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RESEARCH ARTICLE

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Repetitive strikes loading organ culture model to investigate the biological and biomechanical responses of the intervertebral disc

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Abstract

Background: Disc degeneration is associated with repetitive violent injuries. This study aims to explore the impact of repetitive strikes loading on the biology and biomechanics of intervertebral discs (IVDs) using an organ culture model.

Methods: IVDs from the bovine tail were isolated and cultured in a bioreactor, with exposure to various loading conditions. The control group was subjected to physiological loading, while the model group was exposed to either one strike loading (compression at 38% of IVD height) or repetitive one strike loading (compression at 38% of IVD height). Disc height and dynamic compressive stiffness were measured after overnight swelling and loading. Furthermore, histological morphology, cell viability, and gene expression were analyzed on Day 32. Glycosaminoglycan (GAG) and nitric oxide (NO) release in conditioned medium were also analyzed.

Results: The repetitive one strike group exhibited early disc degeneration, characterized by decreased dynamic compression stiffness, the presence of annulus fibrosus clefts, and degradation of the extracellular matrix. Additionally, this group demonstrated significantly higher levels of cell death ($p < 0.05$) and glycosaminoglycan (GAG) release ($p < 0.05$) compared to the control group. Furthermore, upregulation of MMP1, MMP13, and ADAMTS5 was observed in both nucleus pulposus (NP) and

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annulus fibrosus (AF) tissues of the repetitive one strike group ($p < 0.05$). The one strike group exhibited annulus fibrosus clefts but showed no gene expression changes compared to the control group.

Conclusions: This study shows that repetitive violent injuries lead to the degeneration of a healthy bovine IVDs, thereby providing new insights into early-stage disc degeneration.

KEYWORDS
degeneration, intervertebral disc, mechanical load, organ culture, repetitive injury

1 | INTRODUCTION

Lowback pain (LBP) is a prevalent public health problem, affecting a substantial proportion of individuals during their lifetime.[1] LBP places a heavy burden on the global healthcare system and economy.[2] Numerous factors contribute to the occurrence of back pain; within which, disc degeneration plays a key role in the etiology of LBP.[3,4]

The intervertebral disc (IVD) consists of the cartilaginous endplate (CEP), annulus fibrosus (AF) and nucleus pulposus (NP), playing a crucial role in spinal biomechanics.[5] The CEP comprising approximately 60% water, and serves as a vital conduit for nutrient exchange between the NP and the adjacent vertebrae.[6] The AF is composed of 15–25 concentric layers of collagen fibers and contains approximately 65%–70% water content.[7] This unique structural composition enables the IVD to withstand various loads and maintain its mechanical integrity.[8] The abundant water content of the NP, situated centrally within the IVD, facilitates the alleviation of stress by transferring mechanical load to the AF.[7]

Numerous studies have explored intervertebral disc degeneration. It is crucial to choose an appropriate experimental model. In vitro cell culture models simplify conditions but lack the in vivo mechanical cues.[9,10] Animal models resemble humans but face challenges with reproducibility and biological variations.[11,12] Depends on which model. Small animal does not resemble humans at all. Considering these factors, intervertebral disc organ culture has emerged as an appealing approach to compensate the disadvantages associated with in vivo and in vitro conditions.[13,14] Furthermore, bovine caudal discs exhibit notable similarities to mature human discs in essential aspects such as glycosaminoglycan content, cell density, and the absence of notochordal cells.[15,16] Consequently, they are frequently employed as a model for preclinical research.

Mechanical stimulation profoundly influences the synthesis and degradation processes of the IVD. Multiple studies have demonstrated that both supraphysiological loading and repetitive and complex loading have been implicated in the process of disc degeneration.[17,18] In our previous study, in vitro organ culture of bovine caudal discs was conducted. The IVDs were subjected to a supraphysiological mechanical loading. The findings indicated that one strike loading (compression at 50% of IVD height) led to disc degeneration.[19]

Repetitive and high amplitude loading plays a pivotal role in lumbar spine injuries.[20–22] Previous research has demonstrated that the application of repetitive axial, nonviolent loading induces an expedited progression of intervertebral disc degeneration (IVDD) in rabbits.[23] Several researchers have documented that multiple disc puncture injuries initiate a detrimental cycle involving inflammatory responses and matrix degradation, ultimately resulting in progressive disc degeneration.[24] Most studies on the association between disc degeneration and stress injuries primarily focus on chronic stress injuries. However, there is a notable gap in the literature concerning the impact of repetitive acute violent injuries on disc degeneration. In order to delve deeper into the relationship between repetitive violent injuries and disc degeneration, the aim of this study was to examine the biological and biomechanical correlation between repetitive violent injury and IVDD, utilizing the bovine IVD organ culture model.

2 | MATERIALS AND METHODS

2.1 | Extraction of bovine intervertebral disc

Bovine tails were procured from a local abattoir for experimentation. Since the materials used were derived from the surplus of the abattoir and in compliance with Chinese regulations, ethical committee approval was exempted. The isolation of IVDs was performed according to a previously described method.[25] Briefly, following the removal of the surrounding soft tissue, individual IVDs with intact endplates were carefully excised using a band saw. The endplates were subsequently cleaned with phosphate-buffered saline (PBS) via an APEX-PULSE Disposable Pulse Lavage system (Apex, Guangzhou, China). The IVDs were then subjected to a 15-min wash in PBS supplemented with 10% penicillin/streptomycin (Gibco, Waltham, MA) while placed on a shaking table. Subsequently, the IVDs were incubated in a humidified (85%) atmosphere with 5% CO_2 at 37°C using Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Munich, Germany) supplemented with 2% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% ITS, 50 μg/mL L-ascorbic acid, and 0.1% Primocin. Three tails were used. Each group comprised three IVDs obtained from three separate tails. IVDs from the same tail were randomly allocated to three experimental groups: the Physiological Loading control group,

the One Strike Loading model group, and the Repetitive One Strike Loading model group.

2.2 | Cultivation of IVDs and mechanical stress

On Day 1, IVDs underwent physiological loading (0.02–0.2 MPa, 1 Hz/h) within our custom-made bioreactor.[26] The bioreactor was maintained under conditions of 37°C, 85% humidity, and 5% CO_2. On Day 2, IVDs were subjected to mechanical stress (MS) utilizing a universal mechanical tester within a custom-designed incubation chamber,[19] which were maintained at room temperature condition. In the physiological loading group, daily loading occurred at (0.02–0.2 MPa, 1 Hz, 1 h/day) from Day 1 to Day 32, with the remaining time allocated for free swelling in the culture medium. The one strike group underwent MS on Day 2 and received daily loading at (0.02–0.2 MPa, 1 Hz, 1 h/day) from Day 1 to Day 32, with the remainder of the time spent in free swelling in the culture medium. In the repetitive one strike group, loading was conducted daily, excluding Days 2, 5, 8, 11, 14, 17, 20, 23, 26, and 29, with the remaining time allocated for free swelling in the culture medium. The chamber was filled with culture medium to prevent dehydration of IVDs. Based on our previous study,[27] wherein compression at 40% of IVD height was identified as a threshold for irreversible damage, we subsequently selected a compression at 38% of IVD height as the object of this study. In the model group, prior to MS, a 10 N preload was applied for 3 min to ensure reliable contact between the IVD and the load cell, followed by compression at 38% of disc height in 1 s. Following MS injury, the IVDs were cultured with daily physiological loading until Day 32 (long term). For repetitive one strike group, MS was applied on following days: Days 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, and physiological loading was performed for the rest of the day up to Day 32 (long term).

2.3 | Measurement of IVDs height changes

The mean disc height (including the endplates) was measured using calipers at various time points: directly after dissection on Day 0, following overnight free swelling cultures from Day 1 to Day 32, and from Day 1 to Day 31 after loading. Two measurements were conducted per disc to determine the average value, which was subsequently normalized to the initial dimensions on Day 0.

2.4 | Dynamic compressive stiffness

Dynamic compression stiffness was evaluated at specific time points using a custom-designed bioreactor following overnight free swelling on Days 1, 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, and 32.[19] The IVD underwent a 3-min preload, applying a 10% strain determined by the disc height measured before stiffness evaluation, followed by 10 rounds of sinusoidal compression ranging from 5% to 15% strain (10% strain/s). The maximum force (F_{max}) and minimum force (F_{min}) achieved in each round were recorded, while the area of the IVD (S) was measured. The dynamic compression stiffness was then calculated using the formula $(F_{max} - F_{min})/S$. The average from the 10 loading cycles were calculated and used. On Day 1, the compression stiffness of each disc was measured as the baseline for normalization.

2.5 | Histology

On Day 32, IVDs were rapidly fresh frozen and transversely sectioned into 20 μm thick slices and then fixed in 70% methanol, and then changed to 100% methanol. Staining was carried out using 0.1% Safranin-O (Sigma-Aldrich, Munich, Germany) and 0.02% Fast Green (Sigma-Aldrich, Munich, Germany) to visualize proteoglycan and collagen deposition Weigert's hematoxylin staining was performed to observe the distribution of cell nucleus. Imaging was conducted using a light microscope (Kfbio, Ningbo, China). A semiquantitative scoring scheme was employed to assess the degree of degeneration[28] (Table 1).

2.6 | Cell viability

Lactate dehydrogenase (LDH) and ethidium homodimer (ETH) staining was performed to evaluate the cell viability. Distinct regions within

TABLE 1 Histological score grading criteria.

Grade	Histological degeneration
IVD structure	Histology cross section clefts characteristics
0	Normal IVD structure with well-defined annular lamellae, central NP
1	Clefts evidence in IAF, normal NP morphology
2	Clefts evident in IAF, mild clefts in OAF, inverted IAF lamellae with anomalous distortions
3	Bifurcation/propagation of clefts from IAF into NP margins, mild delamination, or concentric tears between lamellae in IAF
4	Propagation of cleft into NP, with disruption in normal NP structure, distortion of annular lamellae into atypical arrangements-severe delamination, separation of translamellar cross bridges
Formation of clefts	
0	No clefts in AF
1	Small clefts area in AF (width of cleft in the range of 90–180 μm)
2	Moderate clefts area in AF (the number of clefts 3, width >180 μm)
3	Moderate clefts area in AF (the number of clefts 3, width >180 μm)
Clefts direction	
0	Clefts were parallel to the AF lamellae
2	Clefts were perpendicular to the AF lamellae

Abbreviations: AF, annulus fibrosus; IAF, inner AF; NP, nucleus pulposus; OAF, outer AF.

the NP, inner annulus fibrosus (IAF), and outer annulus fibrosus (OAF) were selected for analysis. Six randomly selected images (623 × 623 μm) were captured from distinct regions within three specific zones: the NP, the IAF and the OAF in each section. The quantification of live and dead cells was conducted using the ImageJ software. Live cells were identified based on blue staining (LDH) or a combination of blue and red staining (ETH), while cells exhibiting only red staining were classified as dead cells.

2.7 | RNA extraction and gene expression analysis

Gene expression analysis was conducted on histology samples. On Day 32, disc tissue was collected for gene expression analysis. Each disc was labeled in advance according to the natural curvature of the oxtail. After removing the upper and lower endplates, the disc was divided into two parts: one for histological staining and the other for RNA extraction. NP and AF tissues were obtained from each IVD and processed for RNA extraction. Each group underwent experimentation with three samples. For extraction, approximately 150 mg of tissue was digested using 2 mg/mL pronase for 1 h at 37°C. Digestion was halted using FBS, and the tissue was subsequently washed twice with PBS. Remaining PBS was removed, and the tissue was promptly frozen and pulverized in liquid nitrogen using a custom-made pestle device. The resultant powder was combined with 1 mL of TRI Reagent and 5 μL of polyacryl carrier, followed by homogenization using a TissueLyser.[29] Total RNA was extracted and 400 ng of RNA was converted to cDNA, followed by RT-qPCR analysis using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA). Data were analyzed using the $2^{-\Delta\Delta Ct}$ algorithm. The sequences of primers are provided in Table 2.

2.8 | Evaluation of conditioned medium

From Day 1 to Day 33, the medium was collected following free swelling and utilized for the analysis of released GAG and nitric oxide (NO). The content of GAG in the conditioned media was measured using a modified 1,9-dimethyl methylene blue (DMMB) method. The levels of NO and its stable oxidation product, nitrite (NO_2), in the conditioned medium of IVDs was measured using the Griess Reagent kit (Promega, Madison, USA).

2.9 | Statistical analysis

Statistical analyses were conducted utilizing SPSS 22.0 (IBM, Chicago, IL, USA). The Mann–Whitney *U* test was used to assess differences between two groups when the data did not adhere to a normal distribution. Likewise, the Kruskal–Walli's test was utilized to examine differences among three or more groups. A significance level of $p < 0.05$ was considered statistically significant.

TABLE 2 Primers used for RT-qPCR.

MMP1	F:5′-TCCCAGCAGCAACTTCAGAAACA-3′
	R:5′TCCCAGTCACTCTCAGCCCAAA-3′
MMP3	F:5′-GGCTGCAAGGGACAAGGAA-3′
	R:5′-CAAACTGTTTCGTATCCTTTGCAA-3′
MMP13	F:5′-CCATCTACACCTACACTGGCAAAAG-3′
	R:5′-GTCTGGCGTTTTGGGATGTT-3′
ADAMTS4	F:5′-CCCCATGTGCAACGTCAAG-3′
	R:5′-AGTCTCCACAAATCTGCTCAGTGA-3′
ADAMTS5	F:5′-GATGGTCACGGTAACTGTTTGCT-3′
	R:5′-GCCGGGACACACCGAGTAC-3′
IL-6	F:5′-TTCCAAAAATGGAGGAAAAGGA-3′
	R:5′-TCCAGAAGACCAGCAGTGGTT-3′
TNF-α	F:5′-CCTCTTCTCAAGCCTCAAGTAACAA-3′
	R:5′-GAGCTGCCCCGGAGAGTT-3′
COL2	F:5′-GAGCAGCAAGAGCAAGGACAAGA-3′
	R:5′-GCAGTGGTAGGTGATGTTCTGAGAG-3′
COL1	F:5′-AGCGGCGGCTACGACTTGAG-3′
	R:5′-AGCGGCGGCTACGACTTGAG-3′
ACAN	F:5′-AGAAGGACCGATACGAGATCAATGC-3′
	R:5′-AGCGACAAGGAGAGGACACCAT-3′
RPLP0	F:5′-GGCTTTAGGCATCACCACGAAGA-3′
	R:5′-AGGCACACGCTGGCAACATT-3′

3 | RESULTS

3.1 | Changes in IVD height

From Day 1 to Day 13, there were no significant differences in disc height changes between the control and experimental groups after overnight free swelling. Starting from Day 14, the ten strikes group showed a significantly lower disc height compared with the control group (Figure 1A, $p < 0.05$), while no significant difference was observed compared to the one strike group. From Day 3 to Day 32 after free swelling, the disc height in the one strike group did not differ significantly from the control group, but there was a tendency for a decrease in disc height. Furthermore, there was no significant difference in disc height change between the one strike group and the physiological loading group following physiological loading.

3.2 | Dynamic compressive stiffness

At each time point, the dynamic compressive stiffness was normalized to the initial value of the corresponding disc on Day 1 following free swelling. Compared to the physiological loading control group, the ten strikes group exhibited a reduction in dynamic compressive stiffness after the final strike was induced on Day 32 (Figure 1B, $p < 0.05$). Moreover, the dynamic compressive stiffness of the one strike loading

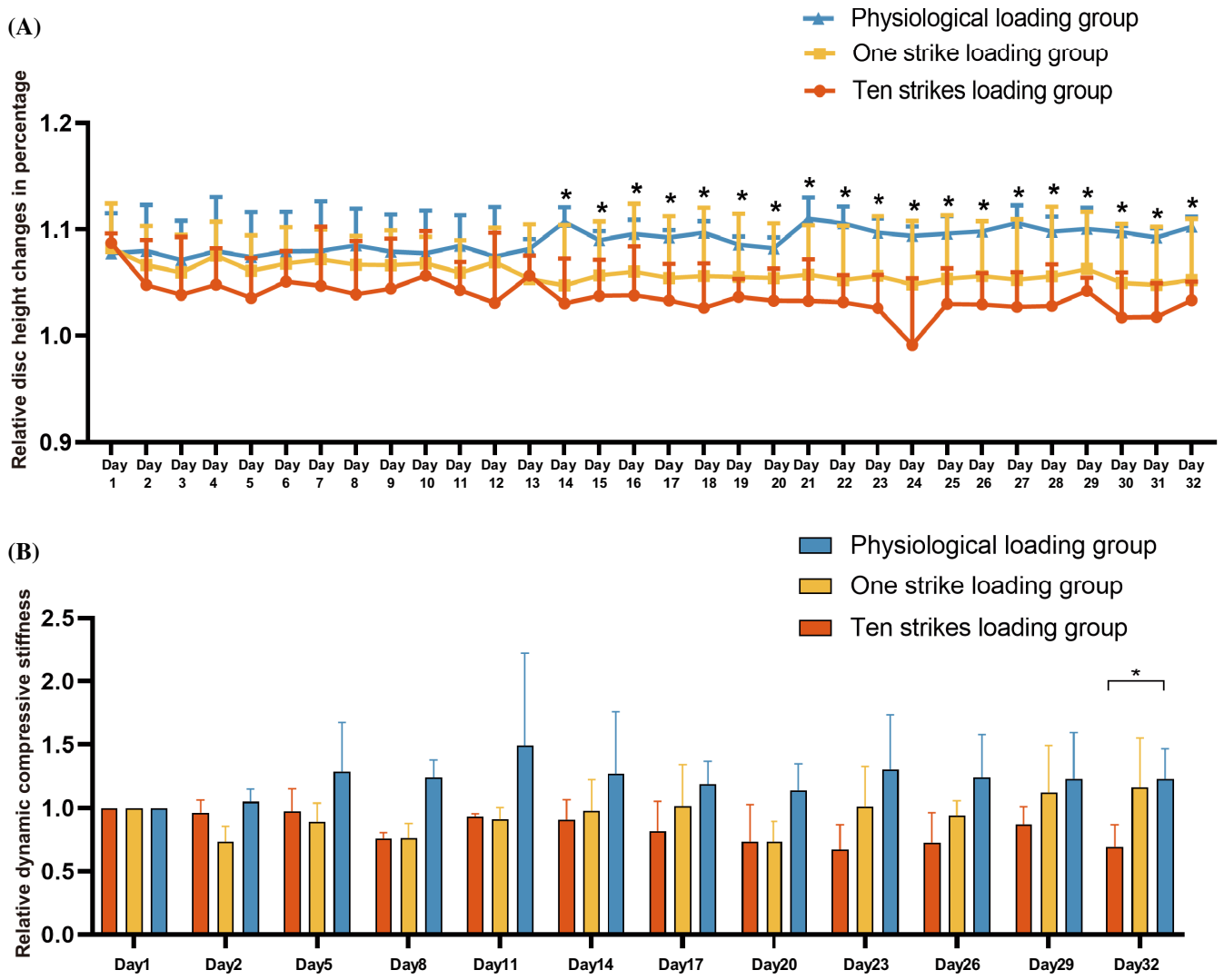

FIGURE 1 (A) Disc height change after swelling. (B) Dynamic compressive stiffness of the IVDs measured at different time points. Data were normalized to original height of each disc on Day 0 after dissection, and dynamic compressive stiffness of each disc on Day 1 after free swelling, Mean + SD; $n = 3$; *$p < 0.05$. Ten strikes loading group versus physiological loading group.

group did not significantly differ from that of the physiological control group.

3.3 | Cell viability

The LDH/ETH staining images for each group are displayed in Figure 2. The ten strikes loading group resulted in significant IVD cell death. The quantification of living cells, dead cells, and cell viability are illustrated in Figure 3. The analysis revealed a significant reduction in the number of living cells within IAF and OAF regions of the IVDs in the ten strikes group on Day 32, compared to the control group and the one strike group (Figure 3A2,A3, $p < 0.05$). In the NP region, there was no significant difference in the number of viable cells between the model group and the control group (Figure 3A1). Figure 3B1–B3 presents the number of dead cells in the NP, IAF, and OAF regions. Specifically, the IAF and OAF regions demonstrated a significantly higher number of dead cells in the ten strikes group compared to the other two groups (Figure 3B2,B3, $p < 0.05$). Notably, in the NP region, there was no significant difference in cell viability between the model group and the control group (Figure 3C1). In the IAF and OAF regions, the one strike group exhibited comparable cell viability to the control group on Day 32 (Figure 3C2,C3). While, the ten strikes group demonstrated significantly reduced cell viability compared to the other two groups (Figure 3C2,C3, $p < 0.05$).

3.4 | Disc morphology

The morphology of cross-section of the disc evaluated by Safranin O/Fast Green staining on Day 32 are shown in Figure 4. The histological scoring criteria used were derived from previous literature.[28] Distinct patterns of clefts were observed in AF of IVD in one strike groups. The control group exhibited mild clefts in both IAF and OAF.

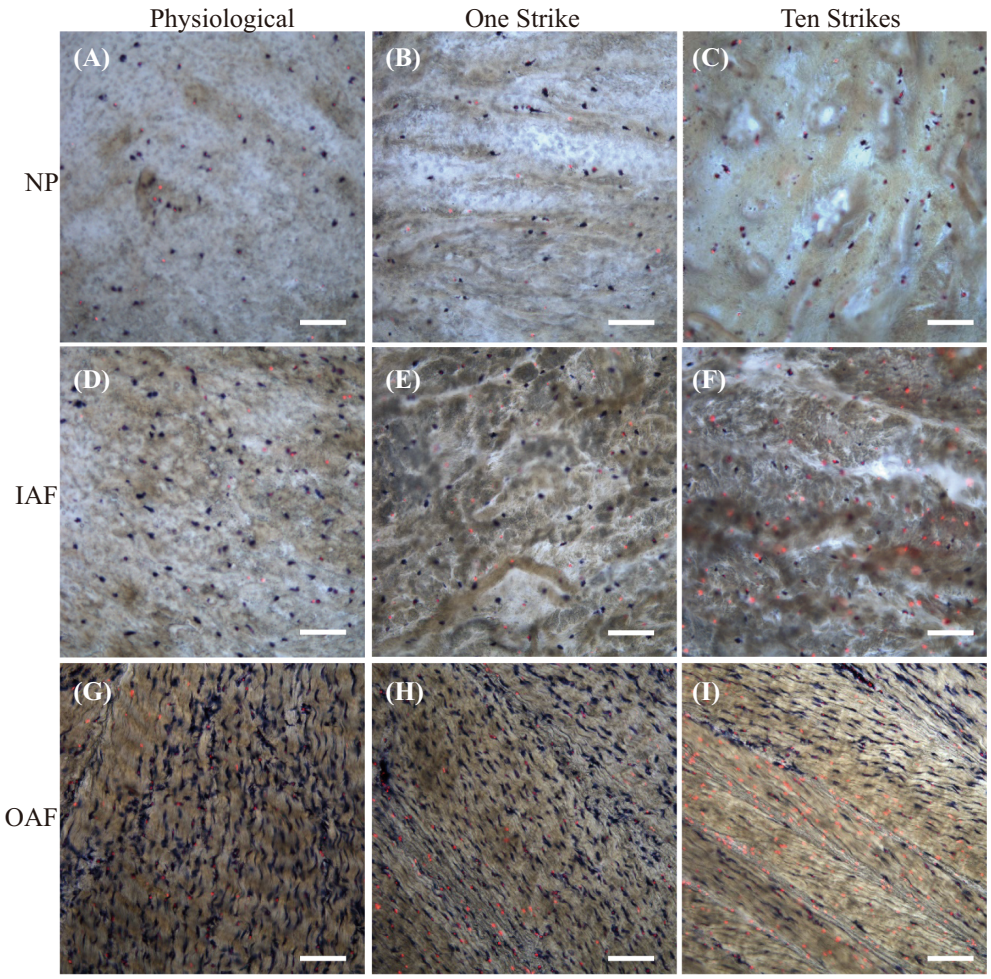

FIGURE 2 Representative LDH/ETH staining images of IVDs collected on Day 32 after physiological loading (A, D, G), on Day 32 after one strike loading (B, E, H), and on Day 32 after ten strikes loading (C, F, I). Cell viability was assessed using LDH/ETH staining (blue and blue/red = alive cell; red only = dead cell). Figures A–C represent NP tissue. Figures D–F represent inner AF (IAF) tissue. Figures G–I represent outer AF (OAF) tissue. Scale bar: 100 μm.

Moreover, the ten strikes group displayed vertical and parallel clefts extending from the IAF to the edge of NP, accompanied by delamination in most samples. Some samples demonstrated cleft propagation into the NP and severe delamination. The ten strikes group scored significantly lower than both the one strike group and the control group (Figure 4J, $p < 0.05$). Furthermore, the physiological load control group scored lower than the one strike group (Figure 4J, $p < 0.05$).

3.5 | mRNA expression in IVD tissue

On Day 32, samples of IVDs were collected from the ten strikes group, one strike group, and control group for gene expression analysis. RT-PCR was used to evaluate the expression levels of various anabolic, catabolic, and proinflammatory genes in NP and AF tissues. In comparison to the control group, the NP tissue in the ten strikes group showed upregulation of MMP1, MMP13, ADAMTS5, and TNF-α genes. COL2 and ACAN genes were downregulated (Figure 5A, $p < 0.05$). Additionally, the AF tissue in the ten strikes group exhibited upregulation of MMP1, MMP13, ADAMTS5, and TNF-α genes, while the one strike group did not demonstrate such upregulation (Figure 5B, $p < 0.05$). No significant disparity in gene expression was observed between one strike group and control group.

3.6 | Evaluation of released NO and GAG

The conditioned medium obtained daily after free swelling of IVD were collected. The levels of secreted NO and GAG in the medium were measured and normalized to the IVD volume. Statistical analysis indicated no significant difference in NO release among the three groups (Figure 6A). However, starting from the 19th day of free swelling in the ten strikes group until the end of the experiment, there was a significant increase in GAG release compared to the control group (Figure 6B, $p < 0.05$). Moreover, no significant difference in GAG release was observed between one strike group and control group.

4 | DISCUSSION

The progression of lumbar disc degeneration is significantly influenced by mechanical overload, most research on the association between disc degeneration and mechanical overload has mainly focused on chronic repetitive injuries. However, there is a lack of studies investigating the effects of repetitive high force loading on IVD. Due to the challenge of acquiring human disc organs, the caudal bovine disc was chosen as the closest in vitro diagnostic model to the human disc.[30] Repetitive compressive stress-induced injuries were performed on the

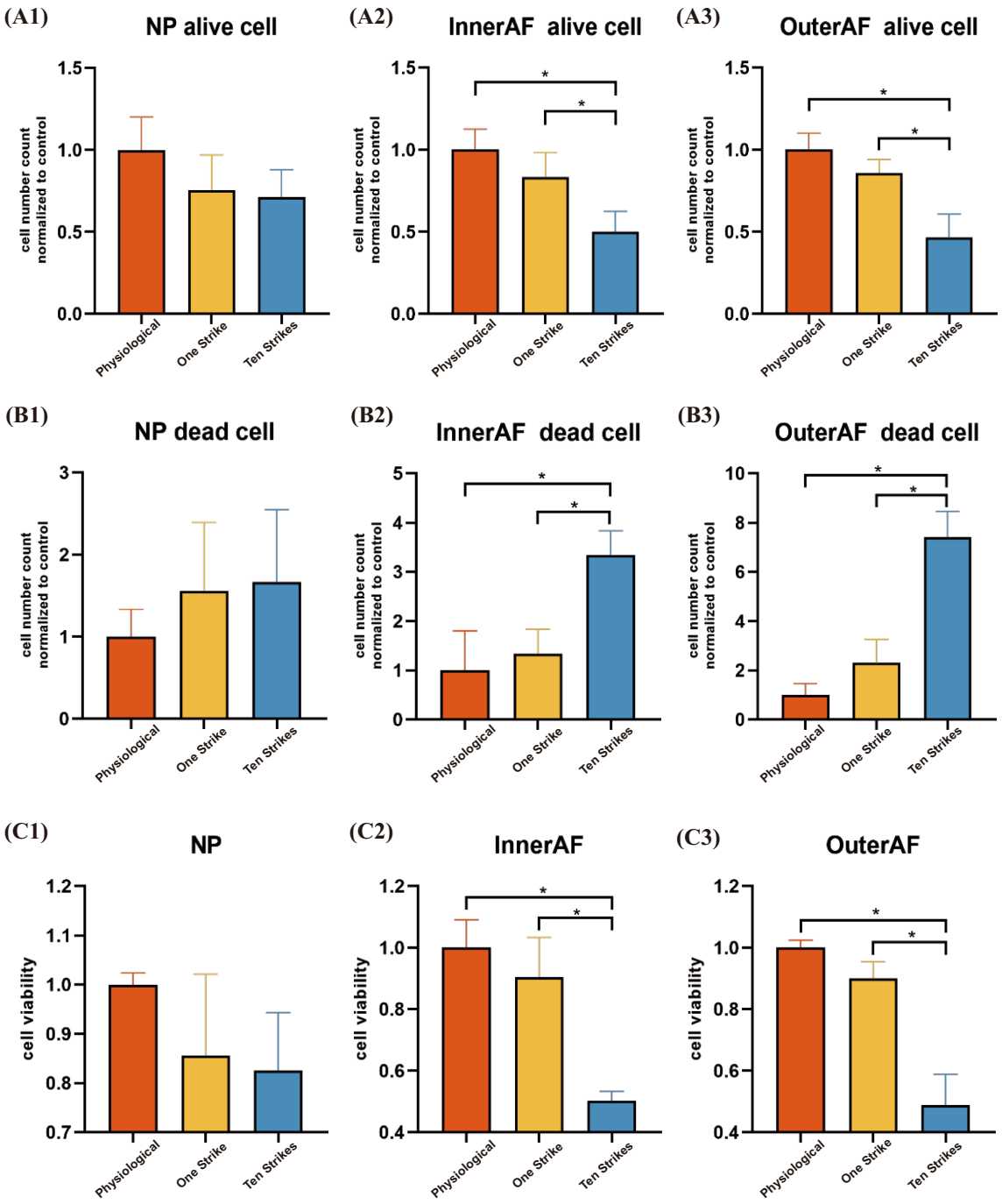

FIGURE 3 The numbers of alive cells, dead cells, and cell viability from different groups. A1, B1, C1 represent NP region; A2, B2, C2 represent inner AF (IAF) region; A3, B3, C3 represent outer AF (OAF) region. the data were normalized to Control group ($n = 3$), Mean + SD, $^*p < 0.05$.

bovine disc organ culture model to simulate the repetitive lumbar spinal trauma. In general, our study provides evidence supporting the correlation between repetitive high force loading and disc degeneration. To our knowledge, we are the first to conduct basic research on the effects of repetitive high force loading on disc degeneration in the IVD organ culture model.

The IVD organ culture bioreactor has long been used in basic and translational research for its advantages of precise control in biochemical and biomechanical aspects and is an indispensable tool for bridging in vitro and in vivo models.[13,31,32] Salzer et al. observed an increase in the metabolic activity of NP tissue with dynamic loading, coupled with a notable reduction in living cell density by the seventh day.[33] Kurakawa et al. reported diminished cell density and viability in nucleus pulposus cells subjected to dynamic compression loading of rat intervertebral discs at 1.5 Hz.[34] Li et al. identified increased NP cell mortality and reduced levels of GAG and type II

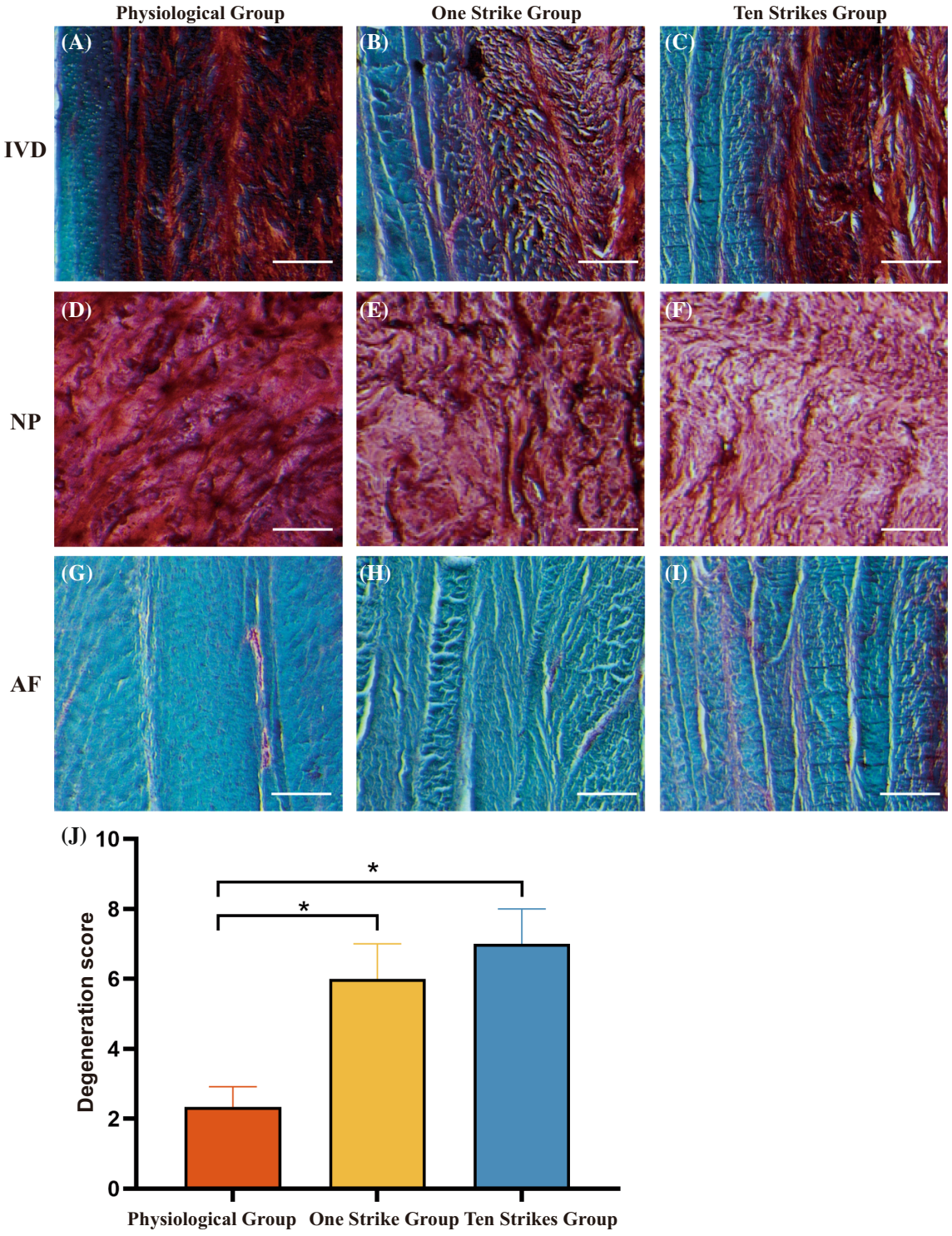

FIGURE 4 Representative Safranin O/Fast green staining images of cross histological sections from IVDs on Day 32 after physiological loading (A, D, G), on Day 32 after one strike loading (B, E, H), and on Day 32 after ten strikes loading (C, F, I). Scale bar: 650 μm. (J) Histological scoring values based on Safranin O/Fast green staining, Mean + SD; $n = 3$; $^*p < 0.05$.

collagen in loading conditions involving 1.3 MPa, 5.0 Hz, and 8 h.[35] Cui identified new epitope peptides formed by proteoglycan and collagen cleavage during IVD degeneration in one strike model, serving as promising biomarkers of early disc degeneration.[36] Meanwhile, it was essential to explore stress and strain in disc degeneration through integrated compression, flexion, and torsion studies in future research.[37] Excessive compression and torsion are key factors in disc degeneration, The decline in IVD height was more prominent under medium and high-stress conditions compared to low-stress conditions. Furthermore, high-stress conditions led to a substantial

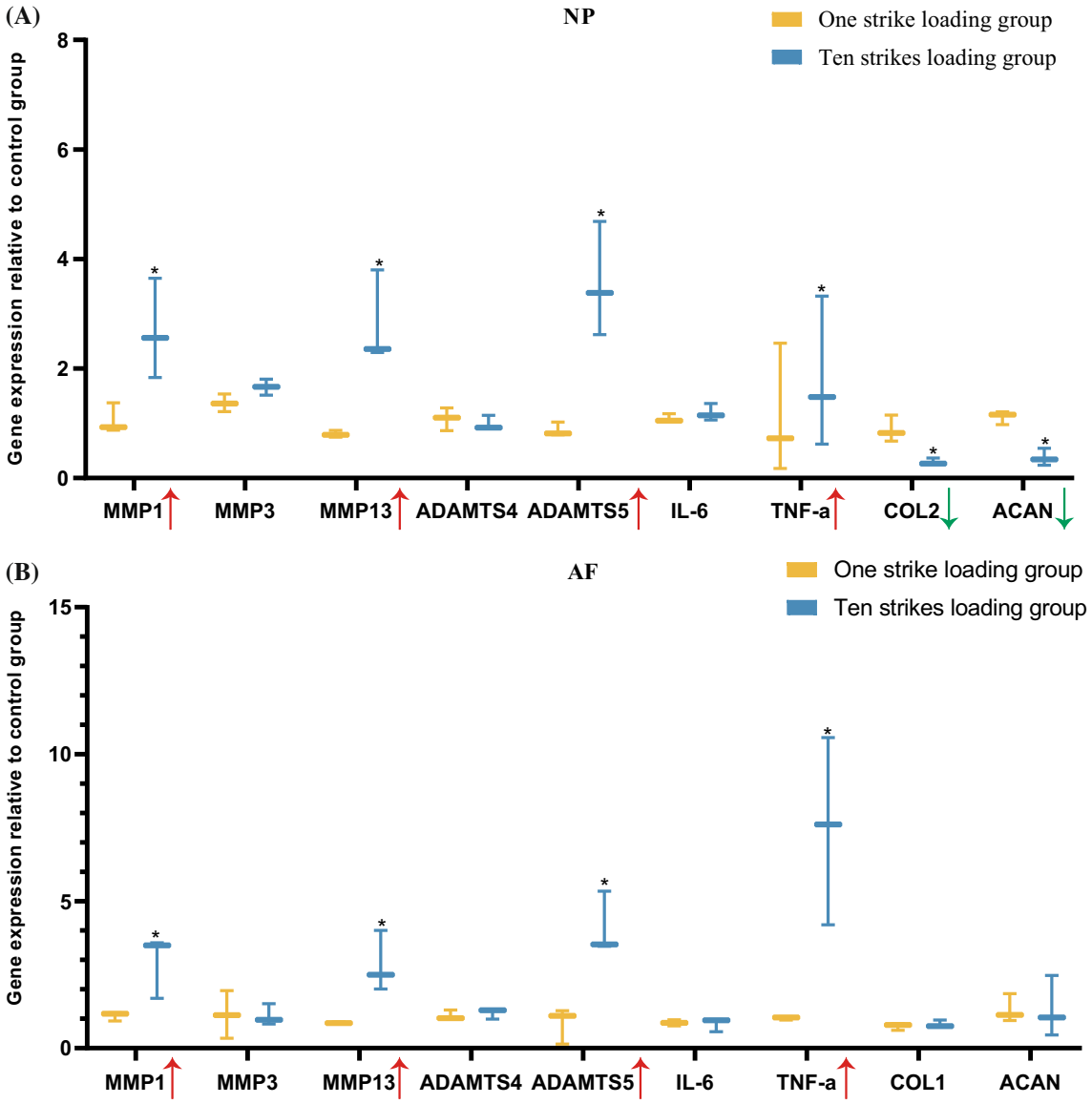

FIGURE 5 Gene expression in NP (A) and AF (B) tissue of one strike loading group and ten strikes loading group normalized to physiological loading group on Day 32. $n = 3$. *$p < 0.05$ versus physiological loading group; ↑ indicates the gene expression was significantly upregulated in ten strikes loading group compared to physiological loading group, ↓ indicates the gene expression was significantly downregulated in ten strikes loading group compared to physiological loading group.

decrease in both cell viability and tissue glycosaminoglycan content. This implies that the concurrent application of compression and torsion exacerbates the development of IVDD.[38] IVD organ culture models were extensively employed in studying disc degeneration due to their reproducibility, measurability, and controllability. Meanwhile, in our previous studies, we consistently demonstrated the reliable application of this apparatus for intervertebral discs in mechanical loading.[19,27,39]

GAGs are essential for maintaining disc height by retaining water. GAGs possess a substantial negative charge density, facilitating electrostatic attraction and binding to water molecules, thereby upholding the hydrated state crucial for the integrity of a healthy intervertebral disc.[40] In a healthy state, the NP contains 90% water, while the AF holds 60%–80% water. However, disc degeneration leads to a gradual decrease in water content.[41] The dynamic compressive stiffness of the IVD serves as an indirect indicator of GAG content changes within the tissue.[42] GAG content in the IVD exhibits a linear relationship with the tissue's swelling stress. During disc degeneration, the GAG content decreases, leading to a lower dynamic compressive stiffness compared to normal disc tissue. Other researchers have also shown that as degeneration progresses, GAG content decreases within the IVD while GAG released into the culture medium increases.[43,44] In our study, we observed a gradual decline in the dynamic compressive stiffness of the IVD in ten strikes group, reaching a significant difference from the physiologically loaded group by Day 32. Moreover, the height of the ten strikes group after free swelling on Day 14 was

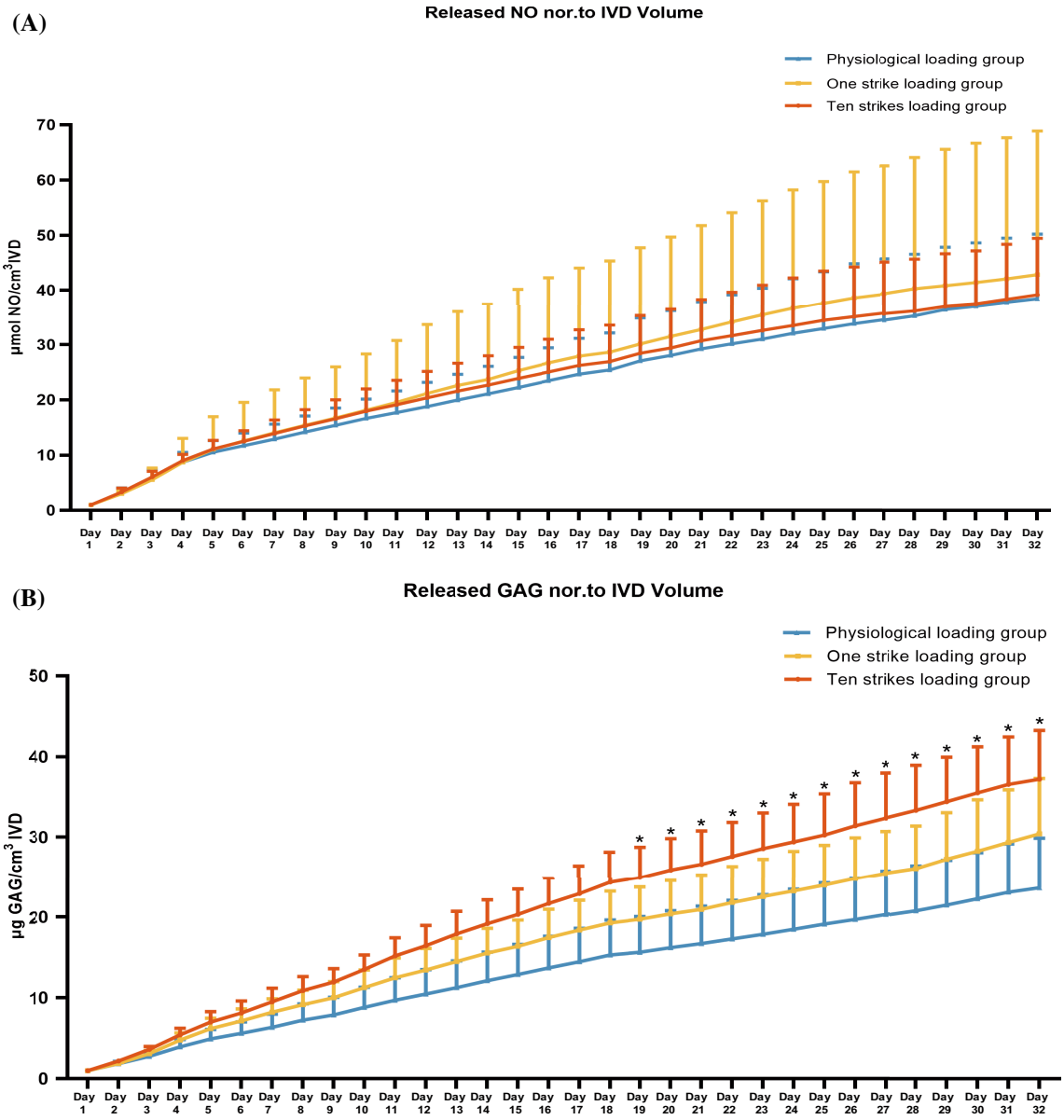

FIGURE 6 Accumulative NO (A) and GAG (B) release in conditioned media of IVDs cultured with physiological loading, one strike loading or ten strikes loading during culture. Mean ± SD; $n = 3$; *$p < 0.05$ versus physiological loading group. GAG; glycosaminoglycan; NO, nitric oxide.

significantly different from control group. However, disc height changes and dynamic compressive stiffness in the one strike group were not significantly different from those in the control group. This difference may be due to the destruction of GAG caused by repeated strikes, but the intervertebral disc absorbs water from the medium as a compensatory effect, resulting in a small change in dynamic compressive stiffness after the first nine strikes. As the loss of GAG increased after ten strikes loading, the IVD absorbed insufficient water to compensate, resulting in a significant difference from the physiologic loading group. Moreover, an increase in GAG release was observed in the conditioned media, suggesting the occurrence of extracellular matrix breakdown in response to repetitive loading.

Biomechanical wear was a significant contributor to disc degeneration.[45] Alterations in the gene expression of the matrix metalloproteinases (MMPs) and metalloproteinases with a thrombospondin motif (ADAMTS) family are crucial in the degradation of the extracellular matrix (ECM). Paul et al.[46] discovered that dynamic overloading resulted in a significant increase in the expression levels of MMP13, MMP14, and ADAMTS5. In our previous one strike loading (compression to 50% of IVD height) organ culture model, supraphysiologic mechanical compression resulted in upregulation of MMP1, MMP3, ADAMTS4, and ADAMTS5 gene expression in NP at short-term culture, MMP1, MMP13 and ADAMTS5 gene expression in AF at long-term culture.[19] But, in this study, we found that ten strikes loading can lead to upregulation of MMP1, MMP13, and ADAMTS5 gene expression in NP and AF tissues at short-term culture. There were no differences in gene expression between the one strike group and the control group at long-term culture. This all demonstrate that

violent injury and mechanical overload have the potential to accelerate the degenerative process of IVD. In conclusion, disc trauma induced various biological changes, including the upregulation of proinflammatory and catabolic gene expression.[47,48] This conclusion has aligned with other organ culture models inducing disc degeneration through altered mechanical conditions.[48–50]

TNF-α plays a key role in the progression of disc degeneration.[51] The injection of TNF-α in an organ culture model of intervertebral disc (IVD), as conducted by Du et al.,[43] induces IVD degeneration. Ulric et al.[24] conducted a study inducing three puncture injuries in the intervertebral discs of rats. The observations revealed that puncture-induced injury exposed the nucleus pulposus (NP) to a novel environment, triggering a cascade inflammatory reaction that ultimately resulted in disc degeneration. However, these findings may not be applicable to the effects of repetitive injury on intervertebral degeneration in early. Through IVD organ culture, our study found that repetitive high force loading can upregulate TNF-α gene expression. However, in our previous study, TNF-a gene expression was not increased.[19] TNF-α can enhance the expression of MMPs and ADAMTS family genes and exerts a suppressive effect on anabolic processes, leading to a reduction in ECM synthesis. Additionally, it augments the catabolic activity within the ECM, thereby accelerating IVD degradation.[52] Hence, it was crucial to contemplate combined anti-catabolic therapy and TNF-α inhibition in disc degeneration treatment.[43,44]

Compressive or traumatic injuries have the potential to induce cell death in IVD. According to Hofmann et al., mechanical overload caused 17% cell death in IVD.[53] In our previous study, we observed 43% cell death in OAF, 10% in IAF, and 14% in NP following one strike loading.[19] Our latest research demonstrated that ten strikes loading resulted in 18% cell death in the NP, 50% in the IAF, and 62% in the OAF. These findings are consistent with our previous research, where a higher post-traumatic cell death was observed in AF compared to NP in IVD. We speculate that mechanical overload increases shear stress in the disc,[54] leading to greater shear stress in the AF tissue due to the pressure-dispersing capabilities of the NP tissue, ultimately resulting in increased cell death. Loading frequency, amplitude, and duration could impact disc damage.[15] In a disc-like organ model, Gewiess illustrated that extended static loading resulted in a 12.1% reduction in cell viability in the annulus fibrosus (AF) and a 4.2% reduction in the nucleus pulposus (NP). Conversely, dynamic loading had no discernible impact on cell viability in the disc.[31] Salzer et al. proposed that dynamic loading acts as an additional stressor, leading to a decrease in nucleus pulposus (NP) cell density.[33] Rosenzweig et al. assessed cell viability in an intervertebral disc bioreactor under three loading conditions: low (0.1–0.3 MPa), medium (0.1–0.3 MPa), and high (0.1–1.2 MPa). They observed that cell viability remained above 80% at low and medium loads, while both nucleus pulposus (NP) and annulus fibrosus (AF) cell viability were impacted by high loads.[55]

This study possesses certain limitations. First, intervertebral discs experience multidirectional forces within their physiologic environment. However, the employed IVD organ culture bioreactor primarily emulates axial compression forces, thereby neglecting the impact of other forces exerted on the disc. In addition, the cartilage endplate model used in our study lacks integration with surrounding tissues (e.g., muscles and ligaments), unlike the in vivo situation, where disc forces may deform differently. Second, in ten strikes group, we conducted only short-term culture; however, the long-term effects of repeated strikes on intervertebral discs need to be observed through extended culture periods.

5 | CONCLUSIONS

The current study shows that subjecting healthy bovine IVDs to ten mechanical compressions resulted in compromised cell viability, elevated expression of catabolic enzymes, and an increased loss of proteoglycans. The utilization of the ten strikes loading bovine IVD model offers valuable insights into the underlying mechanisms of early IVDD induced by repetitive and forceful injuries.

AUTHOR CONTRIBUTIONS

Jiaxiang Zhou, **Zhiyu Zhou**, **Yongming Xi**: Conception and design of study. **Jiaxiang Zhou**, **Hongkun Chen**, **Jianfeng Li**: Acquisition of data. **Zhongyuan He**, **Junhong Li**, **Tao Tang**, **Yukun Du**: Analysis and/or interpretation of data. **Jiaxiang Zhou**: Writing-original draft preparation. **Jianmin Wang**, **Zhengya Zhu**, **Yongming Xi**: Writing-review and editing. **Zhen Li**, **Manman Gao**, **Zhiyu Zhou**, **Yongming Xi**: Supervision. **Zhen Li**, **Manman Gao**, **Zhiyu Zhou**, **Yongming Xi**: Project administration. **Zhiyu Zhou**, **Yongming Xi**: Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

Upon request, interested parties can obtain the data used to support the findings of this study from the corresponding author.

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