

Sex-Specific Effect of Estrogen in TNF-Induced Inflammation in Articular Chondrocytes

Meena Meiyappan^{1,2}, Shawna Lopez¹, Lindsey Hranitsky¹, Paula A Hernandez¹
¹University of Texas Southwestern Medical Center, Dallas, TX. ²Baylor University, Waco, TX
Meena_Meiyappan1@Baylor.edu

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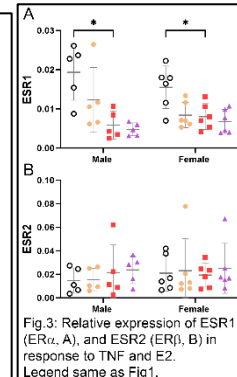
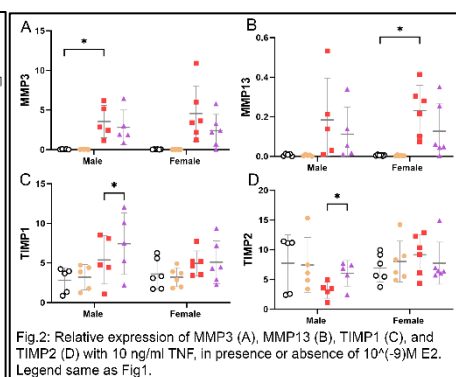
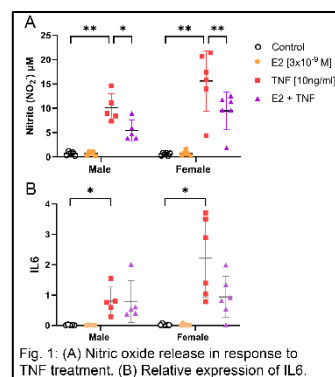
INTRODUCTION: Knee osteoarthritis (OA) is about three times as more prevalent in post-menopausal women, associated with the decrease in systemic levels of Estrogen. Despite the well-documented differences in knee OA incidence and severity between males and females, a deeper understanding on how sex hormones affect the response of chondrocytes to inflammation in a sex-specific manner is needed. 17- β Estradiol (E2) has been shown to reduce the nitric oxide (NO) release induced by IL-1 β in female chondrocytes [1]. Here, we hypothesize that E2 delivers a sex-specific protective effect in chondrocytes reducing the inflammatory response produced by Tumor Necrosis Factor (TNF).

METHODS: Chondrocytes were sourced from bovine knees (5♂, 6♀, 24-30 months old) and seeded as 500,000 cells/well 6-well plates in complete media (DMEM, 10% FBS, 1x antibiotics-antimycotics, 50 ug/ml ascorbic acid). Treatment: when cells reached about 90% confluence, complete media was replaced by estrogen-free media (phenol red-free DMEM, 10% charcoal-stripped FBS, 1x antibiotics-antimycotics, 1x GlutaMAX, 1mM sodium pyruvate, 50 ug/ml ascorbic acid) 24 h prior to treatment. Cells were pre-incubated with or without 3.7×10^{-9} M E2 for 24 h. After that, cells were treated with or without TNF (10ng/ml) for another 24 h in presence or absence of E2. NO release: media was collected to determine NO release using Griess Reagent (Promega). QPCR: Relative expression of IL-6, TIMP1, TIMP2, MMP3, MMP13, ESR1 (Estrogen Receptor α), and ESR2 (Estrogen Receptor β) (BioRad primers, SYBR-green) was calculated using the $2^{-\Delta\Delta C_t}$ method, with YWHAZ as normalizer. Statistical analysis: Two-way ANOVA was used to test the effect of treatment and sex, with Dunnett's correction for multiple comparisons. Significance was set as $p < 0.05$. Analysis was done in Prism GraphPad v.10.

RESULTS: The release of NO increased significantly for both male and female chondrocytes after TNF treatment. E2 was able to reduce the NO production in about 50%, in both male and female cells (Fig. 1A). Although the difference between TNF-treated males and females was not significant, the increase seemed higher in females. Indeed, there was a significant interaction between sex and treatment ($p = 0.026$). Next, we investigated whether E2 was able to prevent the upregulation of catabolic markers induced by TNF in a sex-specific manner. The mRNA expression of the pro-inflammatory cytokine IL-6 was upregulated by TNF in both sexes, and resembling NO data, a significant interaction between sex and treatment was detected for IL-6 ($p = 0.018$; Fig. 1B). E2 was unable to prevent this upregulation. However, female chondrocytes showed a trend towards a prevention ($p = 0.10$; Fig. 1B). Although MMP3 was upregulated in both sexes after TNF treatment, this increase was significant only in male cells (females trended with $p = 0.057$). E2 was unable to prevent TNF-induced MMP3 upregulation (Fig. 2A). The upregulation of MMP13 expression by TNF was significant only in female chondrocytes (males $p = 0.267$) and E2 was unable to prevent it (Fig. 2B). The Tissue Inhibitors of Metalloproteases TIMP1 and TIMP2 were not significantly affected by TNF treatment in either sex. However, E2 + TNF upregulated the expression of both TIMP1 and TIMP2 compared to TNF alone, only in male chondrocytes (Fig. 2C, D). The interaction between sex and treatment trended towards significance for TIMP2 ($p = 0.058$). To investigate if TNF-induced inflammation affected Estrogen receptors α or β , we analyzed changes in gene expression of ESR1 and ESR2. ESR1 expression significantly decreased from control group when TNF was added to both male and female chondrocytes and exhibited no additional change with E2 + TNF (Fig. 3A). On the other hand, the expression of ESR2 was unaffected by any treatment (Fig. 3B).

DISCUSSION: Here we show sex differences in chondrocytes response to TNF-induced inflammation. Both NO release and IL6 showed similar behaviors and significant interactions between sex and treatment. The upregulation of MMP3 and MMP13 was also sex-specific, even though no interaction between sex and treatment was detected. Interestingly, E2 was only able to reduce the NO production, but not to prevent the upregulation of IL6, MMP3, and MMP13, or the downregulation of ESR1. TIMP1 and TIMP2 also showed sex differences, as both were upregulated with the co-treatment of E2 + TNF only in male chondrocytes. These are inhibitors of matrix metalloproteases; therefore, an upregulation may confer protection against catabolism in male cells. Since E2 alone did not affect the expression of TIMP1 and TIMP2, it is possible that E2 acts in conjunction with an additional factor under pro-inflammatory conditions. Future studies include protein expression of TIMPs and inclusion of progesterone and testosterone.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding the role that E2 plays in the inflammatory response of chondrocytes depending on their biological sex can help identify key drivers of the sex differences in the prevalence of knee osteoarthritis. Moreover, this can also help



subjects ongoing hormonal treatments such the transgender population, and females using contraceptives or hormone replacement therapy.

REFERENCES:
[1] Richette et al., 2007