Depth Dependent Impact of Estrogen on Chondrocyte Mitochondrial Function after Physiological Compression of Femoral Condylar Cartilage

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INTRODUCTION: A disproportionate amount of those impacted by osteoarthritis are post-menopausal women, who have a significant decrease in estrogen levels. Because of this population, loss of estrogen has long been considered a contributing factor to cartilage degradation [1]. When physiologic loads are applied, estrogen typically upregulates anabolic genes and downregulates catabolic genes [2]. Additionally, application of estrogen to cartilage explants can prevent cell death in chondrocytes subject to rapid, large strains [3]. However, the response of chondrocytes to mechanical loads is depth dependent in articular cartilage, which may be caused by variations in the stiffness of the surface region versus the middle region of cartilage which creates depth dependent strain fields [4] or caused by depth-dependent variations in chondrocyte behavior [5]. In addition to loss of estrogen, mitochondrial dysfunction and the subsequent apoptosis that can occur have been proposed as mechanisms that lead to osteoarthritis. The depth dependent mitochondrial response of chondrocytes within articular cartilage to estrogen and physiologic loading has yet to be studied. Therefore, the goal of this study is to understand the depth dependent changes in mitochondrial function based on estrogen treatment before and after a physiological compressive load is applied.

METHODS: Porcine knee joints were sterilely dissected to obtain cylindrical explants from lateral and medial femoral condyles, placed in the appropriate control or estrogen pre-compression media, and incubated at 37°C, 5% CO_2. Control media was comprised of DMEM/F12 with L-glutamine (Corning), 10% Fetal Bovine Serum (Gibco) and 1% AB/AM (Thermo Fisher); estrogen media contained an additional 1pM 17β estradiol (Sigma Aldrich). 24 hours after dissection, compressed samples were compressed unconfined in sterile PBS to 30% strain at 20%/s, held at 30% strain for 120 seconds, and then released. All explants were transferred to culture in the appropriate post-compression media for up to 6 days of culture immediately after compression testing, with media changes as appropriate. At the end of the culture period, explants were cut into hemicylinders and stained with 200nM MitoTracker Green (Thermo Fisher) for 50 minutes at room temperature and 10nM Tetramethylrhodamine, methyl ester (TMRM, Thermo Fisher) for 20 minutes. Explants were washed twice in PBS and imaged using a Zeiss LSM 700 Confocal with a 20X objective. MTG was visualized with a 488/518 excitation/emission laser and TMRM was visualized with a 555/585 excitation/emission laser.
Images were analyzed for percent functional mitochondria using a custom MATLAB script to segment cells labeled with each stain (MathWorks). Superficial regions were manually selected as regions of interest to analyze only the top ~200 microns of the cartilage surface. Mitochondrial function was quantified percent of all mitochondria (MTG) sufficiently colocalized with functional signal (TMRM). This study compared 8 experimental conditions, including a compressed and non-compressed case; a pre-compression treatment with estrogen and control medias; and a post-compression treatment with estrogen and control medias. A total of 4-5 explants were tested per condition. Each explant was imaged in 2-5 distinct locations across the plug in both superficial and middle regions, resulting in a minimum of 10 images analyzed per group. Welch's t-test was used for direct comparisons between two groups. ANOVA + Tukey's post hoc was used for comparisons between multiple media conditions.

RESULTS: Analysis of these images demonstrates that mitochondrial dysfunction was depth dependent, with the middle region demonstrating higher functionality than the superficial region in both compressed (+14.42%, $p<0.001$) and non-compressed (+15.96%, $p< 0.0001$) conditions (Figure 1). Compression had no significant impact except in middle region treated only with control media, where compression stimulated mitochondrial function (+13.95%, $p<.05$). Pre-compression application of estrogen does not alter mitochondrial function in this study in any condition. In contrast, post-compression application of estrogen significantly decreases mitochondrial function in the superficial region (CTRL/CTRL – CTRL/ESTR: -17.42%, $p <.05$; ESTR/CTRL-CTRL/ESTR: -18.69%, $p<.05$). This decrease in mitochondrial function was not observed in the middle region.

DISCUSSION: This study demonstrated that estrogen applied after compression significantly decreased mitochondrial dysfunction in the superficial region with little to no effect on the middle region. The increased mitochondrial dysfunction in the superficial region due to estrogen is surprising when compared to existing literature claiming that estrogen has a protective effect against mitochondrial dysfunction and apoptosis. Since these differences were significant in the compressed case but not the uncompressed case, this difference may be attributed to the loading conditions, which were designed to reflect strain and rate of compression during daily activities such as walking, instead of traumatic compression in cases studying post traumatic osteoarthritis. These physiologic loading rates typically enhance anabolic factors [6]. Additionally, estrogen's effects vary with cartilage tissue maturity and sex [7,8] and since the pigs were 5-6 months old (puberty age) and the sex was unknown (but most likely male), this may account for the limited response observed.
The depth dependent impact of estrogen may be attributed to diffusion. Since estrogen's molecular weight is quite large, 272Da, it is likely that low amounts of estrogen diffuse into the middle region [9]. Future research will examine the diffusion of estrogen into cartilage to assess its potential impact.

SIGNIFICANCE/CLINICAL RELEVANCE: Through a fully crossed study of pre-compression estrogen treatment, compression, and post-compression estrogen treatment, we gained insight into the impact of estrogen based on cartilage region and compression. These results improve understanding of progression and treatment of osteoarthritis in estrogen deficient patients.

REFERENCES: [1] Roman-Blas et al (2009) *Arthritis Research & Therapy;* [2] Wang et al (2023) *Bone Research*; [3] Imgenberg et al (2013) *Osteoarthritis & Cartilage*; [4] Bartell et al (2015) *J. Biomechanics*; [5] Chahine et al (2007) *Biomechanics & Modeling in Mechanobiology;* [6] Jeon et al (2012) *Osteoarthritis & Cartilage*; [7] Zhong et al (2010) *Endocrinology*; [8] Kinney et al (2005) *Osteoarthritis & Cartilage;* [9] DiDomenico et al (2018) *Rheumatology*

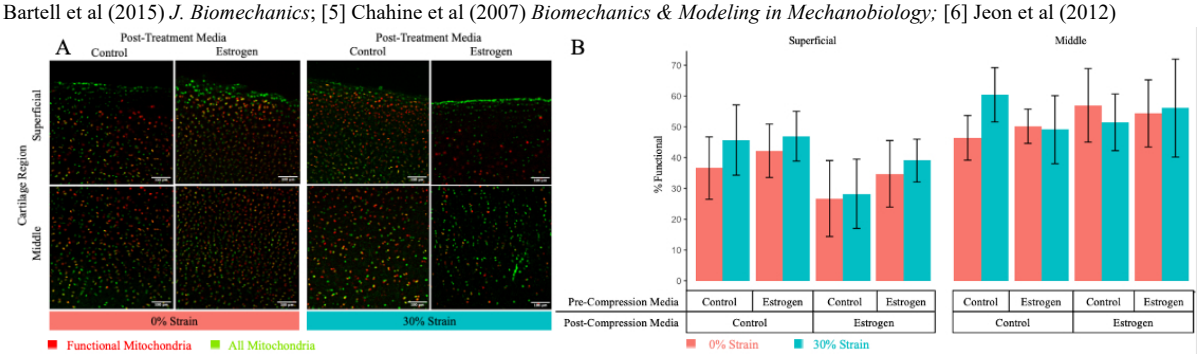

Figure 1: A) Confocal images of cartilage stained with TMRM and MitoTracker green demonstrate decrease in functionality when estrogen is applied 24 hours after compression, with or without compression in superficial region of cartilage. All scale bars are 100um. B) Functionality in each of 8 conditions demonstrates that post-compression application of estrogen decreases mitochondrial function by 17.42% (Control/Control – Control/Estrogen, $p<.05$) and 18.69% (Estrogen/Control – Control/Estrogen, $p<.05$) in the superficial region.