Tendon Pathology Following Menopause Induction in an Aged Murine Model

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INTRODUCTION: Tendon and ligament injuries account for more than half of all reported musculoskeletal injuries in the United States[1], impairing patient's quality of life as basic daily activities are disrupted. While affecting a wide portion of the population, tendon and ligament injuries disproportionately affect older individuals.[2] Further, data suggest females present a more exacerbated shift in tendinopathy with age, exhibiting a ~4-fold increase in Achilles tendon rupture between the ages of 50 and 80 years while males only present a ~1.3-fold increase over the same time-period.[3] Evidence suggests that this sex-specific difference in tendinopathy progression may be due to the systemic reduction of sex-hormones (ex. estrogens and progesterone) following the onset of menopause. Interestingly, clinical studies have demonstrated that post-menopausal women observe an increase in collagen fibril diameter and tendon stiffness in comparison to age-matched females taking estrogen-replacement therapies, implicating the importance of hormone-related regulation of the tendon homeostatic environment during aging. Despite this, the field lacks a fundamental understanding of the role of sex-hormones in modulating tendon pathology, particularly within older female populations.

To address this knowledge gap, we employed an established menopause mouse model utilizing the compound 4-vinylcyclohexene diepoxide (VCD), an ovarian specific toxin that gradually induces atresia in primordial and primary ovarian follicles, arresting follicle maturation and their accompanying sex-hormone production to mimic menopause. Unlike ovariectomy models, which abruptly disrupts sex-hormones via surgical removal of the ovaries, this VCD model replicates the perimenopausal and menopausal transition observed in human populations. Furthermore, by implementing VCD in middle-aged animals we can investigate the effects of menopause on tendon pathology in an age-matched context. We hypothesized that disruption of ovarian follicle function will result in an increase in tendinopathy in aged mice that phenocopies clinical observation, grossly characterized by increase in tendon fibrosis.

METHODS: Animals were acquired from the National Institute of Aging and housed following approved IACUC protocols. Middle-aged C57/BL6 mice (14-16mo) received intraperitoneal injections of VCD in sesame oil for 10 days (160/mg/kg/day) to induce menopause. Vehicle controls were injected with sesame oil (SO). Prior work from our group has validated this model, demonstrating an induction of menopause 115 days after the first injection of VCD in middle-aged mice, characterized by a systemic depletion of 17-β-estradiol and progesterone.[5] Mice were sacrificed 195 days after the start of injections (20-22mo old) to examine the effects of menopause induction on tendon pathology. Biomechanical testing (n=5-7) of Achilles tendon was assessed using a custom tensile device. In brief, the Achilles tendon was isolated and calipers were used to measure the major/minor diameters. Cross-sectional area (CSA) was calculated assuming elliptical geometry. The tendon was pre-loaded to 0.1N and ramped to failure (0.1%/s). Tissue stiffness and modulus were calculated by measuring the slope of linear region of their respective loading curves. Fibril diameters were measured via transmission electron microscopy across six non-overlapping regions for each biological replicate (n=2-3). Pentosidine, an advanced-glycation end-products (AGEs) biomarker, was measured (n=4) using a commercial ELISA kit and normalized to total collagen content, measured by a hydroxyproline assay. Label-free proteomics (n=5) was conducted at the ECM Proteomics core at University of Colorado Anschutz Medical Campus, following established protocols.[6] Statistical testing was conducted using GraphPad Prism with significance established at $p < 0.05$. An unpaired T-Test was performed to evaluate difference in tissue stiffness, modulus, and pentosidine concentration across experimental conditions. Differences in fibril distribution were evaluated using a Kolmogorov-Smirnov Test. For proteomics, peak intensities were normalized by sum and statistically tested using a two-sample t-test using MetaboAnalyst (Ver. 6.0).

RESULTS: TEM reports a shift in collagenous ultrastructure following menopause induction (**Fig. 1A**), characterized by an increase in mean fibril diameter ($p < 0.001$, VCD: n = 3, SO: n =2). We further observed an increase in Pentosidine, an AGE-associated biomarker, following VCD treatments ($p< 0.05$, n = 4). Biomechanical testing (**Fig. 1B**) demonstrates an increase in tissue stiffness ($p <0.05$, n=5-6) and modulus ($p = 0.051$) following menopause induction, but no significant difference in cross-sectional area. Hierarchical clustering (**Fig. 1C**) of the top 25 proteins demonstrates class separation between experimental conditions and significant differences in Periostin (Postn), Tenomodulin (Tnmd), Lamin A (Lmna), Asporin (Aspn), Transthyretin (Ttr), Dermatopontin (Dpt), Filamin C (Flnc) and Collagen Type VI alpha 6 chain.

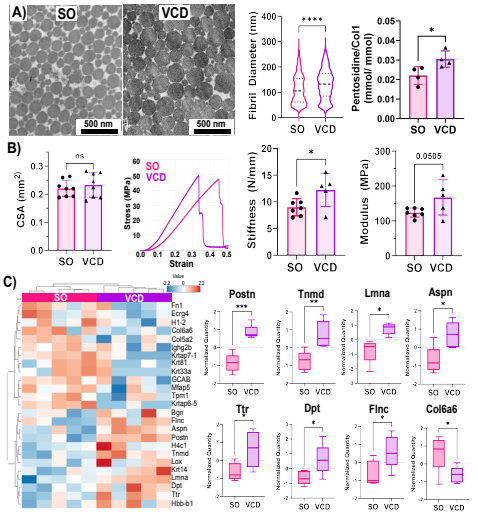

Figure 1: Structure-function relationships following VCD-induced menopause in aged mice. **A)** VCD treatments resulted in an increase in collagen fibril diameter ($p < 0.001$, VCD: n=3, SO: n=2) and pentosidine ($p< 0.05$, n = 4), in comparison to vehicle controls (SO). **B)** Following VCD-induced menopause (n= 5 - 6), we observed no difference in tissue CSA but see an increase in tissue stiffness ($p < 0.05$) and modulus (trending –$p = 0.051$). **C)** Hierarchical clustering of the top 25 proteins identified via label-free ECM proteomics (n=5). Following VCD treatment, we observed an increase in Periostin (Postn), Tenomodulin (Tnmd), Lamin A (Lmna), Asporin (Aspn), Transthyretin (Ttr), Dermatopontin (Dpt), Filamin C (Flnc), and a decrease in Col6a6 in comparison to vehicle controls. Protein intensity normalized to better display relative changes in protein levels across conditions.

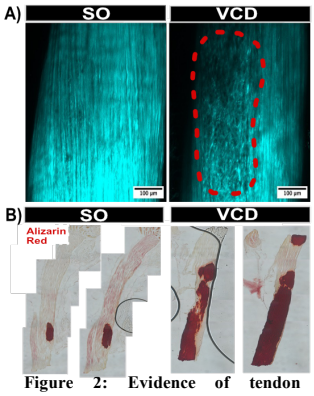

Figure 2: Evidence of tendon calcification. A) Second harmonic imaging shows irregular nodules (red outline) in VCD treated tendons. **B)** Preliminary Alizarin red staining shows heavy ossification throughout the tendon of VCD treated animals.

DISCUSSION: Following the induction of a menopause-like state in aged mice, we observed a clear shift within the underlying structure-function behavior of the Achilles tendon consistent with a pathological fibrotic transition. ECM proteomics corroborates the gross pathological phenotype, reporting significant increases within several proteins of interest, such as Periostin, Asporin, and Dermatopontin, which have been implicated in pathological fibrosis[7], collagen mineralization[8], and ECM organization[9], respectively. Preliminary second-harmonic imaging and Alizarin Red support an increase in tendon calcification following VCD treatments, consistent with reports[10], but additional biological replicates are required. An increase in tendon ossification, in conjunction with increased fibril diameters, would further explain the increase in tissue stiffness observed following VCD treatments. Interestingly, proteomics also identified an increase in Lamin A/C, which has been linked to cellular senescence and suggests loss of ovarian sex-hormones may exacerbate tendon aging. On-going sequencing work aims to identify specific mechanistic pathways that drive menopause associated tendinopathy and validate targets identified by ECM proteomics. Further on-going work aims to address if hormonal supplementation during perimenopause can mitigate tendinopathy progression in these VCD-treated aged mice.

SIGNIFICANCE/CLINICAL RELEVANCE: To our knowledge, this is the first study to leverage a VCD-induced menopause model to investigate the effects of prolonged aberrant sex hormones on tendinopathy in aged mice. This work may provide novel insight into the underlying biological mechanisms that drive tendinopathy within older female populations and uncover new therapeutic targets.

REFERENCES:**1)** Butler et al. (2004) PMID: 15255772 **2)** Smith et al. (2002) PMID: 12485691 **3)** Maffulli et al. (1999) PMID: 10512344 **4)** Hansen et al. (2009), PMID: 18927264 **5)** Gilmer et al., (2024) PMID: 3982079 **6)** McCabe et al. (2023) PMID:36763087 **7)** Naik et al.,(2012) PMID: 23043074 **8)** Kalamajski et al., (2009) PMID: 19589127 **9)** Unamuno et al., (2020) PMID: 32283761 **10)** Wang et al., (2024) PMID: 39193123