Differential Effects of the Antioxidants *N*-Acetylcysteine and Pyrrolidine Dithiocarbamate on Mesenchymal Stem Cell Chondrogenesis

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Abstract

Introduction—Mesenchymal stem cell (MSC) chondrogenesis is associated with increases in intracellular reactive oxygen species (ROS), which may result in oxidative stress that is detrimental to cartilage regeneration. This study evaluated the ability of the antioxidants *N*-acetylcysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) to reduce intracellular ROS, and their effect on MSC chondrogenesis and maturation of cartilage-like extracellular matrix.

Methods—Equine bone marrow MSCs were cultured in serum-supplemented chondrogenic medium with or without NAC or PDTC. ROS was quantified in monolayer after 8 and 72 h of culture. MSCs were seeded into agarose, cultured for 15 days, and analyzed for viable cell density, glycosaminoglycan (GAG) and hydroxyproline accumulation, and collagen gene expression. PDTC cultures were evaluated for oxidative damage by protein carbonylation, and mechanical properties *via* compressive testing.

Results—NAC significantly lowered levels of ROS after 8 but not 72 h, and suppressed GAG accumulation (70%). In secondary experiments using serum-free medium, NAC significantly increased levels of ROS at 72 h, and lowered cell viability and extracellular matrix accumulation. PDTC significantly reduced levels of ROS (~30%) and protein carbonylation (27%), and enhanced GAG accumulation (20%). However, the compressive modulus for PDTC-treated samples was significantly lower (40%) than controls. Gene expression was largely unaffected by the antioxidants.

Conclusions—NAC demonstrated a limited ability to reduce intracellular ROS in chondrogenic culture, and generally suppressed accumulation of extracellular matrix. Conversely, PDTC was an effective antioxidant that enhanced GAG accumulation, although the concomitant reduction in com-

pressive properties is a significant limitation for cartilage repair.

Keywords—Chondrogenesis, Mesenchymal stem cells, Reactive oxygen species, Antioxidant, *N*-Acetylcysteine, Pyrrolidine dithiocarbamate.

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 tissue engineering and regenerative medicine. While populations of MSCs may be prepared using basic cell culture techniques, culture conditions may be modified to enhance the repair potential of MSCs.^{7,32} For cartilage tissue engineering, it is anticipated that MSCs would benefit from improved techniques for promoting robust chondrogenesis and the development of mechanically functional cartilage-like extracellular matrix (ECM).^{25,37,39}

A potential means to improve the cartilage repair potential of MSCs may be through inducing chondrogenesis in the presence of antioxidants. While undifferentiated MSCs in expansion culture maintain a low level of intracellular ROS,⁴³ MSCs undergo a shift in redox status during differentiation, and exhibit different redox profiles depending on the lineage of differentiation.^{5,47,48} For chondrogenesis, progression of differentiation has been associated with downregulation of antioxidants and increased concentrations of intracellular ROS.^{16,40} Elevated levels of ROS may negatively affect the ability of chondrogenic MSCs to secrete repair cartilage. For example, in limb bud cultures depleting endogenous glutathione suppressed the accumulation of cartilaginous ECM.¹³ For synovio-cyte-derived MSCs, upregulation expression of

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antioxidant genes was associated with enhanced chondrogenesis.³¹ Further, the negative effects of oxidative stress on cartilage homeostasis have been well-documented.¹⁴ Therefore, loss of antioxidant capacity may be a limiting factor in the ability of MSCs to regenerate articular cartilage.

Oxidative stress is considered to be a contributing factor in osteoarthritis²² and many other diseases,¹¹ which has led to the investigation of antioxidant therapies. Antioxidants may be readily applied to cartilage tissue engineering strategies involving *ex vivo* culture of cell-seeded constructs. Or, antioxidants may be incorporated into delivery systems designed to localize drugs to cartilage defects.²⁰ Therefore, the objectives of this study were to evaluate the ability of antioxidant supplementation to reduce intracellular ROS, and to determine the effect of antioxidants on MSC chondrogenesis and maturation of cartilaginous 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.¹⁸ Primary studies were conducted with the antioxidants *N*-acetylcysteine (NAC) or ammonium pyrrolidine dithiocarbamate (PDTC), while limited testing was performed with glutathione ethyl ester (GSH-EE). The results of this study demonstrated that antioxidant treatment in the presence of serum can enhance ECM accumulation, but may negatively impact the mechanical functionality of the accumulated neo-cartilage.

MATERIALS AND METHODS

MSC Isolation and Expansion

Bone marrow was aspirated from the iliac crest of 4 2–5 years old horses that were euthanized using an Institutional Animal Care and Use Committee-approved protocol, for reasons unrelated to this study. Isolation and expansion of equine MSCs was conducted as previously described.¹⁷ Colony-forming cultures were established to isolate the MSCs from the bone marrow, after which the MSCs were seeded at 2×10^3 cells/cm² in tissue culture flasks in α -minimal essential medium (Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences, Chicago, IL), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA) and 2 ng/mL fibroblast growth factor-basic (Peprotech, Rocky Hill, NJ). Monolayer cultures proliferated to ~80% confluence over 4 days, and were then expanded through a second passage prior to seeding in chondrogenic culture.

Encapsulation of MSCs in Agarose and Chondrogenic Culture

MSCs were seeded into agarose hydrogel as previously described.¹⁷ Low melting agarose was dissolved in phosphate buffered saline 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.¹⁸ Culture-expanded MSCs were suspended in warm 1.5% (w/v) agarose gel at 12×10^6 cells/mL, which was transferred to the casting molds and then cooled at room temperature. Baseline chondrogenic medium consisted of high-glucose Dulbecco modified Eagle medium supplement with 1% ITS + Premix (BD Biosciences, Bedford, MA), 10 mM HEPES, 0.1 mM non-essential amino acid solution (Thermo Fisher Scientific, Waltham, MA), 37.5 μ g/mL ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), 100 nM Dexamethasone (Sigma-Aldrich, Saint Louis, MO), 5 ng/mL recombinant human transforming growth factor- β 1 (Peprotech, Rocky Hill, NJ).¹⁵ Cultures were supplemented with the antioxidants *N*-acetylcysteine (NAC; Sigma-Aldrich, Saint Louis, MO), glutathione ethyl ester (GSH-EE; Cayman Chemical Company, Ann Arbor, MI) or ammonium pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich, Saint Louis, MO). In NAC cultures, the pH was adjusted to 7.4 by supplementing the medium with 2 μ M sodium hydroxide.

Quantification of Intracellular ROS

MSCs were seeded in 48-well plates at 25×10^3 cells/cm² and incubated overnight in expansion medium. The medium was changed to chondrogenic medium, with or without 5% FBS, and cultured for 8 or 72 h. The cells were labeled in serum-free chondrogenic medium containing 10 μ M 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich, Saint Louis, MO) for 30 min at 37 °C. 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).

Cell Viability

MSC-seeded agarose samples were evaluated for cell viability using a commercial kit CellTiter-Blue® assay (CTB; Promega, Madison, WI), as previously described.⁴¹ Samples were incubated in medium containing CellTiter-Blue at 37 °C for 1 h and 15 min. The reaction was stopped by adding 3% SDS and incubating at room temperature for 15 min, and the medium was analyzed according to the manufacturer's instructions. Data were normalized to sample wet weight.

Quantification of Extracellular Matrix Accumulation

MSCs-seeded agarose samples were digested in proteinase K (Sigma-Aldrich, Saint Louis, MO) in 50 mM Tris HCl, 1 mM CaCl₂ solution at 60 °C overnight. Total accumulated sulfated glycosaminoglycan (GAG) and hydroxyproline were quantified by dimethylmethylene blue⁹ and dimethylamino benzaldehyde³⁸ dye binding assays, respectively. Extracellular matrix accumulation data were normalized to the sample wet weight or CTB absorbance.

Immunohistochemical Staining for Type II Collagen

MSC-seeded agarose samples were fixed in 10% formalin for 48 h, paraffin-embedded, sectioned, and mounted on slides. Sections were de-paraffinized and rehydrated, and then incubated with proteinase K (Sigma-Aldrich, Saint Louis, MO) 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.

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 12,000 × g at 4 °C for 15 min, 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™ 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 house-keeping gene. The primer sequences are listed as follows: Type II collagen (Col2A1), forward primer AAACCATCAACGGTGGCTTCCA and reverse primer GCAATGCTGTTCTTGCACTGGT; Type I collagen (Col1A1), forward primer ATTCCG TGCCTGGCCCCATG and reverse primer GCCT TGGAAACCTTGGGGAC; Type X collagen (Col10A1); forward primer AGGCAACAGCATT ACGACCCAAGA and reverse primer TGAAGC CTGATCCAGGTAGCCTTTG; GAPDH forward primer AAGTGGATATTGTCGCCATCAAT and reverse primer AACTTGCCATGGGTGGAATC.

Protein Carbonyl Content

MSC-seeded agarose samples were pulverized, and then digested for 6 h at 37 °C 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.*³⁵ 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).

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 an Incudyne mechanical testing system.¹⁰ 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 s, followed by 120 s of hold, resulting in an initial increase and subsequent relaxation of compressive stress. Steady-state equilibrium stress was plotted against engineering strain, and the slope of a linear fit was used to obtain the equilibrium modulus.

Statistical Analysis

Data were evaluated for normality using the Shapiro–Wilk test. Data showing a normal distribution were analyzed for paired *t* test or analysis of variance with mixed model using the donor animal ($n = 4$) as a random effect. Pairwise comparisons were analyzed using least squares means with Tukey–Kramer adjustment. Data showing a non-normal distribution were analyzed using the Wilcoxon matched-pairs signed rank test. *P* values less than 0.05 were considered significantly different. Statistical tests for normal distribution, non-parametric testing, and paired *t*-tests were performed using GraphPad Prism 7.02. Analysis of variance was performed using SAS 9.3 software. Data are presented as mean $\pm$ SEM.

Experimental Design

NAC was evaluated at a concentration of 5 mM, which was previously shown to lower the production of ROS during chondrogenesis of ATDC5 cells.¹⁶ Further, low millimolar concentration of NAC have been shown to decrease nitric oxide-induced generation of ROS,³⁰ and interleukin 1 beta-induced expression of matrix metalloproteinase 1,²⁴ in chondrocyte culture. GSH-EE was assessed at 2 or 5 mM. PDTC was tested at 10 μ M. Previously, PDTC has been used in micromolar concentrations *in vitro* to block interleukin-1 beta suppression of Sox9 expression in chondrocytes,²⁹ inhibited growth plate chondrocyte proliferation and differentiation,⁴⁶ and reduce nitrite release from osteoarthritic cartilage.¹ Primary experiments were conducted in culture medium containing 5% FBS. While conventional laboratory methods for inducing MSC chondrogenesis involve culture in serum-free medium,¹⁵ previously we demonstrated that adding serum to chondrogenic medium lowered concentrations of intracellular ROS without suppressing chondrogenesis for adult equine MSC.⁴¹ Therefore, in the current study we largely focused on serum-supplemented medium to lower the potential for oxidative stress. NAC and GSH-EE were also tested in serum-free culture to further explore the effect of each agent on chondrogenesis and/or the concentration of intracellular ROS and cell viability. Quantification of ROS was performed after 8 h or 3 days of culture, while agarose samples were analyzed after 15 days of culture.

RESULTS

N-Acetylcysteine in Serum-Supplemented Medium

Quantification of ROS After 8 h, DCFDA absorbance in NAC cultures was 40% of control cultures (Fig. 1). On day 3, NAC did not significantly affect DCFDA absorbance ($p = 0.23$). **Cell viability and ECM accumulation in agarose** After 15 days of culture, CTB absorbance in NAC cultures was not significantly different from controls ($p = 0.77$; Fig. 2a). When normalized to wet weight, GAG accumulation in NAC cultures was 27% of controls, while hydroxyproline was not significantly different ($p = 0.17$) (Fig. 2b). When normalized to CTB absorbance, GAG accumulation in NAC cultures was 26% of controls, while hydroxyproline was not significantly different ($p = 0.41$) (Fig. 2c).

N-Acetylcysteine in Serum-Free Medium

Quantification of ROS After 8 h, NAC did not significantly affect DCFDA absorbance ($p = 0.11$; Fig. 3). On day 3, DCFDA absorbance in NAC cultures was 50% higher than controls. **Cell viability and ECM accumulation in agarose** After 15 days of culture, CTB absorbance in NAC cultures was 55% of controls ($p < 0.05$, Fig. 4a). Extracellular matrix accumulation was severely suppressed with NAC treatment. When normalized to wet weight, GAG and hydroxyproline accumulation in NAC cultures was 3% and 27% of controls, respectively (Fig. 4b). When normalized to CTB absorbance, GAG and hydroxyproline accumulation were 5% and 47% of controls (Fig. 4c).

FIGURE 1. DCFDA absorbance for 5 mM NAC in serum-supplemented medium. For each timepoint data are normalized to the mean value in control cultures (= 1). Statistical significance is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 2. Cell viability and extracellular matrix accumulation for 5 mM NAC in serum-supplemented medium after 15 days of culture. (a) CellTiter Blue absorbance; (b) Glycosaminoglycan and hydroxyproline accumulation normalized to wet weight; (c) Glycosaminoglycan and hydroxyproline accumulation normalized to CTB absorbance. For each assay statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 3. DCFDA absorbance for 5 mM NAC in serum-free medium. For each timepoint data are normalized to the mean value in control cultures (= 1). Statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

Collagen Gene Expression for N-acetylcysteine in Serum-Free or -Supplemented Medium

The effect of NAC on gene expression of collagens was evaluated in an experiment in which samples were cultured concurrently in the presence or absence of serum (Fig. 5). For Col2A1, interactions between NAC and serum were not significantly different ($p = 0.09$). When considering the main effect of NAC independent of serum, Col2A1 expression in control cultures was approximate 2-fold higher than NAC cultures ($p < 0.05$). For Col1A1, expression was not significantly different between serum-free and serum-supplemented controls ($p = 0.12$). In NAC cultures, Col1A1 expression was 7.0- and 2.4-fold higher than serum-free and serum-supplemented controls, respectively ($p < 0.05$). For Col10A1, neither the interactions between NAC and serum ($p = 0.17$) nor the main effect of NAC ($p = 0.82$) were significant.

Quantification of ROS for Glutathione Ethyl Ester in Serum-Supplemented and Serum-Free Medium

Serum-supplemented medium After 8 h of culture, DCFDA absorbance in 2 and 5 mM GSH-EE was ~ 70% of serum-supplemented controls, respectively ($p < 0.05$, Fig. 6a). On day 3, 2 mM ($p = 0.09$) or 5 mM GSH-EE ($p = 0.40$) did not significantly lower DCFDA absorbance relative to controls. **Serum-free medium** After 8 h of culture, DCFDA absorbance in 2 mM GSH-EE was not significantly different from controls ($p = 0.07$), while 5 mM GSH-EE reduced DCFDA absorbance 42% relative of controls ($p < 0.05$, Fig. 6b). For day 3, data are not reported due to the accumulation of cell debris in cultures and low levels of extracted total protein (35% lower than controls, $p < 0.005$, data not shown) that indicated cytotoxicity. To further confirm the cytotoxicity of GSH-EE in serum-free culture, MSC-seeded agarose was cultured in 2 or 5 mM GSH-EE for 7 days. CTB absorbance normalized to wet weight for 2 mM (201 ± 36 CTB absorbance/mg wet weight) or 5 mM GSH-EE (146 ± 21) was 31 or 50% of that in serum-free controls (290 ± 28) ($p < 0.05$, data not shown).

Pyrrolidine Dithiocarbamate in Serum-Supplemented Medium

Quantification of ROS After 8 h or 3 days of culture, DCFDA absorbance in PDTC cultures were 63 or 73% of controls (Fig. 7). **Cell viability, ECM accumulation and protein carbonylation in agarose** After 15 days of culture, CTB absorbance in PDTC cultures was not significantly different from controls ($p = 0.24$; Fig. 8a). When normalized to wet weight, GAG accumulation in PDTC cultures was 20% higher than controls (Fig. 8b), while hydroxyproline was not significantly different ($p = 0.33$). When normalized to

FIGURE 4. Cell viability and extracellular matrix accumulation for 5 mM NAC in serum-free medium after 15 days of culture. (a) CellTiter Blue absorbance; (b) Glycosaminoglycan and hydroxyproline accumulation normalized to wet weight; (c) Glycosaminoglycan and hydroxyproline accumulation normalized to CTB absorbance. For each assay statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 5. Collagen gene expression for 5 mM NAC in serum-supplemented (FBS) and serum-free (SF) medium after 15 days of culture. For each gene statistical significance ($p < 0.05$) is denoted by different letters. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 6. DCFDA absorbance for GSH-EE in serum-supplemented (FBS) and serum-free (SF) medium. For each timepoint data are normalized to the mean value in control cultures (= 1). On day 3, data are not reported for GSH-EE in serum-free medium due to cytotoxicity. Statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

CTB absorbance, ECM accumulation in PDTC cultures were not different from controls (GAG: $p = 0.76$ and hydroxyproline; $p = 0.41$; Fig. 8c). Protein carbonylation in PDTC samples (0.20 ± 0.014 nmol/mg protein) was 74% of that in control (0.27 ± 0.002 nmol/mg protein) ($p < 0.05$, data not shown).

Collagen Gene Expression and Immunohistochemical Staining for Pyrrolidine Dithiocarbamate in Serum-Supplemented Medium

Samples were analyzed after 15 days of culture. Gene expression Col2A1 ($p = 0.49$), Col1A1 ($p = 0.07$), and Col10A1 ($p = 0.95$) expression were not significantly different between PDTC cultures and controls (Fig. 9a). Immunohistochemical staining Type II collagen was present in PDTC and FBS controls (Fig. 9b). Staining was most intense in pericellular spaces.

Confined Compressive Properties for Pyrrolidine Dithiocarbamate in Serum-Supplemented Medium

On the day of testing two acellular samples were created and evaluated to establish a baseline reference for the agarose gel alone. Equilibrium moduli for acellular samples were 3.5 and 3.9 kPa, resulting in a mean baseline of 3.7 kPa. For MSC-seeded samples cultured for 15 days, the equilibrium modulus for PDTC cultures was 7.3 kPa, which was approximately 60% of controls (12.4 kPa) ($p < 0.01$, Fig. 10).

DISCUSSION

As a precursor of glutathione, NAC is a commonly-used antioxidant³³ that was anticipated to counteract the reduction in endogenous glutathione with progression of chondrogenesis.^{16,40} However, NAC did not suppress levels of intracellular ROS beyond 8 h of chondrogenic culture, and by day 3 functioned as a pro-oxidant in the absence of serum. While the antioxidant properties of NAC have been extensively documented, conditions that promote auto-oxidation of thiols can result in the generation of hydrogen peroxide.²⁶ In particular, serum-free culture has been reported to increase the pro-oxidant behavior of NAC,⁶ which is consistent with the current study. The potential influence of serum-free culture on NAC auto-oxidation may be related to higher levels of ROS, which can potentiate auto-oxidation.⁶ To further support this possibility, the shift from anti- to pro-oxidant

properties of NAC over the first 3 days of serum-free chondrogenic culture coincided with previous reports of early temporal increases in ROS during chondrogenesis.^{16,40} Therefore, it appears possible that changes in the oxidative environment with chondrogenesis in serum-free culture promoted pro-oxidant behavior of NAC.

Previously, we reported that quantification of DCFDA absorbance in monolayer after 24 h of chondrogenic culture was consistent with qualitative ROS staining of MSCs in agarose. However, in the current study DCFDA staining was not analyzed beyond day 3 as 3D culture is conventionally used to induce MSC chondrogenesis, and it was not known if monolayer is reflective of MSC-seeded agarose over multiweek culture. However, several lines of evidence support the potential that NAC acted as a pro-oxidant during multiweek agarose culture. Increases in ROS during the initial days of chondrogenesis have been reported to be at least sustained with subsequent time in culture.^{16,40} Therefore, auto-oxidation of NAC due to high levels of ROS would be expected to persist, and possibly increase over multiweek culture. Elevated levels of ROS may be expected to decrease ECM accumulation, as seen in serum-free or serum-supplemented cultures. For example, ROS has been associated in the reduction of ECM in chondrogenic synovial MSC culture with exposure to pro-inflammatory cytokines,²³ and has been shown to decrease synthesis⁴ and increase degradation⁴² of cartilage proteoglycans. Also, rabbit limb bud cells exposure to the pro-oxidant buthionine sulfoximine suppressed proteoglycan staining in chondrogenic culture.¹³ Lastly, high levels of ROS has been associated with apoptosis,^{21,34} which would be consistent with the reduction in viability in serum-free, NAC cultures in which DCFDA staining was highest on day 3. For these reasons, it appears possible that

FIGURE 7. DCFDA absorbance for 10 μ M PDTC in serum-supplemented medium. For each timepoint data are normalized to the mean value in control cultures (= 1). Statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 8. Cell viability and extracellular matrix accumulation for 10 μ M PDTC in serum-supplemented medium after 15 days of culture. (a) CellTiter Blue absorbance; (b) Glycosaminoglycan and hydroxyproline accumulation normalized to wet weight; (c) Glycosaminoglycan and hydroxyproline accumulation normalized to CTB absorbance. For each assay statistical significance ($p < 0.05$) is denoted by “*”. Data are mean $\pm$ SEM, $n = 4$ animals.

FIGURE 9. Collagen expression for 10 μ M PDTC in serum-supplemented medium after 15 days of culture (a) Gene expression. Data are mean $\pm$ SEM, $n = 4$ animals; (b) Type II collagen immunohistochemistry. Adult equine articular cartilage was used for a control. Bar = 100 μ m.

FIGURE 10. Confined compressive equilibrium modulus for 10 μ M PDTC in serum-supplemented medium after 15 days of culture. The modulus of acellular controls (3.7 kPa) is indicated by the dashed line. The modulus of PDTC samples was significantly lower than controls ($p < 0.05$). Data are mean $\pm$ SEM, $n = 4$ animals.

NAC may have functioned as a pro-oxidant in serum-free and -supplemented cultures over a significant portion of the multiweek cultures.

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.² To address this possibility, we evaluated GSH-EE, a cell permeable derivative of glutathione. Similar to NAC, GSH-EE was cytotoxic with time in serum-free medium, or did not reduce intracellular ROS with 3 days of culture in serum-supplemented medium. These data further indicate that supplementing the glutathione system is not an effective means of reducing intracellular ROS to support 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 after 15 days of culture. These results coincided with a modest increase in GAG accumulation that was associated with a higher cell content, although given the small difference in mean values of the CTB assay (13% higher for PDTC cultures) it is possible that a larger sample size would better resolve the relationship between GAG accumulation and cellular content. In addition to its antioxidant properties,³⁶ PDTC is known to inhibit NF- κ B activation. Activation of NF- κ B by pro-inflammatory cytokines has been reported to decrease ECM accumulation in chondrocyte²⁹ or MSC⁴⁴ cultures. However in the same MSC study suppression of NF- κ B activation in control conditions did not affect ECM accumulation,⁴⁴ which for the current study suggests that potential suppression of NF- κ B activation by PDTC did not influence ECM accumulation.

Despite the differences in ECM accumulation with NAC or PDTC supplementation, Col2A1 expression indicated that chondrogenesis was largely unaffected by culture conditions. For example, while Col2A1 expression was reduced $\sim$ 2-fold after 15 days of culture in NAC, this difference is small compared to the greater than 1000-fold increase in Col2A1 expression previously reported for the progression of equine MSC chondrogenesis between days 0 and 15 in control cultures.¹⁹ Therefore, differences in ECM accumulation appear to be related to the synthesis and assembly of ECM and not suppression of chondrogenic differentiation *per se*. While elevated levels of ROS with chondrogenesis have been associated with hypertrophy,²⁷ Col10A1 expression indicated that changes in levels of ROS with antioxidants in the current study did not promote or inhibit hypertrophic differentiation. In NAC cultures, upregulation of Col1A1 expression suggested differentiation to a more fibrocartilage phenotype, a possibility that is supported by a reduction in

the ratio of GAG/collagen accumulation in NAC cultures (Figs. 1, 2). For PDTC, the p value (0.07) suggests the potential for upregulation Col1A1 with PDTC treatment, although additional studies are needed to better defined the significance of these data.

For cartilage regeneration, the maturation of mechanically-functional ECM is important to ensure that the implant will thrive within the loading environment in the joint. It is well-established that accumulation of ECM in scaffolds seeded with chondrocytes or chondrogenic MSCs results in improved mechanical properties; therefore, it was surprising to find that PDTC samples were significantly softer than controls. The modulus of acellular agarose (3.7 kPa) indicated that the hydrogel itself significantly contributed to the mechanical stiffness of PDTC (7.3 kPa) and control (12.4 kPa) samples. Subtracting the modulus of the acellular agarose, the matrix stiffness in PDTC (3.6 kPa) was approximately 2.5-fold lower than controls (8.7 kPa). This difference would be a significant limitation for cartilage tissue engineering strategies that seek to rapidly recapitulate the mechanical properties of articular cartilage with neo-tissue. While an analysis of ECM structures and assemblies that could explain the unexpectedly low mechanical properties with PDTC treatment is beyond the scope of the study, one possibility is that collagen crosslinking was suppressed by the reductions in ROS. Collagen accumulation and crosslinking play an important role in the maturation of compressive properties in cartilage.⁴⁵ Lysyl oxidase is a collagen crosslinking enzyme that has been shown to improve the mechanical properties of cartilaginous, cell-seeded constructs.²⁵ In endothelial cells, stimulation of lysyl oxidase (LOX) activity with hypoxic culture was suppressed by antioxidants,¹² thereby suggesting a role of ROS. Upregulation of hypoxic-inducible factor (HIF) has been reported to induce expression of LOX and lysyl oxidase-like 2 (LOXL2),⁸ which is notable as it has been postulated that ROS regulates HIF activation.²⁸ Additional studies would be needed to characterize the influence of PDTC on collagen crosslinking, and the extent to which material properties may be affected accordingly.

In conclusion, this study characterized the effect of supplementing 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 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 durability of the secreted ECM.³⁵ However,

such advantages gained by antioxidant treatment may require methods to ensure the mechanical functionality of the ECM, such as supplementing with exogenous LOX or LOXL2 to improve collagen cross-linking and material properties.^{3,25} Further exploration of these conclusions would 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.

CONFLICT OF INTEREST

Suwimol Tangtrongsup declares that she has no conflict of interest. John Kisiday owns stock in Advanced Regenerative Therapies and Regenerative Sciences.

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