vitro. Animal testing of MSC grafts has demonstrated promise for regenerating cartilage, although recent studies have shown that the cartilage repair can be improved if chondrogenic factors are delivered in MSC-seeded scaffolds.[2-4] These data suggest that supplementing the joint environment with chondrogenic factors may be an important aspect of successful cartilage repair by MSCs.

For many years the combination of selected growth factors and the glucocorticoid dexamethasone (Dex) has been used to induce robust MSC chondrogenesis in vitro. When translating these findings to animal studies, the delivery of growth factors has been prioritized, as chondrogenic growth factors are essential to stimulate MSC chondrogenesis in vitro.[5] However, laboratory studies have shown that Dex can significantly enhance growth factor-induced chondrogenesis by supporting ECM accumulation,[6-8] and suppressing catabolism.[8] Further, Dex has shown promise for supporting growth factor-mediated MSC chondrogenesis after

subcutaneous implantation.[9,10] These data suggest that in vivo delivery of Dex may significantly improve MSC cartilage repair.

Effective strategies for delivering chondrogenic factors to support MSC chondrogenesis in vivo should sustain at least a minimum concentration over a critical period of time. While in vitro studies have provided guidelines for dosing and temporal exposure of chondrogenic growth factors for MSCs,[11-14] similar information has not been established for Dex. Therefore, the objective of this study was to investigate the effects of dose and temporal exposure of Dex on MSC chondrogenesis in vitro. We evaluated chondrogenesis of adult equine bone marrow MSCs encapsulated in agarose hydrogel, a model scaffold for studying the biology of bone marrow MSC chondrogenesis in which withholding Dex has been shown to have a negative effect on chondrogenesis.[6,8] Chondrogenesis was evaluated using quantitative measures of ECM accumulation, histology, semi-quantitative gene expression of selected collagens, and alkaline phosphatase. In addition, given that Dex is a potent anti-inflammatory agent, we evaluated prostaglandin E2 (PGE2) secretion and gene expression of selected catabolic enzymes associated with cartilage degradation as an indicator of whether the effects of Dex on MSC chondrogenesis were associated with modulation of inflammation.

2.3 Methods

2.3.1 MSCs isolation and expansion

MSCs were isolated from bone marrow aspirates from the iliac crest of 2-5 year old horses that were euthanized for reasons unrelated to this study. Colony-forming cultures were established to isolate the MSCs from the bone marrow,[15] after which the MSCs were seeded at 2 x 10^3 cells/cm^2 in tissue culture flasks in α-minimal essential medium, 10% fetal bovine serum, and 2 ng/ml

fibroblast growth factor-basic (Peprotech, Rocky Hill, NJ) and cultured to 80% confluence over 4 days. The cells were expanded through a second passage prior to seeding in chondrogenic culture.

2.3.2 Agarose encapsulation and chondrogenic culture

Culture-expanded MSCs were encapsulated in 2% (w/v) agarose gel at $12x10^6$ cells/ml, as previously described.[15] Baseline chondrogenic medium consisted of high-glucose Dulbecco modified Eagle medium supplement with 1% ITS+ Premix (BD Biosciences, Bedford, MA), 37.5μg/ml ascorbate-2-phosphate (Wako Chemicals, Richmond,VA), 5 ng/ml recombinant human transforming growth fator-β1 (Peprotech, Rocky Hill, NJ).[5] Cultures were maintained in 1 or 100 nM Dex (Sigma-aldrich, Saint Louis, MO), or in Dex-free medium, for 15 or 21 days. Culture medium was changed every third day.

2.3.3 Quantification of extracellular matrix accumulation and DNA

Following chondrogenic culture, MSCs-seeded agarose samples were digested in proteinase K (Sigma-aldrich, Saint Louis, MO) in Tris HCl solution at 60°C overnight. DNA was quantified following digestion using the Hoechst dye assay.[16] Total accumulated sulfated glycosaminoglycan (GAG) and hydroxyproline were quantified by dimethylmethylene Blue[17] and dimethylamino benzaldehyde dye[18] binding assays, respectively. Extracellular matrix accumulation data were normalized to the sample wet weight or DNA.

2.3.4 Immunohistochemistry and histology

Samples from 15 days culture were fixed in 10% formalin for 48 hours, paraffin-embedded, sectioned, and mounted on slides. Sections were de-paraffinized and rehydrated prior to staining. Type II collagen Immunohistochemical staining: Samples were incubated with proteinase K (Sigma-Aldrich, Saint Louis, MO) at 37°C for 15 min, and then mouse anti-collagen type II IgG primary antibody using undiluted supernatant (Hybridoma Bank, Iowa City, IA) followed by

donkey anti-mouse IgG secondary antibody conjugated with peroxidase at a 1:500 dilution (Jackson Immunoresearch, West Grove, PA). Antibody detection was performed using VECTOR® NovaRED™ (Vector laboratories, Burlingame, CA). Additional sections were incubated with normal mouse serum at equal concentration to that of the primary antibody as a negative control. Equine cartilage was analyzed in parallel as a control. Toluidine blue: Sections were stained with 0.04% Toluidine blue solution (Electron microscopy sciences, Fort Washington, PA) to detect the accumulation of sulfated proteoglycans.

2.3.5 Prostaglandin E2 levels

Medium from chondrogenic cultures was collected on days 1, 3, 6, 9, 12, 15, 18 and 21, stored at -20°C, and then analyzed for PGE2 concentration using a commercially available enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY). Prostaglandin E2 secretion was normalized to the sample wet weight or DNA.

2.3.6 Alkaline phosphatase

Medium from chondrogenic cultures was evaluated for alkaline phosphatase activity. Media samples were incubated with SIGMAFAST® p-nitrophenyl phosphate substrate solution (Sigma-aldrich, Saint Louis, MO) at 37ºC for 30 min, diluted with 3N NaOH to stop the reaction, and then read spectrophotometrically at 405 nm. Standard curves were created using p-nitrophenol (p-NP, Sigma-aldrich, Saint Louis, MO). Using this protocol, absorbance values for medium samples coincided with 20-100 µM of the p-NP standards. Alkaline Phosphatase activity was normalized to sample wet weight or DNA.

2.3.7 RNA extraction and real-time PCR

MSCs-seeded agarose samples were collected on days 3, 6, 9 and 15 in TRIzol reagent® (Life Technologies, Grand Island, NY) and stored in -80°C. Samples were removed from TRIzol®,

frozen in liquid nitrogen, and pulverized. TRIzol® was first added to the pulverized samples, followed by chloroform at a ratio of 1:5 to the TRIzol®. Samples were centrifuged at 12,000g at 4°C for 15 minutes, and RNA was extracted from the aqueous phase using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with on-column genomic DNase (Qiagen, Valencia, CA). mRNA was reverse transcribed into cDNA using superscript® III first-strand synthesis superMix for qRT-PCR (Life Technologies, Grand Island, NY), and evaluated for type I, II, and X collagen, A disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS4 and 5) and matrix metalloproteinase 1 and 13 (MMP1 and 13) expression using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Forster City, CA). Relative gene expression levels were determined by semi-quantitative real time PCR using TaqMan-based probes and primers for all genes except type X collagen, which was analyzed using primers and Sybr Green (Table 2.1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene.

Table 2.1 Primer and probe sequences

Gene	Primers	Probe
Col1	F: ATTTCCGTGCCTGGCCCCATG R: GCCTTGGAAACCTTGGGGAC	TCCTTCTGGTCCTCGTGGTCTCCCTGG
Col2	F: AAACCATCAACGGTGGCTTCCA R: GCAATGCTGTTCTTGCAGTGGT	AGATGACAACCTGGCTCCCAACACTGCCAA
Col10	F: AGGCAACAGCATTACGACCCAAGA R: TGAAGCCTGATCCAGGTAGCCTTTG	-
ADAMTS4	F: TGTGATCGTGTCATTGGCTCC R: TGTTTGCTGCAGCTAGAACCATC	AGTTTGACAAGTGCATGGTGTGCGGT
ADAMTS5	F: AAGGTGACTGATGGGACCGAATGT R: TTTGAGCCAATGATGCCGTCACAG	AGGCCATACAGTAATTCCGTCTGCGT
MMP1	F: ACTGCCAAATGGACTTCAAGCTGC R: TCTTCACAGTGCTAGGAAAGCCG	CAGGATGTGCTCTACGGATACCCCAAGGAC
MMP13	F: TGATGAAACTTGGACAAGCAGTTCC R: CCTTGGAGTGGTCGAGACCTAAG	AGGCTACAACTTGTTTCTTGTCGCTGCACAC

2.3.8 Experimental design

The effects of Dex concentration on MSC chondrogenesis was evaluated by culturing samples in chondrogenic medium containing a maximum of 100 nM Dex,[5] which is the most commonly used concentration for supporting bone marrow MSC chondrogenesis. From 100 nM, we evaluated the effect of reducing the concentration of Dex by comparing to 1 nM Dex or Dex-free culture. Extracellular matrix accumulation was evaluated after 15 days of culture, while PGE2 secretion was quantified for up to 21 days of culture. The contribution of cyclooxygenase-2 (Cox-2) to PGE2 secretion and chondrogenesis was evaluated using celecoxib (Sigma-Aldrich, Saint Louis, MO), a nonsteroidal anti-inflammatory drug that selectively inhibits Cox-2.[19] Experiments evaluating the effects of timing of administration of Dex were performed using 1 or 100 nM Dex. The effects of Dex withdrawal were evaluated by removing Dex from the culture medium after 1, 3 or 6 days of a 15 day culture period. For the Dex withholding study, 1 nM Dex was added to the culture medium after 1, 2 or 3 days, or 100 nM Dex was added to the culture medium after 3, 4, 5 or 6 days of a 15 day culture period.

2.3.9 Statistical analysis

Data were analyzed for analysis of variance with mixed model using animal as a random effect. Pairwise comparisons were analyzed using least squares means with Tukey-Kramer adjustment. P-value < 0.05 considered statistically significant difference. Statistical test were performed using SAS 9.3 software.

2.4 Results

2.4.1 Preliminary studies

Two experiments were conducted with a small sample size and limited analysis to serve as a basis for designing the primary experiments in this study. First, we evaluated the effect of decreasing 100 nM Dex over several orders of magnitude. When normalized to wet weight, GAG accumulation in 100 nM Dex was not significantly different from 10, 1, or 0.1 nM Dex (P=0.09-1), although in 0.1 nM mean GAG accumulation was approximately 25% lower than 1-100 nM Dex (data not shown). In Dex-free culture, GAG accumulation was significantly lower than 100, 10, and 1 nM, but not 0.1 nM Dex (P=0.09). DNA per wet weight was not significantly different among all Dex conditions (P=0.32-1), and the statistical outcome of GAG accumulation normalized to DNA was not different than wet weight. Given that 1 nM Dex appeared to be the lowest concentration that supported chondrogenesis in the same manner as 100 nM, more comprehensive experiments were conducted for 0, 1 or 100 nM Dex. Next, GAG accumulation as a function of time was evaluated for 0, 1, and 100 nM Dex (data not shown). All cultures showed minimal GAG accumulation on day 3 (~0.1 µg GAG/mg wet weight). On day 7, GAG accumulation was approximately 20% of that on day 15. On days 7 and 15, GAG accumulation in Dex-free cultures was approximately 48% of those in 1 and 100 nM Dex, which were not significantly different (P=1). Given that Dex does not appear to greatly affect the rate at which GAG accumulates with time in chondrogenic culture, subsequent experiments evaluated ECM accumulation on day 15 only.

2.4.2 Dexamethasone titration

Comparisons among 0, 1, and 100 nM Dex were conducted using MSC from 8 horses, with ECM accumulation and histology analyzed after 15 days of culture. Extracellular matrix

accumulation – When normalized to wet weight, GAG (P=0.77) and hydroxyproline (P=0.23) accumulations were not significantly different between 1 nM and 100 nM Dex (Fig. 2.1A). Compared to 1nM and 100 nM Dex, GAG accumulation and hydroxyproline accumulation were approximately 36% and 31% lower in Dex-free cultures, respectively. DNA normalized to wet weight in Dex-free culture was approximately 16% lower than 1 or 100 nM Dex cultures (data not shown). ECM accumulation normalized to DNA (Fig. 2.1B) showed similar statistical results as when normalized to wet weight of samples. Next, the influence of Dex across individual donors was evaluated in a pairwise manner by normalizing GAG and hydroxyproline accumulation in Dex-free to 100 nM Dex cultures for 13 donor horses. GAG accumulation was relatively variable across donors as withholding Dex suppressed GAG accumulation by less than 20% for 4 horses, while for 7 horses GAG accumulation in Dex-free cultures was suppressed by at least 60% compared to 100 nM Dex (Fig. 2.2). Hydroxyproline accumulation in Dex-free cultures was less variable, ranging from 40%-80% of that in 100 nM cultures (data not shown).

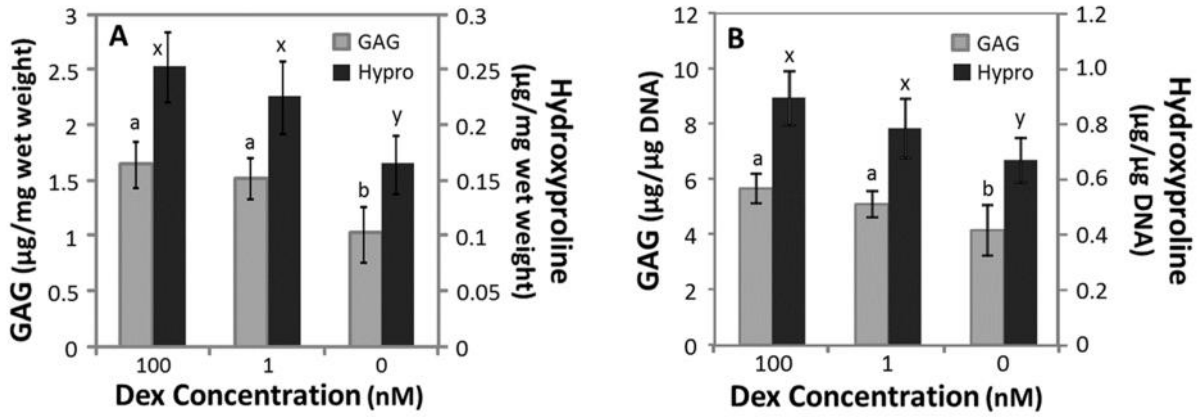

Figure 2.1 GAG and hydroxyproline (Hypro) accumulation after 15 days of culture in chondrogenic medium containing 100, 1 or 0 nM Dex. (A) GAG and hydroxyproline normalized to wet weight of samples. (B) GAG and hydroxyproline normalized to DNA. Data are mean ± SEM, n=8 donor animals. The statistical analysis compared GAG or hydroxyproline accumulation among Dex concentrations, with different letters denote significant difference ($P<0.05$) for each assay.

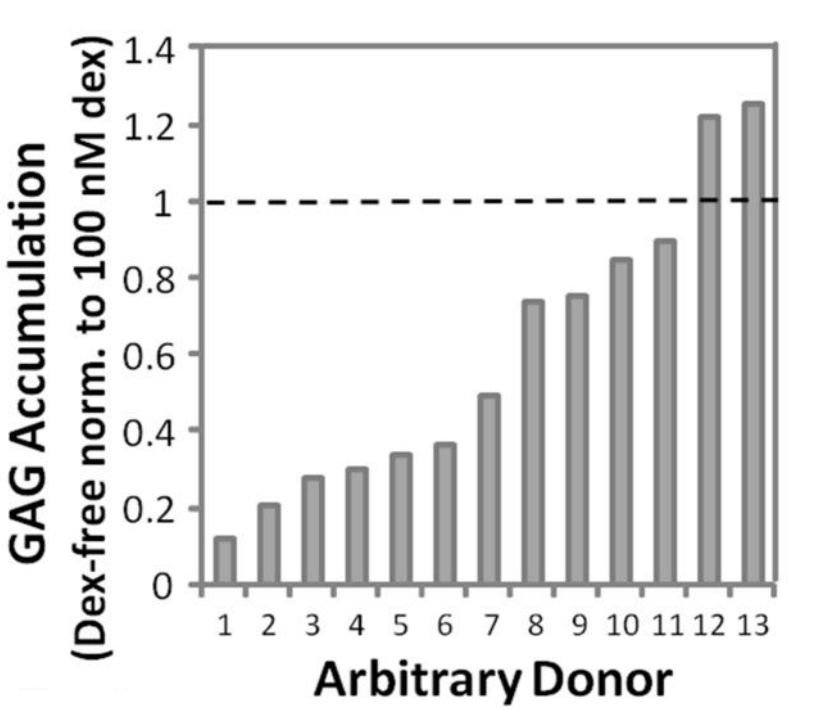

Figure 2.2 GAG accumulation normalized to wet weight from 13 horses after 15 days of culture without Dex or with 100 nM Dex. Data from cultures without Dex were normalized to 100 nM Dex for each individual horse.

2.4.3 Histology

Type II collagen (Fig. 2.3A) and toluidine blue staining (Fig. 2.3B) was present in all conditions from 15 days culture samples. Grossly, the distribution of both stains appeared similar between 1 and 100 nM Dex, with the most prominent staining in pericellular spaces. Staining in Dex-free cultures was largely localized to pericellular spaces. In Fig. 2.3B, toluidine blue staining for Dex-free samples for which GAG accumulation was relatively high (1.9 µg/mg wet weight, Fig. 2.3B(i)) or low (0.27 µg/mg wet weight, Fig. 2.3B(ii)) are presented. Decreasing GAG accumulation in Dex-free cultures was associated with fewer cells that were surrounded by robust pericellular toluidine blue staining.

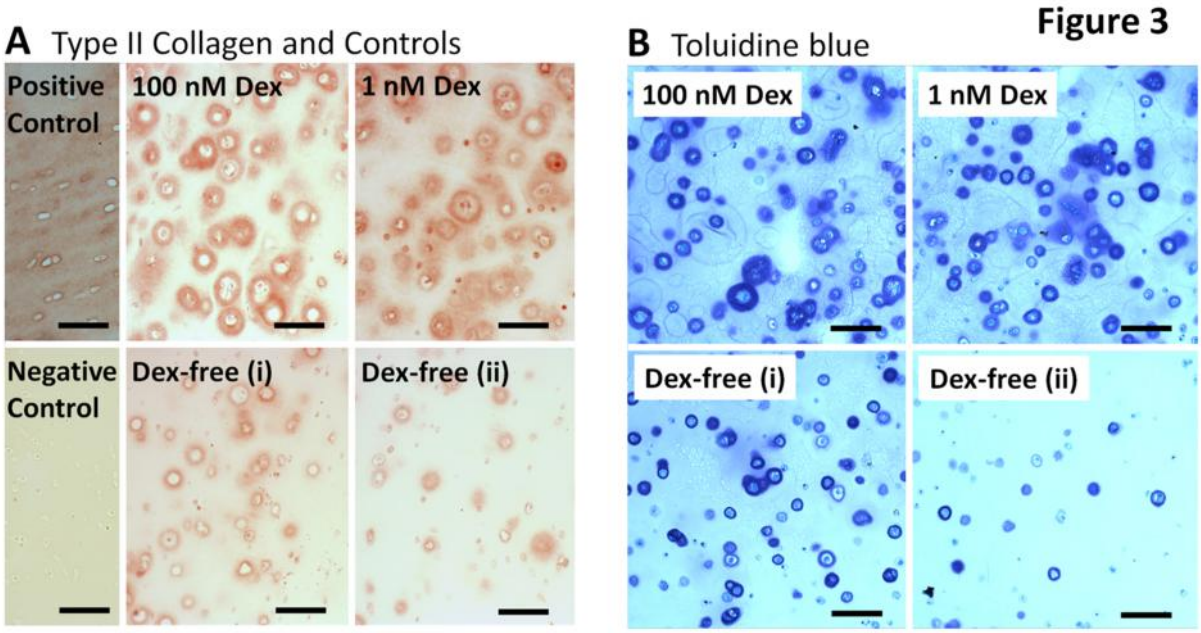

Figure 2.3 Representative staining of MSCs encapsulated in agarose gel cultured in 100, 1 and 0 nM Dex after 15 days of culture. (A) Type II collagen immunohistochemistry, (B) Toluidine blue staining. In Figure 2.3B, representative sections for Dex-free cultures that accumulated a relative high (i) or low (ii) amount of GAG are presented. Controls were performed on equine cartilage. Bar = 100 µm.

2.4.4 Gene expression

Gene expression was evaluated for MSCs from 5 horses after 3, 6, 9 and 15 days of culture (Fig. 2.4A-G). In Figure 2.4A, expression of each gene were normalized to mean expression of day 3 and in Figure 2.4B-G, expressions in Dex-free and 1 nM Dex cultures were normalized to 100 nM Dex at each timepoint. Collagen: Type II and I: In 100 nM Dex (Fig. 2.4A), type II collagen expression increased with time in culture, with an overall 52-fold upregulation between days 3 and 15. Type I collagen expression in 100 nM Dex did not change with time in culture between days 3 and 9 (P=0.58), but on day 15 decreased 2.5-fold relative to day 3. These temporal patterns are consistent with previous reports of collagen gene expression over time during MSC chondrogenesis.[20] When considering relative expression among conditions at each timepoint, type II collagen expression (Fig. 2.4B) was not significantly different between 100 nM and 1 nM Dex, while expression in Dex-free cultures was significantly less than 1 nM or 100 nM Dex (7-fold) on day 3 only. Type I collagen expression (Fig. 2.4C) did not differ between 100 nM and 1 nM Dex cultures, while expression in Dex-free cultures was significantly higher than 100 nM Dex (5-fold) on day 15 only. Type X: In 100 nM Dex (Fig. 2.4A), type X expression increased 13.9-fold between days 3 and 6, but was not significantly different for the rest of the timecourse. The temporal pattern of increasing type X collagen expression with time in culture was consistent with previous reports for human MSCs in pellet culture.[20] When considering relative expression among conditions at each timepoint (Fig. 2.4D), Dex-free and 1 nM Dex cultures were not significantly different from 100 nM through 9 days of culture, although on day 6 type X collagen expression in 1 nM Dex was 16.6-fold higher than Dex-free cultures. On day 15, type X collagen expression in Dex-free and 1 nM Dex cultures was 9.9- and 24-fold higher than 100 nM Dex. MMP: In 100 nM Dex (Fig. 2.4A), MMP13 expression did not change with time in culture (P=0.07-0.95). At each

timepoint, MMP13 was not significantly different between Dex-free and 1 nM Dex cultures (Fig. 2.4E). On days 3 and 6, MMP13 expression in Dex-free or 1 nM Dex cultures was 47- and 24-fold higher than 100 nM Dex cultures, respectively. On day 15, MMP13 expression in Dex-free and 1 nM Dex cultures was approximately 15-fold higher than 100 nM Dex cultures. Across all conditions, MMP1 expression was consistently within the detection limit of the assay on day 3 only. On day 3, the only significant difference was a 56-fold increase in Dex-free culture relative to 100 nM Dex (data not shown). ADAMTS: In 100 nM Dex (Fig. 2.4A), ADAMTS4 expression decrease with time in culture with an overall 4.5-fold downregulation between days 3 and 15, while ADAMTS5 expression did not change with time in culture (P=0.06). At each timepoint, ADAMTS4 and 5 expression was not significantly different between 1 and 100 nM Dex cultures (P=0.28-0.96, Fig. 2.4F-G) except on day3 for ADAMTS4 (2.5-fold). Aggrecanase expression in Dex-free culture was not significantly different from that in 100 nM except for ADAMTS4 on day 3 (3-fold higher), and ADAMTS5 on day 6 (3.8-fold higher).

2.4.5 Alkaline Phosphatase

Alkaline phosphatase activity was evaluated for MSCs from 5 horses. Culture media was analyzed on days 3, 6, 9 and 15, and in Figure 2.5 the results were normalized to the wet weight of the samples. In 100 nM Dex, alkaline phosphatase activity increase for 1.7-fold between day 3 and day 6, and did not change with time for the remainder of the culture period (P=0.68-0.98, Fig. 2.5A). On days 3 and 6, alkaline phosphatase activity in Dex-free and 1 nM Dex was approximately 37% and 60% of those in 100 nM Dex, respectively (Fig. 2.5B). On day 9 (P=0.35) and 15 (P=0.68), alkaline phosphatase activity in 1 nM Dex was not significantly different from 100 nM Dex culture, while Dex-free culture was approximately 38% of that in 100 nM Dex

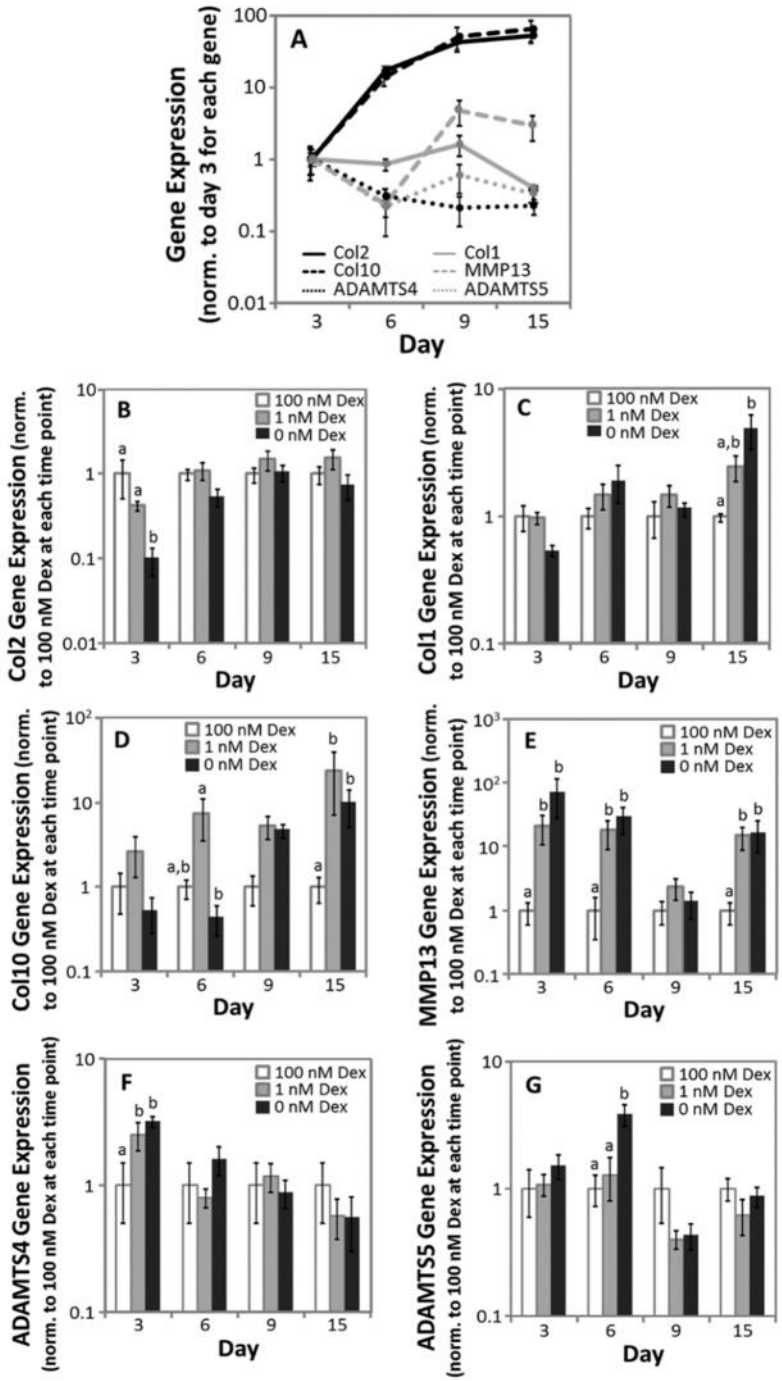

Figure 2.4 Gene expression from days 3, 6, 9 and 15 of cultures. (A) Gene expression over time in chondrogenic culture containing 100 nM Dex (B) Type II collagen, (C) Type I collagen, (D) Type X collagen, (E) MMP13, (F) ADAMTS4, (G) ADAMTS5. Expression was normalized to GAPDH, then (A) to the mean values of expression on day 3 of each gene and (B-G) to the mean values in 100 nM Dex for each timepoint. Data are mean ± SEM, n=5 donor animals. (A) The statistical analysis comparing the expression of each gene over time is presented in the text. (B-G) The statistical analysis compared expression among Dex concentrations at each timepoint, with different letters denoting significant difference ($P < 0.05$)

cultures. Alkaline phosphatase activity normalized to DNA showed similar statistical outcomes as when normalized to wet weight (data not shown).

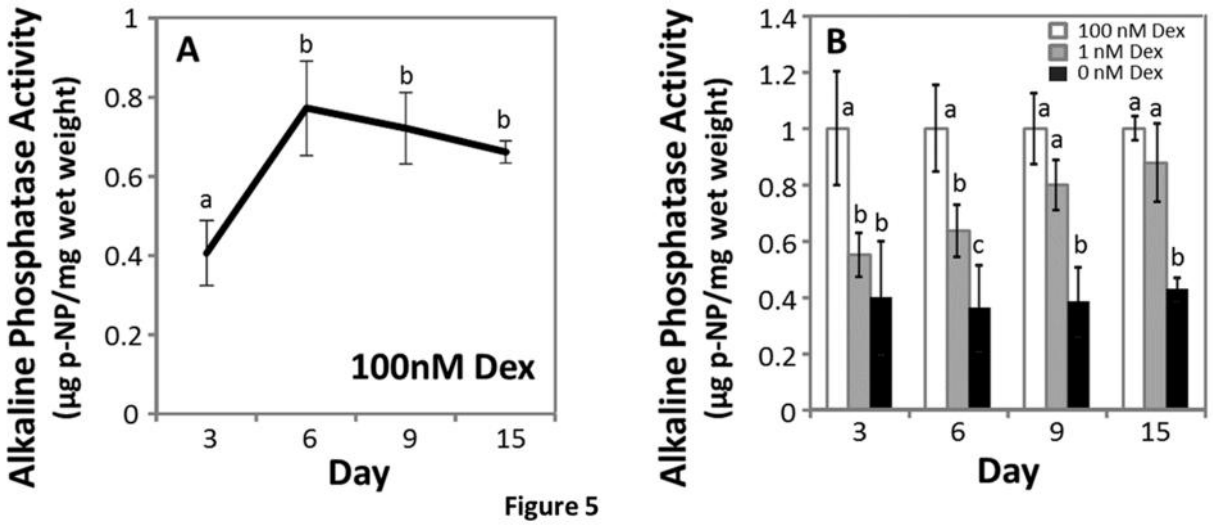

Figure 2.5 Alkaline phosphatase activity. (A) Alkaline phosphatase activity in chondrogenic culture containing 100 nM Dex after day 3, 6, 9 and 15 of culture. (B) Alkaline phosphatase activity in Dex-free or 1 nM Dex relative to 100 nM Dex. Data were normalized to mean values in 100 nM Dex at each timepoint. Data are mean ± SEM, n=5 donor animals. The statistical analysis compared activity among Dex concentrations at each timepoint, with different letters denote significant difference ($P<0.05$).

2.4.5 Prostaglandin E2

PGE2 secreted into the culture medium was quantified between 1 and 21 days of culture for MSCs from 6 horses. In Fig. 2.6, the rate of PGE2 secretion was calculated by dividing the total amount of PGE2 in the medium by the time elapsed since the previous medium change, which was 3 days for all timepoints except for day 1. The rate of PGE2 secretion was normalized to the wet weight of the sample. In 100 nM Dex (Fig. 2.6A) the rate of PGE2 accumulation in the medium was initially low, then increased more than 200-fold by days 3 and 6 of culture. Between days 6 and 9, the rate of PGE2 secretion decreased approximately 30-fold, and did not significantly change for the remainder of the timecourse ($P=0.86$-1). On day 1, PGE2 secretion was significantly

different among all conditions (Fig. 2.6B), with the highest level in Dex-free culture, 122- and 3.5-fold higher than 100 nM and 1 nM Dex, respectively. At subsequent timepoints, PGE2 secretion was not significantly different between Dex-free and 1 nM Dex cultures (P=0.07-0.85). Between days 3 and 21 of culture, PGE2 secretion in 100 nM Dex was 7- to 63-fold lower than Dex-free or 1 nM Dex cultures. PGE2 secretion normalized to DNA showed similar statistical outcomes as when normalized to wet weight (data not shown).

Extracellular matrix accumulation normalized to sample wet weight on day 21 resembled that of Figure 2.1 as GAG (P=0.09) and hydroxyproline (P=0.71) accumulations were not significantly different between 1 nM and 100 nM (data not shown), while GAG and hydroxyproline accumulations in Dex-free culture were 82% and 60% lower than those in 1 nM and 100 nM Dex culture, respectively.

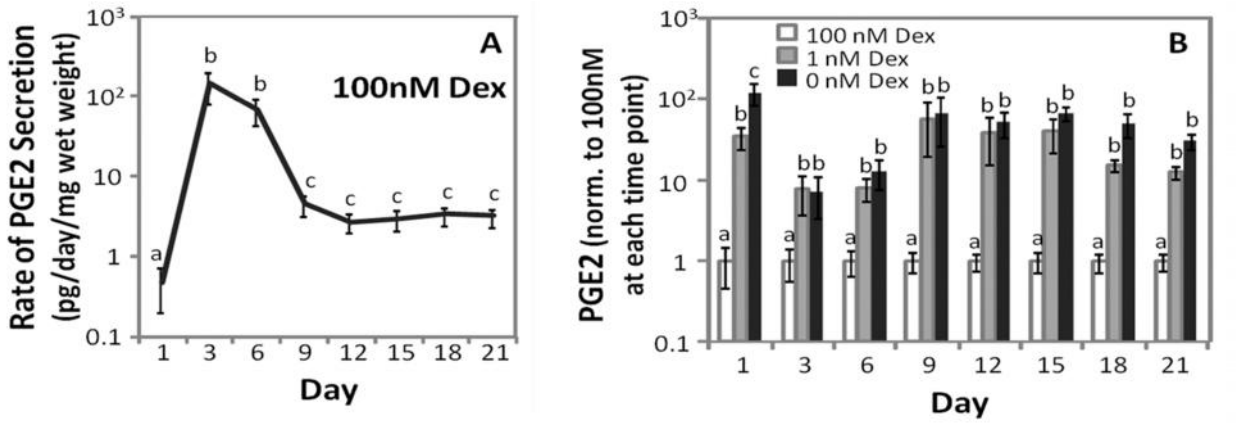

Figure 2.6 Prostaglandin E2 secretion. (A) PGE2 secretion over time in chondrogenic culture containing 100 nM Dex. (B) PGE2 secretion in Dex-free or 1 nM Dex relative to 100 nM Dex. Data were normalized to mean values in 100 nM Dex at each timepoint. Data are mean ± SEM, n=6 donor animals. The statistical analysis compared PGE2 levels among Dex concentrations at each timepoint, with different letters denote significant difference (P<0.05). p-NP: p-nitrophenol.

2.4.6 Celecoxib

Celecoxib was evaluated at a concentration of 10 µM, with a final DMSO concentration of 0.025%, for MSCs from 3 horses. Control cultures were supplemented with 0.025% DMSO. All cultures were maintained in 1 nM Dex. When normalized to wet weight, GAG accumulation in celecoxib cultures (2.24 µg/mg wet weight) was not significantly different than controls (2.04 µg/mg wet weight, P=0.36) (Fig. 2.7A). Hydroxyproline accumulation in celecoxib cultures (0.18 µg/mg wet weight) was not significantly different than controls (0.15 µg/mg wet weight, P=0.6). In control cultures, the rate of PGE2 secretion on days 3 and 6 was 1448 and 463 pg/day/mg wet weight, respectively (Fig. 2.7B), which was at least 500-fold higher than that in celecoxib cultures (0.58 and 0.89 pg/day/mg wet weight). Data normalizing to DNA showed similar statistical outcomes as normalizing to wet weight (data not shown).

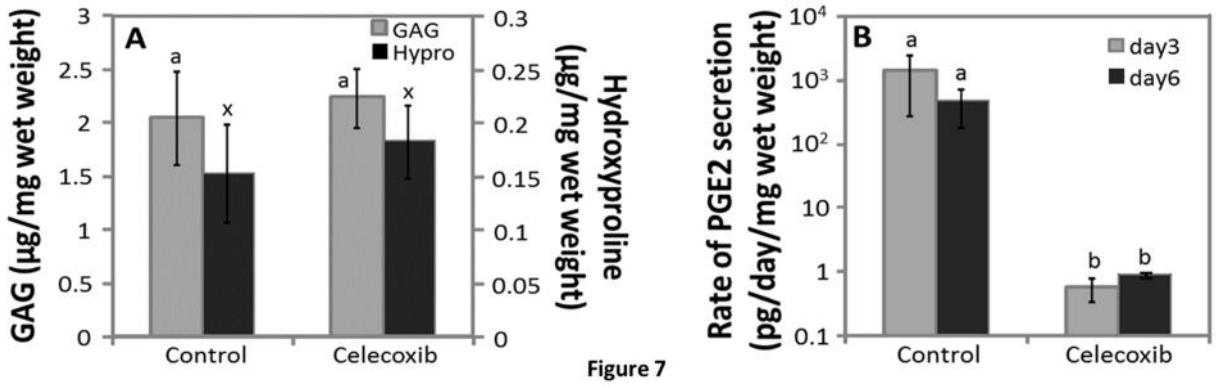

Figure 2.7 ECM accumulation and PGE2 secretion in the presence of 10 µM Celecoxib. (A) GAG and hydroxyproline (Hypro) accumulation after 15 days of culture. (B) PGE2 secretion on days 3 and 6 of chondrogenic culture. Data are mean ± SEM, n=3 donor animals. The statistical analysis compared (A) GAG or hydroxyproline accumulations and (B) Control to celecoxib treatment. Different letters denote significant difference, P<0.05.

2.4.7 Timing of Dex exposure

The effects of withdrawing Dex from chondrogenic culture over time, or temporarily withholding Dex from the start of chondrogenic culture, were tested using a Dex concentration of 1 or 100 nM over 15 day culture period. In both experiments, control cultures were maintained in the presence or absence of Dex for 15 days. Extracellular matrix accumulation in control cultures were consistent with Fig. 2.1 as GAG and hydroxyproline in 1 and 100 nM Dex was higher than in Dex-free cultures (Fig. 2.8A-C). In Fig. 2.8 data were normalized to sample wet weight, which resulted in comparable statistical outcomes when normalized to DNA.

2.4.8 Dex withdrawal

Dex was withdrawn after 1, 3, or 6 days of 15 day culture period. In 1 nM Dex culture, withdrawing Dex after 1 and 3 days resulted in approximately 70% and 51% in GAG and hydroxyproline accumulation relative to controls that received Dex for 15 days (Fig. 2.8A, n=7). Withdrawing Dex after 6 days of exposure resulted in a significant decrease in hydroxyproline, but not GAG (P=0.42), relative to controls that received Dex for 15 days. In 100 nM Dex culture (n=3), withdrawing Dex after 1 and 3 days resulted in GAG accumulation that was approximately 78% of controls that received Dex for 15 days. Hydroxyproline accumulation was not significantly different among conditions (P=0.68), although high animal-to-animal variability was noted in these samples (data not shown).

2.4.9 Dex withholding

In medium containing 1 nM Dex, withholding Dex for up to 3 days did not significantly suppress GAG accumulation relative to the Dex control culture (P=0.19-1, Fig. 2.8B, n=5). Withholding Dex for 3 days was necessary to significantly reduce the accumulation of hydroxyproline relative to Dex controls. Next, for 100 nM cultures Dex was withheld for 3, 4, 5,

or 6 days. Withholding 100 nM Dex for 5 days was necessary to significant reduce the accumulation of GAG. (Fig. 2.8C, n=3). Hydroxyproline accumulation was not significantly different among conditions (P=0.17), although high animal-to-animal variability was noted in these samples. Histology - For both experiments, staining for Type II collagen and toluidine blue was present in all conditions, with ECM accumulating largely in pericellular spaces as in Fig. 2.3A and B (data not shown)

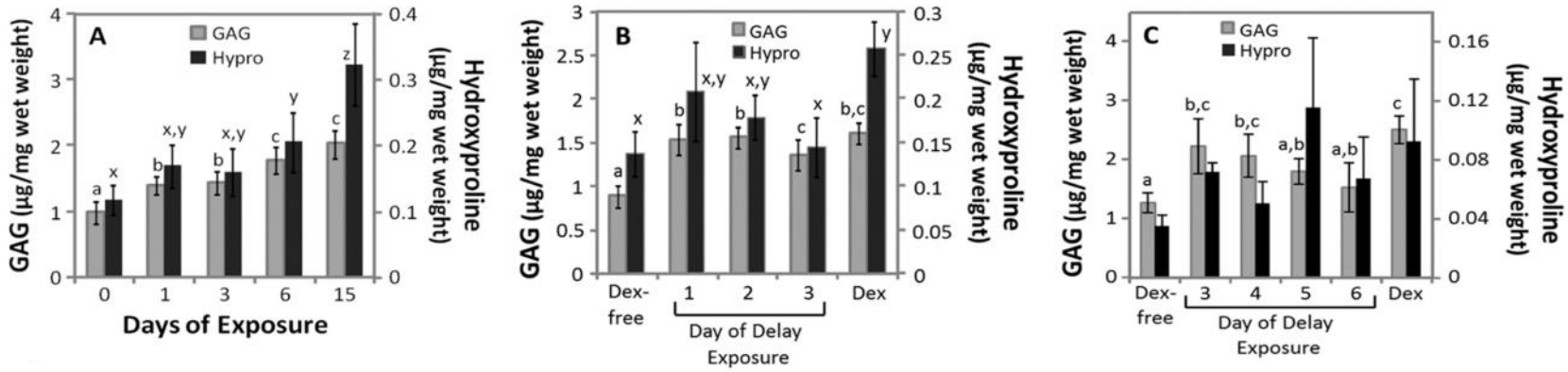

Figure 2.8 GAG and hydroxyproline (Hypro) accumulation after 15 days of culture with withdrawing or withholding Dex during chondrogenic culture. (A) Extracellular matrix accumulation after withdrawing 1 nM Dex over the first 6 days of culture (n=7 donor animals), (B) Extracellular matrix accumulation after withholding 1 nM Dex for up to the first 3 days of culture (n=5 donor animals), (C) Extracellular matrix accumulation after withholding 100 nM Dex for the first 3 to 6 days of culture (n=3 donor animals). Samples were analyzed after 15 day of culture. Data are mean ± SEM. The statistical analysis compared GAG or hydroxyproline accumulation among the different conditions, with different letters denote significant difference ($P<0.05$) for each assay.

2.5 Discussion

Withholding Dex from chondrogenic cultures of adult equine MSCs resulted in a decrease in ECM accumulation of ~40% compared to 100 nM Dex, which was consistent with previous studies involving chondrogenesis of bovine MSCs,[6-8] human bone marrow MSCs in pellet culture,[5] and human adipose-derived stromal cells cultured in alginate hydrogel.[21] Further, the modest increase in type I collagen expression in Dex-free samples on day 15 may suggest greater fibrocartilage-like differentiation in the absence of Dex with time in chondrogenic culture. The effects of withhold Dex was variable across donors in that MSCs from certain donors were minimally affected, while others accumulated only modest amounts of GAG in the absence of Dex. Donor-to-donor variability may be a concern for autologous human therapies as chondrogenesis has been shown to vary greatly among donors in vitro in chondrogenic medium containing 100 nM Dex.[22] As a secondary factor, our data suggests that additional variability may be encountered if Dex is not included in the development of MSC-based cartilage repair therapies.

Suppression of ECM accumulation in Dex-free culture was reversed with 1 nM Dex. Further, measures of cartilage-like phenotypic expression were not significantly different between 1 and 100 nM Dex as both cultures showed robust staining for type II collagen and toluidine blue, while gene expression of type I and II collagen were not significantly different. These data indicate that Dex concentrations may be reduced 100-fold from the conventional laboratory dose of 100 nM while still supporting robust MSC chondrogenesis, which is consistent with reports that Dex concentrations less than 100 nM stimulated robust chondrogenesis in multipotent rat calvaria cells,[23] and sox-9 expression in chondrocytes.[24]

While Dex-free culture resulted in moderate suppression of ECM accumulation, collagen gene expression did not convincingly differentiate between Dex and Dex-free cultures as type II

collagen expression in Dex-free culture was significantly different than 1 nM or 100 nM Dex on day 3 only. While these data may indicate that Dex enhances the rate of differentiation during early chondrogenesis, it is not known whether a lag in type II collagen expression during a period of low ECM synthesis[20] is sufficient to account for the large discrepancies in ECM accumulation between Dex and Dex-free conditions after 15 days of culture. Further, given that gene expression does not necessarily translate to protein synthesis, as documented for aggrecan during MSC chondrogenesis,[15,25] it is possible that post-translational regulation of ECM synthesis may account for the relative low accumulation of GAG and hydroxyproline in the absence of Dex.

A second possibility is that Dex acts to support ECM accumulation through potent anti-inflammatory properties that suppress catabolism in chondrogenic culture. This concept is supported by studies reporting increased aggrecanase activity when Dex was withheld in chondrogenic bovine MSC cultures,[8] and MMP cleavage of aggrecan in human MSC cultures maintained in chondrogenic medium containing 100 nM Dex.[7] When considering gene expression of catabolic enzymes, decreasing or withholding Dex resulted in modest (ADAMTS4) or moderate (MMP13, MMP1) upregulation of gene expression at early timepoints, and moderate upregulation of MMP13 on day 15, although minimal differences between 1 nM Dex and Dex-free cultures on day 3 does not strongly support the differences in ECM accumulation between these two groups. As a second measure of inflammation, we measured PGE2 secretion, which when induced from activation of cox-2 has been associated with degradation in osteoarthritic cartilage,[26] and cartilage when cultured with pro-inflammatory cytokines in vitro.[27,28] In our cultures, large increases in PGE2 secretion with early chondrogenesis followed by decreases to a steady-state rate were consistent with human MSCs in pellet culture.[29] Further, severe suppression of PGE2 synthesis by celecoxib during early chondrogenesis indicated stress- or inflammation-induced activation of

cox-2. Among the Dex conditions tested in this study, cox-2 activation did not appear to significant affect chondrogenesis as increases in PGE2 secretion with reducing or eliminating Dex was not consistent with the effects of Dex on ECM accumulation. While additional assays are necessary to more thoroughly characterize the effects of Dex on inflammation and catabolism, these data suggest that modulation of pathways that influence ADAMTS or MMP gene expression, or cox-2 activation, did not play a critical role in the accumulation of ECM during MSC chondrogenesis.

An ongoing concern about the use of MSCs to resurface articular cartilage defects is the propensity of MSCs to acquire markers of hypertrophy during chondrogenesis. Hypertrophic markers have been identify over time in MSC chondrogenic culture in 100 nM Dex,[30] while reducing the concentration of Dex in culture has been used to promote hypertrophy.[31] In Dex-free culture in the current study, upregulation of MMP13 and type X collagen relative to 100 nM Dex may suggest advanced hypertrophic differentiation in the absence of Dex. One nM Dex did not suppress these markers of hypertrophy, and in fact increased type X collagen expression over Dex-free cultures on day 6. Alkaline phosphatase activity did not suggest increased hypertrophy with reduction or elimination of Dex, although these data are consistent with human MSC pellet cultures in which alkaline phosphatase activity was modestly lower in Dex-free culture relative to 100 nM Dex.[5] By day 15 of chondrogenic culture, moderate levels of alkaline phosphatase secretion may be expected,[30,32] although secretion may increase significantly with additional time in culture.[30,32,33] In addition, beyond 15 days of chondrogenic culture it has been shown that medium conditions that promote hypertrophy can further increase alkaline phosphatase secretion up to 3-fold.[33] Therefore it is possible that additional time in culture, over which alkaline phosphatase secretion may increase with concomitant increases in type I and X gene expression,[30] would better delineate the effect of Dex concentration on hypertrophic markers. While additional assays are

necessary to more thoroughly characterize commitment to hypertrophy, the gene expression data suggest that high levels of Dex may be beneficial to reduce differentiation to this undesirable phenotype. It is possible that Dex influences hypertrophic differentiation through the regulation of cox-2 as suppression of hypertrophic markers has been associated with cox-2 inhibition during chondrogenesis in vitro.[34] This possibility is supported in the current study as gene expression of MMP13 and type X collagen was consistent with PGE2 secretion in that the lowest values were found for 100 nM Dex cultures. While additional studies are necessary to more precisely define the potential relationship between cox-2 activation and hypertrophic differentiation, the persistent PGE2 secretion through 21 days of culture may suggest that multiweek suppression of inflammation leading to cox-2 activation may be effective in suppress hypertrophic differentiation.

Given that the temporal effects of Dex exposure on MSC chondrogenesis have not been reported, we performed experiments in which Dex was withheld or withdrawn to determine how the timing of exposure influence chondrogenesis. At a concentration of 1 nM, Dex could be withheld for up to two days without significantly affecting the accumulation of ECM, while withdrawal studies suggested that Dex plays an important role in ECM accumulation beyond day 6. A similar temporal pattern was noted for 100 nM Dex, although the high variability in hydroxyproline accumulation suggest that a larger samples size is necessary to better delineate the effect of withholding or withdrawing 100 nM Dex. Taken together, these data suggest that strategies for delivering Dex in vivo should prioritized sustained delivery, which is in contrast to chondrogenic growth factors that may be effective in stimulating chondrogenesis with a limited duration of initial exposure.[11-14]

When considering the design of delivery strategies for Dex to support MSC chondrogenesis in vivo, this study indicates that a lower therapeutic bound that is 100-fold less than the

conventional in vitro dose may be sufficient to support robust neo-cartilage development. However, the important issue of whether high levels of Dex function to suppress hypertrophic differentiation in vivo must be determined. With regards to timing of Dex exposure, the need to sustain therapeutic concentrations of Dex for days may prove challenging without advanced techniques for controlled local delivery due to rapid clearing of Dex from the body, even when administered intra-articularly using doses that far exceed the concentrations tested in this study.[35] A secondary consideration for intra-articular dosing of Dex may be that high concentrations that strongly suppress inflammation may have a negative impact as inflammation has been shown to play a supportive role in chondrogenesis.[36,37] In this regard, additional studies are necessary to identify the specific inflammatory pathways that function to support chondrogenesis, and whether those key pathways are modulated by Dex. A limitation of this study is that the effects of Dex was evaluated in a single system consisting of equine bone marrow MSCs in agarose hydrogel, which does not necessarily reflect the response of different species, tissue sources, and scaffolds to Dex. It is likely that the current study does not grossly understate the need for Dex to support ECM accumulation as superior chondrogenesis with Dex concentrations in excess of 100 nM have not been reported. However, it is possible that certain combinations of cells and scaffolds may be less dependent on Dex as laboratory studies have demonstrated robust chondrogenesis in the absence of Dex for human bone marrow MSCs seeded in polyethylene glycol diacrylate[14] or self-assembling peptide hydrogels,[8] or bovine synovium-derived MSCs in pellet culture.[7]

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CHAPTER 3: MODULATING THE OXIDATIVE ENVIRONMENT DURING MESENCHYMAL STEM CELLS CHONDROGENESIS WITH SERUM INCREASES COLLAGEN ACCUMULATION[2]

3.1 Summary

Chondrogenesis of mesenchymal stem cells (MSCs) is induced in culture conditions that have been associated with oxidative stress, although the extent to which the oxidative environment affects differentiation and extracellular matrix (ECM) accumulation is not known. The objectives of this study were to evaluate the oxidative environment during MSCs chondrogenesis in conventional serum-free medium, and the effect of serum-supplementation on intracellular reactive oxygen species (ROS) and chondrogenesis. Young adult equine MSCs were seeded into agarose and cultured in chondrogenic medium, with or without 5% fetal bovine serum (FBS), for up to 15 days. Samples were evaluated for intracellular ROS, the antioxidant glutathione, ECM and gene expression measures of chondrogenesis, and carbonylation as an indicator of oxidative damage. Intracellular ROS increased with time in culture, and was lower in medium supplemented with FBS. Glutathione decreased ~12-fold during early chondrogenesis ($P<0.0001$), and was not affected by FBS ($P=0.25$). After 15 days of culture, FBS supplementation increased hydroxyproline accumulation ~80% ($P=0.0002$); otherwise, measures of chondrogenesis were largely unaffected. Protein carbonylation in chondrogenic MSCs cultures was not significantly different between serum-free and FBS cultures ($P=0.72$). Supplementation with adult equine serum increased hydroxyproline accumulation by 45% over serum-free culture ($P=0.0006$). In

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conclusion, this study characterized changes in the oxidative environment during MSC chondrogenesis, and suggested that lowering ROS may be an effective approach to increase collagen accumulation.

3.2 Introduction

The ability of bone marrow mesenchymal stem cells (MSCs) to undergo chondrogenesis has fostered enthusiasm for tissue engineering approaches to resurface damaged or diseased articular cartilage. Mesenchymal stem cells are isolated and expanded in an uncommitted state, and require exposure to chondrogenic factors to induce differentiation and subsequent secretion of cartilage-like extracellular matrix (ECM)[1]. To ensure that MSCs receive such cues, tissue engineering strategies have been designed to provide controlled exposure to chondrogenic factors during pre-implantation culture[2-5] or as a component of the cell-seeded graft.[6-9]

Current strategies for inducing MSCs chondrogenesis are largely focused on growth factors that stimulate differentiation. However, it has been postulated that additional measures to support the growth and maintenance of repair tissue will increase the likelihood of success.[10] One such measure may be modulation of the oxidative environment. Oxidative stress is generated when cells are unable to neutralize excessive reactive oxygen species (ROS), or are incapable of recycling oxidized biomolecules. The potential for the development of oxidative stress with chondrogenesis was indicated by a chondrogenic cell line (ATDC5), which experienced an increase in ROS with time in culture.[11] For MSCs, the most commonly-used culture medium for inducing chondrogenesis includes transforming growth factor beta (TGF-β),[1] which has been reported to decrease endogenous concentrations of the antioxidant glutathione and increase intracellular ROS.[12] Further, conventional chondrogenic medium does not contain serum. Serum withdrawal is a cell culture technique used to eliminate unknowns associated with serum, synchronize cell cycle,

or induce stress, autophagy, or apoptosis.[13] In particular, serum starvation can increase the production of ROS[14-16], which may occur due to nutrient deprivation.[15] While these data indicate that ROS are generally supportive of MSCs chondrogenesis, the extent to which excess ROS is produced during in vitro chondrogenesis, resulting in oxidative stress, has not been considered.

In chondrocyte cultures, high levels of ROS have been associated reduced synthesis of proteoglycans.[17,18] In mature fibrocartilage, elevated ROS in vivo coincided with increased oxidative damage in the ECM, which was associated with altered mechanical properties and increased susceptibility to degradation.[19] These data suggest that for chondrogenic MSCs, oxidative stress may lead to suboptimal volume and/or quality of the repair tissue. Therefore, the first objective of this study was to characterize the oxidative environment during chondrogenesis in conventional serum-free medium. Next, we evaluated the potential of serum to reduce intracellular ROS, and the concomitant effect on chondrogenesis and oxidative damage. Experiments were conducted using young adult equine MSCs encapsulated in agarose hydrogel, a scaffold that supports robust chondrogenesis of equine MSCs.[20] Initial experiments evaluated intracellular ROS and endogenous concentrations of glutathione over time in serum-free chondrogenic culture. Next, we characterized the ability of fetal bovine serum (FBS) supplementation to reduce intracellular ROS, and the effect on cell viability, ECM accumulation, and extracellular oxidative damage. Finally, the potential to use adult serum as a chondrogenic medium supplement was evaluated by comparing viability and ECM accumulation to serum-free or FBS-supplemented medium.

3.3 Methods

3.3.1 MSCs isolation and expansion

Bone marrow was aspirated from the iliac crest of 4 2-5 year-old mixed breed horses euthanized for reasons unrelated to this study. Colony-forming cultures were established in low glucose Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (GE Healthcare Life Sciences, Chicago, IL) that was heat-inactivated (56°C for 30 minutes), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA) and antibiotic/antimycotic solution (100 unit/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Gibco Amphotericin B) (Thermo Fisher Scientific, Waltham, MA). MSCs were collected after 6-7 days of culture, and were reseeded at 2 x 10^3 cells/cm^2 in minimal essential medium alpha (Thermo Fisher Scientific, Waltham, MA), 10% FBS, 10 mM HEPES, antibiotic/antimycotic solution, and 2 ng/ml fibroblast growth factor-basic (Peprotech, Rocky Hill, NJ).[20] The cultures were incubated for 4 days, and then passaged a second time prior to seeding in chondrogenic culture. Cultures were incubated at 37°C in ambient air plus 5% carbon dioxide. All basal media contained sodium bicarbonate (2.2 g/L) to maintain physiological pH. Previously, equine MSCs obtained using this protocol were capable of trilineage differentiation,[20-22] and express a pattern of cell membrane cluster of differentiation molecules consistent with the MSC phenotype.[22]

3.3.2 Agarose encapsulation and chondrogenic culture

Culture-expanded MSCs were encapsulated in 1.5% (w/v) agarose gel at 12x10^6 cells/ml, as previously described.[20] Chondrogenic medium[1] consisted of high-glucose Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) supplement with 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 µg/ml linoleic acid (ITS+ Premix, BD Biosciences, Bedford, MA, abbreviated in the manuscript

as ‘ITS’), 37.5µg/ml ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), 100 nM dexamethasone (Sigma-Aldrich, Saint Louis, MO) and 5 ng/ml recombinant human TGF-β1 (Peprotech, Rocky Hill, NJ). For certain experiments chondrogenic medium was supplemented with heat-inactivated 5% FBS, with or without ITS. Allogeneic equine serum was obtained from a 5 year-old horse. Blood was collected without anticoagulant, stored at 4˚C for 24 hours, and then centrifuged at 1000g for 10 minutes. Five percent adult equine serum was evaluated in chondrogenic medium containing ITS. Chondrogenic cultures were incubated at 37˚C in ambient air plus 5% carbon dioxide for up to 15 days, with medium changed every third day.

3.3.3 Quantification of extracellular matrix accumulation and DNA content

MSCs-seeded agarose samples were digested in proteinase K (Sigma-Aldrich, Saint Louis, MO) in Tris HCl solution at 60°C overnight. From the digest, total accumulated sulfated glycosaminoglycan (GAG) and hydroxyproline were quantified by dimethylmethylene blue[23] and dimethylamino benzaldehyde dye[24] binding assays, respectively. DNA was quantified using the Hoechst dye assay as previously described.[25] Extracellular matrix accumulation was normalized to the wet weight or DNA content of each sample.

3.3.4 Cell viability

MSC-seeded agarose samples were evaluated for viability using the CellTiter-Blue® assay (Promega, Madison, WI) per the manufacturer’s instructions. Sample were incubated in medium containing CellTiter-Blue at 37ºC for 1 hour and 15 min, and then the reaction was stopped by adding 3% SDS and incubating at room temperature for 15 minutes. Aliquots of medium were analyzed for absorbance at 490 nm (excitation) and 529 nm (emission) wavelengths on a microplate reader. Data were reported as CTB absorbance normalized to the wet weight of the sample.

3.3.5 Evaluation of intracellular ROS

Qualitative staining

MSC-seeded agarose samples were fluorescent labeled with CellROX® green reagent (Thermo Fisher Scientific, Waltham, MA) in chondrogenic culture medium at 37ºC for 45 minutes. The samples were washed, and intracellular fluorescence was imaged using an Olympus IX83 Inverted Microscope. Images were collected using an exposure time of 1 second.

Quantification

MSCs were seeded in 48-well plates at 25x103 cells/cm2, and incubated overnight in expansion medium. The medium was changed to serum-free chondrogenic medium or chondrogenic medium plus 5% FBS, and cultured for an additional 24 hours. The cells were labeled in serum-free chondrogenic medium containing 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich, Saint Louis, MO) for 30 minutes at 37ºC. Cultures were washed three times with phosphate buffered saline, and the cells were lysed using a solution of 50 mM Tris, 150 mM NaCl, and 1% Triton X-100. Cell lysates were analyzed at 490 nm (excitation) and 529 nm (emission) wavelengths on a microplate reader. Background absorbance, obtained from lysate from cells incubated without DCFDA during the labeling period, was subtracted from labeled cultures. Data were reported as DCFDA absorbance normalized to the total protein content of the cell lysates determined with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

3.3.6 Glutathione content

MSC-seeded agarose samples were analyzed for total glutathione or oxidized glutathione (GSSG) using a commercial kit (GSH/GSSG-Glo™ Assay (Promega, Madison, WI)) according to the manufacturer's instructions. Data were normalized to the wet weight of the samples. Total

glutathione and GSSG were used to calculate for ratio of reduced/oxidized glutathione (GSH/GSSG ratio).

3.3.7 Protein carbonyl content

Protein extraction - MSC-seeded agarose samples were pulverized, and then digested for 6 hours at 37ºC with 0.5 unit/ml of chondroitinase ABC (Sigma-Aldrich, Saint Louis, MO). Soluble proteins were extracted using 1 M NaCl according to the methods of Sharft et. al.[19] As a control, articular cartilage samples from 2-5 year-old horses were incubated in 40 unit/ml collagenase for 2 hours 30 minutes at 37ºC, subjected to 9 freeze-thaw cycles,[19] and then processed as performed for MSCs-seeded agarose. To compare the ECM extraction efficiency of 1M NaCl, extract from MSCs cultured in chondrogenic medium containing ITS and 5% FBS or articular cartilage were evaluated for hydroxyproline content. In addition, samples that were not treated with chondroitinase ABC were subjected to NaCl extraction, which was then evaluated for GAG content. Carbonyl content - NaCl extraction solutions were diluted 6-fold with water, and carbonylation was quantified using a commercial kit (OxiSelect Protein Carbonyl Fluorometric Assay (Cell Biolabs, San Diego, CA)).

3.3.8 RNA extraction and real-time PCR

MSC-seeded agarose samples were collected in TRIzol reagent® (Life Technologies, Grand Island, NY) and stored at -80°C. Samples were removed from TRIzol®, cooled in liquid nitrogen, and pulverized. Five hundred microliters of TRIzol® was first added to the pulverized samples, followed by chloroform at a ratio of 1:5 to the TRIzol®. Samples were centrifuged at 12,000g at 4°C for 15 minutes, and RNA was extracted from the aqueous phase using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with on-column genomic DNase (Qiagen, Valencia, CA). mRNA was reverse transcribed into cDNA using

superscript® III first-strand synthesis system for RT-PCR (Thermo Fisher Scientific, Waltham, MA), and evaluated for aggrecan, type I, II, and X collagen, and matrix metalloproteinase 13 (MMP 13) using the Biorad CFX96^{TM} Real-Time PCR Detection System (Biorad, Hercules, CA). Relative gene expression levels were determined by semi-quantitative real time PCR using primers and SYBR Green (Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene.

Table 3.1 Forward and reverse primers

Gene	Forward Primers	Reverse Primers
Aggrecan	ACCACTTTACTCTTGGCGTTTG	GCGAGTTGTCAGGGTCTGAA
Collagen I	ATTTCCGTGCCTGGCCCCATG	GCCTTGGAAACCTTGGGGAC
Collagen II	AAACCATCAACGGTGGCTTCCA	GCAATGCTGTTCTTGCAGTGGT
Collagen X	AGGCAACAGCATTACGACCCAAGA	TGAAGCCTGATCCAGGTAGCCTTTG
MMP13	TGATGAAACTTGGACAAGCAGTTCC	CCTTGGAGTGGTCGAGACCTAAG
GAPDH	AAGTGGATATTGTCGCCATCAAT	AACTTGCCATGGGTGGAATC

3.3.9 Immunohistochemical and histological staining

MSC-seeded agarose samples were fixed in 10% formalin for 48 hours, paraffin-embedded, sectioned, and mounted on slides. Sections were de-paraffinized and rehydrated prior to staining. *Type II collagen immunohistochemical staining*: Samples were incubated with proteinase K (Sigma-Aldrich, Saint Louis, MO) solution at 37°C for 15 min. Sections were exposed to mouse anti-collagen type II IgG primary antibody using undiluted supernatant (Hybridoma Bank, Iowa City, IA), followed by donkey anti-mouse IgG secondary antibody conjugated with peroxidase at a 1:500 dilution (Jackson Immunoresearch, West Grove, PA).

Antibody detection was performed using VECTOR® NovaRED™ (Vector laboratories, Burlingame, CA). Additional sections were incubated with normal mouse serum at equal concentration to that of the primary antibody as a negative control. Equine cartilage was analyzed in parallel as a positive control. *Toluidine blue*: Sections were stained with 0.04% Toluidine blue solution (Electron Microscopy Sciences, Fort Washington, PA).

3.3.10 Statistical analysis

Each experiment was conducted using MSCs from 4 horses. Data were evaluated for normality using the Shapiro-Wilk test. Data that showed a normal distribution were analyzed for analysis of variance with mixed model using animal as a random effect. Pairwise comparisons of medium conditions were analyzed using least squares means with Tukey-Kramer adjustment. For glutathione content, medium condition and time were considered main effects, and pairwise comparisons of main effect and their interactions were performed. Data showing a non-normal distribution were analyzed using Wilcoxon matched-pairs signed rank test. P-values less than 0.05 were considered statistically significant. Statistical tests for normal distribution and non-parametric test were performed using GraphPad Prism 7, and correlation and parametric tests were performed using SAS 9.3 software. Data are presented as mean ± SEM.

3.4 Results

Throughout the manuscript and in figures serum-free chondrogenic medium is denoted as 'ITS', chondrogenic medium plus 5% FBS as 'ITS/FBS', and chondrogenic medium containing 5% FBS but without ITS as 'FBS'.

3.4.1 CellRox staining in ITS medium

CellRox staining was evaluated after 1 or 6 days of culture. Intracellular fluorescence in samples that were not labeled with CellRox was not grossly detectable (data not shown).

Intracellular ROS staining was faint after 1 day of culture (Figure 3.1, representative images of all samples). By day 6, intracellular ROS staining had increased compared to day 1.

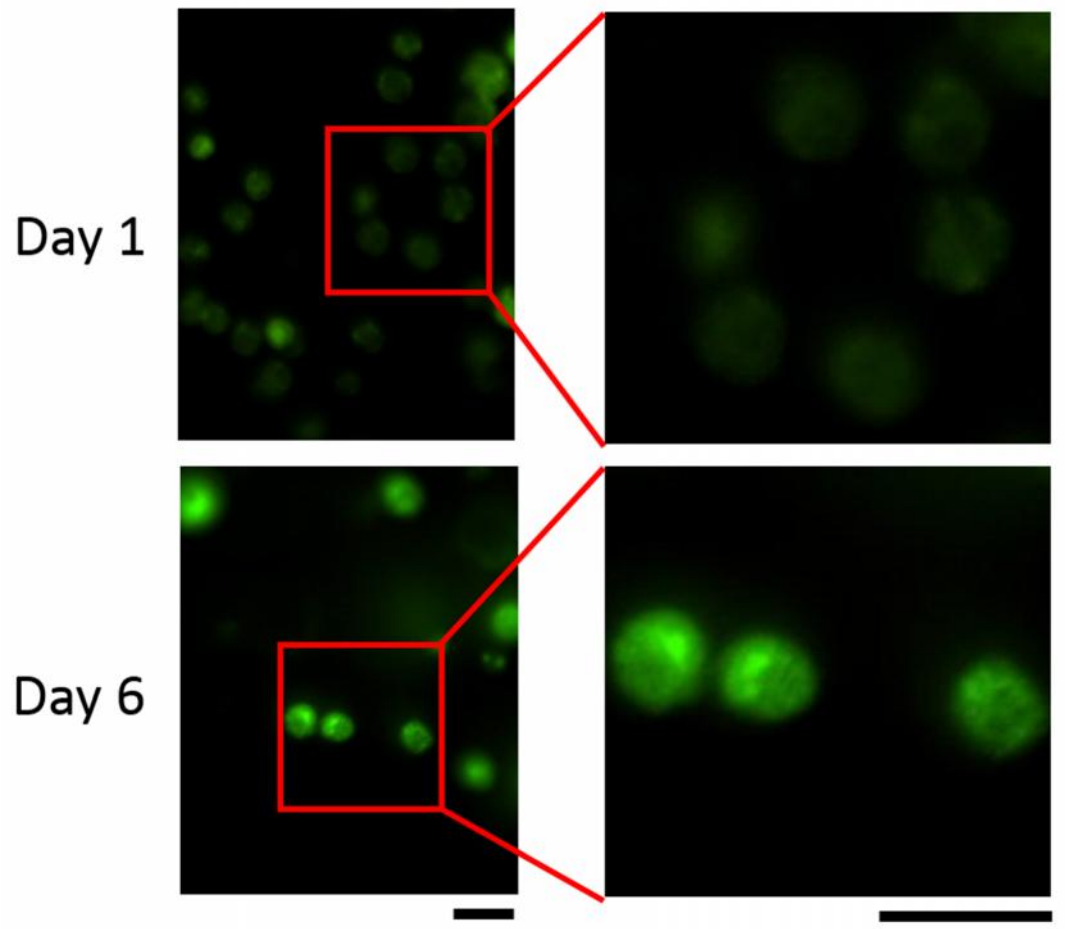

Figure 3.1 CellRox staining for intracellular reactive oxygen species in serum-free chondrogenic culture (ITS). Fluorescent images were captured after 1 or 6 days of culture. Bar = 25 µm.

3.4.2 Glutathione content in ITS or ITS/FBS medium

Total glutathione and GSSG were evaluated after 1 or 6 days of culture (Figure 3.2). Individual pairwise comparisons are not reported as the interactions between medium and time were not significant (P=0.25). Independent of medium conditions, total glutathione decreased ~12-fold between days 1 and 6 of culture (P<0.0001). On day 1, the ratio of GSH/GSSG in ITS/FBS

medium was 50% higher than ITS culture (P=0.001). GSSG was below the detection limit of the assay on day 6.

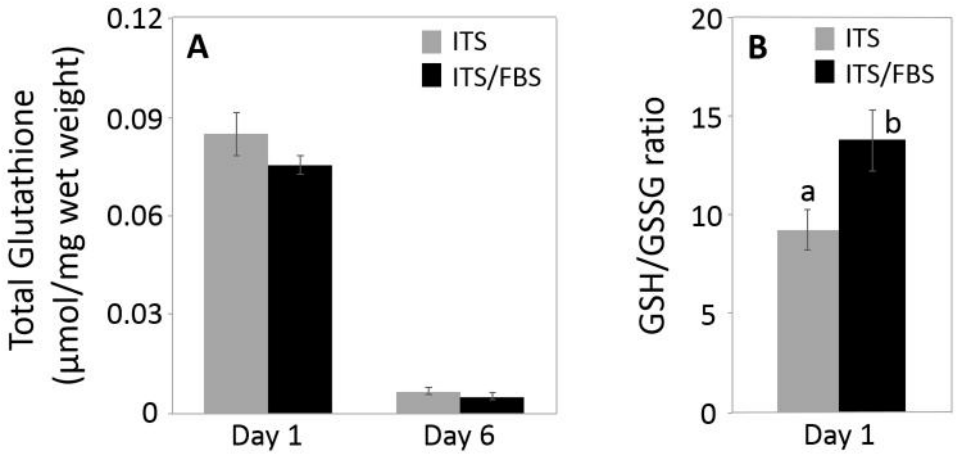

Figure 3.2 Glutathione content in samples cultured in ITS or ITS/FBS medium. (A) Total glutathione was evaluated after 1 and 6 days of culture. Independent of medium conditions, total glutathione decreased ~12-fold between days 1 and 6 of culture ($p<0.0001$) (B) Ratio of reduced-to-oxidized glutathione (GSH/GSSG) after 1 day of culture. Day 6 is not reported due to low levels of GSSG that were below the detection limit of the assay. Data are mean ± SEM, n=4 donor animals. For each assay different letters denote significant differences ($P<0.05$).

3.4.3 Detection of intracellular ROS in ITS or ITS/FBS medium

DCFDA: Monolayer cultures were analyzed after 1 day of incubation in chondrogenic media. The level of intracellular ROS in ITS cultures (0.62 ± 0.10 DCFDA absorbance/mg protein) was 2.6-fold higher than ITS/FBS cultures (0.24 ± 0.03 DCFDA absorbance/mg protein) ($P<0.001$, data not shown).

CellRox: Samples were evaluated immediately after encapsulating MSCs in agarose, and then after 3, 9 or 15 days of chondrogenic culture (Figure 3.3). On day 0 intracellular staining was faint. In ITS medium intracellular staining increased over time, in a manner that resembled the short timecourse presented in Figure 3.1. In ITS/FBS medium intracellular staining increased slightly with time in culture, and to a much lower extent than in ITS culture.

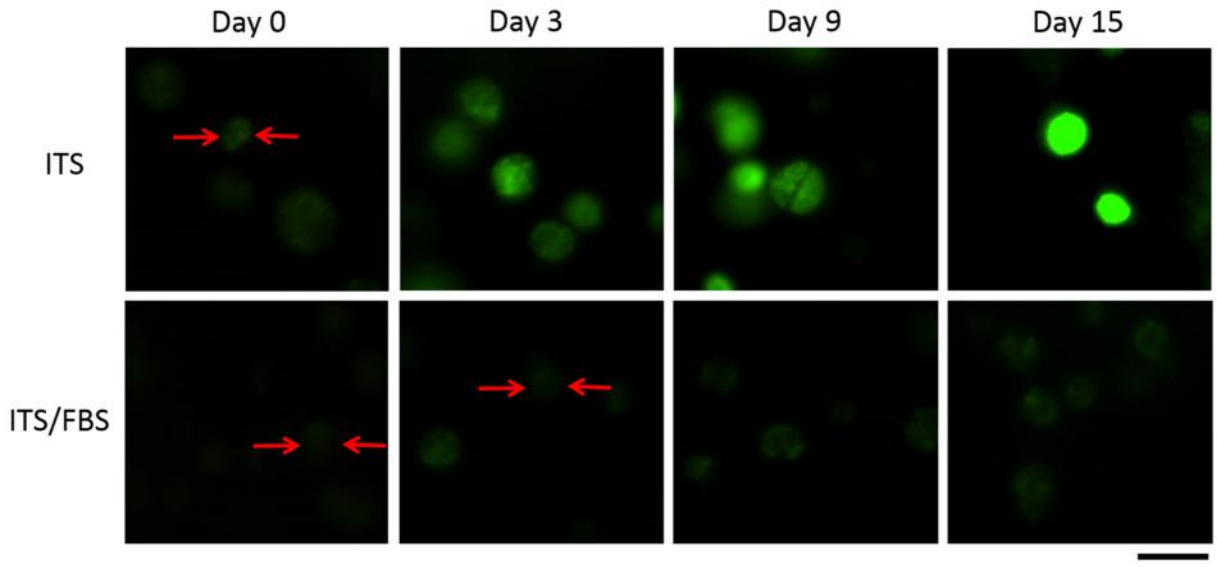

Figure 3.3 CellRox staining for intracellular reactive oxygen species in ITS or ITS/FBS medium. Fluorescent images were captured after 0, 3, 9, and 15 days of culture. Bar = 25 µm.

3.4.4 Cell viability in ITS, ITS/FBS, or FBS medium

Cell viability was evaluated after 7 and 15 days of culture. Each timepoint was statistically analyzed separately due to the absence of a control that would account for potential variability that may result from running the assay on different days. On day 7, the viable cell density in ITS (394 ± 35 CTB absorbance/mg wet weight) was not significant different than ITS/FBS (467 ± 100 CTB absorbance/mg wet weight, P=0.63, data not shown). On day 15, the viable cell density in ITS (302 ± 19 CTB absorbance/mg wet weight) was not significant different than ITS/FBS (348 ± 3 CTB absorbance/mg wet weight). For FBS samples, the viable cell density was 7.8-fold (50 ± 18 CTB absorbance/mg wet weight; P=0.01) and 3.7-fold (82 ± 27 CTB absorbance/mg wet weight; P=0.0004) lower than ITS cultures on day 7 and 15, respectively.

3.4.5 Extracellular matrix accumulation in ITS, ITS/FBS, or FBS medium

ECM accumulation was evaluated after 15 days of culture. When normalized to wet weight, mean GAG and hydroxyproline accumulation in ITS cultures was 1.8 and 0.22 µg/mg wet weight,

respectively (Figure 3.4A). In ITS/FBS cultures, GAG accumulation was not significantly different than ITS (P=0.99), while hydroxyproline was 83% higher than ITS cultures (P=0.0002). In samples cultured in FBS medium GAG and hydroxyproline accumulation were relatively low, approximately equivalent to 2% and 20% of that in ITS cultures, respectively (P=0.0016 and P=0.0001, respectively). Glycosaminoglycan and hydroxyproline accumulation in FBS samples were significantly lower than ITS/FBS cultures (P=0.0001 and P<0.0001, respectively). DNA was quantified from digests that were analyzed for ECM. For each sample, DNA normalized to wet weight was plotted against previous measures of cell viability (data not show). Correlation analysis resulted in a correlation coefficient of 0.92, which indicated that DNA is reflective of the viable cell content. Normalizing ECM accumulation to DNA did not change the outcome of the statistical analysis, with ECM accumulation in FBS cultures approximately 25% of ITS or ITS/FBS (Figure 3.4B).

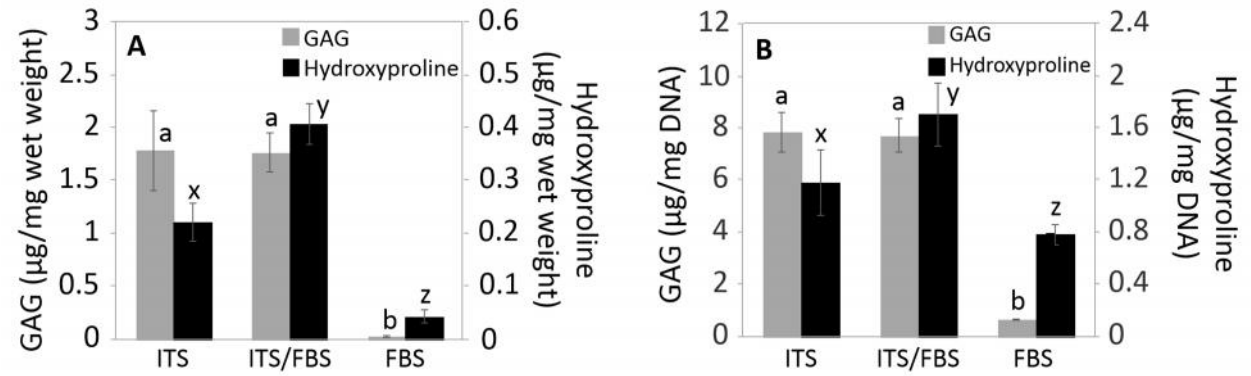

Figure 3.4 Glycosaminoglycan and hydroxyproline accumulation in ITS, ITS/FBS, or FBS medium after 15 days of culture. (A) GAG and hydroxyproline normalized to wet weight; (B) GAG and hydroxyproline normalized to DNA. Data are mean ± SEM, n=4 donor animals. For each assay, different letters denote significant differences (P<0.05).

3.4.6 Protein carbonylation in ITS or ITS/FBS medium

Samples were analyzed after 15 days of culture. *GAG and hydroxyproline content of NaCl extraction:* For MSC-seeded agarose cultured in ITS/FBS medium, the GAG and hydroxyproline content in NaCl extracts were 0.175 ± 0.027 and 0.0081 ± 0.0007 µg/µg total protein, respectively. For adult cartilage, the GAG and hydroxyproline content in NaCl extracts were 0.226 ± 0.027 and 0.0064 ± 0.0009 µg/µg total protein, respectively. These data indicate that the concentration of the major components of cartilage ECM were comparable between chondrogenic culture and native cartilage. *Carbonyl content*: In chondrogenic MSCs cultures, the carbonyl content was not significantly different between ITS and ITS/FBS medium (P=0.72, Figure 3.5). Protein carbonylation in articular cartilage extract was approximately 3.45-fold less than chondrogenic MSCs cultures (P=0.0002 and P<0.0001).

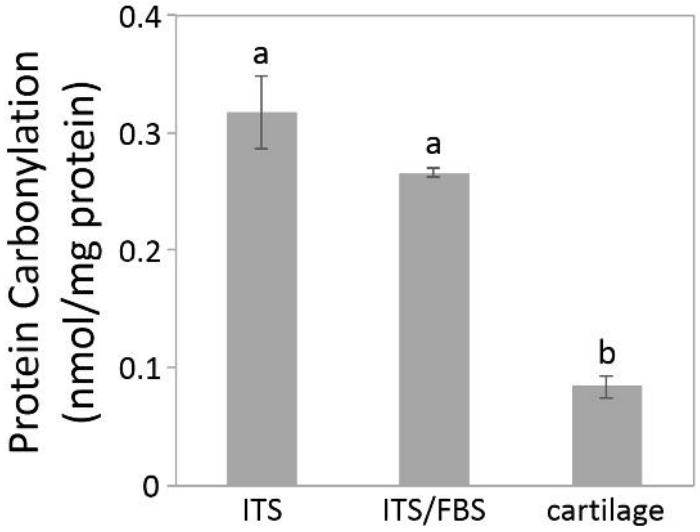

Figure 3.5 Extracellular protein carbonylation in ITS or ITS/FBS medium after 15 days of culture. Adult equine articular cartilage was analyzed in parallel as a control. Data are mean ± SEM, n=4 donor animals. Different letters denote significant differences (P<0.05).

3.4.7 Histological staining in ITS or ITS/FBS medium

Samples were analyzed after 15 days of culture. Toluidine blue (Figure 3.6A) and type II collagen staining (Figure 3.6B) was present in all samples. Relatively intense staining was localized to pericellular spaces. Interterritorial staining for type II collagen appeared to be higher for ITS/FBS cultures.

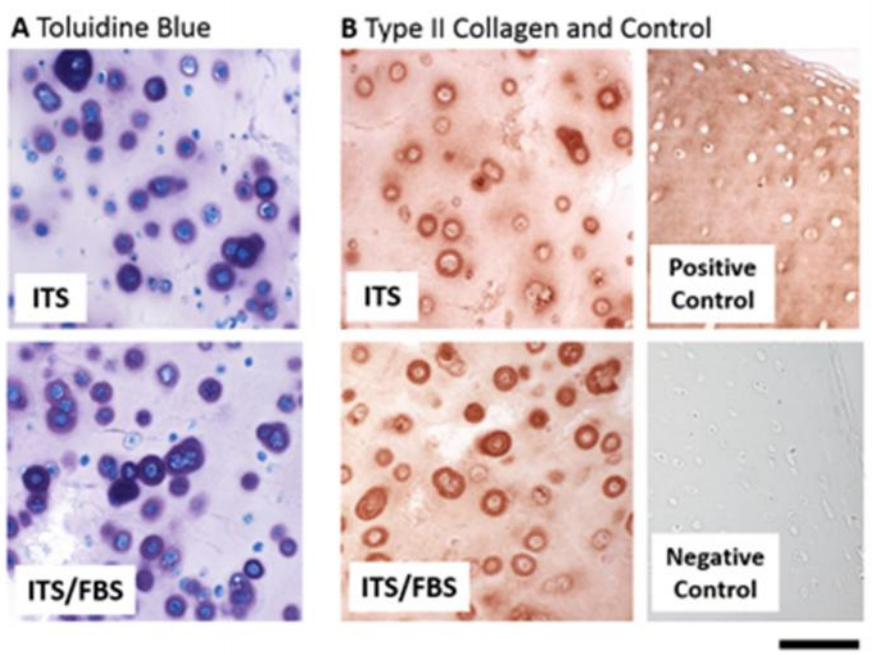

Figure 3.6 Histological staining for samples cultured in ITS or ITS/FBS for 15 days. (A) Toluidine blue; (B) Type II collagen immunohistochemistry. Adult equine articular cartilage was used for a control. Bar = 100 μm.

3.4.8 Gene expression in ITS or ITS/FBS medium

Gene expression was analyzed after 15 days of culture. Type I (P=0.12) and X collagen (P=0.18), and MMP13 (P=0.36) expression were not significantly different between ITS and ITS/FBS cultures (data not shown). Aggrecan and Type II collagen expression in samples cultured in ITS/FBS medium was 3.1-fold (P=0.04) and 2.8-fold (P=0.01) lower than ITS samples, respectively (data not shown).

3.4.9 Extracellular matrix accumulation in medium supplemented with adult equine serum

Extracellular matrix accumulation was evaluated after 15 days of culture. Cell viability and ECM accumulation in chondrogenic medium supplemented with ITS and allogeneic adult equine serum were compared to ITS and ITS/FBS culture. Viability was not significantly different among culture conditions (P=0.7, data not shown). GAG accumulation was not significantly different among culture conditions (P=0.34, Figure 3.7). Hydroxyproline accumulation in adult serum cultures was 45% higher than ITS (P=0.0006), and 15% lower (P=0.014) than ITS/FBS (Figure 3.7).

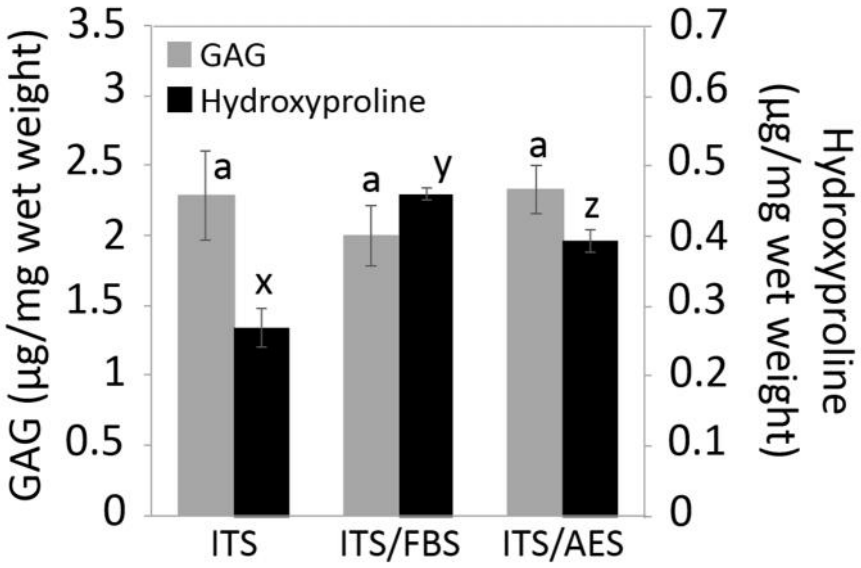

Figure 3.7 Glycosaminoglycan and hydroxyproline accumulation in ITS or ITS/FBS medium, or medium supplemented with ITS and 5% adult equine serum (ITS/AES). Data are mean ± SEM, n=4 donor animals. For each assay, different letters denote significant differences (P<0.05).

3.5 Discussion

Serum-free medium has been widely used to induce MSCs chondrogenesis in vitro, resulting in robust secretion of GAG- and type II collagen-rich ECM. In this study, such chondrogenesis was associated with early increases of intracellular ROS that persisted over 15

days of culture, which is consistent with temporal patterns reported for chondrogenesis of ATDC5 cells.[11] In addition, increasing ROS may explain previous reports of increased prostaglandin E2 (PGE2) secretion with progression of chondrogenesis,[26,27] given that a relationship between ROS and PGE2 has been demonstrated in chondrocytes.[28] The reduction in glutathione (Figure 3.2A) during early MSCs chondrogenesis suggests that increases in intracellular ROS are at least in part due to downregulation of endogenous antioxidants, which has been previously reported for MSCs chondrogenesis and superoxide dismutase.[29] Moderate levels of ROS can act as important secondary messengers that influence stem cell survival, migration, and differentiation.[30] For example, ROS-generating NADPH oxidase that supports chondrogenesis[11] has been associated with the PI3K pathway,[31] which has also been shown to support chondrogenesis.[32,33] Therefore, elevated levels of ROS may play an important role in MSCs chondrogenesis.

Given that the striking increase in intracellular ROS with chondrogenesis suggests the potential for oxidative stress, we investigated the effect of decreasing ROS by supplementing the medium with FBS. In this study, FBS proved to be an effective antioxidant based on the increased ratio of reduced-to-oxidized glutathione during early chondrogenesis (Figure 3.2B), and lowering of intracellular ROS relative to serum-free culture (Figure 3.3). The biological significance of the reduction in ROS with FBS here is supported by experiments using different cell types in which 2-5-fold changes in ROS with serum-starvation were associated with induction of apoptosis[14,16,34] or autophagy[35]. For chondrogenesis, the most significant effect of FBS was a ~80% increase in collagen accumulation without a concomitant increase in proteoglycan content (Figure 3.4), which is consistent with reports that ROS can differentially affect the synthesis of collagen and proteoglycans.[36] Otherwise, the reduction in ROS with FBS did not greatly affect most chondrogenic assays. Differences in intracellular ROS did not translate to extracellular oxidative

damage (Figure 3.5), although intracellular ROS may not predict extracellular oxidative damage as redox modulation can differ between intra- and extra-cellular spaces.[37] Taken together, these data indicate that MSCs are largely effective in managing the oxidative environment associated with chondrogenesis.

An important aspect of our experimental design was that FBS was added to chondrogenic medium containing ITS. This approach differs from early exploration of serum as a stand-alone basal supplement for chondrogenic medium, which for bone marrow MSCs failed to support stable formation of pellet cultures.[1] For synovium-derived MSCs seeded in alginate, serum-supplementation alone resulted in moderately lower type II collagen and aggrecan gene expression related to serum-free, ITS supplemented medium.[38] These results were consistent with low cell viability and ECM accumulation in FBS medium here. Taken together it appears that ITS, and perhaps specifically insulin,[39,40] plays a critical role in supporting MSCs chondrogenesis. Fetal bovine serum has been investigated in combination with ITS for synovium-derived MSCs in pellet culture, where it moderately decreased ECM accumulation, and lowered type II collagen gene expression and immunohistochemical staining.[41,42] These results may differ from the current study based on the different tissue source for MSCs, or the use of pellet instead of hydrogel cultures.

MSC chondrogenesis may be particular vulnerable to extracellular oxidative stress as differentiation has been reported to coincide with decreases in superoxide dismutase gene and protein expression,[29] which has been shown to be downregulated by TGF-β.[43] This potential is supported by the finding that protein carbonylation in chondrogenic MSCs hydrogels was significantly higher than age-matched articular cartilage (Figure 3.5). Elevated oxidative damage has been associated with pathological changes in mature tissue such as intervertebral disc fibrocartilage with aging,[19] or heart valves with disease.[44] However, additional information is

needed to determine whether the extent to which oxidative damage is increased in chondrogenic MSCs is sufficient to compromise cartilage repair.

For the culture of MSCs for clinical applications, the use of FBS has raised concerns about an immune response induced by xenogenic materials. Therefore, while increasing collagen accumulation with FBS supplementation of chondrogenic medium is compelling, the use of species-matched or autologous serum in place of FBS would alleviate a major concern. For chondrocytes, adult serum has been reported to be equivalent or superior to FBS for expansion, redifferentiation, and ECM accumulation in three dimensional culture.[45,46] Here, collagen accumulation more closely resembled bovine cartilage explant cultures in which proteoglycan synthesis increased among serum-free, adult serum, and FBS cultures.[47] While adult serum did not maximize collagen accumulation, the 45% increase over serum-free culture (Figure 3.7) may be considered advantageous compared to the further modest gains supported by FBS. Additional studies are needed to more rigorously characterize the effect of adult serum on chondrogenesis, including potential variability among individuals. In addition, it is possible that concentrations of serum that are lower than the 5% used in this study may be equally or more effective in supporting chondrogenesis.[46,48,49]

In conclusion, this study identified temporal changes in the oxidative environment during MSCs chondrogenesis, and the potential benefit of adding antioxidants to existing methods of inducing differentiation. Further, the evaluation of serum as an antioxidant here may be particularly important for tissue engineering strategies that seek to induce chondrogenesis in vivo as serum is a component of synovial fluid. For these results there are several limitations to consider. Given that serum-starvation may have a broad effect across cell functions,[13] the use of antioxidants may more precisely correlate the influence of ROS on chondrogenesis. We evaluated

young adult equine MSCs based on the use of 2-5 year-old horses for animal models of cartilage resurfacing. However, in humans the need for cartilage healing is prevalent through mid-adulthood, and older cells may be less capable of tolerating the oxidative environment during chondrogenesis due to a loss of antioxidant capacity with aging.[50-52] Chondrogenic cultures were maintained in ambient air, which is particularly relevant to oxidative stress as ambient oxygen is hyperoxic for bone marrow MSCs,[53] and may promote the generation of ROS and oxidative damage as demonstrated in murine monolayer culture.[54] Reduced oxygen has been shown to enhance ECM accumulation in chondrogenic MSCs cultures,[55,56] and based on the current study the positive effect of low oxygen may be in part due to reduced levels of ROS. In this case, it is possible that adding serum in low oxygen chondrogenic culture would lower the production of ROS below a critical level for supporting chondrogenesis, as observed for antioxidant treatment of ATDC5 cultures.[11] While extracellular oxidative damage may compromise the repair potential of chondrogenic MSCs, a more comprehensive analysis is needed to better understand the degree to which components of the ECM are affected, and the effect of such oxidative modifications on the maturation of neo-cartilage in vivo. Such studies would better define the potential impact of modulating ROS to improve the use of MSCs to heal cartilage defects.

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CHAPTER 4: AMMONIUM PYRROLIDINEDITHIOCARBAMATE INCREASES GAG CONTENT BUT DECREASES MECHANICAL PROPERTY OF SERUM-SUPPLEMENT MESENCHYMAL STEM CELL CHONDROGENESIS[3]

4.1 Summary

Mesenchymal stem cells (MSCs) experience an increase in intracellular reactive oxygen species (ROS) with chondrogenesis in vitro, although the extent to which elevated levels of ROS are detrimental to chondrogenic MSCs is not known. The objectives of this study were to evaluate the ability of antioxidants to reduce intracellular ROS during MSC chondrogenesis, and the effect on differentiation and maturation of cartilage-like extracellular matrix (ECM). Young adult equine MSCs were cultured in chondrogenic medium containing N-acetylcysteine (NAC), glutathione ethyl ester (GSH-EE) or pyrrolidine dithiocarbamate (PDTC). The antioxidants were primarily evaluated in the presence of 5% fetal bovine serum, although in some cases additional testing was conducted in serum-free medium in which baseline levels of ROS are relatively high. Intracellular ROS was quantified with up to 3 days in monolayer culture, while chondrogenesis was evaluated by seeding MSCs in agarose hydrogel and culturing for 15 days. In serum-supplemented cultures NAC transiently lowered the concentration of intracellular ROS, while in serum-free medium NAC acted as a pro-oxidant with time in culture. In both cases NAC suppressed ECM accumulation in agarose cultures. Similarly, GSH-EE was not an effective antioxidant in chondrogenic culture. In serum-supplemented culture PDTC reduced concentrations of intracellular ROS. In agarose, PDTC modestly enhanced ECM accumulation and lowered oxidative damage, but unexpectedly decreased compressive properties. In conclusion,

[3] Submitting as a peer-review manuscript to Free Radical Biology and Medicine.

supplementing the glutathione antioxidant system was not effective in lowering the production of ROS during MSC chondrogenesis, and was detrimental to the accumulation of cartilaginous ECM. PDTC demonstrated that reducing intracellular ROS during chondrogenesis can enhance ECM accumulation and lower oxidative damage. However, the concomitant reduction in compressive properties is a significant limitation as robust mechanical integrity is an important factor in the success of cell-seeded constructs for cartilage repair.

4.1 Introduction

The ability to readily isolate and culture-expand bone marrow mesenchymal stem cells (MSCs) has generated enthusiasm for the use of this multipotent cell type for regenerative medicine and tissue engineering. While the most frequent means of preparing MSCs for experimental therapies is limited to culture-expansion, ongoing efforts to optimize culture conditions or induce differentiation may significantly improve the repair potential of MSCs. For cartilage tissue engineering, it is anticipated that the use of MSCs would benefit from improvements upon current techniques for promoting chondrogenesis and the development of mechanically functional cartilage-like extracellular matrix (ECM).

A potential means to improve upon the cartilage repair potential of MSCs may be through modulation of reactive oxygen species (ROS). In bone marrow, MSCs reside in a niche that maintain an undifferentiated state[1,2] that is thought to minimize oxidative stress.[2] In culture, undifferentiated MSCs has been shown to have low level of intracellular ROS due to an effective ROS scavenging and high levels of total intracellular glutathione.[3] Mesenchymal stem cells undergo a shift in redox status during differentiation, and exhibit different redox profiles depending on the lineage differentiation pathway.[4-6] For chondrogenesis, progression of differentiation has been associated with downregulation of antioxidants and increased

concentrations of ROS. The importance of ROS during chondrogenesis was demonstrated in studies in which knockdown of NADPH oxidase, a major source or ROS, completely suppressed survival and differentiation of ATDC5 and primary chondrocytes.[7] Increases in ROS have been associated with stimulation of AKT and ERK signaling, leading to the enhanced expression of SOX-9, and type II collagen and proteoglycan accumulation.[7] However, overproduction of ROS may lead to suboptimal MSCs differentiation,[8] which was indicated for chondrogenesis as lowering intracellular ROS coincided with improved ECM accumulation. Therefore, moderate reductions in intracellular ROS during chondrogenesis may be an effective approach to maximizing the cartilage repair potential of MSCs.

Conventional laboratory methods for inducing MSC chondrogenesis involve culture in serum-free conditions, which has been associated with high levels of intracellular ROS. Consistent with these data, we demonstrated that adding serum to chondrogenic medium was sufficient to lower the accumulation of intracellular ROS, which enhanced accumulation of ECM. To further evaluate the potential to reduce ROS during chondrogenesis, the object of this study was to evaluate the effect of antioxidants on differentiation and maturation of cartilage-like ECM. Experiments were conducted using young adult equine MSCs cultured in monolayer or encapsulated in agarose hydrogel, a scaffold that supports robust chondrogenesis of equine MSCs.[9] Chondrogenesis was evaluated in the presence of three antioxidants, N-acetylcysteine (NAC), glutathione ethyl ester (GSHEE) or ammonium pyrrolidinedithiocarbamate (PDTC). In this study, we demonstrate that PDTC is the most effective antioxidant in chondrogenic culture, and is capable of enhancing ECM, but negatively impacts the mechanical functionality of the accumulated neo-cartilage.

4.2 Materials and methods

4.2.1 MSCs isolation and expansion

Bone marrow was aspirated from the iliac crest of 4 2-5 years old horses that were euthanized for reasons unrelated to this study. Colony-forming cultures were established to isolate the MSCs from the bone marrow,[9] after which the MSCs were seeded at 2 x 10^3 cells/cm^2 in tissue culture flasks in α-minimal essential medium, 10% fetal bovine serum, and 2 ng/ml fibroblast growth factor-basic (Peprotech, Rocky Hill, NJ) and cultured to 80% confluence over 4 days. The cells were expanded through a second passage prior to seeding in chondrogenic culture.

4.2.2 Agarose encapsulation and chondrogenic culture

Low melting agarose was dissolved in phosphate buffered solution and sterile filtered. Casting molds were created by transferring 2.4 ml of warm 1% (w/v) agarose to 35 mm petri dishes and cooled at room temperature to initiate gelation. Six mm diameter wells were created in the agarose using a biopsy punch. For compression testing, MSC-seeded agarose samples were cast in 3.2 mm thick, 6 mm diameter stainless steel molds, as previously described.[9] Culture-expanded MSCs were suspended in warm 1.5% (w/v) agarose gel at 12x10^6 cells/ml, which was transferred to the casting molds and then cooled at room temperature. Baseline chondrogenic medium (ITS) consisted of high-glucose Dulbecco modified Eagle medium supplement with 1% ITS+ Premix (BD Biosciences, Bedford, MA), 37.5μg/ml ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), 100 nM Dexamethasone (Sigma-aldrich, Saint Louis, MO), 5 ng/ml recombinant human transforming growth factor-β1 (Peprotech, Rocky Hill, NJ),[10] with our without 5% FBS. Cultures were supplemented with the antioxidants N-acetylcysteine (NAC; Sigma-Aldrich, Saint Louis, MO) or glutathione ethyl ester (GSHEE; Cayman *Chemical Company*, Ann Arbor, MI) or

ammonium pyrrolidinedithiocarbamate (PDTC; Sigma-Aldrich, Saint Louis, MO). In NAC cultures, the pH was adjusted to 7.4 by adding 2 ul sodium hydroxide/ml of medium.

4.2.3 Quantification of extracellular matrix accumulation

Following 15 days of chondrogenic culture, MSCs-seeded agarose samples were digested in proteinase K (Sigma-aldrich, Saint Louis, MO) in Tris HCl solution at 60°C overnight. Total accumulated sulfated glycosaminoglycan (GAG) and hydroxyproline were quantified by dimethylmethylene blue[11] and dimethylamino benzaldehyde dye[12] binding assays, respectively. Extracellular matrix accumulation data were normalized to the sample wet weight.

4.2.4 Quantitative measurement of ROS

MSCs were seeded in 48-well plates at $25x10^3$ cells/cm^2, and incubated overnight in expansion medium. The medium was changed to serum-free chondrogenic medium or chondrogenic medium plus 5% FBS, and cultured for 8 or 72 hours. The cells were labeled in serum-free chondrogenic medium containing 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich, Saint Louis, MO) for 30 minutes at 37ºC. Cultures were washed three times with phosphate buffered saline, and the cells were lysed using a solution of 50 mM Tris, 150 mM NaCl, and 1% Triton X-100. Cell lysates were analyzed at 490 nm (excitation) and 529 nm (emission) wavelengths on a microplate reader. Data were normalized to the total protein content of the cell lysates determined with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

4.2.5 RNA extraction and real-time PCR

MSC-seeded agarose samples were collected in TRIzol reagent® (Life Technologies, Grand Island, NY) and stored at -80°C. Samples were removed from TRIzol®, cooled in liquid nitrogen, and pulverized. Five hundred microliters of TRIzol® was first added to the pulverized samples, followed by chloroform at a ratio of 1:5 to the TRIzol®. Samples were centrifuged at

12,000g at 4°C for 15 minutes, and RNA was extracted from the aqueous phase using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with on-column genomic DNase (Qiagen, Valencia, CA). mRNA was reverse transcribed into cDNA using superscript® III first-strand synthesis system for RT-PCR (Thermo Fisher Scientific, Waltham, MA), and evaluated for aggrecan, type I, II, and X collagen using the Biorad CFX96^{TM} Real-Time PCR Detection System (Biorad, Hercules, CA). Relative gene expression levels were determined by semi-quantitative real time PCR using primers and SYBR Green. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The primer sequences are listed as follows: Type II collagen, forward primer AAACCATCAACGGTGGCTTCCA and reverse primer GCAATGCTGTTCTTGCAGTGGT; Type I collagen, forward primer ATTTCCGTGCCTGGCCCCATG and reverse primer GCCTTGGAAACCTTGGGGAC; Type X collagen; forward primer AGGCAACAGCATTACGACCCAAGA and reverse primer TGAAGCCTGATCCAGGTAGCCTTTG; GAPDH for ward primer AAGTGGATATTGT-CGCCATCAAT and reverse primer AACTTGCCATGGGTGGAATC.

4.2.6 Protein carbonyl content

Protein extraction – MSC-seeded agarose samples were pulverized, and then digested for 6 hours at 37ºC with 0.5 unit/ml of chondroitinase ABC (Sigma-Aldrich, Saint Louis, MO). Soluble proteins were extracted using 1 M NaCl according to the methods of Sharft et. al..[13] NaCl extraction solutions were diluted 6-fold with water, and carbonylation was quantified using a commercial kit (OxiSelect Protein Carbonyl Fluorometric Assay (Cell Biolabs, San Diego, CA)). Data were normalized to total protein content measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

4.2.7 Cell viability

MSC-encapsulated in agarose sample were evaluated for cell viability after 15 days of chondrogenic cultures using a commercial kit CellTiter-Blue® assay (Promega, Madison, WI). Sample were incubated in medium containing CellTiter-Blue at 37ºC for 1 hour and 15 min. The reaction was stopped by adding 3% SDS and incubating at room temperature for 15 minutes, and the medium was analyzed according to the manufacturer's instructions. Data were normalized to sample wet weight.

4.2.8 Compressive properties

Four mm diameter plugs were cored from MSC-seeded agarose samples that were cast in stainless steel molds. The equilibrium moduli of the plugs were measured in radially confined uniaxial compression using a Incudyne mechanical testing system.[14] A porous platen was used to apply 4 sequential 5% ramp-and-hold compressions to each plug from 10% to 30% strain. Each 5% compression was applied over 30 seconds, followed by 120 seconds of hold, resulting in an initial increase and subsequent relaxation of compressive stress. The ratio of the steady-state equilibrium stress to the engineering strain was used to compute the equilibrium modulus.

4.2.9 Immunohistochemistry and histology

Samples were fixed in 10% formalin for 48 hours, paraffin-embedded, sectioned, and mounted on slides. Sections were de-paraffinized and rehydrated prior to staining. *Type II collagen Immunohistochemical staining*: Samples were incubated with proteinase K (Sigma-Aldrich, Saint Louis, MO) at 37°C for 15 min, and then mouse anti-collagen type II IgG primary antibody using undiluted supernatant (Hybridoma Bank, Iowa City, IA) followed by donkey anti-mouse IgG secondary antibody conjugated with peroxidase at a 1:500 dilution (Jackson Immunoresearch, West Grove, PA). Antibody detection was performed using VECTOR® NovaRED™ (Vector

laboratories, Burlingame, CA). Additional sections were incubated with normal mouse serum at equal concentration to that of the primary antibody as a negative control. Equine cartilage was analyzed in parallel as a control. *Toluidine blue*: Sections were stained with 0.04% Toluidine blue solution (Electron microscopy sciences, Fort Washington, PA) to detect the accumulation of sulfated proteoglycans.

4.2.10 Statistical analysis:

Data were analyzed for paired t-test or analysis of variance with mixed model using animal as a random effect. Pairwise comparisons were analyzed using least squares means with Tukey-Kramer adjustment. P-value < 0.05 considered statistically significant difference. Statistical tests for paired t-test and analysis of variance were performed using GraphPad Prism 7.02 and SAS 9.3 software, respectively.

4.2.11 Experimental design

NAC was evaluated at a concentration of 5 mM, GSH-EE was assessed at 2 mM or 5 mM, while PDTC was tested at 10 µM PDTC. Preliminary testing indicated that higher doses of NAC or PDTC were at least partially cytotoxic (data not shown). NAC and GSH-EE were tested in presence or absence of serum, while PDTC was evaluated in serum-containing medium only. N-acetylcysteine and PDTC cultures were evaluated for cell viability and ECM accumulation. In addition, PDTC cultures were evaluated for protein carbonylation as a measure of oxidative damage and compressive properties.

4.3 Results

4.3.1 NAC in serum-supplemented medium – quantification of ROS, cell viability, and ECM accumulation.

Quantification of ROS – After 8 hours, DCFDA absorbance in NAC cultures was 40% of control cultures (Figure 4.1A). On day 3, NAC did not significant affect DCFDA absorbance (P=0.23). *Cell viability and ECM accumulation* – After 15 days of culture, viable cell density in NAC cultures was not significantly different from controls (P=0.77; Figure 4.1B). GAG accumulation in NAC cultures was 27% of controls (Figure 4.1C), while hydroxyproline was not significantly different (P=0.17).

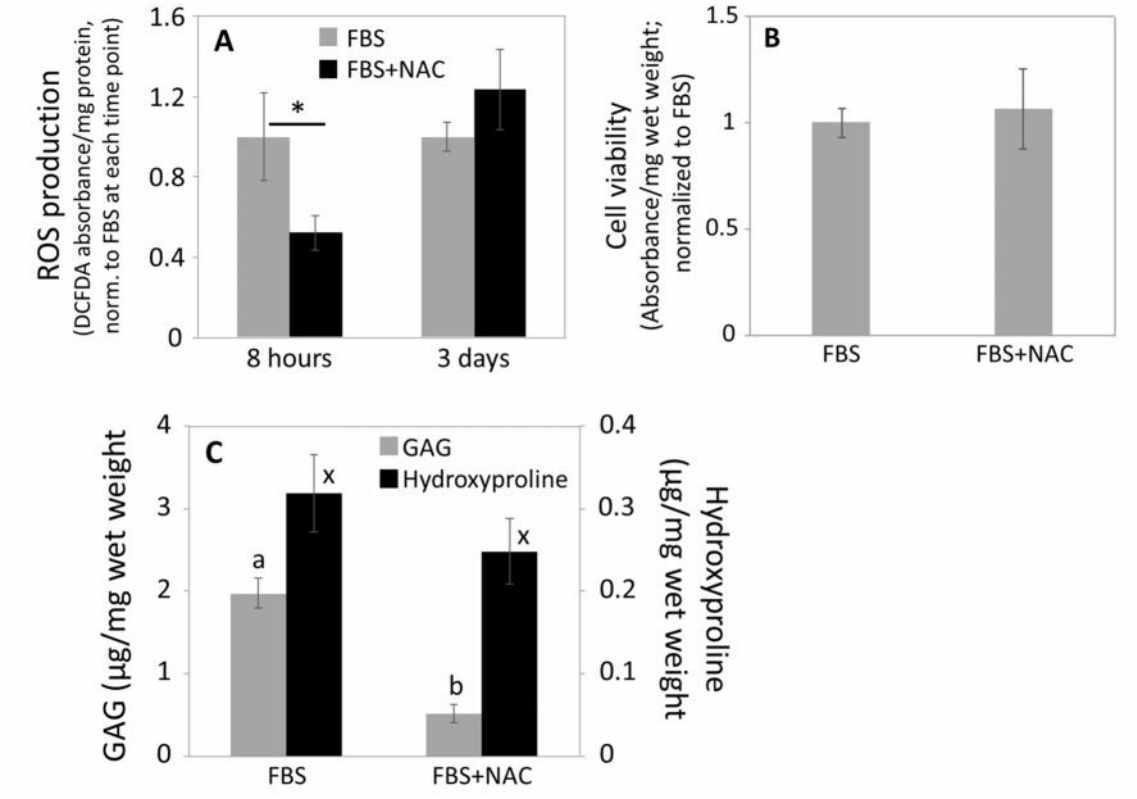

Figure 4.1 MSCs chondrogenic culture in serum-supplement medium (FBS) with 5 mM N-acetylcysteine (NAC). (A) Quantification of ROS by DCFDA after 8 hours and 3 days of culture; (B) Cell viability after 15 days of culture; (C) Glycosaminoglycan and hydroxyproline accumulation after 15 days of culture, different letters denote significant differences for each assay (P<0.05). Data are mean ± SEM, *P<0.05, n=4 donor animals.

4.3.2 NAC in serum-free medium – quantification of ROS, cell viability, and ECM accumulation.

Quantification of ROS – After 8 hours, NAC did not significant affect DCFDA absorbance (P=0.11; Figure 4.2A). On day 3, DCFDA absorbance in NAC cultures were 50% higher than controls. *Cell viability and ECM accumulation* – The viable cell density in NAC cultures was 55% of that in controls (Figure 4.2B). In serum-free culture ECM accumulation was severely suppressed with NAC treatment. GAG and hydroxyproline accumulation in NAC cultures was 3% and 27% of controls, respectively (Figure 4.2C).

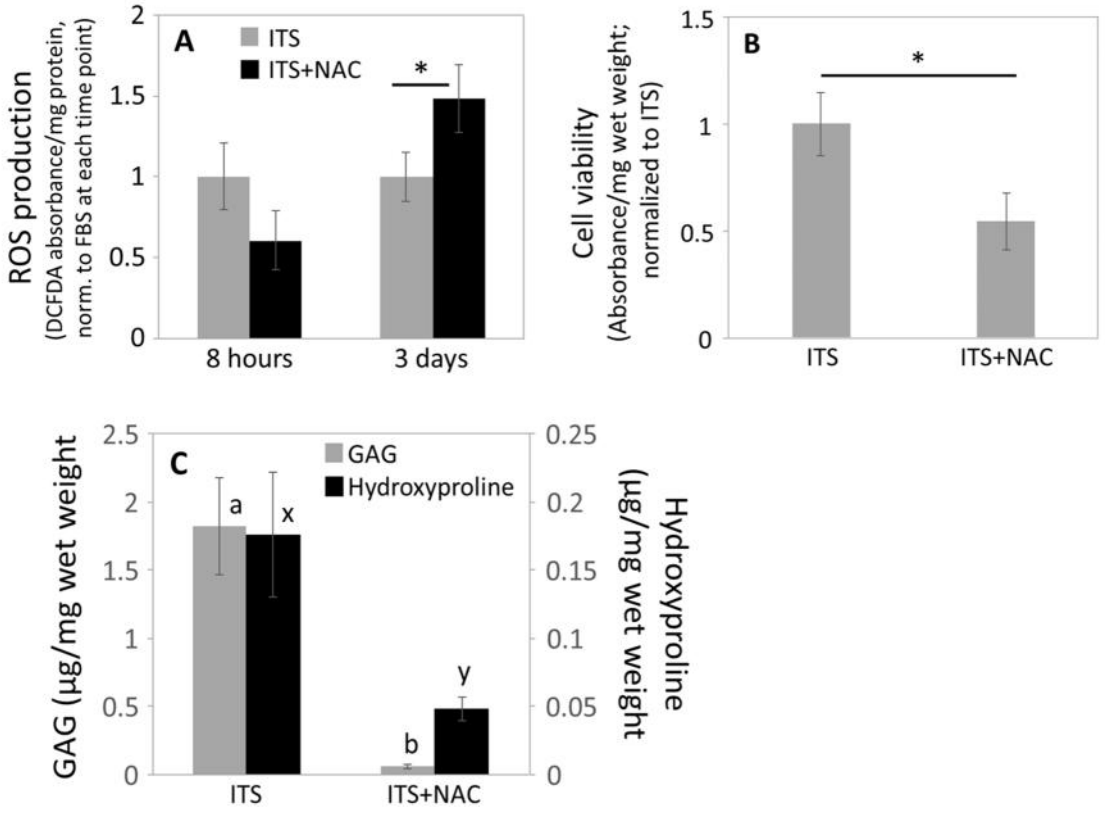

Figure 4.2 MSCs chondrogenic culture in serum-free medium (ITS) with 5 mM N-acetylcysteine (NAC). (A) Quantification of ROS by DCFDA after 8 hours and 3 days of culture; (B) Cell viability after 15 days of culture; (C) Glycosaminoglycan and hydroxyproline accumulation after 15 days of culture, different letters denote significant differences for each assay (P<0.05). Data are mean ± SEM, *P<0.05, n=4 donor animals.

4.3.3 NAC in serum-free or -supplemented medium

Gene expression – Gene expression was evaluated after 15 days for culture. Type X collagen expression in were not significantly different among cultures with or without NAC (Figure 4.3). There is no interaction between serum and NAC on type II collagen. When consider only the effect of NAC factor, NAC did not affect type II collagen in serum-supplement culture (P=0.57) while decrease by 2.7-fold in serum-free cultures. With significance in interaction between serum and NAC, expression of type I collagen was not different between serum-supplement and serum-free cultures (P=0.78). NAC increase type I collagen expression by 2.4-fold and 7-fold in serum-supplement and serum-free cultures, respectively.

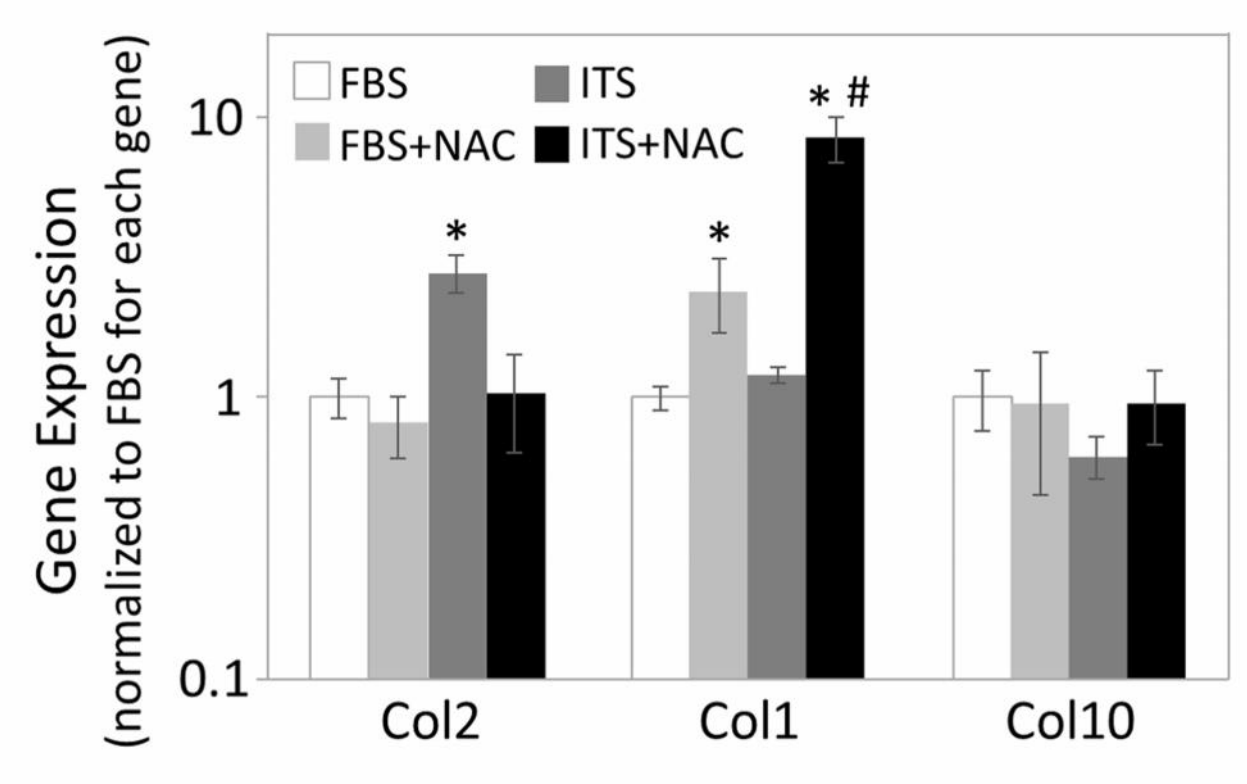

Figure 4.3 Gene expression after 15 days of MSCs chondrogenic culture in serum-supplement (FBS) and serum-free (ITS) medium with 5 mM N-acetylcysteine. Data are mean ± SEM, n=4 donor animals. *P<0.05, compared to FBS of each gene; #P<0.05, compared to FBS+NAC; +P<0.05; compare to ITS

4.3.4 Quantification of ROS for GSH-EE in serum-supplemented and serum-free medium.

8 hours of culture: In serum-free cultures, DCFDA absorbance in 2 mM and 5 mM GSH-EE was 30% of serum-free controls. In serum-supplemented medium, DCFDA absorbance in 2 mM and 5 mM GSH-EE was 25% and 43% of serum-supplemented controls, respectively. *3 days of culture*: In serum-free cultures, data for 2 mM and 5 mM GSH-EE are not reported due to the accumulation of cell debris in cultures and low levels of isolated total protein that indicate cell toxicity. In serum-supplemented cultures, supplementation with 2 mM or 5 mM GSH-EE did not significantly affect DCFDA absorbance (P=0.99).

4.3.5 PDTC in serum-supplemented medium – Quantification of ROS, cell viability, ECM accumulation, and protein carbonylation.

Quantification of ROS – After 8 hours or 3 days of culture, DCFDA staining in PDTC cultures were 63% or 73% of control cultures (Figure 4.4A). *Cell viability and ECM accumulation* – After 15 days of culture, the viable cell density in PDTC cultures was not significantly different from controls (P=0.24; Figure 4.4B). GAG accumulation in PDTC cultures was 20% higher than controls (Figure 4.4C), while hydroxyproline was not significantly different (P=0.33). In PDTC samples, protein carbonylation was 73% of that in control cultures (Figure 4.4D).

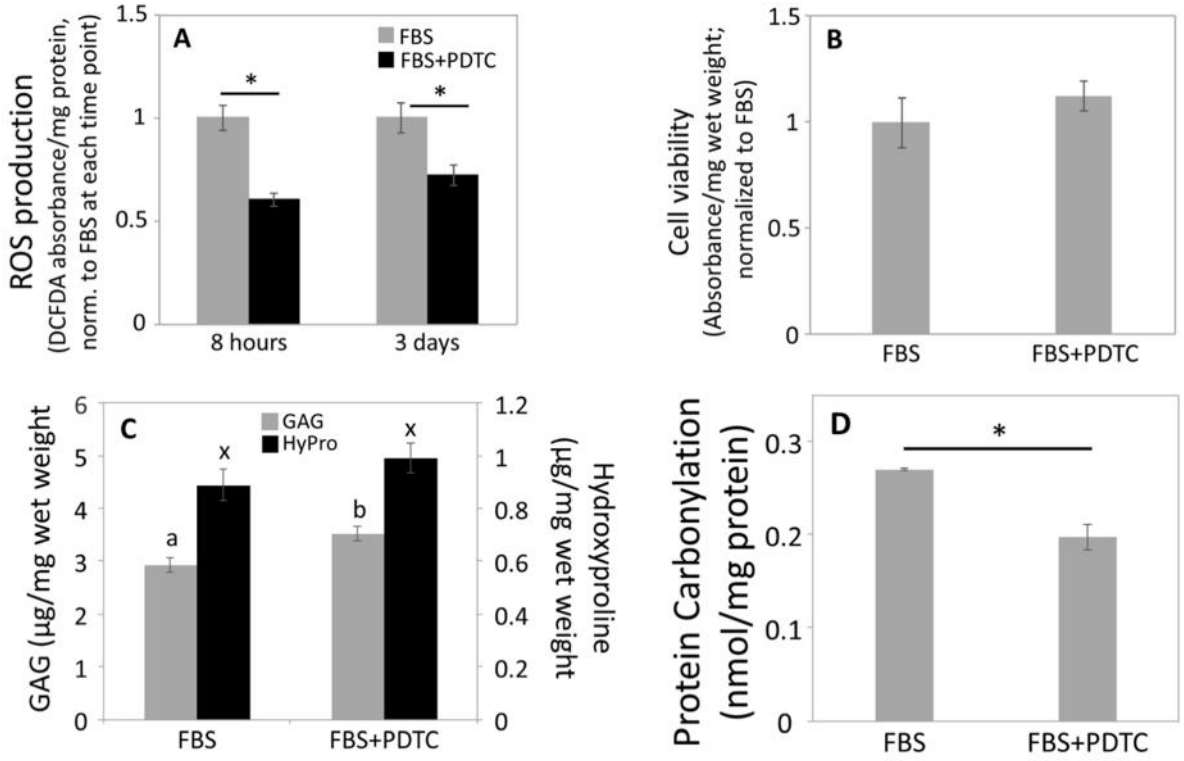

Figure 4.4 MSCs chondrogenic culture in serum-supplement medium (FBS) with 10 µM ammonium pyrrolidinedithiocarbamate (PDTC). (A) Quantification of ROS by DCFDA after 8 hours and 3 days of culture; (B) Cell viability after 15 days of culture; (C) Glycosaminoglycan and hydroxyproline (hypro) accumulation after 15 days of culture, different letters denote significant differences for each assay ($P<0.05$); (D) Extracellular protein carbonylation after 15 days of culture. Data are mean ± SEM, *$P<0.05$, n=4 donor animals.

4.3.6 Collagen gene expression and immunohistochemical staining for PDTC in serum-supplemented medium.

Samples were analyzed after 15 days of culture. *Gene expression* - Type II ($P=0.12$) and X ($P=0.93$) collagen expression were not significantly different between PDTC cultures and controls (Figure 4.5A). Type I collagen expression for PDTC cultures was 3-fold higher than FBS samples. *Immunohistochemical staining* – Type II collagen was present in PDTC and controls. Staining was most intense in pericellular spaces (Figure 4.5B).

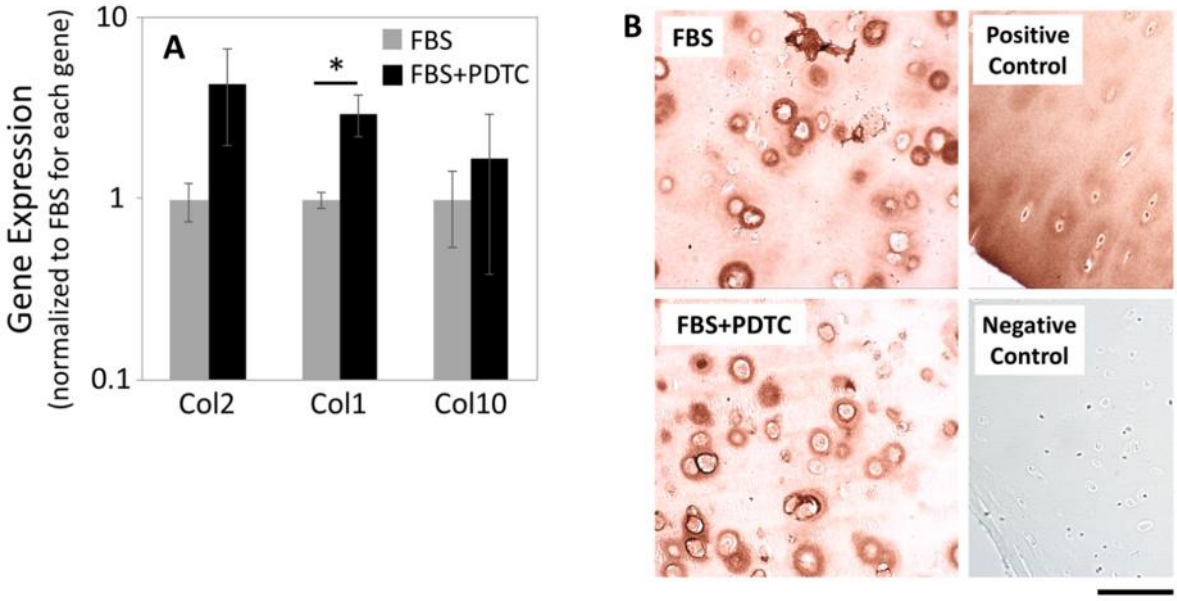

Figure 4.5 MSCs chondrogenic culture in serum-supplement medium (FBS) with 10 µM ammonium pyrrolidinedithiocarbamate (PDTC) after 15 days of culture. (A) Gene expression; (B) Representative sample of Type II collagen immunohistochemistry. Adult equine articular cartilage was used for a control. Bar = 100 µm.

4.3.7 Mechanical properties for PDTC in serum-supplemented medium.

Acellular samples created and evaluated on the day of testing resulted in an equilibrium modulus of approximate 3 kPa. The equilibrium modulus for samples cultured in PDTC was 7.3 kPa (Figure 4.6), which was approximately 60% of control cultures (12.4 kPa) ($P<0.01$).

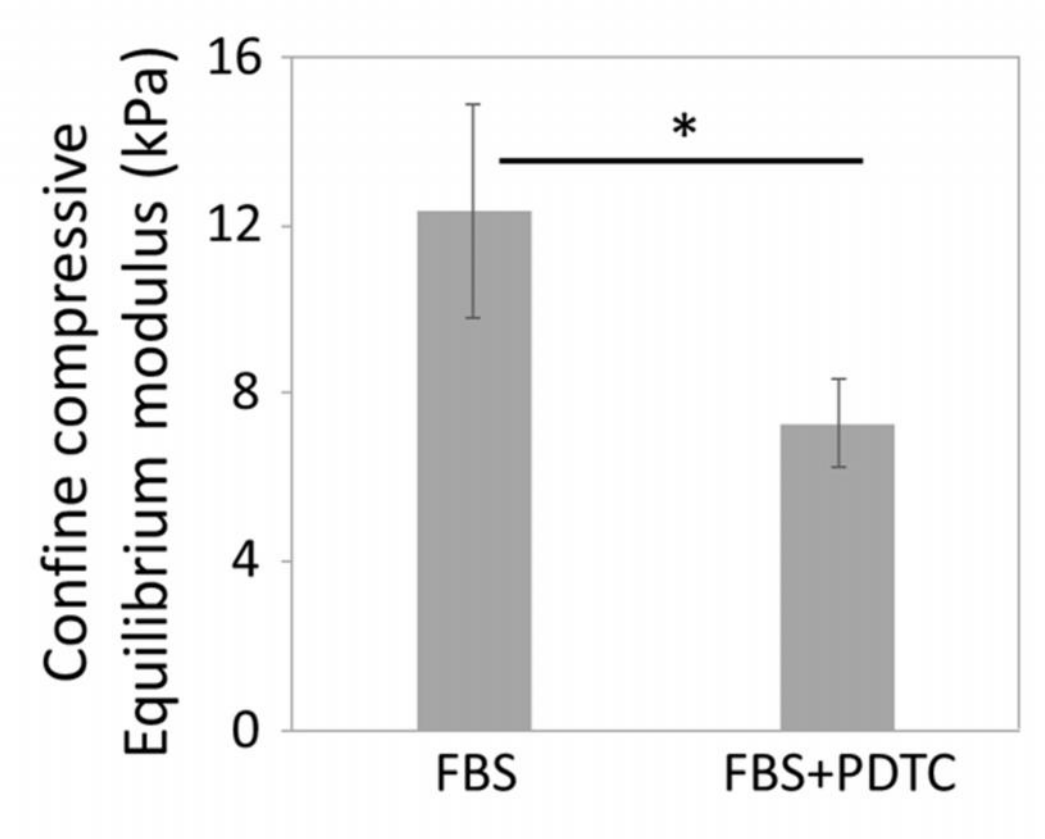

Figure 4.6 Mechanical properties of MSCs chondrogenic culture in serum-supplement medium (FBS) with 10 μM ammonium pyrrolidinedithiocarbamate (PDTC) after 15 days of culture. Data are mean ± SEM, *P<0.05, n=4 donor animals.

4.4 Discussion

As the first step in evaluating the effect of lowering the concentration of ROS during chondrogenesis we evaluated the commonly-used antioxidant NAC. N-acteylcysteine is a precursor of glutathione, which is the substrate of glutathione peroxidase and peroxiredoxin in oxidizing hydrogen peroxide. Supplementation of the glutathione system appeared to be particularly important for chondrogenesis as large decreases in endogenous glutathione have been reported with progression of differentiation. However, NAC did not suppress levels of intracellular ROS beyond 8 hours of chondrogenic culture, and further functioned as a pro-oxidant in the absence of serum. While the antioxidant properties of NAC have been extensively described,

presence of ROS can lead to auto-oxidization of NAC, thereby generating hydrogen peroxide.[15-17] This may be potentiated by the absence of serum, which has been shown to promote auto-oxidation.[18]

Intracellular ROS were quantified in chondrogenic monolayer cultures, which we previously reported to be generally consistent with qualitative staining of MSCs in agarose during early chondrogenesis. However, the limited ROS analysis in the current study did not encompass additional temporal changes in intracellular ROS that may occur during multiweek culture, which is conventionally performed prior to evaluation of chondrogenesis. Extracellular matrix accumulation and cell viabilities in agarose cultures suggests the potential that NAC largely functioned as a pro-oxidant in chondrogenic conditions. For example, in the presence of serum, NAC decreased GAG but not hydroxyproline accumulation. Such differential regulation of GAG and hydroxyproline was previously reported when intracellular ROS was evaluated as a function of serum-supplementation. In serum-free culture, severe suppression of ECM accumulation is consistent with high levels of ROS in chondrocyte culture, while loss of viability is consistent with ROS-induced apoptosis.[19,20]

A possible explanation for the limited antioxidant properties of NAC may be the presence of TGF-β, which can be a potent inhibitor of glutathione synthesis.[21] To address this possibility, we evaluated a cell permeable derivative of glutathione. However, similar to NAC, GSH-EE was cytotoxic in serum-free medium, or did not reduce intracellular ROS with three days of culture. Therefore, these data indicate that modulation of the glutathione system is not an effective means of reducing intracellular ROS during chondrogenesis.

Unlike NAC, PDTC was an effective antioxidant in chondrogenic conditions as indicated by the moderate reductions in ROS during early chondrogenesis, and lower extracellular protein

carbonylation in agarose samples. While PDTC stimulated a ~20% increase in GAG accumulation, this improvement was modest compared to the 80% increase in hydroxyproline accumulation previously reported for serum-supplementation. Further, the increase in GAG accumulation with PDTC coincided with an unexpected decrease in compressive properties. A possible explanation for the low mechanical properties with PDTC treatment is that the reduction in ROS decreased expression of the collagen crosslinking enzyme lysyl oxidase (LOX),[22,23] which has been shown to play an important role in crosslinking in cartilage tissue engineering.[24] Taken together, these data suggest that suppression of ROS in serum-supplemented culture does not benefit the maturation of ECM secreted by chondrogenic MSCs. To address this problem, the application of exogenous LOX might be an alternative improvement of mechanical property of MSCs chondrogenesis. Studies indicated that exogenous LOX promote collagen cross-linking, improve the tensile properties and enhanced integration between native-to-engineering cartilage.[22,25]

Despite the differences in ECM accumulation among NAC, PDTC, and control cultures, gene expression of type II collagen was largely similar. Given that type II collagen is anticipated to increase approximately 10,000-fold with multiweek chondrogenic culture, the effects of NAC and PDTC appear to be related to the synthesis and assembly of ECM and not chondrogenic differentiation per se. For example, in NAC cultures reduced ECM accumulation may have resulted from high levels of ROS that suppressed synthesis and/or enhanced degradation, as reported for chondrocyte culture. In particular, ROS itself can degrade proteoglycans and collagen.[26-28] For PDTC it is possible that improved GAG accumulation resulted from inhibition of NF-kB,[29] which has been implicated in pro-inflammatory loss of chondrogenesis and ECM accumulation.[29,30] However, the suppression of NF-kB in conventional serum-free culture did not affect MSCs chondrogenesis,[30] which suggests that NF-kB inhibiting properties of PDTC did not

influence the findings of this study. Similarly, modest to no changes in type I or X collagen expression indicated that antioxidants did not strongly promote or inhibit fibrocartilaginous or hypertrophic differentiation. In particular, elevated levels of ROS with chondrogenesis have been associated with hypertrophy, although it appears that simple suppression of ROS is not sufficient to obtain a more articular cartilage-like phenotype.

In conclusion, this study characterized the effect of altering the oxidative environment during chondrogenesis with commonly-used antioxidants in research and medicine. The findings for NAC and GSH-EE lend caution that compounds generally regarded as antioxidants may not performs as such in chondrogenic culture, especially in conventional medium that lacks serum. PDTC demonstrated the ability to reduce ROS during early chondrogenesis and improve the quantity of GAG accumulation. Further, the reduction in oxidative damage with PDTC may improve the functionality and durability of the secreted ECM. However, such advantages gained by antioxidant treatment may require methods to ensure the mechanical functionality of the ECM. Further exploration of these conclusions would greatly benefit from a more extensive temporal analysis of intracellular ROS to determine the exact influence of antioxidants over multiweek culture, and how cellular responses may change over time accordingly.

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CHAPTER 5: SUMMARY

Bone marrow-derived mesenchymal stem cell (MSCs) have been investigated for cartilage tissue engineering based on an ability to undergo chondrogenesis and secrete cartilaginous extracellular matrix (ECM). Based on these properties, MSC-seeded graft may be used to replace lost or degraded hyaline cartilage, and restore a smooth, lubricated surface to provide low friction articulation and facilitate the transmission of loads to the underlying subchondral bone. This dissertation research focuses on modulating inflammation and oxidative environment during MSC chondrogenesis to better understand the biology of MSC chondrogenesis and provide guidelines for improving cartilage tissue engineering.

Dexamethasone is glucocorticoid that is used in combination with TGF-β to induce MSC chondrogenesis in vitro and in vivo. Conventionally, induction of chondrogenesis in vitro involves multiweek exposure to 100 nM dexamethasone, which may be difficult to translate in vivo. Our result indicated that 1 nM dexamethasone is sufficient to support MSC chondrogenesis, although higher concentrations of dexamethasone may be necessary to suppress undesirable hypertrophic differentiation as shown by type X collagen and MMP13 expression. Given the anti-inflammatory property of dexamethasone, we investigated the effect of dexamethasone concentration on prostaglandin E2 (PGE2), which is secreted by MSCs in an inflammatory environment. In medium containing 1 nM dexamethasone, PGE2 secretion was significantly higher than cultures containing 100 nM dexamethasone. However, complete suppression of PGE2 secretion using a COX-2 inhibitor did not affect MSC chondrogenesis, thereby indicating that PGE2 secretion is a by-product of MSC chondrogenesis. Timing exposure of dexamethasone introduce in MSCS

chondrogenic cultures suggested that early exposure to dexamethasone was not critical, while sustained exposure of at least a week appears to be necessary to maximize ECM accumulation.

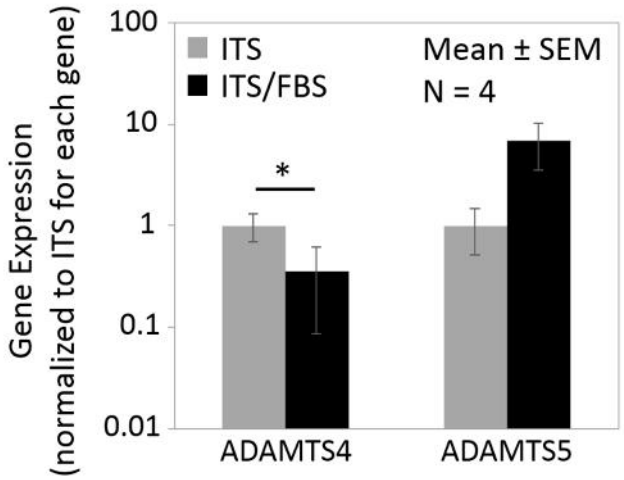
To further investigate the inflammatory response leading to upregulation of PGE2 secretion we investigated the oxidative environment during progression of MSC chondrogenesis. Conventionally, MSC chondrogenesis is induced in serum-free medium, which has been shown to stimulate the production of reactive oxygen species (ROS). We confirmed that intracellular ROS increased with time in serum-free chondrogenic culture, and concentrations of the antioxidant glutathione decreased. Supplementing chondrogenic medium with fetal bovine serum (FBS) lowered intracellular ROS, which coincide with increase in collagen accumulation. However, serum-supplement did not affect ECM protein carbonylation, the indicator of protein oxidative damage. Adult serum was also capable of stimulating collagen synthesis over serum-free cultures, which is important as the use of xenogeneic materials is a longstanding concern for human tissue engineering strategies. In summary, this study characterized changes in the oxidative environment during MSC chondrogenesis, and suggested that lowering ROS may be an effective approach to increase collagen accumulation.

Given that elevated levels of intracellular ROS may adversely affect extracellular matrix synthesis by chondrogenic MSCs, next we evaluated whether antioxidants could further improve extracellular matrix accumulation. In this study, we evaluated three antioxidants, N-acetylcysteine (NAC), glutathione ethyl ester (GSHEE), or ammonium pyrrolidinedithiocarbamate (PDTC). We found that NAC and GSHEE were not effective antioxidants in serum-supplemented chondrogenic medium, while in serum-free medium NAC was a pro-oxidant. Further, NAC decreased extracellular matrix accumulation in chondrogenic cultures. PDTC proved to be an effective antioxidant as indicated by decreased concentrations of intracellular ROS and extracellular

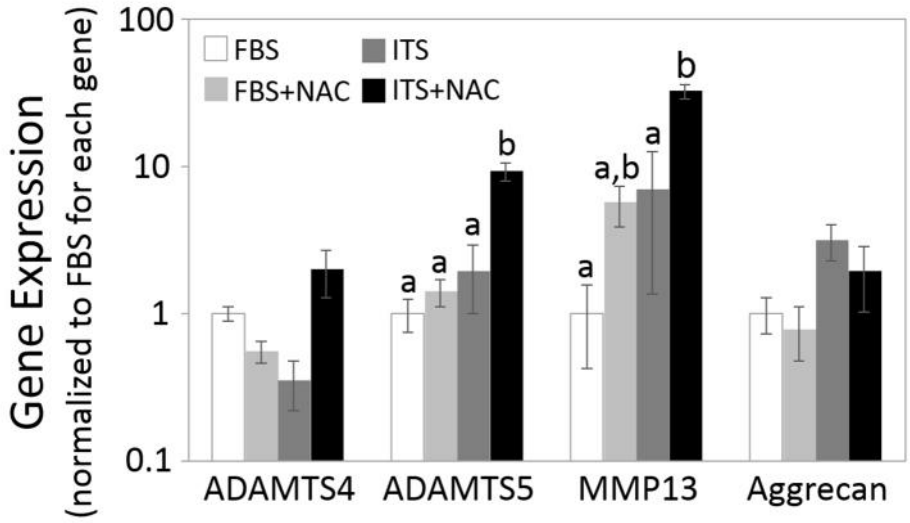
oxidative damage. In addition, PDTC increased GAG accumulation. However, PDTC unexpectedly decrease the mechanical properties of MSC-seeded constructs. In summary, this study indicated that lowering intracellular ROS can improve ECM accumulation in cultures of chondrogenic MSCs, but the translation value of antioxidant therapies for cartilage tissue engineering may be severely reduced if the mechanical integrity of secreted neo-tissue is compromised.

The research described in this dissertation provides new information regarding the biology of MSCs chondrogenesis and the involvement of inflammation and ROS in the maturation of extracellular matrix in constructs seeded with chondrogenic MSCs. We discovered new parameters for the use of dexamethasone for supporting robust chondrogenesis, and demonstrated the effects of modulating the oxidative environment. As cartilage tissue engineering is still a challenge field that will require ongoing innovation to achieve the goal of full-thickness cartilage repair, this laboratory research can be used as a basis for predifferentiation of MSCs, or induction of chondrogenesis after implantation, in both animal and human patients.

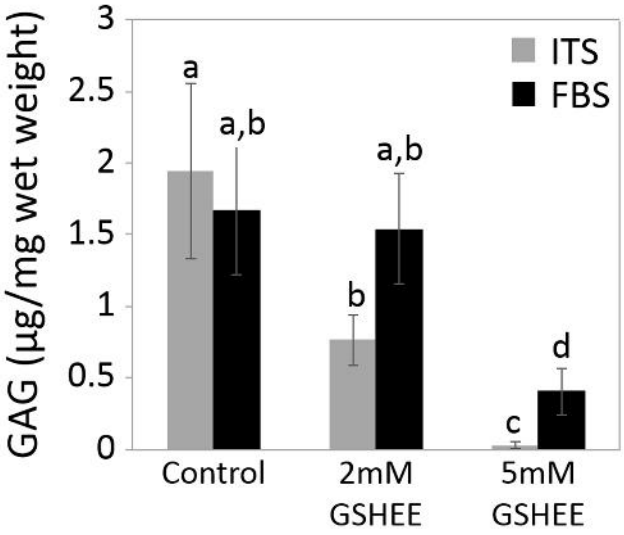
APPENDIX



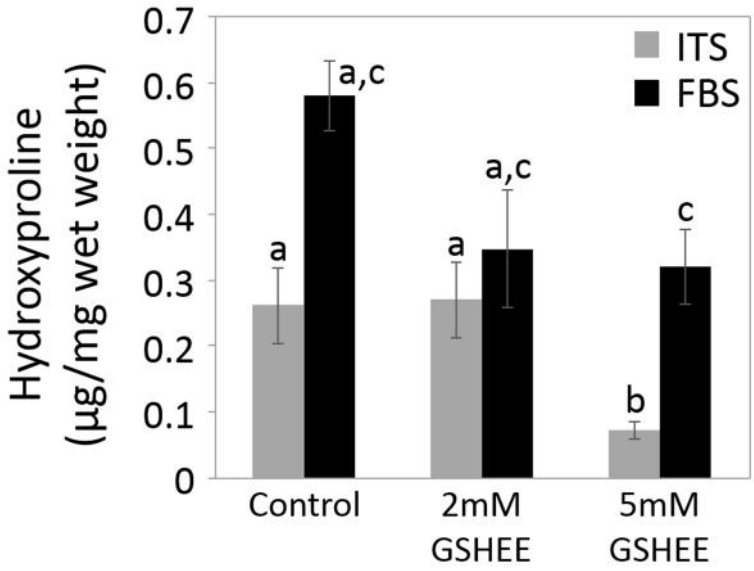
Appendix figure 1 Gene expression of ADAMTS4 and ADAMTS5 after 15 days of MSCs chondrogenic culture in serum-free (ITS) and serum-supplement (ITS/FBS) medium. Data are mean ± SEM, n=4 donor animals, *P<0.05.



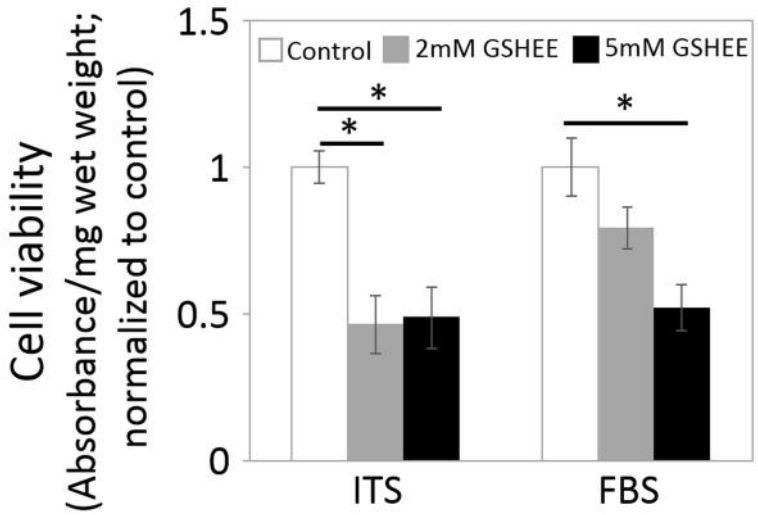
Appendix figure 2 Gene expression of ADAMTS4, ADAMTS5, MMP13, and aggrecan after 15 days of MSCs chondrogenic culture in serum-supplement (FBS) and serum-free (ITS) medium with 5 mM N-acetylcysteine. Data are mean ± SEM, n=4 donor animals, different letters denote significant differences for each assay (P<0.05).



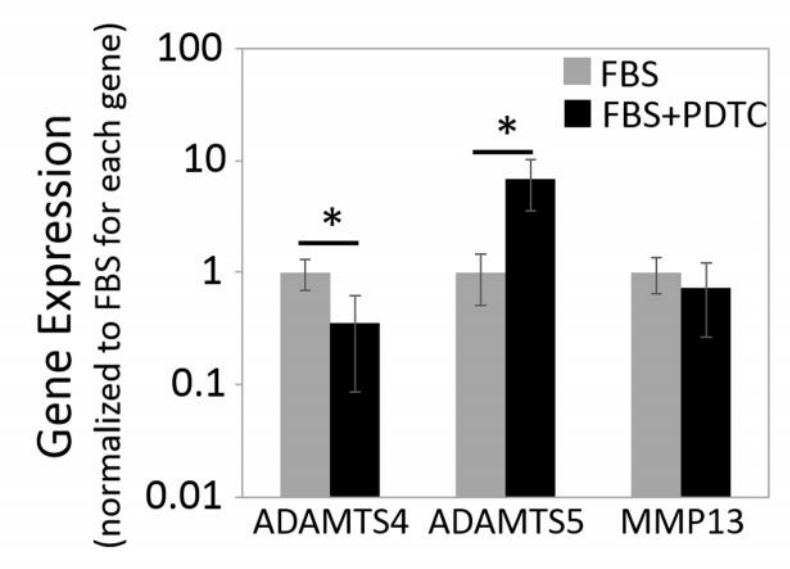
Appendix figure 3 Glycosaminoglycan accumulation after 15 days of MSCs chondrogenic culture in serum-free (ITS) and serum-supplement medium (FBS) with 2 or 5 mM glutathione ethyl ester (GSHEE). Data are mean ± SEM, n=4 donor animals, different letters denote significant differences (P<0.05).



Appendix figure 4 Hydroxyproline accumulation after 15 days of MSCs chondrogenic culture in serum-free (ITS) and serum-supplement medium (FBS) with 2 or 5 mM glutathione ethyl ester (GSHEE). Data are mean ± SEM, n=4 donor animals, different letters denote significant differences (P<0.05).



Appendix figure 5 Cell viability after 15 days of MSCs chondrogenic culture in serum-free (ITS) and serum-supplement medium (FBS) with 2 or 5 mM glutathione ethyl ester (GSHEE). Data are mean ± SEM, n=4 donor animals, different letters denote significant differences (P<0.05).



Appendix figure 6 Gene expression of ADAMTS4, ADAMTS5, and MMP13 after 15 days of MSCs chondrogenic culture in serum-supplement (FBS) medium with 10 µM ammonium pyrrolidinedithiocarbamate (PDTC). Data are mean ± SEM, n=4 donor animals, *P<0.05.