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Age-Associated Changes in the Response of Tendon Explants to Stress Deprivation is Sex-Dependent

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Abstract

The incidence of tendon injuries increases dramatically with age, which presents a major clinical burden. While previous studies have sought to identify age-related changes in extracellular matrix structure and function, few have been able to explain fully why aged tissues are more prone to degeneration and injury. In addition, recent studies have also demonstrated that age-related processes in humans may be sex-dependent, which could be responsible for muddled conclusions in changes with age. In this study, we investigate short-term responses through an ex vivo explant culture model of stress deprivation that specifically questions how age and sex differentially affect the ability of tendons to respond to altered mechanical stimulus. We subjected murine flexor explants from young (4 months of age) and aged (22–24 months of age) male and female mice to stress-deprived culture conditions for up to one week and investigated changes in viability, cell metabolism and proliferation, matrix biosynthesis and composition, gene expression, and inflammatory responses throughout the culture period. We found that aging did have a significant influence on the response to stress deprivation, demonstrating that aged explants have a less robust response overall with reduced metabolic activity, viability, proliferation, and biosynthesis. However, age-related changes appeared to be sex-dependent. Together, this work demonstrates that the aging process and the subsequent effect of age on the ability of tendons to respond to stress-deprivation are inherently different based on sex, where male explants favor increased activity,

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apoptosis and matrix remodeling while female explants favor reduced activity and tissue preservation.

Keywords

tendon; aging; explant; sex; flexor; tenocyte

Introduction:

Musculoskeletal disorders and injuries disproportionately affect the aging population, resulting in pain, disability, and substantial declines in quality of life. Tendon and ligament injuries are no exception, with the incidence of rotator cuff tears present in greater than 25% of the population over the age of 60.¹ These injuries can be complicated by the poor healing capacity of the tissue, which results in a fibrous, disorganized scar tissue at the tendon-bone junction that is associated with high rates of re-injury. Furthermore, these acute injuries are most often preceded by chronic age-related degeneration of the tissue, which is still not well understood. With the aging population rising dramatically each year and support for an increasingly active lifestyle to improve cardiovascular outcomes, there is a major clinical need to understand how age-related changes may influence overall tendon health, and potentially how these changes leave aged tendons susceptible to degeneration and injury.

Previous studies by our laboratory and others aimed to understand age-related injuries focused on measuring alterations in tissue composition, structure, and function that naturally occur with age. Assessments of quasi-static mechanical function in aged tendons do not paint a clear picture, as age-related changes appear to vary based on the specific tendon studied, the protocol used to assess changes, and the boundary conditions (gripping, testing environment, etc.) for experimentation.²⁻⁵ However, measures of dynamic tissue function through fatigue loading⁶, dynamic macroscopic testing,^{7,8} and measures of dynamic responses at the fiber^{9,10} and fibril¹¹ levels all suggest a diminished mechanical function in the aging population. In addition, changes in extracellular matrix structure and composition with age have also been detected. Specifically, collagen fibril morphology is altered in aged rodent tendons, cell density is significantly reduced, and both enzymatic and non-enzymatic crosslinking is increased.¹²⁻¹⁴ However, these results are often heavily dependent on the tissue that is being tested and on the ages defined as 'young' and 'old' in each study.

Many of the studies described above provide insight into how young and aged tendons differ in their structure, composition, and mechanical function at a particular point in time, but inherently these studies are static and do not tell us about biological function. The primary function of the resident cell population, tenocytes, is to remodel this extracellular matrix in response to various external stimuli to adapt to functional demands and to repair matrix damage caused by repetitive loading. Therefore, it is likely that changes with age in the extracellular matrix are preceded by cellular adaptations. Although tendons typically are thought to have very low matrix turnover at homeostasis, tenocytes become metabolically active, begin to proliferate and work to actively remodel the matrix in response to exercise.¹⁵⁻¹⁹ In the case of mechanical injury by either overloading or unloading, this response shifts

to a degenerative process marked by upregulation of matrix degradation and increased inflammation.²⁰⁻²⁴ While studies have found that the injury response of aged rodent tendons is impaired,^{2,25} few have investigated the ability of aged cells to regulate tissue homeostasis.

Furthermore, recent evidence identifying age-related changes in aged tendons has also shown that the aging process itself may be sex-dependent. Sex discrepancy in the incidence of human tendon injuries are site-specific; males are more prone to Achilles tendon ruptures by as much as 19:1,²⁶ but most reports do not suggest that sex is a factor in rotator cuff tears.^{27,28} There are sex discrepancies in other age-related musculoskeletal disorders such as osteoporosis,²⁹ osteoarthritis,³⁰ and frailty.³¹ However, the evidence regarding sex-related differences in tissue function is mixed, with some studies showing large differences, and some showing none at all.^{2,8,32} With respect to aging, one recent study identified differential and opposite expression patterns in engineered tendons created from male and female human mesenchymal stem cells, providing initial insight into inherent sex differences that may be critical for understanding degenerative tendon disease.³³ Furthermore, aging does appear to have a sex-dependent effect on tissue healing in rodent tendons and other tissues.³⁴ Increased evidence of sex-dependent aging processes would further motivate the need for sex-based therapeutics in treating tendon injuries.

Therefore, the overall purpose of the present study was to understand how the response to altered mechanical stimulus and the re-establishment of tissue homeostasis is altered in aging, and to investigate sex-related differences in this process. Using an explant culture system which preserves native matrix architecture, we can investigate the biological response of tenocytes to altered mechanical stimuli without disrupting the extracellular environment or hierarchical load transfer mechanisms. In this study, we focused on understanding how aged tendon explants differentially respond to the removal of mechanical stimulus via stress-deprived culture conditions. We hypothesized that aged tendon explants would have reduced cell activity and matrix composition initially, and also have reduced capacity to actively respond to a loss of mechanical tension. We also hypothesized that the effect of aging on the ability of tendon explants to respond to stress deprivation may be sex-dependent based on recent studies describing differential healing based on sex.³⁴⁻³⁶

Materials and Methods:

Study Groups and Tissue Harvest

Flexor digitorum longus (FDL) tendon explants were harvested from approximately 120 young (4 months of age) and aged (22–24 months of age) male and female C57BL/6 mice (n=30 mice per group, Fig. 1). While sexual maturity occurs at approximately one month of age in C57BL/6 mice, tendon structure and mechanical function is typically considered mature around 3–6 months of age.^{9,37} In this study, our ‘young’ group is considered skeletally mature and represents a human age equivalency of 20–30 years of age.³⁸ Our ‘aged’ group represents a human equivalency of 60–70 years of age, which is a clinically relevant population for tendon degeneration and is also within the established period where biomarkers of old age, such as senescence, are typically detected.³⁹ Three of the groups (young male, young female, aged male) were obtained from the National Institute of Aging’s Aging Rodent Colony per an approved MIT CAC protocol (#0618–061-21). Aged

female mice were obtained from an unrelated study per an approved Northeastern animal use protocol (NU-IACUC #16–1136-R). Young male explant data on viability, metabolism, biosynthesis, proliferation, and matrix composition were gathered as part of a previous study (In Supplemental Data⁴⁰) and are reproduced here for comparison with other study groups.

Tendons were harvested from both hind limbs of each mouse, but care was taken to ensure that tendons from the same animal were not used for the same assay. Directly following sacrifice as previously described,⁴⁰ a 10-millimeter segment from the intrasynovial portion of the tendon was collected and placed directly into culture in stress-deprived conditions (no mechanical stimulus) for up to 7 days. Cultures were maintained in a standard culture medium consisting of low glucose Dulbecco's Modified Eagle Media (1 g/L (Corning Life Sciences, Tewksbury, MA)) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Pittsburgh, PA), 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25µg/ml amphotericin B (Sigma). Medium was changed every other day ('M' in Fig. 1) and spent medium was collected on days 1 and 3 to measure release of inflammatory cytokines ('CR'). Explants were also collected to assess changes in tendon health ('TH') including viability, metabolism, biosynthesis, composition, and gene expression ('GE').

Explant Cell Viability, Cell Density, and Cell Metabolism

Cell viability was assessed via live/dead staining in 1x PBS containing fluorescein diacetate (FDA; 4 mg/ml, Sigma) and propidium iodide (1 mg/ml, Sigma) for viable and non-viable cells, respectively. Tendons (n=4/group/day) were imaged using a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus Scientific Solutions, Waltham, MA) at 10x magnification to obtain confocal z-stacks for each sample. Composites were then created by averaging by maximum intensity to produce a single image containing multiple layers of cells. On average, stacks of images penetrated approximately 30% into the depth of the tissue. Viability was then quantified using Fiji⁴¹ via point selection of fluorescent maxima. Percentage cell viability was calculated from number of viable cells normalized to the total number of cells, while cell density was calculated from the total number of cells (live+dead) per region-of-interest (ROI) area (µm²). Importantly, viability imaging was performed on image stacks approximately 100 µm into the depth of the tissue and is representative primarily of internal tenocytes and does not include surface cells, as would other biochemical assays. Explant cell metabolism was assessed via the resazurin reduction assay as previously described.^{40,42} Following a 3-hour incubation in the medium, intensity of the reduced product, resorufin, was measured at 530/590nm. Values were then normalized to daily control wells with no explants, such that a value below 1 in presented data would represent a culture well with no living tissue.

Matrix Biosynthesis and Composition of Explants

Synthesis of sulfated glycosaminoglycans (sGAG) and DNA (as a measure of cell proliferation) was measured by 24-hour incorporation of ³⁵S-sulfate (20 µCi/mL) and ³H-thymidine (1 µCi/mL), respectively (Perkin-Elmer, Norwalk, CT). Following termination, explants (n=5/group/day) were washed and digested with proteinase K (5mg/ml) (Roche, Indianapolis, MN) for 18 hours. Radiolabel incorporation was measured using a liquid scintillation counter (Perkin-Elmer) and represents the capacity for DNA or protein (sGAG

in this case) synthesis, or the rate of synthesis over a particular amount of time, rather than the total amount of cell proliferation or synthesized protein. sGAG content was measured from the same digested explants using the dimethylmethylene blue (DMMB)⁴³⁻⁴⁶ assay, as described previously. Total double-stranded DNA content, including DNA from live cells and dead cells that have not yet been cleared, serves as a snapshot measure of total cellularity at each time point in the tissue and was measured using the PicoGreen dye-binding assay.⁴⁷⁻⁴⁹ Biochemical data (sGAG content, sGAG synthesis) and cell proliferation data are presented normalized to DNA content, but raw (non-normalized) data are also available for reader interpretation in the Supplemental Data section (see below).

Gene Expression from Explants

Whole explants were also harvested from each group at days 0 (baseline), 1 and 7, flash frozen with liquid nitrogen, and stored at -80°C for analysis of gene expression as described previously (n=4–6/group/day).⁵⁰⁻⁵² For extraction of total RNA, samples were pulverized and homogenized in TRIzol reagent and then separated using phase-gel tubes (Qiagen, Chatsworth, CA). The supernatant was then purified following the Qiagen RNeasy mini kit protocol (Qiagen). Total RNA was reverse transcribed using the AmpliTaq-Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA) and real-time PCR was performed using the Applied Biosystems 7700HT instrument with SYBR Green Master Mix (Applied Biosystems). Primer pairs and sequences are listed in Supplemental Data (Supplemental Table S1). Briefly, we measured tendon-specific markers (scleraxis, tenomodulin), as well as markers of major extracellular matrix components (collagens 1, 3, decorin, biglycan, fibromodulin), matrix degradation (mmp-3 and mmp-13), inflammatory responses (IL-1 β , IL-6, and TNF- α), apoptosis (caspase-3, caspase-9), and senescence (p16/INK, p19/ARF, p53). Expression for each gene was calculated from the threshold cycle (Ct) value, and normalized to the internal housekeeping gene GAPDH and to day 0 controls to calculate the fold change. All calculations were performed in log space to maintain symmetry about zero and for better comparison between up- and down-regulated values, as described previously.⁵³⁻⁵⁵

Inflammatory Cytokines Released To Medium

To assess inflammatory responses between study groups, spent medium collected from explants was analyzed using the Meso Scale Discovery Mouse ProInflammatory 7-Plex Tissue Culture assay (Meso Scale Discovery, Rockville, MD). This allowed for quantification of protein levels of seven different cytokines (IL-10, IL-6, IL-12p70, IFN- γ , IL-1 β , KC/GRO (mouse analog of CXCL1), and TNF- α) in a single medium sample (n=5 samples/group/day). The concentration of cytokines present in the medium was measured at days 1 and 3 in culture and pooled for comparisons between age and sex. It's important to note here that there is minimal cell crawl out from these explants during the 7-day culture window and therefore cytokine production is limited to cells within the explants.

Statistical Evaluation

Targeted statistical evaluations, rather than the full-factorial study, were performed in order to specifically test the hypotheses of this study. This study focused on age-related differences and therefore comparisons were only made within each sex when considering the

effect of age (young male vs. old male, young female vs. old female). Additionally, sex-related differences presented in the Supplemental Data (Supplemental Figures S3, S4) were only analyzed within each age group (young male vs. young female, old male vs. old female). All statistical comparisons were made using a two-way ANOVA with effects for time in culture and group (age OR sex), followed up with Bonferroni-corrected post-hoc tests to determine differences at each point in culture. For all comparisons, significance was denoted at $*p<0.05$ and a trend at $\#p<0.1$.

Results:

We first investigated whether there were any sex-related or age-related differences in tendon properties at baseline immediately after tendon excision, prior to the start of stress-deprived explant culture conditions (Fig. 2). There were no significant differences in explant metabolism at baseline between any of the groups (Fig. 2A). However, age-related changes were present in male explants, with increased total DNA (trend, Fig. 2B), decreased cell viability (trend, Fig. 2D) and decreased cell density (Fig. 2E). There were no significant sex- or age-related differences in sGAG content (Fig. 2C), although raw data suggested increased sGAG content in aged compared to young males (trend, Supplemental Fig. S1). No significant age-related changes were found at baseline in female explants, and no significant sex-related differences were found in either age group.

Explant viability over the course of the 7-day culture period was significantly altered with both age and sex. Young female and aged female explants remained at approximately 40% viability for the duration of the culture period and there was no significant difference between the two groups (Fig. 3A-C). However, cell density (live + dead cells per ROI area) was reduced in the aged female explants compared to young explants at days 1 and 3 in culture (Fig. 3C). Significant age-related changes were found in male explants. While young male explants remained viable at approximately 70% live cells for the entire culture period (Fig. 3D,F), aged male explants exhibited reduced viability at the initial dissection (D0, Fig. 2D) and remained at approximately 30% viability throughout the culture period (Fig. 3E,F). In addition, cell density was reduced in aged male explants compared to young explants at almost every time point in culture (Fig. 3F).

In male explants, several age-related differences were found in the response to stress-deprivation in terms of tendon biological activity. Young explants generally increase in metabolic activity, matrix biosynthesis, and cell proliferation over the course of the weeklong culture period (Fig. 4A,B,D). In contrast, aged explants exhibited a less robust response over the culture period compared to young explants in all three of these parameters, especially at days 3–7 in culture. While sGAG content (normalized to DNA) was higher in aged explants throughout the culture period (Fig. 4C), this was largely attributed to decreased cellularity (i.e., DNA content) over time in culture, which was further exacerbated in aged male explants towards the end of culture (Fig. 4E). It's important to note that over the course of the culture period there is no significant decrease in DNA content for young male explants, and that the most significant reduction in DNA content occurs during the first day of culture for aged male explants (likely due to clearance of cells that were lost during

harvest). Raw data showed similar trends, with decreased cell proliferation and sGAG biosynthesis in aged males (Supplemental Fig. S2).

There were fewer age-related differences in the response to stress deprivation in female explants (Fig. 5, Supplemental Fig. S2). There were no significant differences between young and aged female explants in their metabolic response to stress deprivation (Fig. 5A). Raw data showed increased proliferation and sGAG synthesis in aged explants (Supplemental Fig. S2), but when normalized per DNA content, age-related differences were not strong, with significant differences only at days 3 and 5 in proliferation (Fig. 5B,D). Furthermore, there was little effect of age on changes in tissue composition over time (Fig. 5C,E). sGAG content was decreased in aged female explants at days 3 and 5 while DNA content was increased in aged explants at day 5 only. When comparing between the two sexes, female explants (both young and aged) exhibited a reduced response to stress deprivation when compared to young male explants (Supplemental Fig. S3). Conversely, aged male explants had reduced cellular activity compared to aged female explants, mounting a less robust response overall (Supplemental Fig. S4).

Analysis of gene expression revealed both age- and sex-related differences between groups in the response to stress deprivation (Fig. 6). Collagen 1 was generally downregulated early and upregulated later in culture, particularly in male explants (Fig. 6A). However, young female explants downregulated collagen 1 expression throughout the stress-deprived culture period. Decorin expression was either unchanged or increased in young or aged male explants, but female explants downregulated decorin expression throughout culture, particularly in aged explants (Fig. 6B). Other matrix proteins (collagen 3, biglycan, fibromodulin) all showed similar results (Supplemental Fig. S5). Of note, decorin and biglycan expression were significantly increased with age in female explants only at baseline (day 0, not shown).

Scleraxis was unchanged in young male and female explants, but significantly downregulated in aged female explants and upregulated in aged male explants (Fig. 6C), suggesting sex-related differences in aging. Tenomodulin was downregulated in all groups at every point during the stress deprived culture (Supplemental Fig. S5), but also significantly downregulated at baseline in aged male explants compared to young male explants (day 0, not shown). Generally, expression of MMP-13 was downregulated early on and then upregulated later in the culture period. However, male explants showed significant upregulation of MMP-13 expression at day 7 which was much weaker or not present in young and aged females, respectively (Fig. 6D). Expression of MMP-3 was upregulated in young and aged male explants, but this result was much weaker for young female explants, and aged female explants showed no change in MMP-3 expression at all (Supplemental Fig. S5).

Young male explants, young female explants, and aged female explants all downregulated expression of three pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α)- in response to stress deprivation (Fig. 6E-G). This response was not present aged male explants for any of the pro-inflammatory markers. Caspase-9 and caspase-3 were both upregulated in aged male explants following a 7-day exposure to stress deprivation (Fig. 6H,I). On the contrary, both

we felt it was imperative to show early changes that may influence later culture. However, this highlights an important unanswered question regarding the effect of this initial cell death on the rest of the tissue explants.

The influence of signals released from dead/dying cells on the rest of the living tenocytes is currently unknown, and this remains an important limitation of the study. In other cell types, it's been shown that apoptotic cells can cause both apoptosis and proliferation in adjacent cells.⁶⁰⁻⁶² Apoptotic cells are also capable of inducing metabolic changes in living cells which are not induced by necrosis⁶³. On the other hand, necrotic cells release their cellular contents and unidentified 'danger signals' upon disruption of the cell membrane, which can act as a chemoattractant and stimulate inflammatory responses.⁶⁴ Unfortunately, we have not yet directly identified the mechanism of cell death in this model. Given no evidence of apoptosis early in the culture period in both the present study and our previous work,⁴⁰ as well as the idea of death being caused by mechanical removal of the tissue, we hypothesize that this initial cell death is necrotic. Since explant culture is without a circulating system by which to attract a large exogenous inflammatory response and inconsistencies with the resident endogenous inflammatory response (Fig. 7), we do not believe this initial cell death is causing negative responses but recognize that this cell death may not necessarily replicate the *in vivo* condition. We also did not find any literature to suggest that apoptosis or necrosis would trigger increased metabolic activity or matrix synthesis and there was no loss in viability in any of the groups following the initial harvest. Nevertheless, the relationship between programmed and non-programmed cell death and the function of adjacent living cells is an important gap in our knowledge that current studies are focused on.

Despite this initial cell death, aged male explants still demonstrated many of the changes previously found in 2D stem cell culture in response to the loss of mechanical stimulus, including reduced proliferative capacity, reduced capacity to synthesize matrix and reduced metabolic activity (Fig. 4). These age-related changes were not accounted for solely by the discrepancy in initial cell viability, and represent a diminished response of the remaining living cells to stress deprivation. Given that these changes were not different at baseline (Fig. 2), this finding suggests that a disruption in homeostasis is required to demonstrate age-related differences, similar to concepts hypothesized recently by others.² This could have serious implications for the response to mechanical overload, as well as processes involved in repair.

Another significant finding in our study concerns sex-related differences in the response to stress deprivation. Female explants did not exhibit the same responses to stress-deprivation as did male explants. In young mice, female explants showed a reduced response to stress deprivation compared to explants from young males (Fig. 3,4; Supplemental Fig. S3,4), specifically in metabolic activity, matrix biosynthesis, and proliferative capacity. Interestingly, these differences were inverted in aged mice, where female explants demonstrated increased responses compared to male explants, although all three groups (young female, aged female, and aged male) revealed decreased response compared to young males. These findings, however, could be interpreted in multiple ways. On one hand, a less robust response to stress deprivation suggests reduced matrix breakdown, reduced inflammatory responses, and reduced apoptosis, thus preserving the matrix immediately

after mechanical injury. This is supported by the observed changes in mRNA expression levels, where the markers associated with these responses are downregulated in young female explants (Fig. 6). On the other hand, the less robust response to stress deprivation could also mean reduced repair responses which have been associated with aging.^{2,65} More recent studies actually suggest superior healing in females over males in Achilles tendon.³⁴ Regardless of age, our data suggest differential pathways in males and females, supporting matrix turnover in males (increased matrix degeneration, apoptosis) but tissue preservation in females (decreased inflammation, apoptosis, matrix degeneration).

When looking at the interaction between age- and sex-related differences rather than each factor independently, it appears that the aging process itself appears to be sex-dependent. While large differences appear in the response to stress deprivation in aging male explants, the differences in female explants are much weaker. Furthermore, there are very few differences with the response to stress deprivation in young and aged female explants when looking at the profile of mRNA levels across different critical pathways. This has been suggested recently by several studies performing transcriptomic analysis on engineered and native tendons from young and old mixed sex human donors.^{33,66} These studies reported many changes similar to our results, such as increased signaling in MMP and p53 pathways in aged males but not aged females. Importantly, since our work investigates responses to stress deprivation in an explant culture model, the absence of systemic inflammatory, metabolic, or hormonal changes leads to the conclusion that male and female cells within tendon appear to be inherently different in their responses to stimuli (or lack thereof). This is particularly interesting in the case of inflammatory responses as stress deprivation elicited differential release of cytokines into the culture medium with both age and sex. Due to a limited panel of inflammatory cytokines (many of which were below detectable limits), it is difficult to speculate on whether aging induces different inflammatory profiles but our data does confirm that this process does appear to be sex-dependent similar to previous studies.^{66,67} This could have potential implications for recruiting systemic inflammatory responses to the injury site in the case of a focal defect or tear, as well as for the ability of the local and infiltrating cells to perform adequate tissue repair.

One of the more intriguing findings in our study focuses on the induction of senescence due to stress-deprivation, specifically in expression of p16^{INK}, p19^{ARF}, and p53 which play a role in cell cycle arrest and cellular senescence.⁶⁸⁻⁷⁰ We did not find increased expression of any of these markers at baseline in aged male or female tendons. However, in response to stress deprivation, we found that aged male explants showed upregulation of all three markers while young and aged female explants showed downregulation or no changes in expression (Fig. 6). Aged females may have increased p53 expression compared to young females, but the response was much weaker than aged males and not statistically significant given our sample size. We originally thought that aged explants would have reduced cellular activity due to senescence, but that appears to be the case only for males. Young and aged females showed downregulation of many cellular processes to limit metabolic responses, but the underlying mechanisms and pathways are still unknown.³³ In addition to senescence, there are a number of additional biological pathways that are altered with aging that could impact overall tissue homeostasis, which are outlined nicely in a review by López-Otín and colleagues, but are not addressed here.⁷¹ Several studies have examined stem cell

populations from aged tendons, reporting reduced cell numbers as well as a reduced capacity to proliferate, migrate, and differentiate.^{56,57,72} However, the effect of these biological changes on the ability of resident tenocytes to respond to various stimuli is still relatively unexplored and presents many avenues for future work.

While new findings concerning the role of aging in the ability of tendons to respond to altered mechanical stimulus are reported here, there are some considerations to be made regarding the interpretation of this work. First, this study does not take into account differences between male and female mice that could alter inherent cellular behavior. Although we did not observe any dramatic differences between groups at baseline, we did not measure animal activity, muscle size, or hormonal changes. With respect to hormonal status, female mice approach the equivalent of human perimenopause by 9 months of age and are reproductively senescent by 12 months of age.⁷³ Therefore the female mice used in this study are all 'post-menopausal' and, therefore, age-appropriate for the clinical population targeted in tendon injuries (65+). However, we did not take into account hormonal differences in young populations, and in the future could consider additional groups to address this. Second this work is primarily concerned with the initial response to stress deprivation and focuses on early changes in cellular activity. We also did not investigate functional declines that could occur later in culture (after 2 weeks, based on our pilot data) and therefore can only speculate on how these early changes in cellular responses could influence remodeling downstream. While we made every effort to collect explants for gene expression around similar times of day, we did not control for cell cycle or circadian phases which could be altered with aging. This is an ongoing avenue of investigation, specifically as inherent circadian phases of gene expression have been shown to control matrix synthesis and breakdown.⁷⁴

Finally, while most studies have investigated age-related changes in cell behavior using 2D culture of tendon stem cells, our studies utilize 3D explant cultures which have a heterogeneous cell population that has not yet fully been characterized. Our studies are specifically designed to investigate total native tissue response, mimicking all of the matrix connections and cell populations that would be present *in vivo*. An understanding of the individual cell types present within such tendon explants and their relative contributions to our results will be critical for identification of potential therapeutic interventions in the future. Moreover, our work is focused primarily on bulk tissue analyses but we recognize that there may be tremendous cell-to-cell heterogeneity in responses to stress deprivation that are influenced by both cell cycle/circadian changes and the environment. A better understanding of how age-associated changes in the microenvironment may influence the ability of tenocytes to respond to mechanical stimulus would also be beneficial to understanding the mechanism of age-related declines in tissue biology as reported here and this is the focus of future studies.

To our knowledge, this is the first study to identify short-term changes in the response to altered mechanical stimulus in aging tendons, and to isolate remarkable sex-dependent changes in the response of aging tendon explants. While our study has focused on a simple model for altering mechanical homeostasis through stress deprivation, we hypothesize that increased mechanical loading may also reveal sex-dependent changes associated with aging.

^{23,75,76} Future studies will seek to investigate this hypothesis, and to understand how the homeostatic mechanical ‘set-point’ may be altered in aged tendons, leaving them more prone to repetitive or overload-induced injury. We are also interested in site-specific alterations in tissue responses, as many studies have shown that age- and disease-related effects are tissue-dependent.^{2,47,48} It stands to reason that aging processes may progress differently depending on the functional requirements of particular tissues, as suggested by the incidence of site-specific injuries at different ages as described earlier. Regardless, we believe these studies provide a critical piece of the puzzle in understanding age-related tissue degeneration, spanning the gap between two-dimensional cell culture studies that are lacking extracellular matrix interactions and *in vivo* studies that are complicated by systemic aging processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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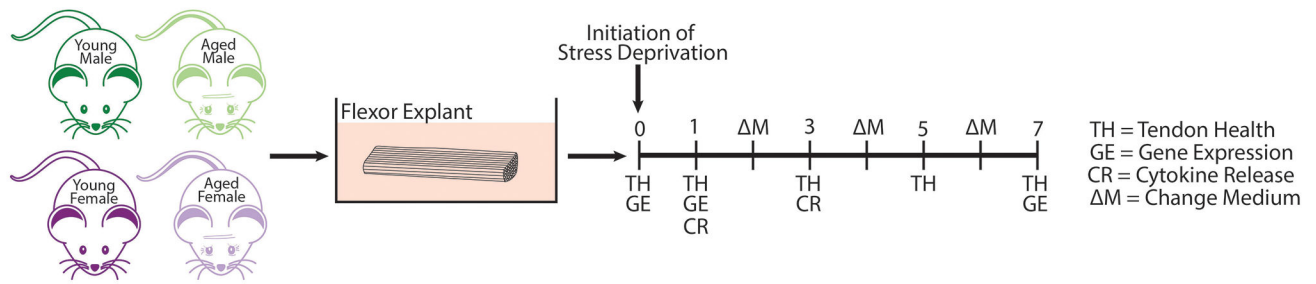


Figure 1. Overall study design:

Study groups (young male, young female, aged male, aged female), 7-day culture period, and assays performed are all depicted. ‘TH’ represents measures of tendon health, including viability, matrix composition, cell proliferation and matrix biosynthesis, ‘GE’ represents measurement of explant mRNA expression, ‘CR’ represents measurement of inflammatory cytokines released into medium, and ‘M’ represents times when medium was changed.

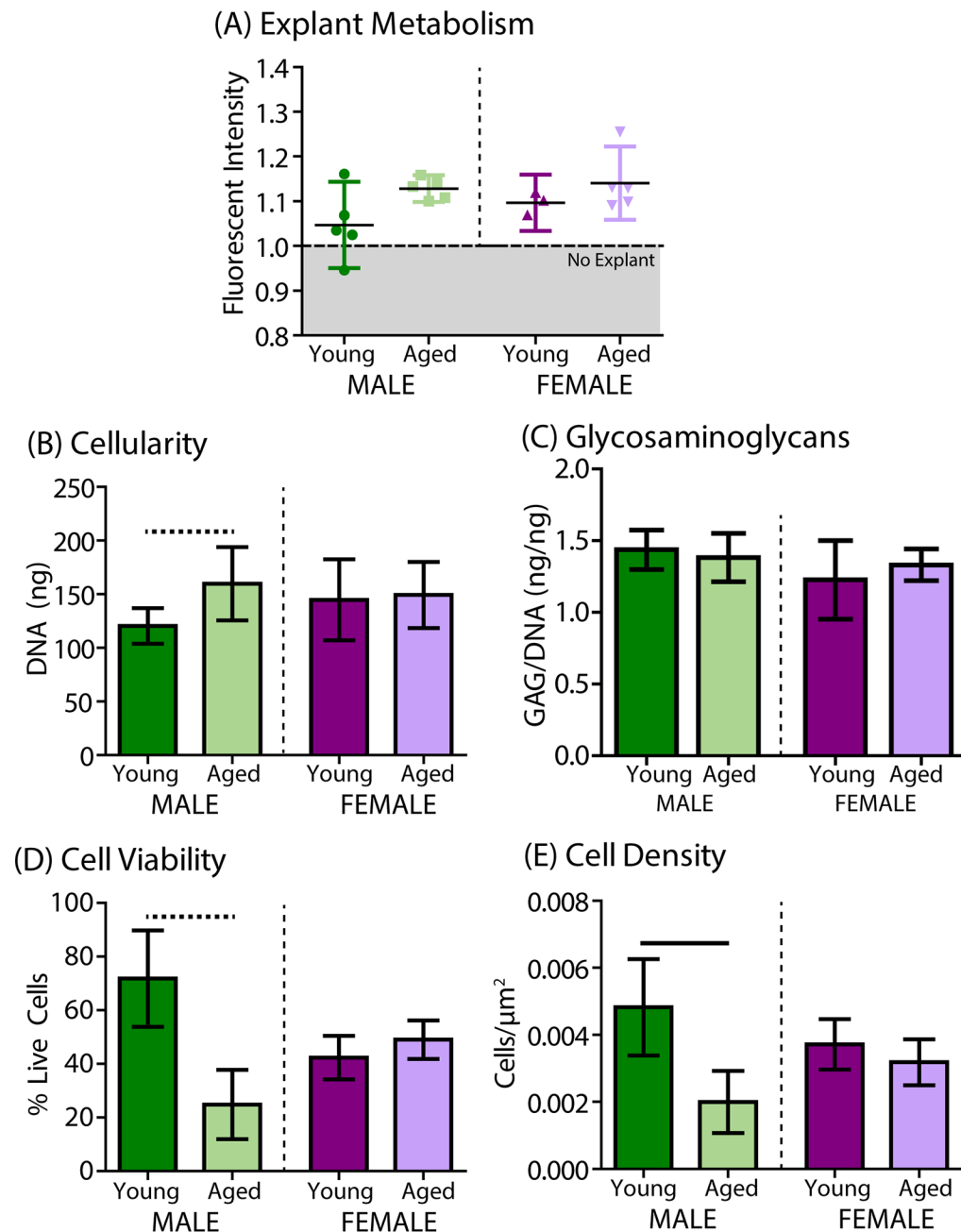


Figure 2. Sex-related and age-related differences at baseline immediately after tendon excision and before culture:

Few baseline differences were found when comparing day 0 flexor explants from young and aged, male and female mice. No significant differences with aging were found in female explants in (A) explant metabolism, (B) cellularity, (C) glycosaminoglycans, (D) cell viability, and (E) cell density. Cellularity was slightly increased (trend, B), while cell viability (trend, D) and cell density (E) were reduced in aged compared to young male explants. All data are expressed as mean \pm 95% confidence interval, where solid bars represent significant comparisons ($p < 0.05$) and dashed bars represented trends ($p < 0.1$).

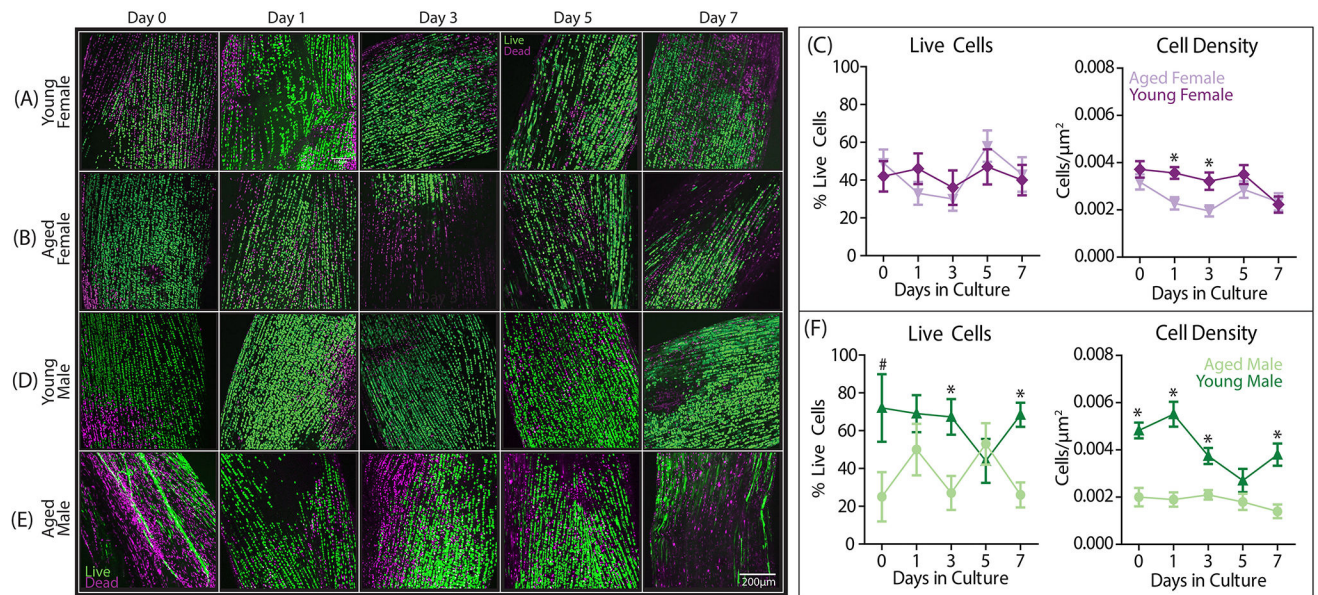


Figure 3. Cell viability of (A) young female, (B) aged female, (D) young male, and (E) aged male days 1, 3, 5, and 7 in culture via live/dead imaging:

Images are representative of approximately 4-6 tendons and each image is a merged z-stack of multiple layers of cells visualizing approximately 110 μm into the depth of the tissue (total depth ~200-300 μm). (C,F) Quantification through ImageJ was performed to calculate cell viability and cell density for all groups. Data are presented as mean ± standard error, where statistical significance is denoted by stars (*) at p<0.05 and a trend is denoted by hash marks (#) at p<0.1.

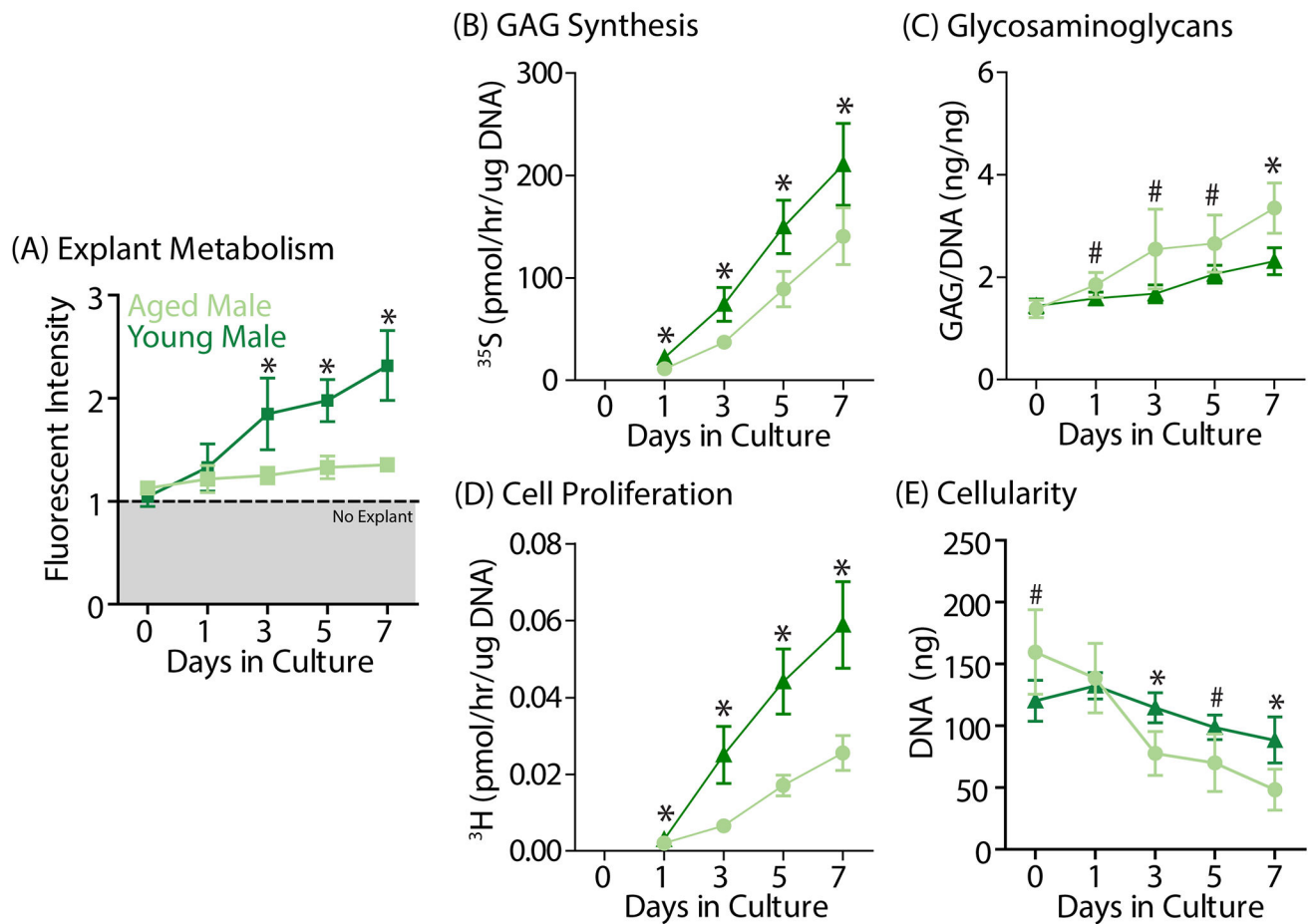


Figure 4. Age-related changes in the response of male explants to stress deprivation: In males, aged explants had reduced (A) metabolic activity and (B) sGAG biosynthesis in response to stress deprivation compared to young explants. (C) sGAG content was higher in aged explants compared to young explants. However, (D) cell proliferation and (E) cellularity were also both significantly reduced in aged explants compared to young explants. Data are presented as mean \pm 95% confidence interval where statistical significance is denoted as * p <0.05 and a trend is denoted as # p <0.1.

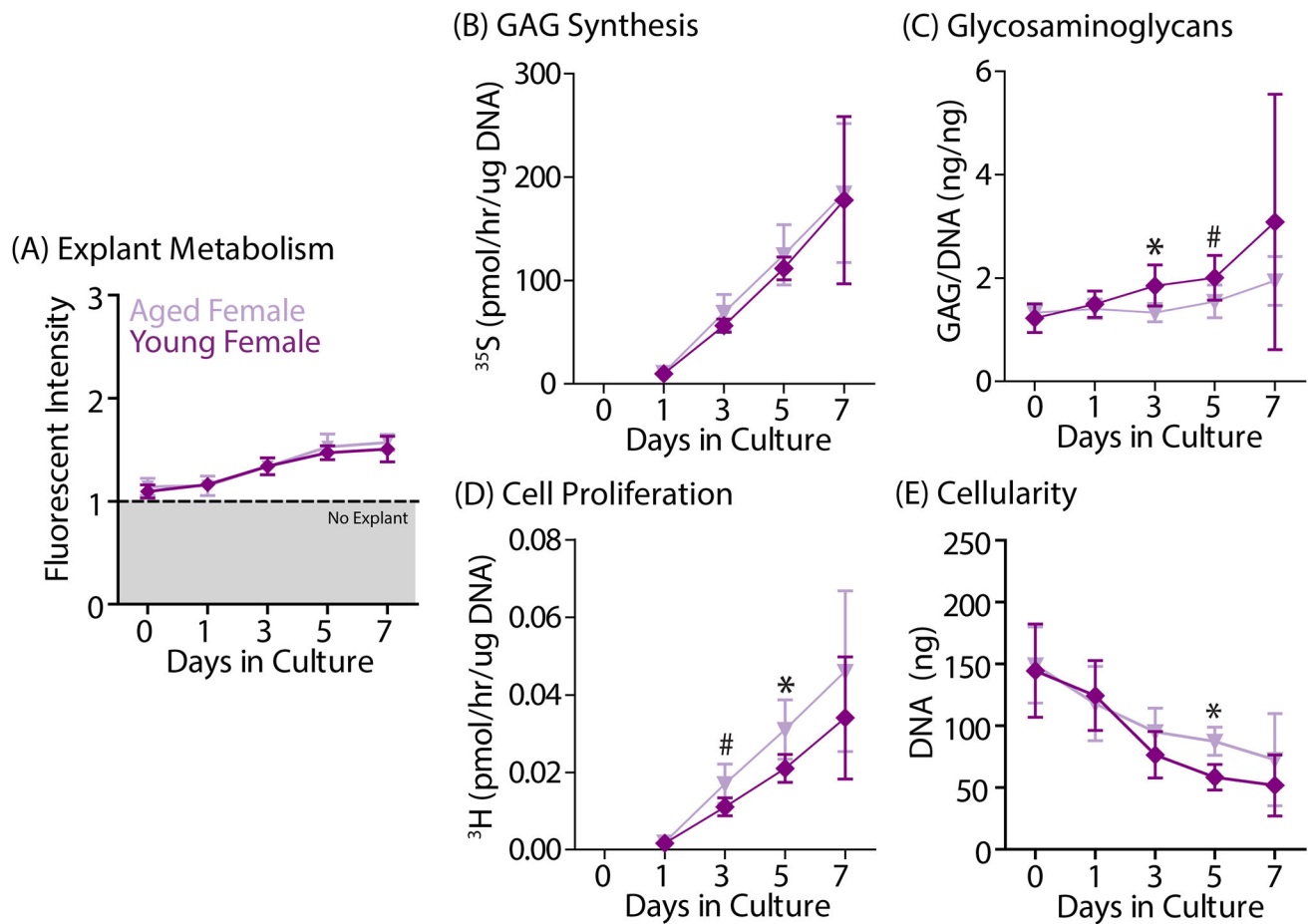


Figure 5. Age-related changes in the response of female explants to stress deprivation:

In females, there were no significant effects of aging on the response to stress deprivation in terms of (A) metabolic activity and (B) sGAG biosynthesis comparing young and aged explants. (C) sGAG content was slightly higher in young explants compared to aged explants at days 3 and 5 in stress-deprived conditions. However, (D) cell proliferation and (E) cellularity were both reduced in young explants compared to aged explants, although the differences were only present in the middle of culture and the differences were smaller. Data are presented as mean \pm 95% confidence interval where statistical significance is denoted as * $p < 0.05$ and a trend is denoted as # $p < 0.1$.

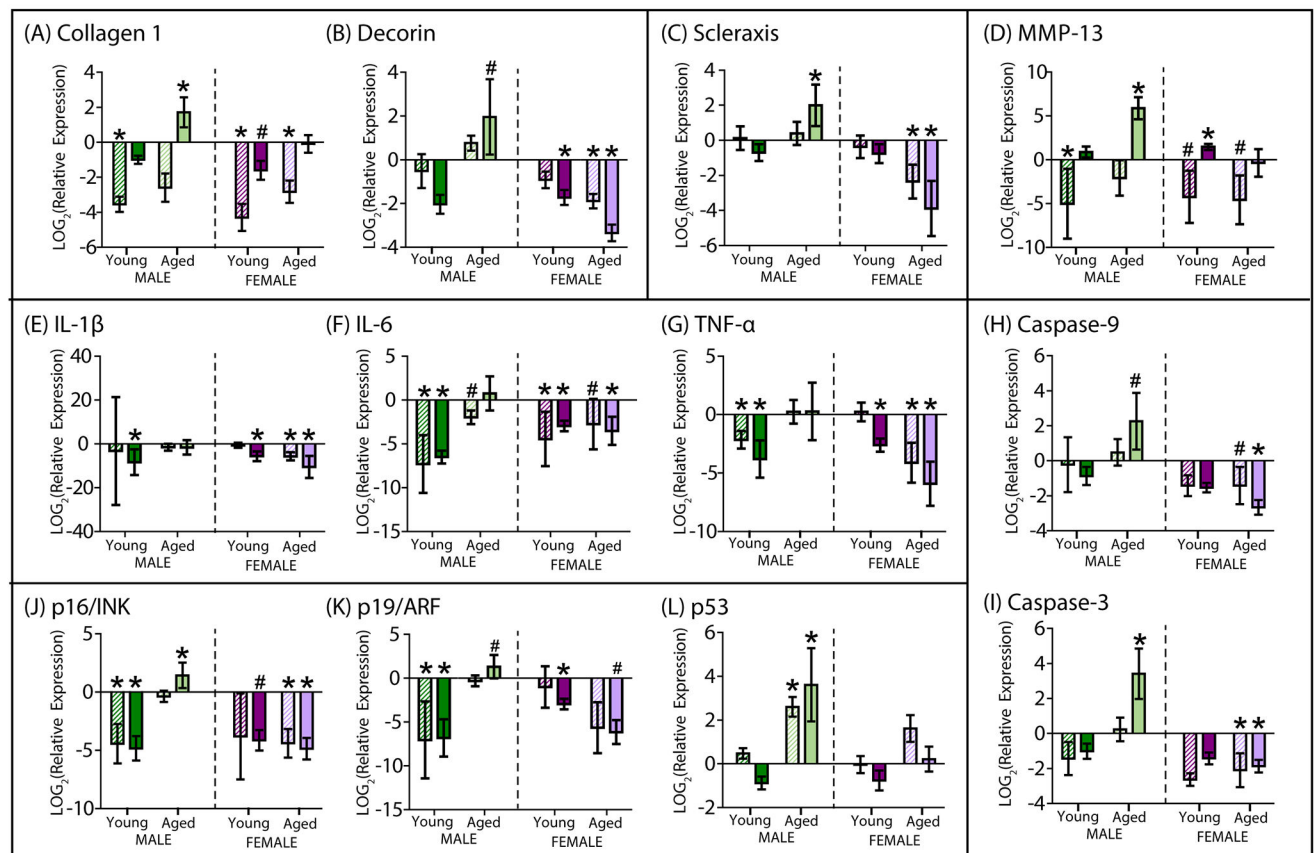


Figure 6. Age- and sex-related differences in flexor tendon explant gene expression: mRNA levels of (A) collagen 1, (B) decorin, (C) scleraxis, (D) matrix metalloproteinase-13, (E) IL-1 β , (F) IL-6, (G) TNF- α , (H) caspase-9, (I) caspase-3, (J) p16^{INK4a}, (K) p19^{ARF}, and (L) p53. Expression levels are normalized to GAPDH and then to day 0 controls to calculate the fold change. Diagonal striped bars represent day 1 data while solid bars represent day 7 data for each group. All data are presented as mean \pm 95% confidence interval. Stars (*) represent significant comparisons with day 0 control levels where $p < 0.05$ and hash marks (#) represent trends where $p < 0.10$.

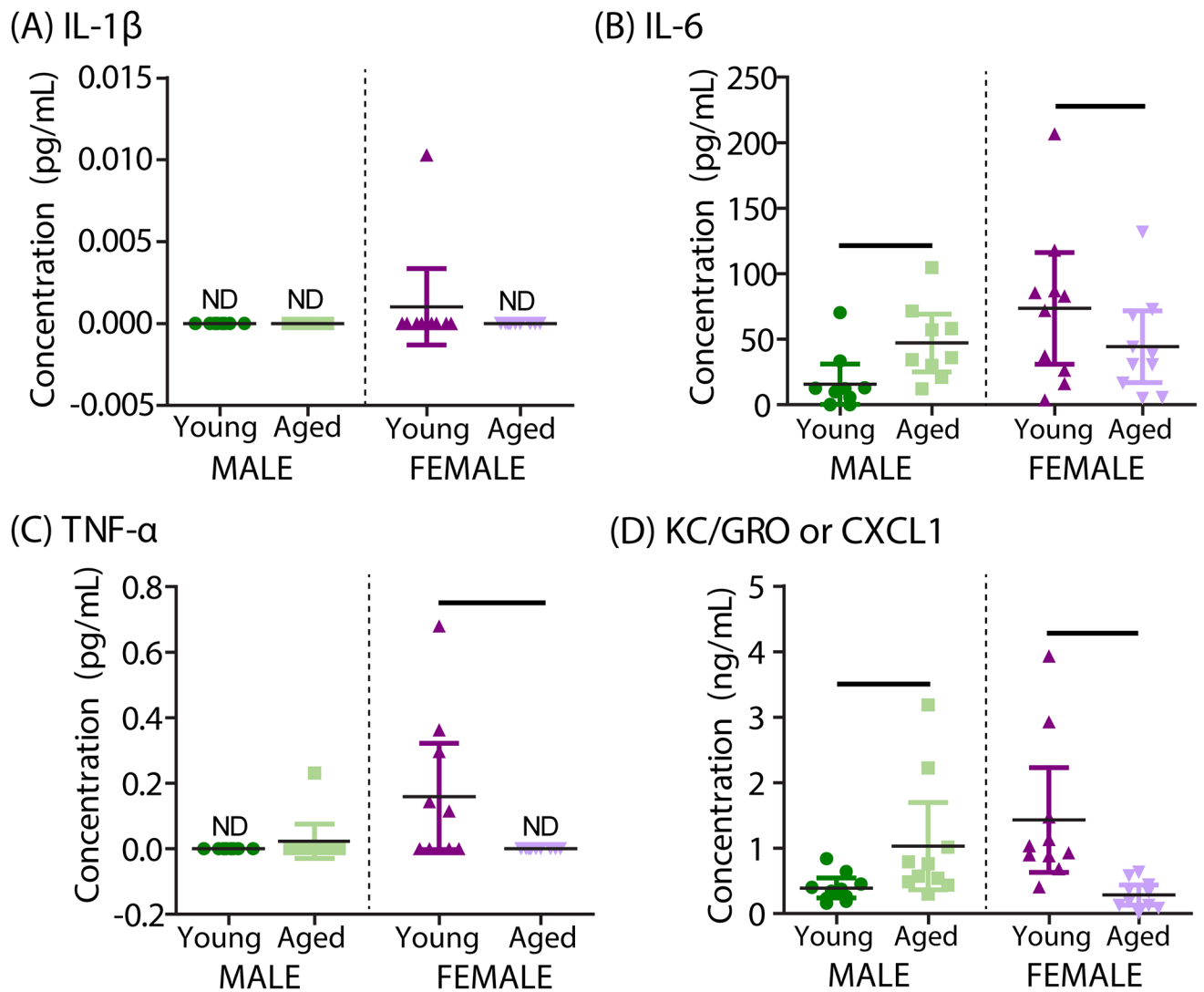


Figure 7. Inflammatory cytokines released by tendon explants to culture medium: Concentration of cytokines (A) IL-1 β , (B) IL-6, (C) TNF- α , and (D) KC/GRO or CXCL1 (mouse analog of IL-8) in the culture medium for young and aged, male and female FDL explants in stress deprived conditions. Concentrations were measured at days 1 and 3 during the one-week culture period and presented as pooled data here. All data are expressed as mean \pm 95% confidence interval and bars represent significant comparisons where $p < 0.05$. 'ND' is listed where concentrations were below the detection limit of the assay and therefore not detected.