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Sex differences in the biomechanical and biochemical responses of caudal rat intervertebral discs to injury

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Abstract

Background: Intervertebral disc degeneration (IDD) is a major cause of low back pain (LBP) worldwide. Sexual dimorphism, or sex-based differences, appear to exist in the severity of LBP. However, it is unknown if there are sex-based differences in the inflammatory, biomechanical, biochemical, and histological responses of intervertebral discs (IVDs).

Methods: Caudal (Coccygeal/Co) bone-disc-bone motion segments were isolated from multiple spinal levels (Co8 to Co14) of male and female Sprague–Dawley rats. Changes in motion segment biomechanics and extracellular matrix (ECM) biochemistry (glycosaminoglycan [GAG], collagen [COL], water, and DNA content) were evaluated at baseline and in response to chemical insult (lipopolysaccharide [LPS]) or puncture injury ex vivo. We also investigated the contributions of Toll-like receptor (TLR4) signaling on responses to LPS or puncture injury ex vivo, using a small molecule TLR4 inhibitor, TAK-242.

Results: Findings indicate that IVD motion segments from female donors had greater nitric oxide (NO) release in LPS groups compared to male donors. HMGB1 release was increased in punctured discs, but not LPS injured discs, with no sex effect. Although both male and female discs exhibited reductions in dynamic moduli in response to LPS and puncture injuries, dynamic moduli from female donors were higher than male donors across all groups. In uninjured (baseline) samples, a significant sex effect was observed in nucleus pulposus (NP) DNA and water content. Female annulus fibrosus (AF) also had higher DNA, GAG, and COL content (normalized by dry weight), but lower water content than male AF. Additional injury- and sex-dependent effects were observed in AF GAG/DNA and COL/DNA content. Finally, TAK-242 improved the dynamic modulus of female but not male punctured discs.

Conclusions: Our findings demonstrate that there are differences in rat IVD motion segments based on sex, and that the response to injury in inflammatory, biomechanical, biochemical, and histological outcomes also exhibit sex differences. TLR4 inhibition protected against loss of mechanical integrity of puncture-injured IVD motion segments, with differences responses based on donor sex.

KEYWORDS
biomechanics, extracellular matrix, injury, inflammation

1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is a major cause of low back pain (LBP) worldwide. In 2017, it was estimated that, globally, 577 million people experienced LBP at least once in their lifetime.[1] Furthermore, healthcare costs attributed to LBP have soared since earlier studies estimated at least $100 billion spent annually worldwide.[2] No effective treatment currently exists. Conventional treatments, such as spinal fusion or disc replacement with implants, appear to be inadequate at decreasing pain and reconstituting the native intervertebral disc (IVD) biomechanical and biological environment.[3] However, it is known that IVD is a key player in the degenerative cascade, and that pro-inflammatory cytokines and mediators, such as nitric oxide (NO), have been shown to be triggers and mediators of IDD.

Sexual dimorphism, or sex-based differences, appear to exist in levels of LBP, based on a greater prevalence and severity of musculoskeletal pain in women compared to men. According to the Global Burden of Disease Studies in 2019, the prevalence of LBP globally was 7.64% worldwide; however, when stratified by sex, 8.83% of women experienced LBP compared to 6.42% of men.[4] Additionally, in Spain, women reported a greater prevalence (17.8%) of LBP than men (11.3%).[5] There are cultural and gender differences (e.g., manual labor job or one's likelihood to go to a doctor) that can influence the data on the onset and severity of LBP. Pregnancy may also be a factor for women leading to a 50% incidence rate in LBP and exacerbating the development of degenerative spinal spondylolisthesis.[6,7] Sex hormones, such as estrogen, may also contribute to the onset of LBP and IDD.[7–9] Interestingly, postmenopausal women above the age of 50 are more susceptible to lumbar IDD than age-matched males.[10] However, men are more susceptible to IDD in their youth, most likely due to increased mechanical stress and physical injury from an increased likelihood to participate in high-impact physical activities.[11–13] IDD typically becomes apparent for males in the second decade of life, 10 years earlier than in women. Nevertheless, Wang et al. showed that elderly women had more severe IDD than elderly men at all lumbar levels.[13]

Despite this clinical evidence, it is unknown if there are differences in IVD composition between sexes. Biomechanics analysis of the human spine has shown that female specimens demonstrate a greater overall range of motion in all planes compared to males, regardless of the severity of degeneration[7,14]; however, this may be due to many factors including differences in spinal anatomy, vertebral anatomy, or tissue laxity. Meanwhile, T1ρ values for the nucleus pulposus (NP) taken from magnetic resonance images of lumbar discs suggest that males may have higher amounts of proteoglycan content than females.[15] Studying sex-based differences among humans can be difficult due to cultural and gender biases. Research animals, however, live in a controlled environment, allowing for the study of sexual dimorphism while minimizing societal influences.

Few studies to date have evaluated the effect of sex on IVD response to injury. In a set of studies by Mosely et al., disc degeneration was simulated in vivo using an IVD annular puncture injury with findings indicating that rats exhibit sexual dimorphism in injury responses. While both sexes had decreased cellularity and increased fibronectin at injury sites, females had an increased degeneration grade in the outer annulus fibrosus (AF) compared to males. Meanwhile, male IVDs had greater mechanical properties, while females had reduced second-harmonic generation (SHG) intensity, suggestive of collagen (COL) density,[16] compared to males post-puncture injury, suggesting that male IVDs exhibited improved healing compared to female punctured IVDs. A follow-up study on pain behavior found that male rats showed increased allodynia compared to females after IVD puncture injury.[17] In mice, a study showed that a high advanced glycation end products (AGE) diet resulted in increased compressive stiffness and torque range for females than for males.[18] In SPARC-null mice, while similar inflammatory responses between sexes in degenerated IVDs were reported, increased voluntary running was observed to reduce inflammatory mediator release (CXCL1 and CXCL5) ex vivo only in female mice.[19] To our knowledge, not many studies have studied sex differences in animal models ex vivo, and of those that have, their results were consistent with in vivo findings.[20,21] One advantage of using an ex vivo approach is that by removing tissues and cells from the complex interactions with other cells, hormones, neurotransmitters, nutrients, and environmental exposures in vivo, which themselves vary in living organisms by sex, we are able to isolate sex-based differences in a tissue of interest, the IVD. Therefore, investigating the effect of donor sex ex vivo can help define IVD-specific differences that are not dependent on regional or systemic responses to injury.

The goals of this study were to evaluate the response of male and female IVD motion segments to puncture injury or inflammatory stimulation with lipopolysaccharide (LPS) ex vivo and to investigate the contributions of Toll-like receptor 4 (TLR4) signaling to injury responses. A growing body of evidence suggests that TLR4 is involved in the pathogenesis of the IVD.[22–24] TLR4 expression in the IVD increases with degeneration severity and mediates catabolic and inflammatory processes.[23–26] Furthermore, damage-associated molecular patterns (DAMPS), such as high mobility group box-1 (HMGB1) or fibronectin fragments, have been shown to have degenerative effects on disc cells including increased expression of inflammatory cytokines and matrix-degrading enzymes by signaling through TLRs.[27–29] HMGB1 can serve as an agonist of TLR4 and lead to downstream NF-κβ activation.[30] However, it is unknown if IVD puncture injury responses are mediated through TLR4. To evaluate this, we assessed sex-based differences in response to injury with the presence of a small molecule TLR4 inhibitor, TAK-242. We hypothesized

that donor sex would be a significant contributor to the inflammatory response, as well as biomechanical, biochemical, and histological properties of the IVD motion segment when subjected to chemical or physical injury. Subsequently, we expected that TAK-242 would modulate the inflammatory response and improve biomechanical, biochemical, and histological outcomes post-injury through inhibition of the TLR4 pathway.

2 | METHODS

2.1 | Sample isolation and tissue culture of discs

The use of animals in this work was approved by the Institutional Animal Care and Use Committee at Columbia University. Caudal (Coccygeal/Co) bone-disc-bone motion segments (four to six per animal) were isolated from multiple spinal levels (Co8 to Co14) of eight male and eight female Sprague–Dawley rats (200–350 g, 3-month old). Cuts were made from mid vertebral body to separate levels, and samples were washed in 1× phosphate-buffered saline with 1% antibiotic-antimycotic (AA) and then briefly submerged in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 1% AA (basal media). Motion segments were then cultured in chemically defined media (CM: phenol-free DMEM without L-glutamine, 100 µg/mL sodium pyruvate, 50 µg/mL L-proline, 1% AA, 1% ITS Premix, and a 4 mM concentration of 3:1 Glutamax:Glutamine) for 24 h.[31] After the 1-day equilibration period, samples were allocated into one of the three injury groups: untreated (CM alone), LPS (1 µg/mL LPS in CM), or puncture (CM alone). Samples were cultured for either 6 days (short term) or 38 days (long term) to investigate changes in extracellular matrix (ECM) biomechanics, biochemistry and histology. Motion segments were allocated consistently by spinal level for short-term and long-term studies (Figure 1).

2.2 | Chemical stimulation and puncture injury of discs

LPS (Sigma-Aldrich) was suspended in sterile deionized water by sonication (1 mg/mL) and diluted in CM to 1 µg/mL. Inflammatory stimulation was simulated by culturing motion segments with LPS (1 µg/mL in CM) throughout the study. For short-term culture, CM ± LPS was replenished at Days 2 and 4, while for long-term culture, CM ± LPS was replenished at Days 3, 6, 10, 13, 17, 20, 24, 27, 31, and 38. Physical injury was simulated with a one-time puncture injury, made using a 20-gauge needle inserted 4 mm into the IVD and held in place for 15 s before the start of the culture duration in CM.

2.3 | TLR4 inhibition

A separate cohort of animals was used to determine the effect of TAK-242 on the response of IVD to injury. Five male and five female

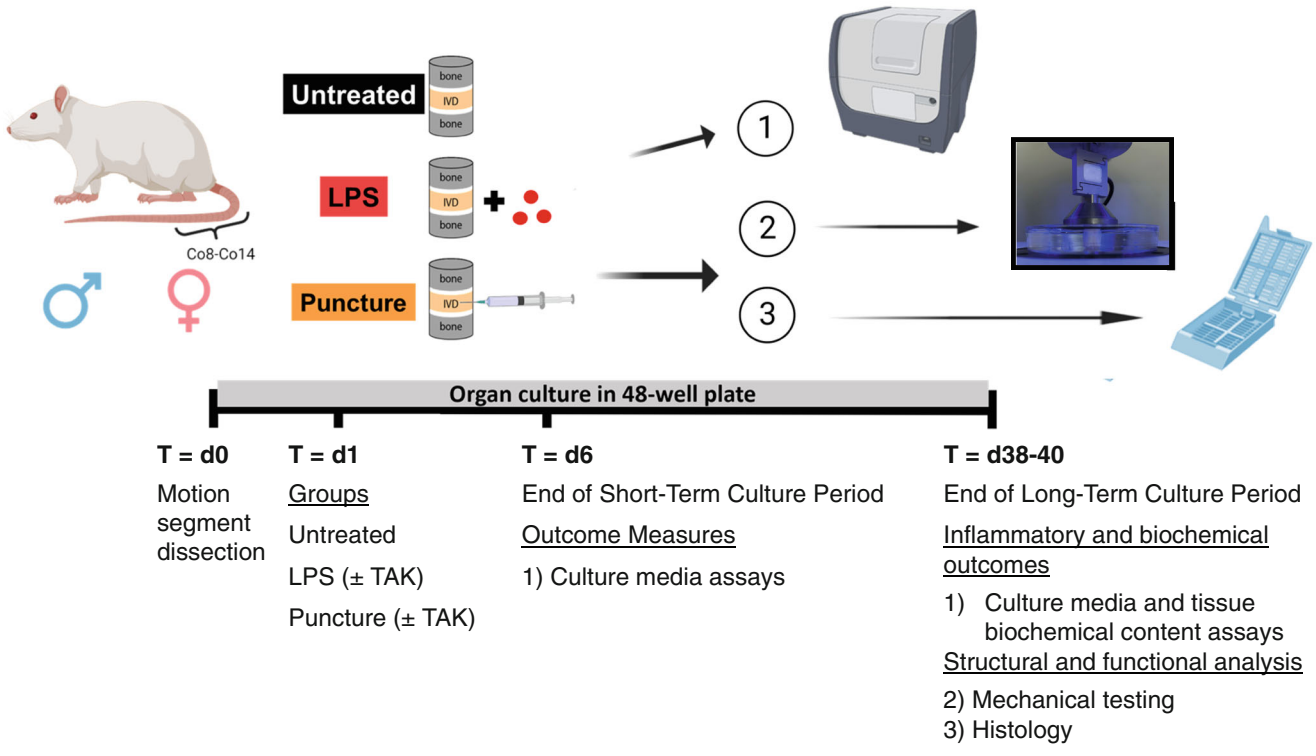

FIGURE 1 Experimental plan of short-term and long-term motion segment culture period. Created with BioRender.com.

rats were used to isolate $N = 5$ caudal motion segments per animal as described above. The motion segments were exposed to puncture or LPS injury as previously described. Samples were then cultured in CM ± TAK-242 for up to 38 days in culture. TAK-242 (TLR4 inhibitor, EMD Millipore) was solubilized in DMF (Thermo Scientific) (25 mmol/L) and diluted to a working dose of 1 μM to block TLR4 signaling in the motion segments. The motion segments were allocated to the following groups: puncture (P), puncture + TAK (PT), LPS (LPS), or LPS + TAK (LPST). For long-term culture, CM ± TAK was replenished in these groups at Days 3, 6, 10, 13, 17, 20, 24, 27, 31, and 38.

2.4 | Disc supernatant analysis

Tissue culture media were collected every 2 days (short term), or every 3–4 days (long term), and stored for analysis. Media supernatant was analyzed for levels of NO using the Griess Reagent System (Promega), lactate dehydrogenase (LDH) with the Cytotoxicity Detection Kit (Roche), and glycosaminoglycan (GAG) loss into the media with the 1,9-dimethylmethylene blue (DMMB) assay (Sigma-Aldrich) (Farndale et al., 1986). For the 6-day time point, levels of HMGB1 (Tecan) in the media were also measured. Supernatant levels were normalized to motion segment wet weight to reduce the effects of segment variability (Figure S6D).

2.5 | Mechanical testing

Using digital fluoroscopy, disc height, and cross-sectional area were measured before discs were subjected to unconfined compression testing at the end of the long-term study (Days 38–40) to normalize mechanical testing results. Radiographic analysis of IVDs was performed using a BenchTop Labscope (Glenbrook Technologies). Pre-loading images were acquired, and the radii and disc heights of samples were analyzed using ImageJ.[32] For radius measurements, three equally spaced measurements were taken across the width of each IVD and averaged (Figure S6A). For height measurements, five equally spaced measurements were taken across the length of each IVD and averaged (Figure S6B). Preconditioning consisted of 20 cycles of 2 N applied at 0.1 Hz, followed by a creep load of 2 N (equivalent to 1 rat body weight) until equilibration (20 min). Dynamic and equilibrium moduli were calculated using a custom MATLAB code (Figure 1).

2.6 | Biochemical content of IVDs

Post mechanical-testing, whole IVDs (encompassing the NP and AF regions) were dissected, and their wet weights were measured (Figure S6D). Subsequently, the NP and AF regions were separated, and individual wet weights of each region were measured (Figure S6E,F). Samples were then stored at −80°C until lyophilization. Samples were lyophilized for 48 h using a benchtop freeze dryer (Labconco), and dry weights of separated NP and AF regions were measured. Tissue water content (%) was calculated based on the weights ((wet weight − dry weight)/wet weight). Samples were then digested in Papain (0.02% [v/v], P3125, Sigma-Aldrich) in a pH = 6.0 digest buffer (sodium acetate anhydrous) (S2889, Sigma-Aldrich), cysteine hydrochloric acid (HCl) (C9768, Sigma-Aldrich), ethylenediaminetetraacetic acid (EDTA) (E6758, Sigma-Aldrich) in a 60°C water bath for 24 h. Quantification of DNA, GAG, and COL content from the tissue digests was measured using PicoGreen dsDNA Assay Kit (P11496, ThermoFisher), DMMB, and orthohydroxyproline (OHP) assay with a 1:7.64 OHP-to-collagen mass ratio,[33] respectively. DNA content was measured to assess the cellular content of the treated groups versus controls. Furthermore, DNA content was used to normalize GAG and collagen content to inform differences observed with cellular density or activity.

2.7 | Histology

To evaluate changes in IVD morphology, short- and long-term discs were fixed in 10% neutral buffered formalin phosphate overnight at 4°C post-mechanical testing. Motion segments were then decalcified in 14% EDTA for 2 weeks and cut longitudinally along the vertebral bodies. Decalcified discs were paraffin-embedded and sectioned (7 μm). Slides were stained with Safranin O with a Fast Green counterstain or Picrosirius Red indicating GAG or COL content, respectively. Images were taken using a Zeiss Axio Observer Z1 and an Axiocam 503 color camera and graded for the histopathologic features using the recent ORS Spine section initiative scale.[34]

2.8 | Statistical analysis

Data were assessed for normality using the Shapiro–Wilk test. Two-way analysis of variances (ANOVAs) were performed for effect of injury (untreated, LPS, puncture), sex (male, female), and interaction between injury and sex. Group comparisons were performed with Holm–Šídák post hoc tests. When warranted by the result of the normality test, a nonparametric Kruskal–Wallis ANOVA with injury (untreated, LPS, and puncture), sex (male and female), and their interactions were performed using the Dunn's multiple comparisons test for group comparisons. Within each injury type, two-way ANOVAs were also performed to identify the effect of TAK-242 (with or without), sex (male and female), and interaction between TAK-242 and sex on injury response. Group comparisons were performed using Holm–Šídák post hoc tests. When warranted by the result of normality test, a nonparametric Kruskal–Wallis ANOVA within each injury type was performed to determine the effect of TAK-242 (with or without), sex (male and female), and their interactions, with Dunn's multiple comparisons test used for group comparisons. In all tests, $p < 0.05$ was considered statistically significant. The main effects p values (injury or treatment, sex, and interaction) are presented above each subfigure in bold if any of the terms had $p < 0.05$. Group comparisons are presented in the figures using symbols (*,†, a, or b). A significant interaction term indicates that the effect of injury (or treatment) depends on donor sex. Analyses were performed with GraphPad (9.5.0).

3 | RESULTS

3.1 | Cumulative release of inflammatory markers over time

At 6-day post-injury, HMGB1 release into the media was found to be dependent on injury type ($p = 0.0018$), but not sex ($p = 0.47$, Figure 2A). Therefore, looking at pooled male and female data, HMGB1 release from punctured discs (2.2 ng/g tissue) was greater than untreated (0.24 ng/g, $p = 0.002$) and LPS injured (0.19 ng/g, $p = 0.002$) discs (Figure S1A). The HMGB1 release in male-punctured discs was also significantly greater than LPS injured discs ($p = 0.03$). In female discs, a trend toward increased release of HMGB1 was observed in punctured versus LPS discs (Figure 2A). Cumulative NO release into the media also showed a significant effect of injury ($p < 0.0001$), but not sex ($p = 0.18$) or their interaction ($p = 0.057$, Figure 2B). LPS injury of discs led to the greatest short-term NO release into the media compared to the untreated (male: $p = 0.04$, female: $p < 0.0001$) and punctured (male: $p = 0.054$, female: $p < 0.0001$) discs (Figure 2B). Additionally, female-LPS injured motion segments released significantly greater levels of NO compared to male-LPS injured motion segments ($p = 0.027$, Figure 2B).

Long-term culture of male and female motion segments led to the greatest amount of NO release into the media from LPS injured discs compared to untreated discs in both sexes ($p = 0.006$ and $p = 0.0002$, respectively, Figure 2C–E). After 40 days in culture, male-untreated ($p = 0.007$) and male-punctured ($p = 0.001$) discs released significantly greater NO levels compared to their female counterparts (Figure 2E). Male discs appeared to release significantly higher levels at NO starting at Day 27 of culture ($p = 0.049$, Figure 2C), while female discs were responsive to LPS injury early on, at Day 3 ($p = 0.019$, Figure 2D), contributing to significantly higher levels of NO in female-LPS discs compared to both female untreated and female-punctured discs throughout the majority of the culture period (Figure 2D). Initially, female-LPS (Day 3) and punctured (Day 13) discs appeared to release more NO due to injury; however, this effect dissipated over time (Day 38) (Figure 2D).

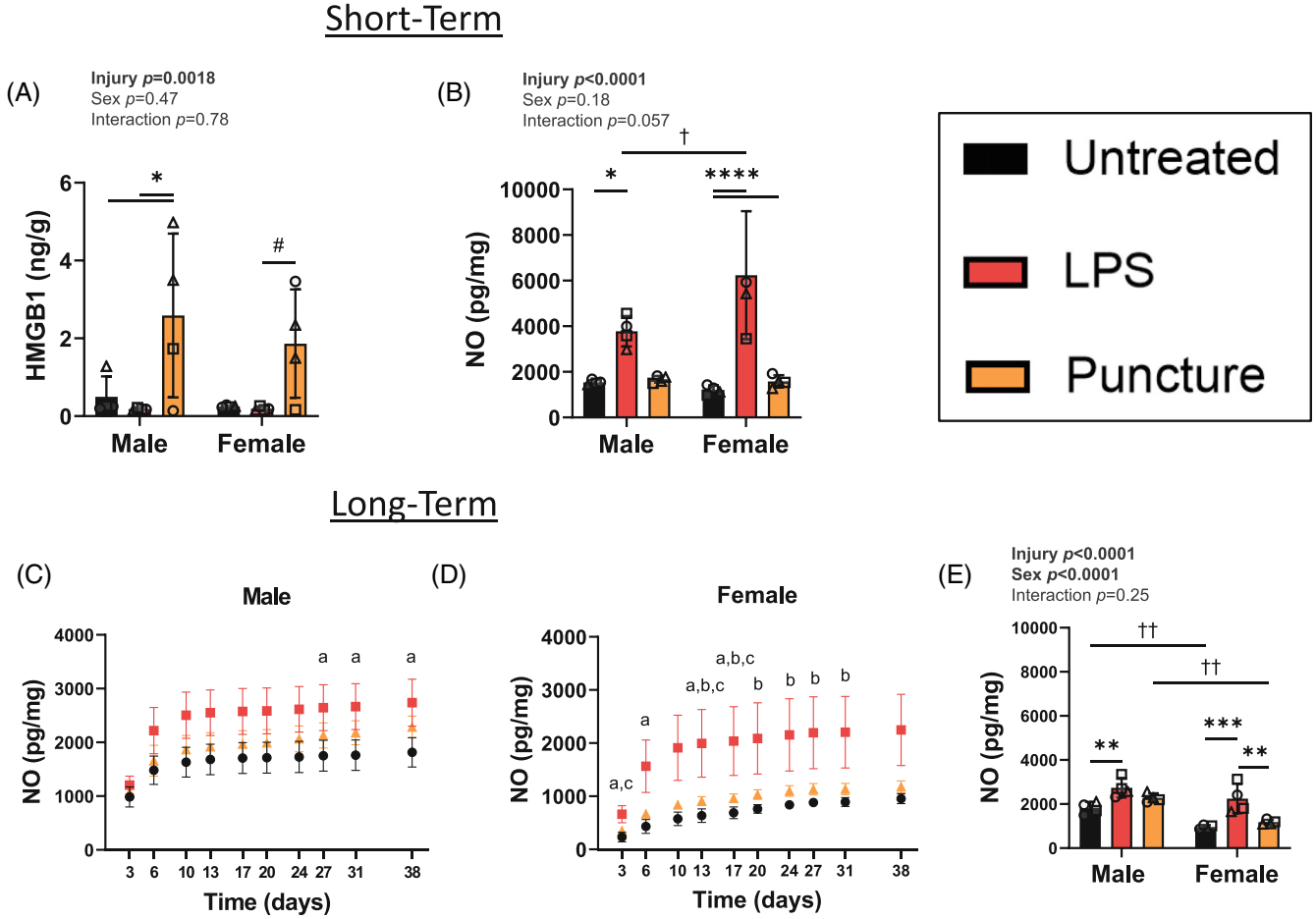

FIGURE 2 Cumulative short-term release of (A) HMGB1 and (B) NO into disc media $^{*}p < 0.05$ (each symbol represents a different donor in each sex). Long-term release of NO into the media from (C) male and (D) female motion segments in each group. (E) Day 38 cumulative NO release ($N = 4$, each symbol represents a different donor in each sex). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $^{a}p < 0.05$ for untreated versus LPS, $^{b}p < 0.05$ for untreated versus puncture, $^{c}p < 0.05$ for LPS versus puncture, $\#p < 0.1$ for post hoc between injury, and $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ for post hoc between sex). LPS, lipopolysaccharide; NO, nitric oxide.

3.2 | Mechanical properties

Both injury ($p < 0.0001$) and sex ($p < 0.0001$) were significant contributors to the dynamic modulus of discs (Figure 3A). Sex differences were observed in the dynamic modulus of untreated motion segments (male: 4.5 ± 0.5 MPa, female: 5.0 ± 0.6 MPa, $p = 0.0039$, Figure 3A). This sex-dependent difference remained when LPS or puncture injury was applied to the motion segments ($p = 0.0027$ and $p = 0.0081$, respectively). In response to injury, both male and female LPS and punctured motion segments exhibited similar decreases in dynamic modulus compared to their respective untreated discs ($p < 0.0001$, Figure 3A). The equilibrium modulus of motion segments was also dependent on both injury ($p = 0.001$) and sex ($p = 0.017$, Figure 3B). However, only punctured discs from female donors exhibited a significant decrease in equilibrium modulus compared to untreated discs ($p = 0.0021$, Figure 3B).

3.3 | Tissue water content

Whole IVD water content was not significantly dependent on injury ($p = 0.70$) or sex ($p = 0.34$) (Figure 3C). For NP water content, sex ($p = 0.010$), but not injury ($p = 0.62$), was a significant factor (Figure 3D). AF water content exhibited a significant effect of injury ($p = 0.008$) and the interaction term ($p = 0.002$) (Figure 3E). Male-untreated AFs had higher water content than female-untreated AFs ($p = 0.001$, Figure 3E). Injury from LPS and puncture significantly increased AF water content compared to untreated discs in female donors ($p = 0.0006$ and $p = 0.0006$, respectively, Figure 3E) but not in male donors.

3.4 | Biochemical content of injured discs

In untreated groups, sex was not a significant factor in NP biochemical content. In pooled analyses, injury was a significant contributor to NP

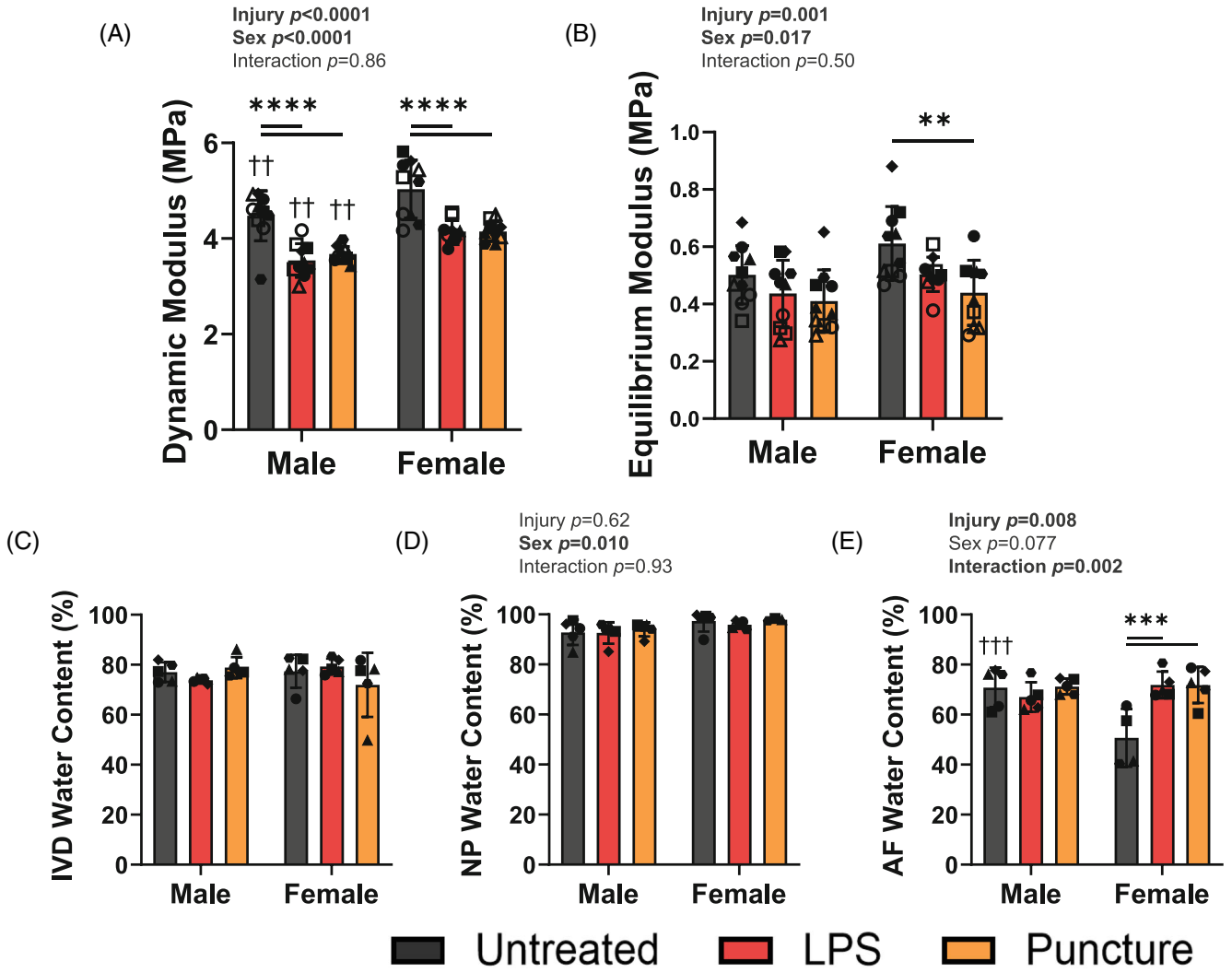

FIGURE 3 Mechanical properties of long-term untreated, LPS, and puncture-treated motion segments: (A) dynamic modulus ($N = 10$) and (B) equilibrium modulus ($N = 10$) and water content of (C) IVD ($N = 5$), (D) NP alone ($N = 3$–5), and (E) AF alone ($N = 4$–5). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ for post hoc between injury; $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ for post hoc between sex. Each symbol represents a different donor in each sex. AF, annulus fibrosus; IVD, intervertebral disc; LPS, lipopolysaccharide; NP, nucleus pulposus.

biochemical content where GAG/DW decreased ($p = 0.033$, Figure S1G), and COL/DNA increased in punctured discs compared to untreated controls ($p = 0.012$, Figure S1J). Sex was a significant factor in NP DNA/DW ($p = 0.001$), where the puncture group had lower DNA content in male NP compared to female NP ($p = 0.007$, Figure 4A). Injury was a significant factor in NP COL/DNA ($p = 0.038$); however, no significant difference between injuries was found (Figure 4E). NP COL/DNA increased slightly in puncture versus untreated, though this difference was not significant ($p = 0.091$) (Figure 4E). No significant differences in NP biochemical content were observed in LPS injured groups versus untreated.

The biochemical content of AF in untreated groups was significantly different in male and female discs. The DNA/DW of male AF was lower than female AF, regardless of injury ($p < 0.0001$, Figure 4F). Untreated male discs also had significantly lower GAG/DW and COL/DW content in the AF compared to untreated female discs ($p = 0.03$ and $p < 0.0001$, Figure 4G,H). In the AF region, injury ($p = 0.0007$) and sex ($p < 0.0001$) were significant factors in DNA/DW content, where LPS and puncture injury led to a decrease in DNA content compared to untreated female, but not male, AF ($p = 0.0015$ and $p = 0.0091$, Figure 4F). When assessing changes in GAG content, LPS decreased GAG/DW versus untreated in female donors ($p = 0.045$, Figure 4G), while puncture injury significantly decreased GAG/DW content in both sexes compared to their respective untreated group (male: $p = 0.004$, female: $p < 0.0001$, Figure 4G). Moreover, puncture led to lower GAG/DW than LPS injured AF in both sexes (male: $p = 0.0015$, female: $p = 0.0003$, Figure 4G). Injury was not a significant factor in COL/DW content ($p = 0.053$, Figure 4H) of male or female discs.

Normalizing ECM content by DNA content led to further injury- and sex-dependent effects. The GAG/DNA content in AF was greater in male than female discs, regardless of injury ($p \leq 0.0002$, Figure 4I). Sex, injury, and interaction effects were significant in AF GAG/DNA content ($p < 0.0001$, $p < 0.0001$, and $p \leq 0.0011$, Figure 4I). Male-LPS injured AFs exhibited a higher GAG/DNA compared to both male-untreated and male-punctured AF, respectively ($p < 0.0001$ and

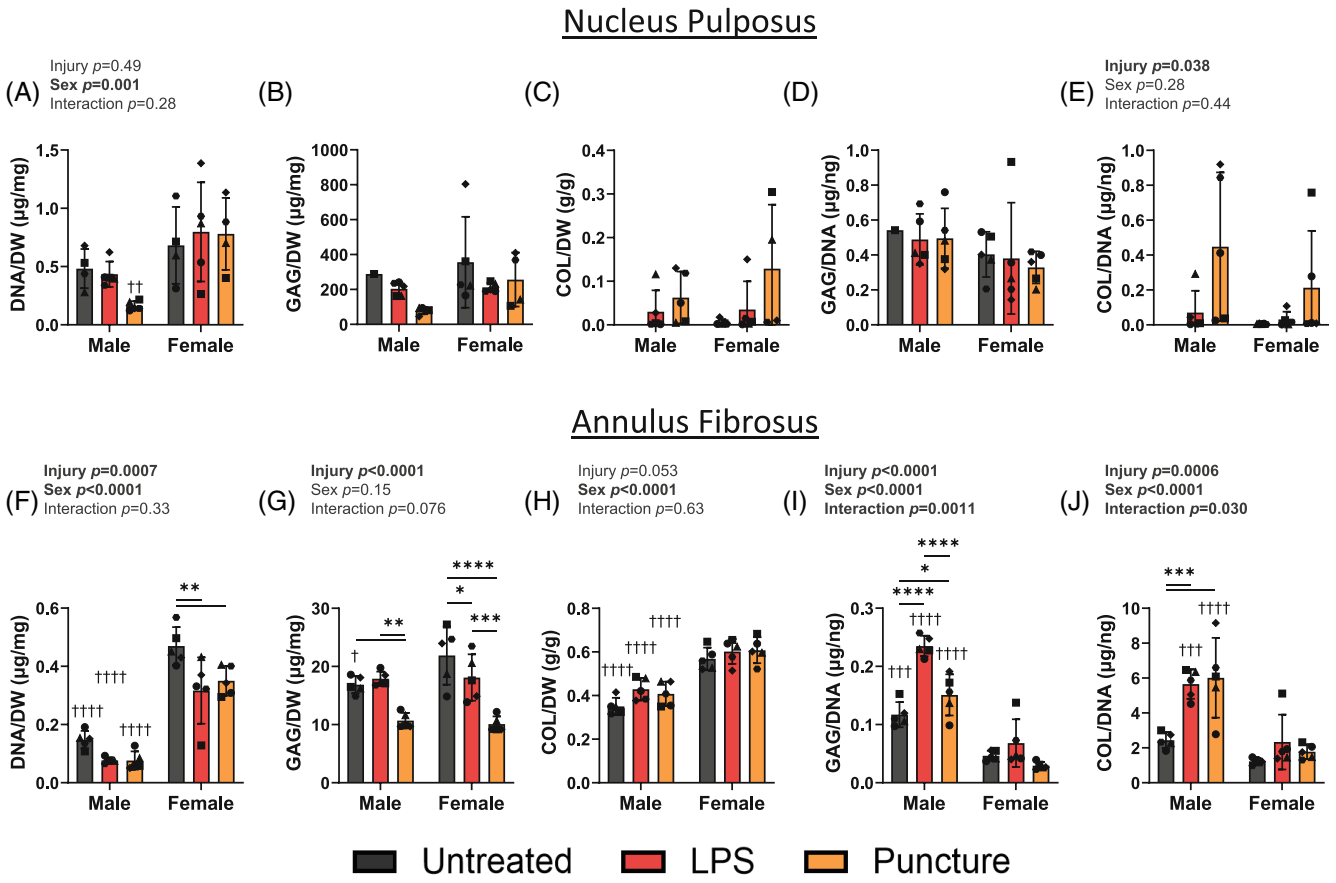

FIGURE 4 Biochemical content of long-term cultured motion segments separated by NP (A–E) and AF (F–J) regions. Biochemical content of the NP region of the disc is displayed as (A) DNA/dry weight (DW), (B) GAG/DW, (C) COL/DW, (D) GAG/DNA, and (E) COL/DNA ($N = 4$–5 for all except for COL/DW and COL/DNA graphs where samples for male untreated were lost or undetectable). Biochemical content of the AF region of the disc is displayed as (F) DNA/DW ($N = 5$), (G) GAG/DW ($N = 5$), (H) COL/DW ($N = 5$), (I) GAG/DNA ($N = 5$), and (J) COL/DNA ($N = 5$). For NP COL/DW and NP COL/DNA graphs, ANOVA was only conducted on LPS and puncture groups for both sexes due to missing samples. Salvaged male untreated was used for DNA and GAG analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ for post hoc between injury; $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ for post hoc between sex. Each symbol represents a different donor in each sex. AF, annulus fibrosus; ANOVA, analysis of variance; LPS, lipopolysaccharide; NP, nucleus pulposus.

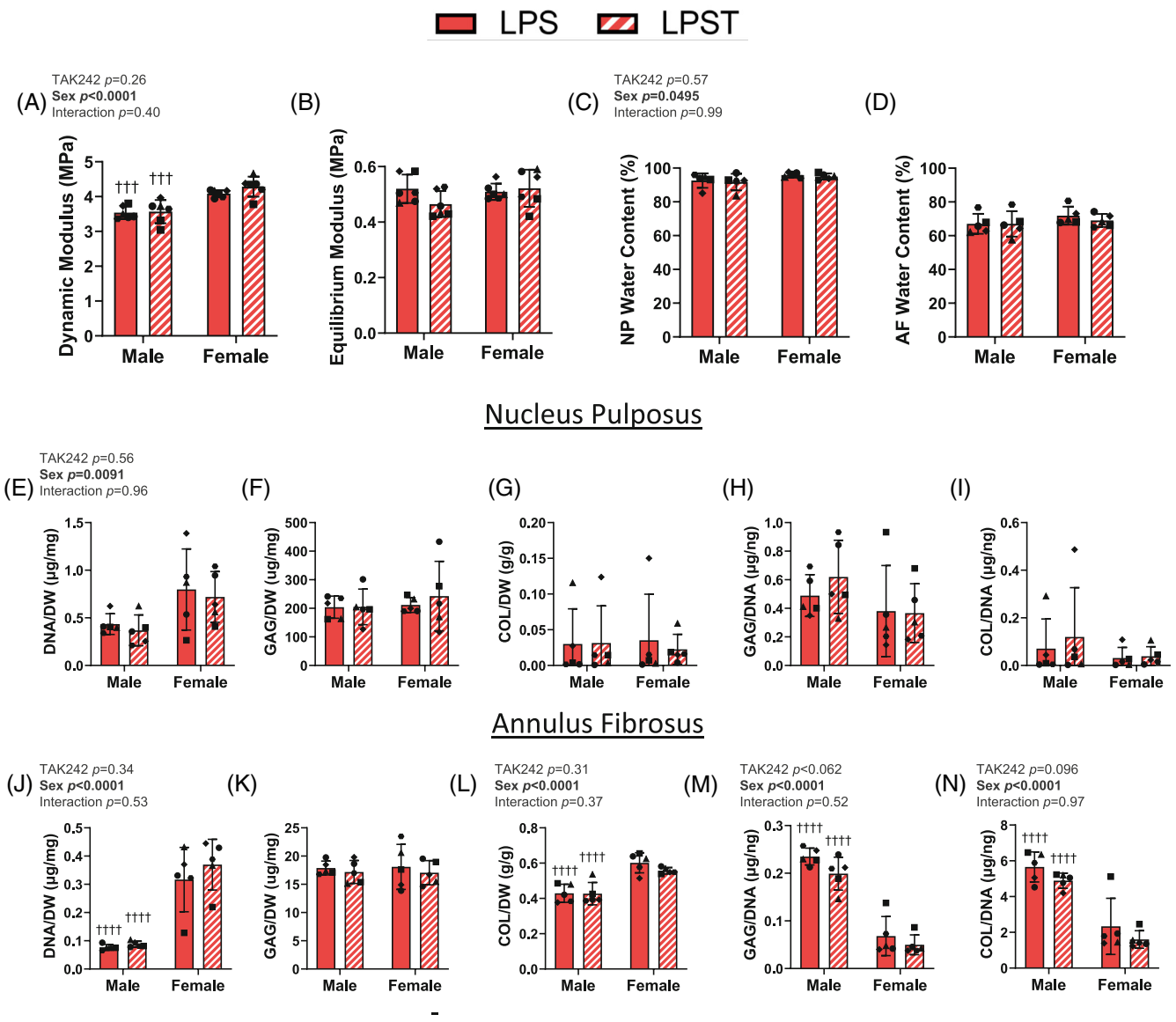

FIGURE 5 Mechanical properties of long-term LPS and LPST treated motion segments: (A) dynamic modulus ($N = 6$) and (B) equilibrium modulus ($N = 6$). Water content percentage of (C) NP ($N = 5$) and (D) AF ($N = 5$). Biochemical content of long-term cultured motion segments separated by NP (E–I) and AF (J–N) regions. Biochemical content of the NP region of the disc is displayed as (E) DNA/DW ($N = 5$), (F) GAG/DW ($N = 5$), (G) COL/DW ($N = 5$), (H) GAG/DNA ($N = 5$), and (I) COL/DNA ($N = 5$). Biochemical content of the AF region of the disc is displayed as (J) DNA/DW ($N = 5$), (K) GAG/DW ($N = 5$), (L) COL/DW ($N = 5$), (M) GAG/DNA ($N = 5$), and (N) COL/DNA ($N = 5$). $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ for post hoc between sex. Each symbol represents a different donor in each sex. AF, annulus fibrosus; DW, dry weight; LPS, lipopolysaccharide; LPST, LPS + TAK; NP, nucleus pulposus.

$p < 0.0001$, Figure 4I). Male-punctured AF also had greater GAG/DNA compared to male-untreated AF ($p = 0.044$, Figure 4I). These differences were not observed in AF GAG/DNA in samples from female donors. In AF COL/DNA, injury ($p = 0.0006$) and sex ($p < 0.0001$) effects were observed in addition to interaction effects ($p = 0.030$), where LPS and punctured discs had greater COL/DNA than untreated AF ($p = 0.0006$ and $p = 0.0003$, Figure 4J) in samples from male but not female donors. There was, however, significantly higher COL/DNA in male-LPS injured and punctured discs compared to corresponding female discs (LPS: $p = 0.0005$, puncture: $p < 0.0001$, Figure 4J).

3.5 | TAK-242 treatment of LPS injured and punctured discs

In LPS injury groups, sex-($p < 0.0001$), but not TAK-treatment ($p = 0.26$), was a significant factor in the dynamic modulus of discs (Figure 5A). Female-LPS and female-LPST discs exhibited higher dynamic moduli compared to their male counterparts ($p = 0.0009$ and $p = 0.0001$, Figure 5A). For NP water content, sex was a significant factor ($p = 0.050$), while TAK was not ($p = 0.57$, Figure 5C). However, there were no TAK- or sex-dependent differences in equilibrium modulus or AF water content in LPS and LPST discs (Figure 5B,D).

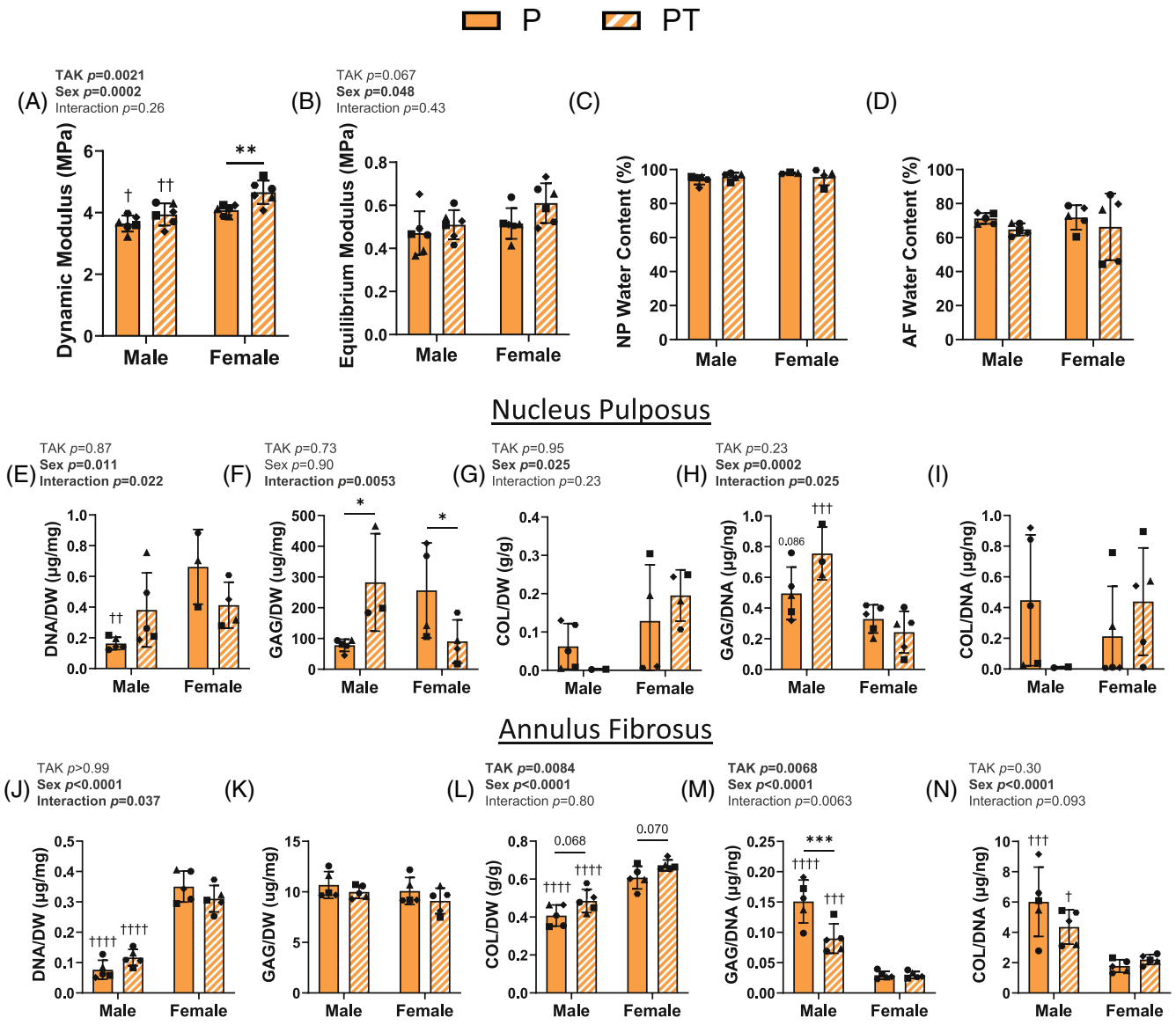

FIGURE 6 Mechanical properties of long-term P and PT treated motion segments: (A) dynamic modulus ($N = 6$) and (B) equilibrium modulus ($N = 6$). Water content percentage of (C) NP ($N = 5$) and (d) AF ($N = 5$). Biochemical content of long-term cultured motion segments separated by NP (E–I) and AF (J–N) regions. Biochemical content of the NP region of the disc is displayed as (E) DNA/DW ($N = 3$–5), (F) GAG/DW ($N = 3$–5), (G) COL/DW ($N = 2$–5), (H) GAG/DNA ($N = 3$–5), and (I) COL/DNA ($N = 2$–5). Biochemical content of the AF region of the disc is displayed as (J) DNA/DW ($N = 5$), (K) GAG/DW ($N = 5$), (L COL/DW ($N = 5$), (M) GAG/DNA ($N = 5$), and (N) COL/DNA ($N = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ for post hoc between TAK injury, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ for post hoc between sex. Each symbol represents a different donor in each sex. AF, annulus fibrosus; DW, dry weight; NP, nucleus pulposus; P, puncture; PT, puncture + TAK.

In the NP region of discs, there was no effect of TAK between LPS NP and LPST NP in DNA/DW, GAG/DW, COL/DW, GAG/DNA, and COL/DNA (Figure 5E–I). Sex is a significant contributor in NP DNA/DW ($p = 0.0091$), but there are no NP male–female differences ($p > 0.095$, Figure 5E). Sex also did not affect NP GAG/DW, COL/DW, GAG/DNA, or COL/DNA (Figure 5F–I). In the AF region of LPS and LPST discs, there appeared to be no effect of TAK in DNA/DW, GAG/DW, COL/DW, GAG/DNA, and COL/DNA content (Figure 5J–N). However, sex was a significant contributor to AF DNA/DW ($p < 0.0001$), with lower levels found in male versus female, independent of TAK treatment ($p < 0.0001$, Figure 5J). Sex was also a significant factor in COL/DW ($p < 0.0001$, Figure 5L) with lower levels in male versus female samples, independent of TAK treatment (Figure 5L). When normalizing GAG or COL by DNA content, both GAG/DNA (LPS: $p < 0.0001$, LPST: $p < 0.0001$) and COL/DNA (LPS: $p < 0.0001$, LPST: $p < 0.0001$) were significantly greater in male versus female samples, independent of TAK treatment group (Figure 5M,N).

In the puncture injury groups, both TAK treatment ($p = 0.0021$) and sex ($p = 0.0002$) were significant factors contributing to the dynamic modulus (Figure 6A). Male P ($p = 0.023$) and male PT ($p = 0.0011$) discs exhibited lower dynamic moduli compared to their

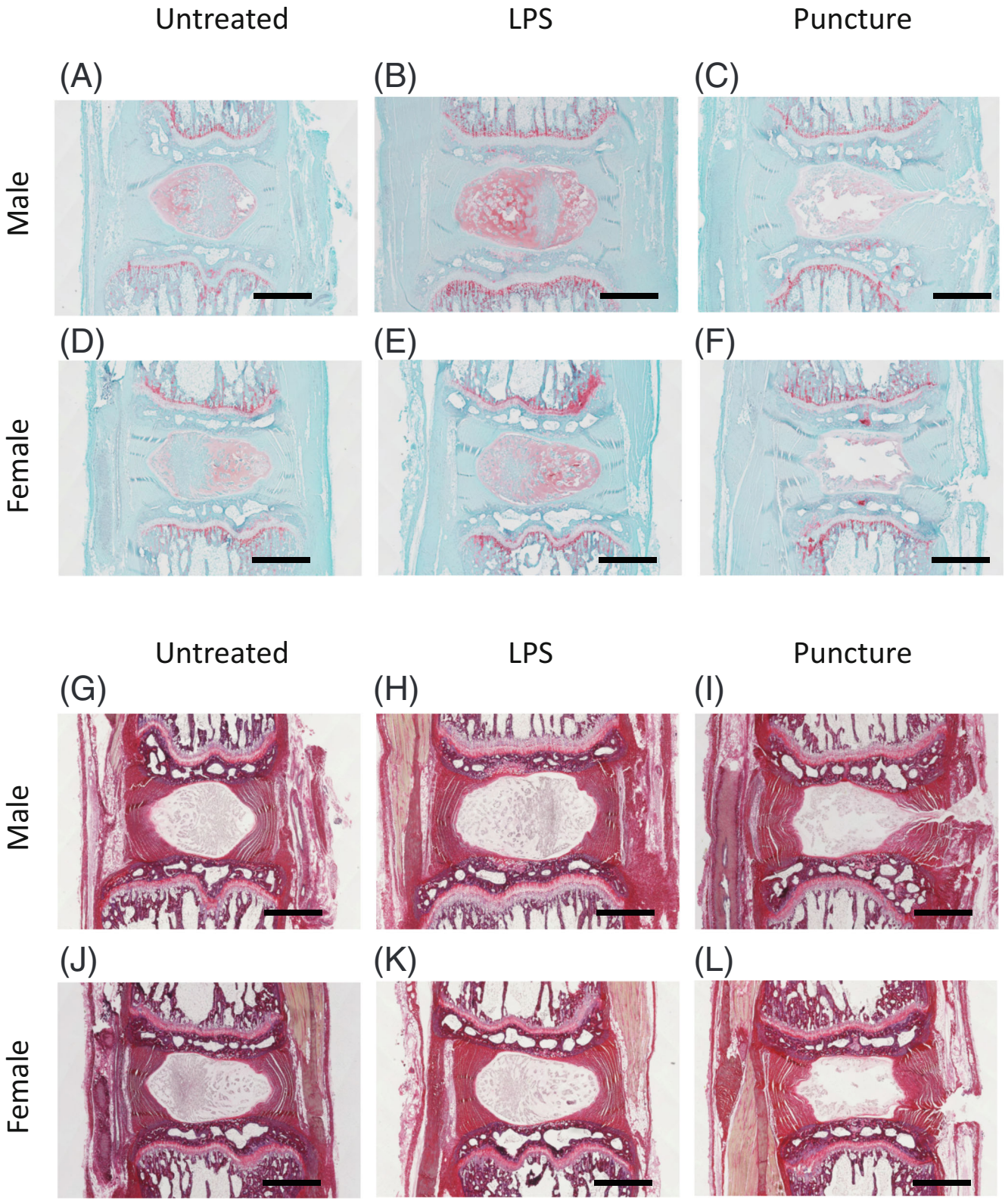

FIGURE 7 Safranin O with Fast Green staining of short-term male (A) untreated, (B) LPS, and (C) puncture and female (D) untreated, (E) LPS, and (F) puncture discs. Picrosirius Red staining of short-term male (G) untreated, (H) LPS, and (I) puncture and female (J) untreated, (K) LPS, and (L) puncture discs. Scale bar: 1 mm. LPS, lipopolysaccharide.

female counterparts (Figure 6A). Additionally, TAK treatment increased dynamic moduli values for female ($p = 0.007$), but not male discs ($p = 0.11$, Figure 6A). There was significant sex- ($p = 0.048$), but not TAK-dependent ($p = 0.067$), effects on equilibrium modulus of punctured discs (Figure 6B). Both sex and TAK did not affect NP or AF water content of P and PT discs (Figure 6C,D). In the NP region of

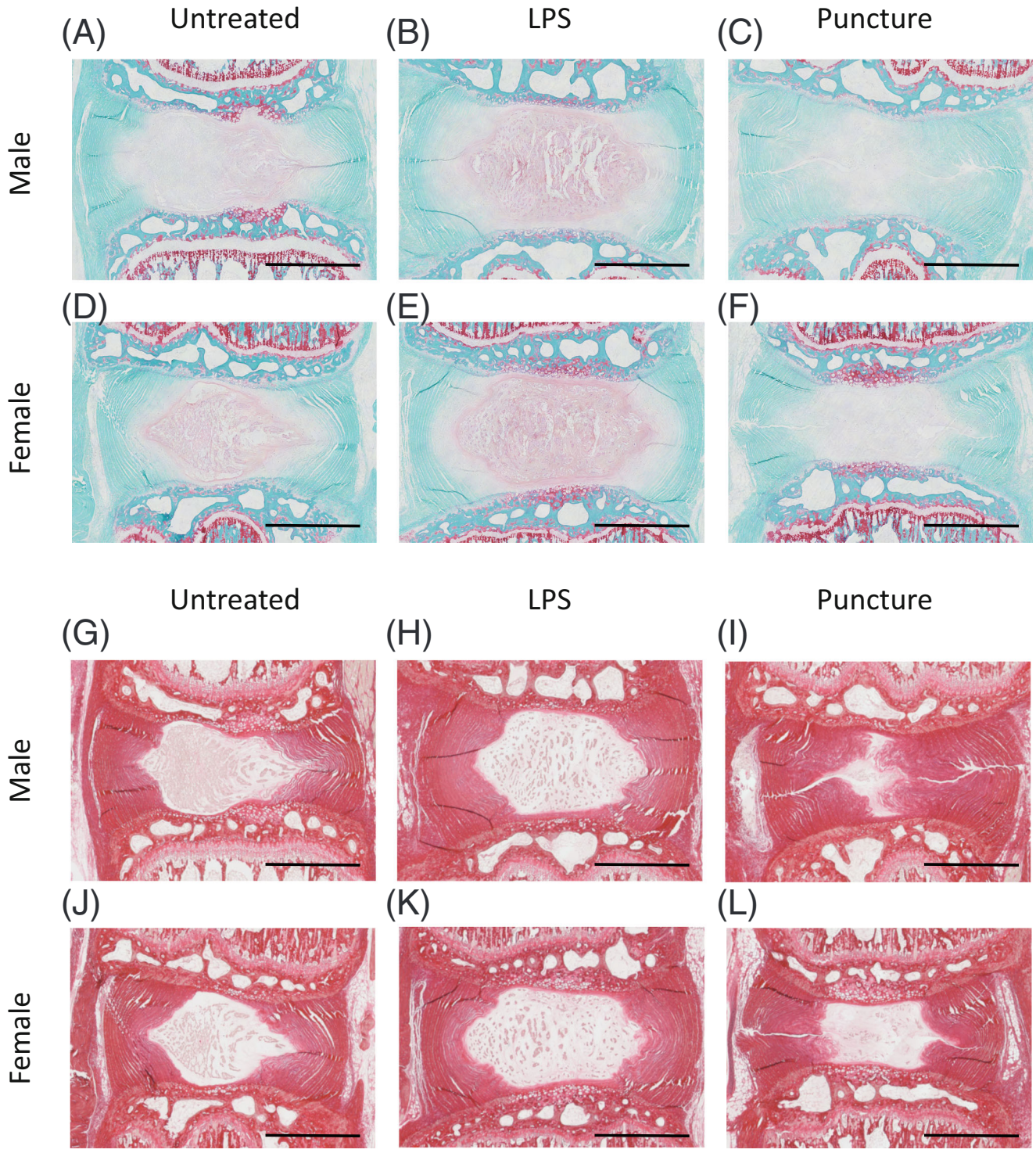

FIGURE 8 Safranin O with Fast Green staining long-term male (A) untreated, (B) LPS, and (C) puncture and female (D) untreated, (E) LPS, and (F) puncture discs. Picrosirius Red staining of long-term male (G) untreated, (H) LPS, and (I) puncture and female (J) untreated, (K) LPS, and (L) puncture discs. Scale bar: 1 mm. LPS, lipopolysaccharide.

discs, there appears to be no effect of TAK between P and PT in DNA/DW, GAG/DW, COL/DW, GAG/DNA, or COL/DNA (Figure 6E–I). The NP DNA/DW showed a significant effect of sex ($p = 0.011$) and sex–TAK interaction ($p = 0.022$), with lower levels of male-NP DNA/DW in punctured discs than in corresponding female discs ($p = 0.0046$, Figure 6E). Furthermore, there were significant interaction terms in NP GAG/DW ($p = 0.0053$, Figure 6F), where TAK treatment increased punctured male-NP GAG/DW ($p = 0.049$) but decreased female-NP GAG/DW ($p = 0.049$, Figure 6F). Sex was a significant factor in COL/DW ($p = 0.025$, Figure 6G), but no

differences in group comparisons were observed (Figure 6G). Sex ($p = 0.0002$, Figure 6H) and sex–TAK interaction effects ($p = 0.025$) were significant in GAG/DNA, with higher levels of GAG/DNA in male versus female TAK-treated groups ($p = 0.0004$, Figure 6H).

Sex ($p < 0.0001$) and sex–TAK interaction ($p = 0.037$) were significant factors in AF DNA/DW, where female P and PT have higher DNA/DW content than males ($p < 0.0001$, Figure 6J). TAK ($p = 0.0084$) and sex ($p < 0.0001$) were significant factors in AF COL/DW ($p < 0.0001$, Figure 6L) with lower levels in male versus female samples, independent of treatment (Figure 6L). Increasing trends in AF COL/DW were observed in both male and female samples due to TAK treatment (Figure 6L). AF GAG/DNA had significant contributions from sex ($p < 0.0001$), TAK ($p = 0.0068$), and sex–TAK interactions ($p = 0.0063$, Figure 6M), where male GAG/DNA is higher than female counterparts, regardless of TAK treatment (P: $p < 0.0001$, PT: $p = 0.0005$, Figure 6M). TAK significantly decreased AF GAG/DNA versus puncture in male ($p = 0.0009$), but not female samples (Figure 6M). When normalizing collagen by DNA content, sex ($p < 0.0001$, Figure 6N) remains a significant contributor to AF COL/DNA, with higher levels in male versus female, independent of TAK treatment (Figure 6N).

3.6 | Histological analysis

Histological staining of short- and long-term LPS and punctured discs exhibited injury-dependent differences based on Safranin O and Picrosirius Red staining. In untreated discs, the shape of the NP in male (Figures 7A,G and 8A,G, Figure S3) and female discs (Figures 7D,J and 8D,J) appeared oval with mild distortions, and exhibited similarities in vacuolated NP cell morphology and proteoglycan staining (Figures 7A,D and 8A,D). With LPS injury, male (Figures 7B,H and 8B,H) and female discs (Figures 7E,K and 8E,K) had additional distortions to NP shape along with decreases in vacuolated NP cell morphology. However, there appears to be higher proteoglycan staining intensity in the NP region of LPS-treated discs (Figures 7B,E and 8B,E) compared to respective untreated controls (Figures 7A,D and 8A,D). Puncture injury caused the greatest disruption to disc structure in both male (Figures 7C,I and 8C,I) and female discs (Figures 7F,L and 8F,L). In the puncture group, the NP became irregular in shape and smaller in area in males (Figures 7C,I and 8C,I) and females (Figures 7F,L and 8F,L). After puncture injury, loss of NP cellularity was also observed; the NP-AF border appeared more interrupted, and the AF appeared more distorted with greater evidence of fissures (Figures 7C,F,I,L and 8C,F,I,L). Puncture injury significantly increased the average degeneration grade compared to untreated male and female discs in both short- and long-term cultures (Figures S3A and S4A). Short-term, male-punctured discs also had a higher average degeneration score in the endplate category than their female counterparts (Figure S3J). In long-term culture, however, no sex differences in histological grading were observed based on the grading of Safranin O and Picrosirius Red staining (Figure S4).

4 | DISCUSSION

This study identified sex-based differences in male and female caudal IVDs from the rat, exhibited by differences in mechanical properties, namely dynamic and equilibrium moduli, ECM biochemical content, and inflammatory response. Female untreated discs had a higher dynamic modulus compared to male untreated discs. AF of female untreated discs also had lower water content and greater DNA content suggesting greater cellularity, in addition to higher levels of GAG and COL content than in male untreated AF. The higher ECM biochemical content, thus, may be contributing to the higher dynamic modulus of female discs compared to males. Moreover, chemical injury using LPS led to a greater inflammatory response in female discs than in male discs in the short-term study, yet this difference in response is lost when looking at the long-term results. In contrast, a greater inflammatory response was seen in males compared to females in the long-term study after puncture injury. This suggests that the IVD inflammatory response to injury is sex- and time-dependent. One advantage of using this ex vivo approach is that it isolates sex-based differences that are specific to the IVD motion segment, the organ of interest, rather than responses dependent on systemic interaction with injury (e.g., immune system or hormone responses).

The findings of the current study extend earlier findings on sex effects in rat IVDs. Mosley et al. identified some baseline distinct sex differences in IVD height where males had greater baseline IVD height than females. Likewise, additional sex-based differences were reported due to an in vivo 3× puncture injury.[35] While both sexes had decreased cellularity and increased fibronectin at injury sites, females had an increased degeneration grade in the outer AF compared to males. Meanwhile, male IVDs had greater torsional stiffness, torque range, and viscoelastic creep responses, while females had reduced SHG intensity compared to males post-puncture injury, suggesting that male IVDs exhibited improved healing compared to female punctured IVDs.[35] In the current study, the response to injury, physical (puncture) or chemical (LPS), also exhibited differences between sexes. Indeed, we saw more of a sex-based response when discs were subjected to injury. Female IVDs, compared to male IVDs, appear to be more biologically responsive to LPS stimulation, indicated by their increased NO release compared to their respective untreated controls, and more susceptible to mechanical degradation, as evidenced by a significant decrease in equilibrium moduli in female-punctured compared to female untreated. However, both male and female discs were mechanically responsive to chemical and puncture injuries with decreased dynamic moduli. Chemical injury using LPS led to an increase in NO release into the media compared to untreated discs, while punctured discs did not. Given the short 12-h half-life of NO,[36] our sensitivity to observe changes in NO levels in short- versus long-term experiments was varied given the different time points of media collections in the experiments. Furthermore, during the short-term experiments, we see a higher release from female LPS discs than their male counterparts. Thus, it appears that females are more sensitive to LPS treatment in the short-term experiment than males.

However, this may not be evident in the long-term experiment due to the longer duration to first or between media collections.

HMGB1 release was increased in punctured discs, with no sex-based effect, suggesting that cell necrosis due to puncture injury may be contributing to HMGB1 release.[37] Stratifying by sex further magnified injury effects on HMGB1 release. Interestingly, LPS stimulation did not induce significant HMGB1 release, which differs from prior findings on LPS-injured human NP cells,[28] indicating that presence of complex ECM may be regulating HMGB1 responses. Thus, HMGB1 release appears to be dependent on disc injury. Additionally, the daily level of HMGB1 release was so minute by Day 6 of culture, rendering the disc release of HGMB1 below the measurement range of the assay. Like LPS, HMGB1 can serve as an agonist of TLR4 and lead to downstream nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) activation.[38]

Both LPS and puncture injury led to a significant decrease in dynamic modulus in male and female discs. In addition, puncture injury also reduced AF GAG content. Korecki et al. previously used a bovine IVD organ culture loading model to examine the effect of needle puncture injury on disc mechanics and biology. They observed decreased dynamic modulus due to needle puncture injury,[39] which is consistent with the findings of this study. Interestingly, they did not detect changes in GAG release into the media. Our results demonstrate an increase in GAG loss into the media with puncture injury, along with decreased GAG content in the AF post-puncture injury. The results suggest that the AF was more responsive to changes with injury, specifically GAG content, compared to the NP response to injury.

However, when looking at GAG production per AF DNA content, we see an increase in GAG content of male LPS injured and punctured AFs compared to male untreated AF suggesting higher ECM content potentially due to lower cellular density in males. However, we do not see the same response to injury in samples from female donors suggesting that injury response is sex-dependent. A similar effect is observed when looking at COL production per AF DNA, where there is an increase in COL/DNA in male LPS injured and punctured AF compared to their female injured counterparts. The higher GAG/DNA and COL/DNA content post-injury in male AF compared to female AFs also indicated a greater biosynthetic response post-injury, suggesting a greater ECM remodeling capabilities in motion segments isolated from male donors compared to female donors. This supports the prior finding of male IVDs having improved ECM properties compared to female IVDs as reported by greater SHG intensity in the outer AF post-puncture injury,[35] which represents COL integrity,[16] COL organization,[40] and COL fibril diameter.[41] Lower biosynthetic responses of AF motion segments appear to be primarily driven by the higher cellularity of female AF, where cells may not be responding biosynthetically to the same degree post-injury as in the male AF. However, in our study, female discs appeared to have healthier histological features than male discs 38 days post-puncture. In addition to remodeling differences in response to injury, the higher levels of AF COL and dynamic modulus in uninjured female IVDs may have protected against injury insult, compared to samples from male donors, and thus lowered the overall need to drive biosynthetic responses. Future studies are needed to directly measure the biosynthetic response of male and female AF cells and their cellular sensitivity to injury.

TAK-242 is a small molecule inhibitor of TLR4 signaling that intracellularly interferes with interactions between TLR4 and its adaptor molecules.[42] Surprisingly, TAK-242 was more effective at improving the structural and functional properties of punctured discs than LPS-stimulated discs. Since HMGB1 can serve as an agonist of TLR4 and lead to downstream NF-κβ activation, the greater effectiveness on punctured IVDs may be due to the drug's blocking of TLR4 signaling through the HMGB1 ligand. Direct LPS stimulation may have overpowered the potential protective effects of TAK-242. Additionally, the puncture injury may have facilitated better diffusion of the TAK-242 drug into the motion segment, whereas the structural integrity of LPS injured discs was maintained, thus limiting diffusion of TAK-242 throughout the motion segment. TAK-242 appeared to improve the dynamic modulus of female punctured, but not male discs, which may be evidenced by greater levels of COL/DW (albeit not significant) in female NP and AF compared to punctured only discs. When comparing IVD geometry alone, females generally had a lower IVD radii, heigh, volume, and motion segment wet weight than males. There were also differences in ECM dry weight; female AF dry weights were smaller than their male counterparts, while NP dry weights were similar between male and female. Thus, by normalizing the GAG and COL content to wet weight or dry weight of separated NP and AF components, we are accounting for variation due to sex, size, or biological variability in the biochemical content of the ECM. Water content is also reported normalized to the original weight of the NP or AF, therefore, accounting for variability in size due to sex or biological variation. Ultimately, observations of differences in biochemical content presented normalized to wet or dry weight represent differences in the biochemical content, independent of the variation in size between male and female donors. In mechanical testing, we observed clear differences in the size of male and female motion segments, and thus incorporated the size differences in our mechanical testing (as normalizing factors).

Limitations of this study include the inherent differences in male and female anatomy and physiology, such as bone density, animal size, and hormone levels. Additionally, different animal donors were used for the short-term and long-term experiments, so biological variability was observed with different study durations. Furthermore, the long-term ex vivo culture is limited by a lack of perfusion mimicking bodily fluids and vascularization, potentially leading to non-physiologic, deleterious effects on IVD cells. We may have lost some cartilage-like phenotype, based on GAG content changes in our culture system; thus, GAG retention using media supplements like ascorbic acid, could be explored in future studies. Future directions include determining how sex hormones, such as estrogen and testosterone, can influence inflammatory, biochemical, and biomechanical responses after injury.[8] This study also supports the notion for future preclinical research to consider separate male and female cohorts, as there are baseline differences in the IVD, as well as distinct sex-dependent responses to physical and chemical injury.

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