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# LOW-ENERGY MECHANICAL IMPACTS TO ARTICULAR CARTILAGE INCREASE AT LEAST ONE ANABOLIC PROTEIN IN CHONDROCYTES

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## INTRODUCTION

Acute joint trauma resulting from accidents or sports injuries may trigger a cascade of degenerative events within cartilage leading to post-traumatic osteoarthritis (PTOA), a significant cause of morbidity in aging populations[1]. Many studies link PTOA to increases in collagen and proteoglycan degradation[2], decreases in protein synthesis, changes from collagen type II to type I synthesis[3], and apoptosis and necrosis of chondrocytes[4], among other mechanotransducive responses. Such changes result from deleterious responses from either chondrocytes (mature cells in articular cartilage) or chondroprogenitor cells (multipotent cells capable of chondrogenic differentiation)[5].

We used genetic markers to observe activity of both chondrocytes and progenitor cells. In particular, we observed chondrocyte proliferation using the protein Ki67[6], which marks active phases expressed during the cell cycle[7]. We also observed changes in the pivotal transcription factor Sox9, which serves as a master regulator of cartilage formation and differentiation, and is a widely accepted marker of chondrocytes and chondrogenic progenitor cells[8]. We also studied activation of phosphorylated Epidermal Growth Factor Receptor (pEGFR), which is a tyrosine kinase receptor with multiple roles in development, homeostasis, and disease[9].

It is well established in the current literature that mechanical impact or compression injury of articular cartilage results in changes in gene expression that lead to matrix degradation and catabolic cellular responses; however, many of these studies generate visible macroscale damage to the articular surface of cartilage[10,11]. Especially in early disease stages, when damage may not be visible and when microcracks in the network of collagen[12] likely form, how mechanical factors affect cell function remains unknown. In this study, we aimed to determine the mechanotranducive response of chondrocytes to low-energy impacts to cartilage.

## METHODS

In total we tested 98 full-thickness cylindrical specimens (Ø 3 mm) with both cartilage and subchondral bone intact from two bovine medial femoral condyles, received on ice within 48 hours from slaughter (Animal Technologies, Inc., Tyler, TX). We assigned specimens to one of three impact groups (none, 1.5 mJ/mm$^3$, 3.2 mJ/mm$^3$) and measured the time-course (0, 24, 48, 72 hours post-impact) localization of Sox9, Ki67, and pEGFR via immunohistochemistry.

*Mechanical Impact Test*: We impacted the articular surface of specimens from the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups in unconfined compression using a drop tower with a flat metal platen (Ø 12.4 mm)[12]. We impacted samples at 0.5 m/s[12]. We calculated the actual impact energy density ($E_{\text{imp}}$) applied to each specimen using

$$E_{\text{imp}} = \frac{m v_{\text{imp}}^2}{2\pi r^2 t}, \quad (1)$$

where $m$ is the total mass applied, $v_{\text{imp}}$ is the velocity of the load carriage at the moment of impact, $r$ is the specimen radius, and $t$ is the specimen thickness. We measured the acceleration and force at a sampling rate of 100,000 Hz. Post-impact we rinsed samples in PBS. For the control group, the specimens rested in PBS for the duration of the test.

*Cell Culture and Fixation*: We placed the specimens in the 0-hour time-course groups in 4% paraformaldehyde (Sigma, St. Louis, MO). We placed the remaining specimens in 1 mL of sterile media comprised of DMEM/F12 (Gibco, Gaithersberg, MD), 0.05 mg/mL ascorbic-acid-2-phosphate (Sigma), 0.1% bovine serum albumin (Sigma), 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 100 units/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) for culture at 37˚C and 5% $CO_2$ for 24, 48, or 72 hours post-impact and changed this daily. Once we removed these specimens from culture, we fixed them

in 4% paraformaldehyde (Sigma) for four days, and decalcified using 14% EDTA with $NH_4OH$ (Sigma) for four days at 4 ˚C with rocking.

*Histology and Immunohistochemistry*: We stained matrix proteoglycans with 1% aqueous Safranin O (Sigma) counterstained with Weigert's Iron Hematoxylin (Poly Scientific, Bay Shore, NY) and 0.02% aqueous Fast Green (Fisher Scientific, Hampton, NH). For immunohistochemistry, we de-paraffinized, rehydrated, and incubated a subset of slides with citrate antigen retrieval buffer followed by 3% hydrogen peroxide, blocking solution, and overnight incubation with primary antibodies in blocking buffer[13]. We diluted the following primary antibodies to 1:1000: rabbit anti-Ki67 (Abcam, Cambridge, MA); rabbit anti-Sox9 (Abcam); and rabbit anti-pEGFR (Abcam). We incubated slides with a biotinylated anti-rabbit secondary antibody which we detected using a Vectastain Elite ABC kit (Vector Laboratories) and chromogenic detection with DAB (Vector)[13].

*Imaging and Image Analysis*: We imaged slides using a Nikon Eclipse E800 light microscope and obtained full cross-sections using a 4x objective, and three to five images of central regions of the immunohistochemically stained slides with 0-600 pixels of overlap using a 20x objective. We excluded sample edges. We used images from specimens stained with Safranin O to qualitatively assess the integrity of the articular surface. We recreated full-thickness cross-sections from the 20x images using Fiji's Grid/Collection Stitching Plugin[14] for ImageJ (NIH, Bethesda, MD), and determined boundaries for the superficial (SZ), middle (MZ), and deep zones (DZ) using morphology of the lacunae and the cellular arrangement. For each antibody, we quantified positive and negative cellular staining within each zone, and calculated the percent positive cells.

*Statistical analyses*: We tested for normal distributions of the percent positive cells for each antibody using the Shapiro-Wilk Test for normality. We used separate two-way ANOVAs to evaluate the effects of impact and time on the percent positive cellular localization of each antibody within each zone and included impact level and time as fixed effects, and both the thickness of each specimen and the cow identifier as covariates. We used post-hoc tests to evaluate significant differences among treatment combinations for interactions, and used separate linear regressions to investigate interactions between impact energy density, first Piola-Kirchhoff stress, and engineering strain with the percent positive cells.

## RESULTS

In Fig.1, we show distributions of the percent positive cells for each antibody separated by through-thickness zone and time in culture. We found no significant differences in the interaction of impact group and time as predictors for zonal response of percent positive cells for Ki67 and pEGFR. However, we found that positive cellular localization of anti-Sox9 in each zone varied with respect to time. For anti-Sox9, we found statistically significant differences ($p < 0.05$) in SZ at 48 hours, and MZ and DZ at 0, 24, and 72 hours. We did not find statistically significant differences in the total number of cells for any antibody and zone at any time. We also found significant inverse relationships between Sox9 localization in the MZ and DZ for all three predictors.

## DISCUSSION

This study is the first to demonstrate changes in Sox9, a master regulator of chondrogenesis, in response to low-energy impact loading. We used low-energy impacts that are below the threshold that induces cell death, which can be as low as 5 mJ/mm$^3$ [15]. We selected 1.5 and 3.2 mJ/mm$^3$ as non-injurious, low-energy impacts that have 25% and 40% probability of microcracking the network of collagen[12]. Alternatively, we found mechanical stimuli that positively influence mechanotransducive responses. Here mechanotransduction refers to the processes through which cells sense and respond to mechanical stimuli by converting them to biochemical signals that elicit specific cellular responses. We identified mechanical impacts that upregulated Sox9, and determined that impact energy density is a better predictor of positive cellular Sox9 than first-Piola-Kirchoff stress or engineering strain. Additionally, our low-energy impacts did not alter cell proliferation or pEGFR signaling.

Although there are no definitive cellular markers for chondroprogenitors, they express Sox9 among other markers and migrate towards damaged articular cartilage matrix. We distinguished between chondroprogenitors and mature chondrocytes by migration inferred by comparing proliferation with the total number of cells in each through-thickness zone. Since the number of cells present in any zone did not change with increased Sox9, the changes we observed may come from mature, non-migratory chondrocytes. Thus, low-energy impacts may have no effect on chondroprogenitor cells, but may affect mature chondrocytes.

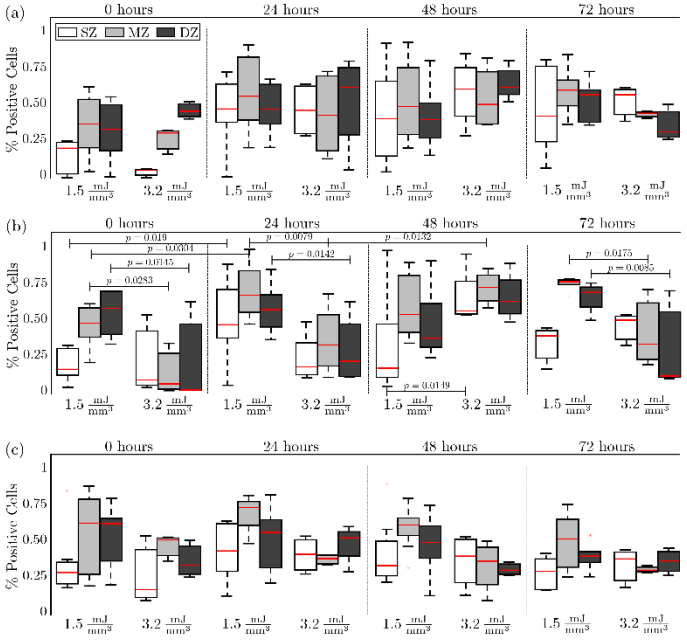


**Figure 1: Distributions of the percent positive cells in the SZ, MZ, and DZ of (a) Ki67, (b) Sox9, and (c) pEGFR.**


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## REFERENCES


[1] Ryan, J et al., *J Tissue Eng Regen Med*, 3:107-16, 2009. [2] Setton, L et al., *J Orthop Res*, 13:473-82, 1995. [3] Lahm, A et al., *Arch Orthop Trauma Surg*, 130:557-64, 2010. [4] Chen, C et al., *J Orthop Res*, 19:703-11, 2001. [5] Seol, D et al., *Arthritis Rheum*, 64:3626-37, 2012. [6] Muinoz-Lopez, E et al., *Open Orthop J*, 6:150-9, 2012. [7] Scholzen, T et al., *J Cell Physiology*, 182:311-22, 2000. [8] Healy, C et al., *Dev Dyn*, 215:69-78, 1999. [9] Seol, D et al., *Arthritis Rheum*, 64:3626-37, 2012. [10] Lee, JH et al., *Arthritis Rheum*, 52:2386-95, 2005. [11] Chan, PS et al., *J Orthop Res*, 23:1146-51, 2005. [12] Kaleem, B et al., *Osteoarthritis Cartilage*, 25:544-53, 2017. [13] Fisher, M et al., *Bone*, 37:741-50, 2005. [14] Preibisch, S et al., *Bioinformatics*, 25:1463-5, 2009. [15] Duda, G et al., *Clin Orthop Relat Res,* 393:302-9, 2001.