Cyclic mechanical tension reinforces DNA damage and activates the p53-p21-Rb pathway to induce premature senescence of nucleus pulposus cells

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Abstract. Intervertebral disc (IVD) degeneration (IDD) is a widely recognized contributor to low back pain. Mechanical stress is a crucial etiological factor of IDD. During the process of IDD, a vicious circle is formed between abnormal mechanical stress and the damage of disc structure and function. Notably, the pathological process of IDD is mediated by the phenotypic shift of IVD cells from an extracellular matrix anabolic phenotype to a catabolic and pro-inflammatory phenotype. Therefore, the effects of mechanical stress on the initiation and progression of IDD depend on the mechanobiology of IVD cells. Recently, disc cell senescence was identified as a new hallmark of IDD. However, the senescent response of disc cells to mechanical stress remains unknown. In this study, we found that prolonged exposure of cyclic mechanical tension (CMT) with unphysiological magnitude generated by the Flexercell tension system markedly induced premature senescence of nucleus pulposus (NP) cells. CMT augmented the DNA damage of NP cells, but did not affect the redox homeostasis of NP cells. Moreover, the p53-p21-retinoblastoma protein (Rb) pathway was activated by CMT to mediate the CMT-induced premature senescence of NP cells. The findings are beneficial to understanding the mechanism of disc cell senescence and the mechanobiology of disc cells further. It suggests that prolonged abnormal mechanical stress accelerates the establishment and progression of disc cell senescence and consequently impairs the structural and functional homeostasis of IVDs to cause IDD. Preventing the pro-senescent effect of mechanical stress on IVD cells is a promising approach to delay the process of IDD.

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Abbreviations: IVD, intervertebral disc; IDD, intervertebral disc degeneration; NP, nucleus pulposus; AF, annulus fibrosus; SA-β-Gal, senescence-associated β-galactosidase; ECM, extracellular matrix; DDR, DNA damage response; SIPS, stress-induced premature senescence; Rb, retinoblastoma protein; CMT, cyclic mechanical tension; LBP, low back pain; CEP, cartilage endplate ROS, reactive oxygen species; Msr, methionine sulfoxide reductase; FBS, fetal bovine serum; PBS, phosphate-buffered saline

Key words: cyclic mechanical tension, Flexercell tension system, DNA damage, disc cell senescence, the mechanobiology of disc cells, intervertebral disc degeneration

Introduction

Low back pain (LBP) is a major threat to our health and society due to the huge socio-economic burdens and the high incidence of disability and 60-80% of the population suffer from LBP at some point in their lives. LBP is exhausting our limited medical resources (1,2). However, our understanding of pathophysiology of LBP remains limited.

A widely recognized contributor to LBP is intervertebral disc (IVD) degeneration (IDD). The degree of disc degeneration positively correlates with the severity of LBP (3,4). The structure of degenerative discs is characterized by a loss of water and proteoglycans in nucleus pulposus (NP), annulus fibrosus (AF) tears and cartilage endplate (CEP) calcification. The etiological factors of IDD are involved in aging, infection, diabetes, trauma and genetic predisposition (5-10). Mechanical stress is one of the major causes of IDD (11-13). Spine resists multidirectional mechanical loadings in daily life, which contributes to maintaining the structure and function of discs. However, when mechanical stress is overloaded, it accelerates the initiation and progression of IDD. As also, disc degeneration causes the disturbed stress distribution in discs, which generates mechanical stress concentration. As a result, a vicious circle is formed between mechanical loadings and disc structure to promote the process of IDD (12).

In consideration of that the pathological process of IDD is mediated by the phenotypic shift of disc cells from an extracellular matrix (ECM) anabolic phenotype to a catabolic and pro-inflammatory phenotype (14,15), the effects of mechanical stress on the structure and function of IVDs are proposed to depend on the relationship between mechanical behavior and

disc cell functions that is known as the mechanobiology of disc cells (12,13). Flexercell tension system (Flexcell International Corp., Hillsborough, NC, USA), a system that applies cyclic mechanical tension (CMT) on cells *in vitro*, is widely used to investigate the mechanobiology of disc cells. CMT has been reported to regulate the matrix metabolism, cytokines production, cytoskeleton organization and apoptosis of disc cells (16-19). Elucidating the mechanobiology of disc cells in detail contributes to understanding the roles of mechanical stress in the pathogenesis of IDD in depth.

Disc cell senescence is a new hallmark of disc degeneration (20,21). The growth of senescent disc cells is irreversibly arrested. As a consequence, the decrease in the number of viable and functional disc cells in IVDs caused by cell death can not be compensated by disc cell proliferation. Furthermore, the senescence-associated secretory phenotype (SASP) of disc cells is characterized by an increased secretion of pro-inflammatory cytokines, ECM proteases and chemokines. Thus, senescent disc cells promote matrix degradation of discs and induce the pro-inflammatory cytokine storm in the micro-environment of discs, which accelerates the establishment and progression of IDD (22,23). The triggers of disc cell senescence involve telomere erosion, oxidative stress, cytokines and DNA damage (24-26). Interestingly, abnormal mechanical loadings caused by prolonged upright posture have been found to promote disc cell senescence in rat IVDs, and then to accelerate the progression of IDD (27,28), suggesting that mechanical stress is a crucial trigger of disc cell senescence. Revealing the role of mechanical stress in disc cell senescence benefits our understanding of the pathogenesis of IDD.

Focusing on NP cells, they are exposed to various mechanical stresses, including compression, shear stress, hydrostatic pressure as well as tension (29,30). However, there have been no studies investigating the effect of mechanical stress on NP cell senescence so far. Therefore, in this study, we applied a physiological CMT (5% elongation, 5% CMT) and an unphysiological CMT (20% elongation, 20% CMT) to rat NP cells using a FX-5000T Flexercell tension plus system. CMT with 20% elongation is regarded as unphysiological due to that the physiological limit of IVD area change caused by mechanical stress is known as 15% (16,19,31). Senescence-associated β-galactosidase (SA-β-gal) staining and BrdU incorporation were performed to investigate NP cell senescence after CMT stimulation. The DNA damage and redox state of NP cells were evaluated after CMT application. Moreover, we also examined the activation of senescence-associated molecular pathways in NP cells subjected to CMT. This study elucidated the role of mechanical stress in the senescence of NP cells, providing a novel insight into the causes and molecular mechanism of disc cell senescence.

Materials and methods

Ethics statement. This study was approved by the Ethics Committee of Xinqiao Hospital. All protocols were in accordance with the ethical standards set by the Declaration of Helsinki.

Antibodies. The mouse monoclonal anti-rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-47724), p53 (sc-126), p21 (sc-6246), p16 (sc-1661) and the rabbit polyclonal anti-rat retinoblastoma protein (Rb, sc-50) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The rabbit monoclonal rabbit anti-rat phospho-histone γ-H2A.X (Ser139) antibody was obtained from Cell Signaling Technology (#9718; Danvers, MA, USA). The donkey polyclonal anti-mouse IgG Alexa Fluor® 647-conjugated secondary antibody (AP192SA6) and the goat polyclonal goat anti-rabbit IgG (H+L) Alexa Fluor® 647-conjugated secondary antibody (AP187SA6) were purchased from Merck Millipore (Billerica, MA, USA). The goat polyclonal anti-mouse IgG (H+L) horseradish peroxidase (HRP)-conjugated secondary antibody (ZB2305) and the goat anti-rabbit IgG (H+L) HRP-conjugated secondary antibody (ZB2301) were purchased from ZSGB-BIO (Beijing, China).

Isolation and culture of rat NP cells. Caudal spines were aseptically excised from adult (3-month-old) male Sprague-Dawley rats (Laboratory Animal Research Center of Daping Hospital, Chongqing, China) after sacrificed by peritoneal injection of excessive pentobarbital sodium. The gelatinous NP tissues were separated from caudal discs (C1-C10), and then, were digested in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Invitrogen, Carlsbad, CA, USA) containing 0.2% type II collagenase (Sigma, St. Louis, MO, USA) for 2 h at 37˚C. After being passed through a 70 μm cell mesh to remove tissue debris, the single-cell suspension was centrifuged at 100 x g for 5 h and the supernatant was removed. The cellular pellet was resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). Isolated NP cells were plated in 25 cm^2 culture flasks (Corning, Inc., Corning, NY, USA) at 37˚C and 5% CO_2. The medium was replaced twice a week. When confluent, the cells were subcultured. The cells at the second passage were used in the experiments.

Application of CMT on cultured NP cells. NP cells were seeded on a 6-well flexible silicone membrane BioFlex™ plates coated with collagen type I (Flexcell International Corp., McKeesport, PA, USA) at a density of $2x10^5$ cells/well. After reaching 70-80% confluence, the cells were starved with serum-free DMEM/F12 for 24 h for synchronization and then stretched using a FX-5000T Flexercell tension plus system (Flexcell International Corp.) in DMEM/F12 medium containing 10% FBS at 37˚C and 5% CO_2. CMT with 5% elongation and the CMT with 20% elongation at a frequency of 1 Hz for 6, 12, 24 or 48 h were delivered as per the protocol. The cells cultured in the same plates under the same conditions were kept static to serve as the control. The morphology of cells was observed and imaged using a phase contrast microscope (Olympus, Tokyo, Japan).

SA-β-gal staining. The activity of SA-β-gal in NP cells was stained using a SA-β-gal staining kit (#9860; Cell Signaling Technology) according to the protocol provided by the manufacturer. Briefly, NP cells cultured in BioFlex™ plates were washed using phosphate-buffered saline (PBS) and fixed with 2% formaldehyde for 25 min at room temperature. After rinsing with PBS, the cells were incubated with the staining solution containing X-gel (1 mg/ml) at 37˚C for 12 h. Then, the mean percentage of SA-β-gal-positive cells in nine random

Table I. Primer sequences used in the real-time PCR analysis.

Target gene	Forward primer	Reverse primer
p53	GGGAATCTTCTGGGACGGGACA	CTGGTGGGCAGTGCTCTCTTTG
p21	CTGCCTGGTTCCTTGCCACTTC	GCTCTGGACGGTACGCTTAGGT
p16	CGTCGTGCGGTATTTGCGGTAT	GCGTTGCCAGAAGTGAAGCCA
Rb	AGCAGCCTCAGCCTTCCATACT	TGTTCTGGCTCTGGGTGGTCAG
MsrA	GGCAATGACTGTGGCACGCA	CCTCTCGGATGTCGGTGGTGAT
MsrB1	TCCTGTGGCAAGTGTGGCAATG	TGACTGAGGCTGGAGTGGTTGG
MsrB2	AGCAAGGCAGACTGGCAGAAGA	GGGCTATCACAGCACACGCAAT

MsrbA, methionine sulfoxide reductase A; Msrb1, methionine sulfoxide reductase B1; Msrb2, methionine sulfoxide reductase B2.

fields per well was determined using a phase-contrast microscope (x200 magnification; Olympus).

BrdU incorporation assay and DNA damage assay. For BrdU incorporation assay, NP cells were incubated with BrdU (1 μg/ml; BD Biosciences, San Jose, CA, USA) at 37˚C and 5% CO_2 for 2 h after cyclic stretch, and then, were fixed with 70% of ethanol. For DNA damage assay, NP cells were washed using PBS and then were fixed with 4% paraformaldehyde for 30 min after cyclic stretch. The rounded silicon membranes were separated from the BioFlex™ plates, and were cut into minor sectors. Next, the sectorial membranes were attached to the bottom of culture dishes. After rinsing with PBS, NP cells on the sectorial membranes were incubated with 1 ml HCl (2 mol/l) at room temperature for 30 min for BrdU incorporation assay. After permeabilization and antigen blocking, cells in culture dishes were incubated with a mouse monoclonal anti-rat BrdU (B8434, 1:500 dilution; Sigma) and the primary antibody against histone γ-H2A.X (1:400, a DNA damage marker) overnight at 4˚C. After washing, the cells were incubated with the Alexa Fluor 647 dye-conjugated secondary antibodies (AP192SA6, 1:400 dilution and AP187SA6, 1:400 dilution) respectively in the dark and then stained with DAPI (0.1 mg/ml; Sigma). Cells without incubation with primary antibodies served as the negative control. Images in three random fields were obtained using a confocal microscope (x200 magnification; Lecia, Weltzlar, Germany). The mean percentage of BrdU-positive cells was calculated. The mean integrated density (nuclear area x mean gray value, MID) of γ-H2A.X-expressing cells was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Reactive oxygen species (ROS) measurement. The ROS production of NP cells was measured using 2',7'-dichlorfluorescein-diacetate (DCFH-DA) (D6883; Sigma). DCFH-DA was oxidized by ROS to generate the highly fluorescent dichlorofluorescein (DCF). After CMT application, NP cells were isolated with trypsin and were centrifuged at 100 x g for 5 min. Next, the cells were resuspended using PBS containing H_2DCF-DA (25 μM) and were incubated at 37˚C and 5% CO_2 for 30 min. After incubation, the cells were washed with serum-free DMEM/F12 medium three times. The mean fluorescence intensity (MFI) was analyzed using a flow cytometer (Beckman-Coulter, Pasadena, CA, USA).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from stretched and static control NP cells using 1 ml TRIzol reagent (Takara Bio, Shiga, Japan). RNA quality and quantity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). One microgram RNA was reverse transcribed using a Prime Script RT Reagent kit (Takara Bio) according to the manufacturer's protocols. Real-time quantitative PCR was performed in triplicate on a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Thermo Scientific) with SYBR® Premix Ex Taq™ II (Takara Bio). The 20 μl reaction mixtures (10 μl SYBR, 6 μl H_2O, 0.4 μl ROX, 0.8 μl forward primer, 0.8 μl reverse primer and 2 μl cDNA) was amplified under the following conditions: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. PCR products were subjected to melting curve analysis. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (32). GAPDH was the internal reference gene. We measured the relative expression of p53, p21, p16, Rb, methionine sulfoxide reductase A (MsrbA), Msrb1 and Msrb2 in NP cells. Mean Ct values were normalized to that of GAPDH. The primers of genes investigated in this study are listed in Table I.

Western blot analysis. Total proteins were extracted from NP cells using a protein extraction reagent (Thermo Fisher Scientific). Protein concentration was quantified using BCA method (Beyotime, Shanghai, China). Cell lysates mixed with loading buffer (Invitrogen) were electrophoresed on 10% (w/v) sodium dodecyl sulfate-polyacrylamide (SDS) gels and transferred to polyvinylidenefluoride membranes (Millipore). The membranes were blocked using 5% milk proteins in Tris-buffered saline containing 0.1% Triton X-100 (TBST) at 37˚C for 1 h and then incubated with primary antibodies against GAPDH (1:1,000 dilution), p53 (1:700 dilution), p21 (1:500 dilution) and Rb (1:700 dilution) overnight at 4˚C, followed by incubation with the HRP-conjugated secondary antibodies (ZB2301, 1:400 dilution and ZB2305, 1:400 dilution) respectively at 37˚C for 1 h. Proteins were detected using ECL Western Blotting Detection Reagent (Thermo Scientific).

Statistical analysis. All measurements were performed in three replicates at least. Data are presented as mean ± standard error of the mean (SEM). For comparisons between two independent groups, the two-tailed Student's t-test

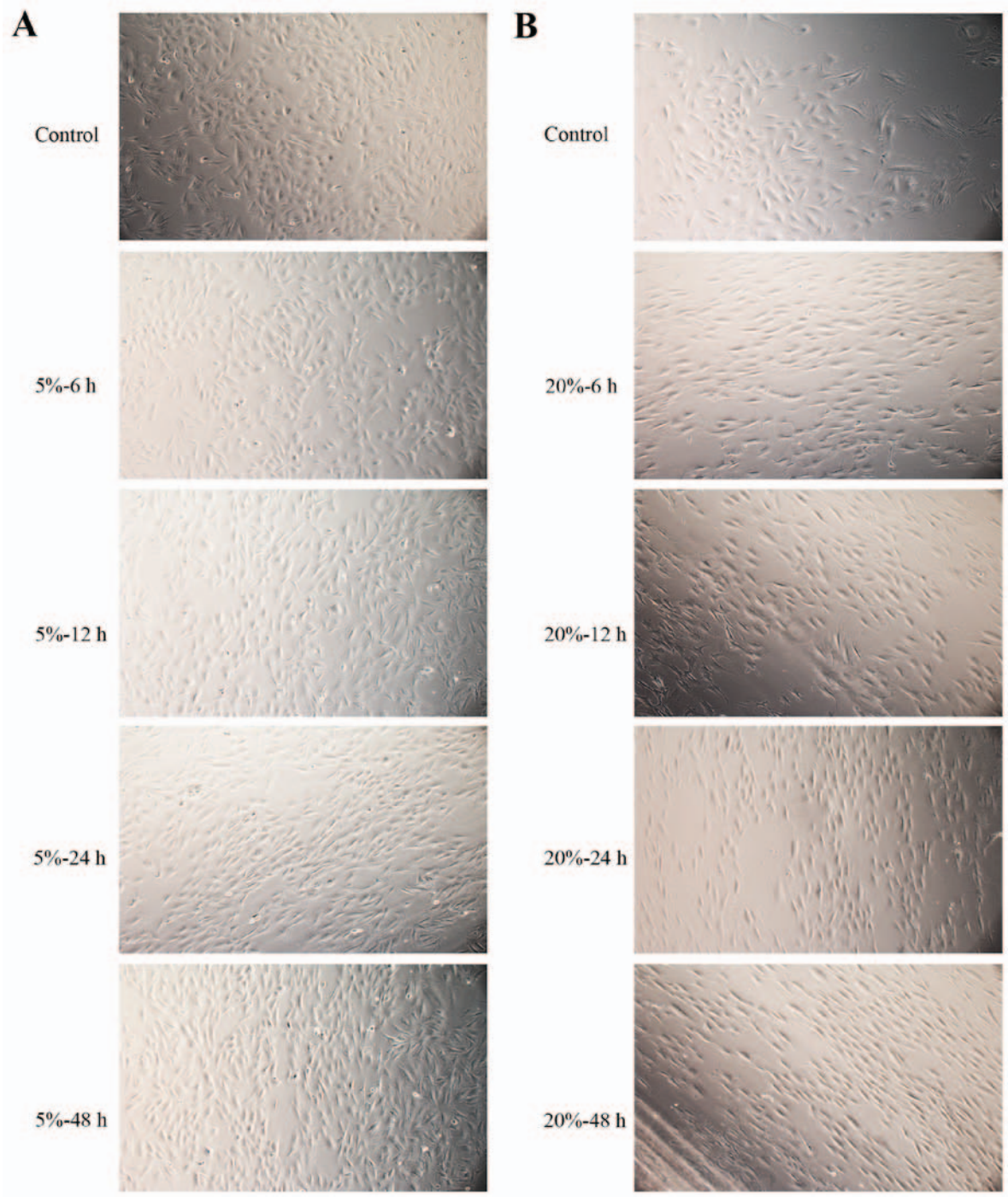

Figure 1. The morphology of nucleus pulposus (NP) cells after the application of cyclic mechanical tension (CMT, x200 magnification). (A) The morphology of NP cells after the application of CMT with 5% elongation for different duration. (B) The morphology of NP cells after the application of CMT with 20% elongation for different duration. Control, NP cells that were cultured under the same conditions and were kept static; 5%, CMT with 5% elongation; 20%, CMT with 20% elongation.

was used. Differences between three or more groups were statistically tested by one-way ANOVA and least significant difference (LSD) multiple comparisons. The data of RT-qPCR assays were statistically tested using Kruskal-Wallis nonparametric analysis and Mann-Whitney U post-hoc tests as described previously (33,34). Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS version 22.0 software programs (International Business Machines Corp., Amonk, NY, USA). $P<0.05$ was considered to indicate statistical significance.

Results

Morphology of NP cells after CMT application. With the duration of 20% CMT increasing, NP cells gradually aligned in a certain direction. The morphology of NP cells changed from a polygonal morphology into a spindle-like morphology (Fig. 1B). However, these changes were not obvious in NP cells subjected to 5% CMT (Fig. 1A). Furthermore, NP cells attached well on the silicon membrane after the application of 5% CMT and 20% CMT for 48 h (Fig. 1).

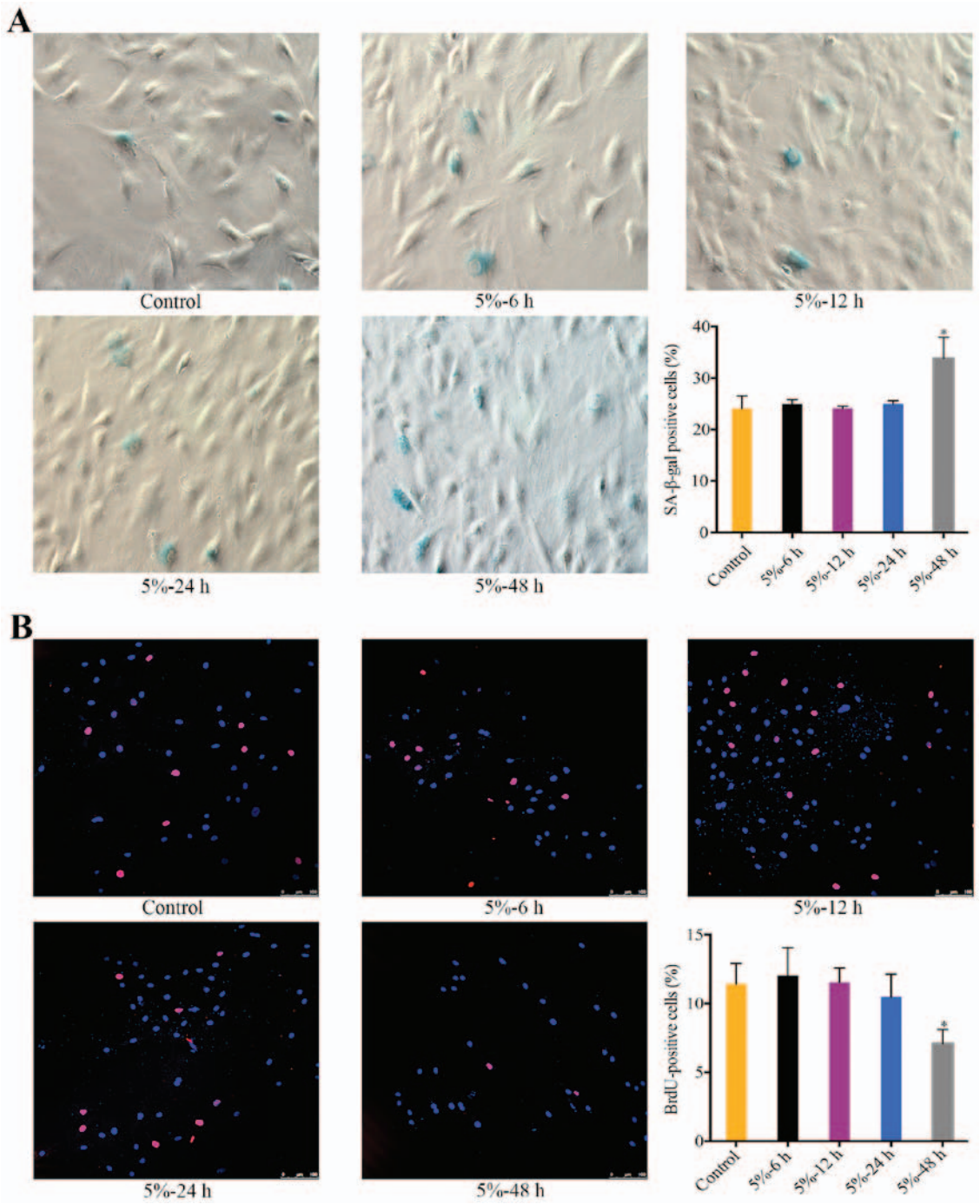

Figure 2. The effect of cyclic mechanical tension (CMT) with 5% elongation on the senescence of nucleus pulposus (NP) cells. (A) Senescence-associated β-galactosidase (SA-β-gal) staining and the percentage of SA-β-gal-positive cells in NP cells subjected to CMT with 5% elongation for different duration. (B) Immunofluorescence staining of BrdU and the percentage of BrdU-positive cells in NP cells subjected to CMT with 5% elongation for different duration. *P-value <0.05 compared with the control, error bars represent standard error of the mean (SEM). Control, NP cells that were cultured under the same conditions and were kept static; 5%, CMT with 5% elongation.

The effect of 5% CMT on the senescence of NP cells. There was a significant increase in the percentage of SA-β-gal-positive NP cells after the application of 5% CMT for 48 h (P<0.05). During 6 to 24 h, the percentage of SA-β-gal-positive cells was stable and not significantly different from the control (Fig. 2A). The effect of 5% CMT on the cell cycle of NP cells was analyzed by performing BrdU incorporation assays. Consistent with the results of SA-β-gal staining, the percentage of BrdU-positive cells showed slight changes without statistical significance after 5% CMT application for 6 to 24 h (Fig. 2B). The percentage of BrdU-positive cells was significantly higher than that in the control after 5% CMT application for 48 h (P<0.05) (Fig. 2B).

The effect of 20% CMT on the senescence of NP cells. Exposure of NP cells to 20% CMT resulted in a significant increase in the percentage of SA-β-gal-positive cells in a duration-dependent manner (P<0.05) (Fig. 3A). Starting at 12 h after the application of 20% CMT, the percentage of BrdU-positive NP cells gradually declined with the dura-

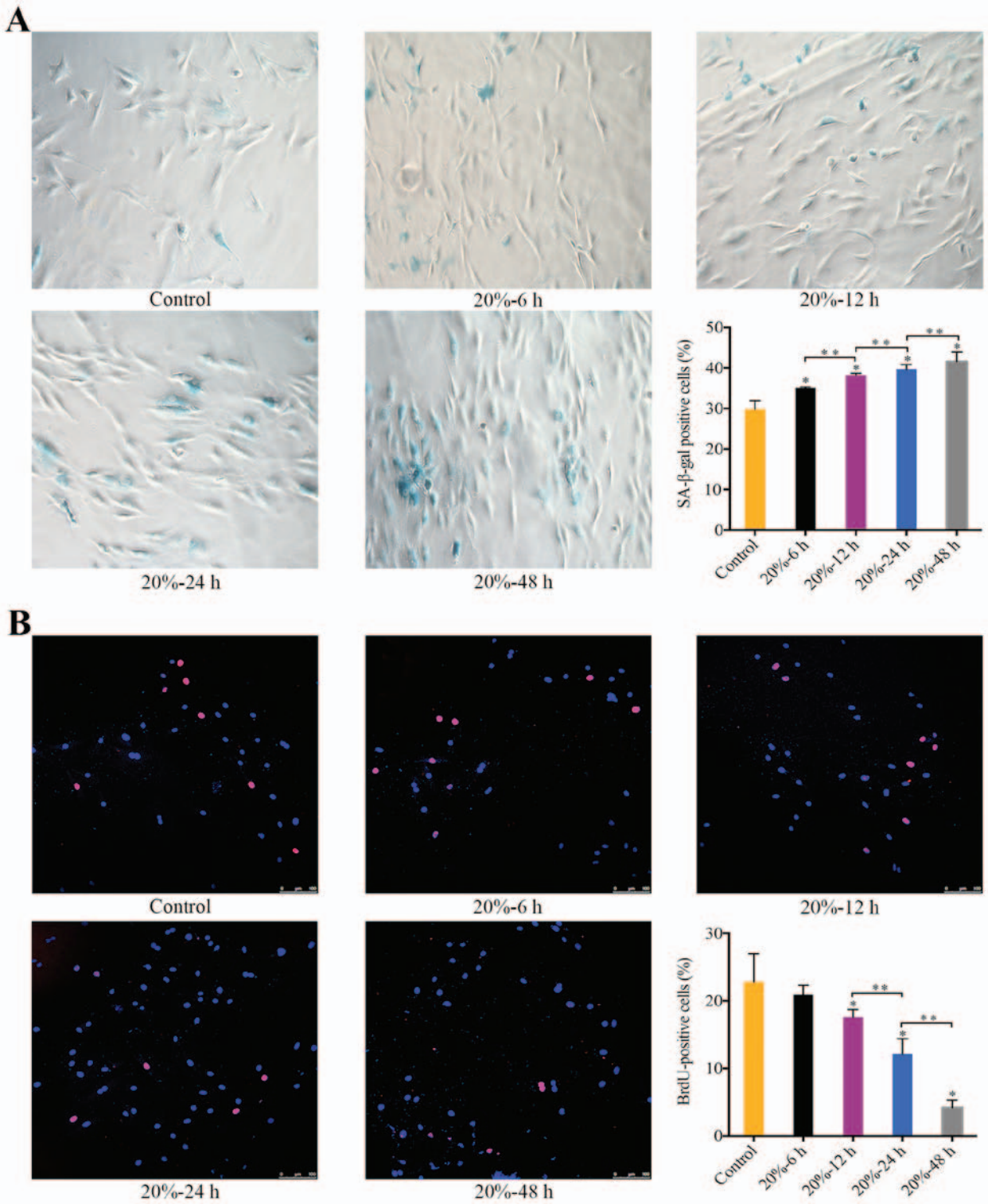

Figure 3. The effect of cyclic mechanical tension (CMT) with 20% elongation on the senescence of nucleus pulposus (NP) cells. (A) Senescence-associated β-galactosidase (SA-β-gal) staining and the percentage of SA-β-gal-positive cells in NP cells subjected to CMT with 20% elongation for different time-periods (B) Immunofluorescence staining of BrdU and the percentage of BrdU-positive cells in NP cells subjected to CMT with 20% elongation for different time-periods. *P-value <0.05 compared with the control; **P-value <0.05, error bars represent standard error of the mean (SEM). Control, NP cells that were cultured under the same conditions and were kept static; 20%, CMT with 20% elongation.

tion of 20% CMT increasing (P<0.05) (Fig. 3B). The results suggest that the premature senescence of NP cells subjected to 20% CMT is more prominent than that subjected to 5% CMT. Thus, we assessed the molecular mechanism of the mechanical stress-induced premature senescence of NP cells under 20% CMT.

CMT reinforced the DNA damage of NP cells. In consideration of that DNA damage is an internal trigger of NP cell senescence (23), we investigated the expression of γ-HAX foci in the nuclei of NP cells subjected to 20% CMT using immunofluorescence assays, which revealed the DNA damage in the nuclei of NP cells. The MID of γ-H2A.X-positive cells

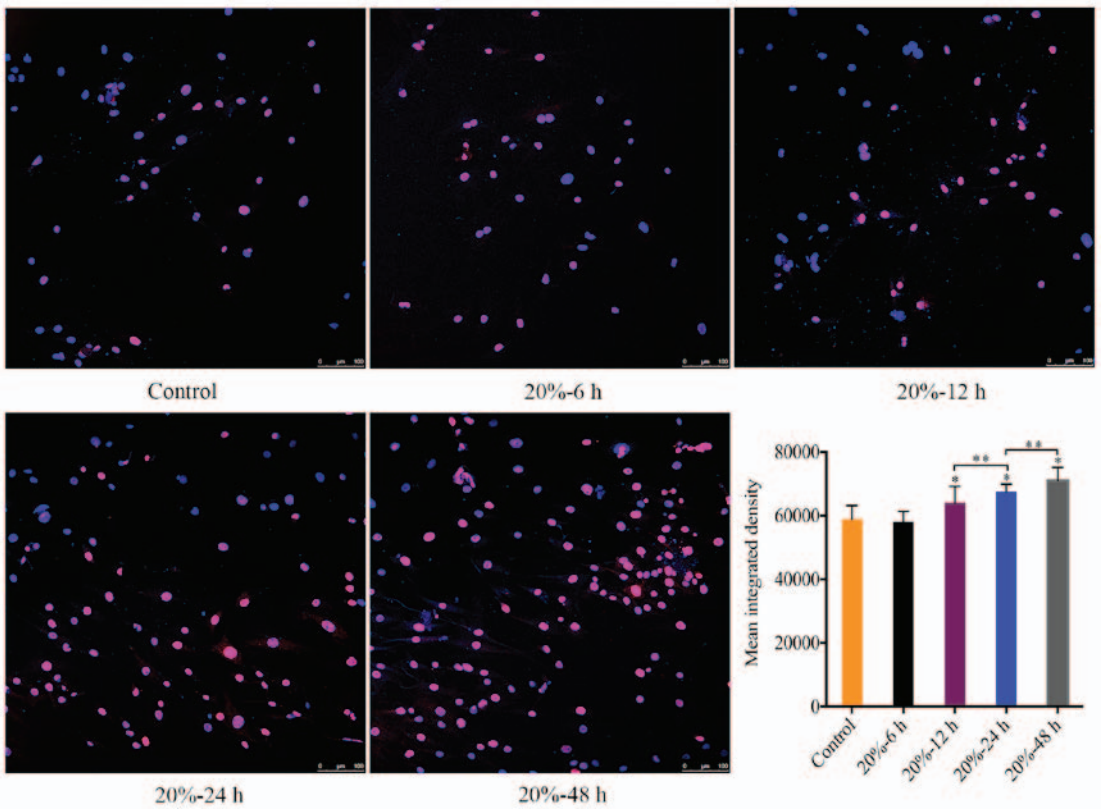

Figure 4. DNA damage of nucleus pulposus (NP) cells subjected to cyclic mechanical tension (CMT) with 20% elongation. Immunofluorescence staining of γ-H2A.X and the mean integrated density (nuclear area x mean gray value) of γ-H2A.X-expressing cells in NP cells subjected to CMT with 20% elongation for different time-periods. *P-value <0.05 compared with the control; **P-value <0.05, error bars represent standard error of the mean (SEM). Control, NP cells that were cultured under the same conditions and were kept static; 20%, CMT with 20% elongation.

was calculated using ImageJ. The MID of γ-HAX-positive cells gradually increased (P<0.05) (Fig. 4) with the duration of CMT increasing since the application of CMT for 12 h.

The redox state of NP cells is not affected by CMT. Oxidative stress caused by excessive ROS generation is also an essential trigger of NP cell senescence (23,24). A previous study demonstrated that the DNA damage of senescent fibroblasts is partially ROS-dependent (35). Therefore, we measured ROS levels in NP cells after 20% CMT application. Notably, CMT had little effect on the ROS production of NP cells although the duration of 20% CMT increased to 48 h (Fig. 5A). On the other hand, Msr is responsible for repairing the oxidative damage of proteins through the reduction of methionine residues in proteins, and is a newly identified oxidative stress marker of disc cells (36). However, the results of RT-qPCR analysis showed that the expression of MsrA, MsrB1 and MsrB2 in NP cells were not regulated by 20% CMT significantly, suggesting that the intracellular redox state of NP cells is not affected by CMT (Fig. 5B). Oxidative stress is possibly not involved in mediating the inductive effect of CMT on the DNA damage and premature senescence of NP cells.

CMT activates the p53-p21-RB signaling pathway in NP cells. Concerning the molecular mechanism of NP cell senescence, the p53-p21-Rb pathway and the p16-RB pathway are essential molecular pathways that induce the cell cycle arrest of disc cells (25,37). Nevertheless, their roles in the CMT-induced premature senescence of NP cell were unclear. Therefore, PCR analysis and western blot analysis were performed to determine the expression of p53, p21, p16 and Rb in NP cells subjected to 20% CMT for 24 h. The result showed that CMT significantly upregulated the expression of p53, p21 and Rb rather than p16 in NP cells (P<0.05) (Fig. 6A). Consistent with PCR analysis, western blot analysis confirmed the upregulation of p53, p21 and Rb in NP cells induced by the application of 20% CMT for 24 h. The expression of p16 in NP cells was prominent and was not regulated by CMT (Fig. 6B).

Discussion

To our knowledge, this is the first study investigating the effect of CMT on the senescence of NP cells. Disc cell senescence is a newly identified cellular event during the process of IDD (22,23). The accumulation of senescent disc cells in degenerative discs has been identified in previous studies. The number of senescent disc cells in degenerative IVDs is positively correlated with the severity of disc degeneration (20,21,38). Therefore, elucidating the roles of disc cell senescence in the initiation and progression of IDD contributes to understating the pathogenesis of IDD better. Although IVD is a tissue with low cell density, disc cells are homeostasis maintenance cells in discs and regulate the ECM homeostasis of discs to maintain the structure and function of IVDs (39). Senescent disc cells are replication exhausted, resulting in a decrease in the

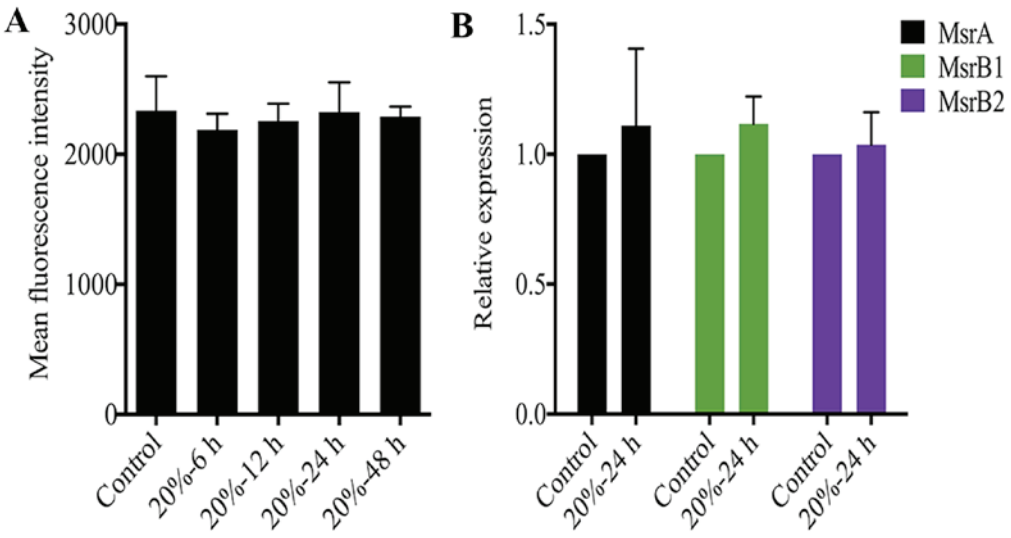

Figure 5. The effect of cyclic mechanical tension (CMT) with 20% elongation on the redox state of nucleus pulposus (NP) cells. (A) The production of ROS in NP cells subjected to CMT with 20% elongation for different duration. (B) The quantitative PCR analysis of methionine sulfoxide reductase A (MsrbA), Msrb1 and Msrb2 in NP cells subjected to CMT with 20% elongation for 24 h. Error bars represent standard error of the mean (SEM). Control, NP cells that were cultured under the same conditions and were kept static; 20%, CMT with 20% elongation.

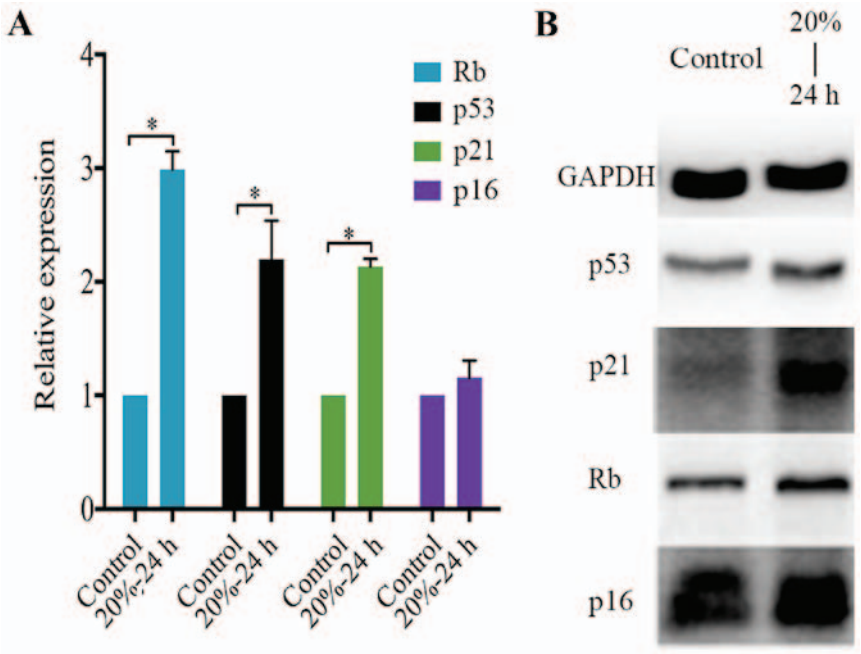

Figure 6. The activation of the p53-p21-retinoblastoma protein (Rb) pathway induced by cyclic mechanical tension (CMT) with 20% elongation. (A and B) The quantitative PCR analysis and western blot analysis of p53, p16, p21 and Rb in nucleus pulposus (NP) cells subjected to CMT with 20% elongation for 24 h. *P-value <0.05 error bars represent standard error of the mean (SEM). Control, NP cells that were cultured under the same conditions and were kept static; 20%, CMT with 20% elongation.

number of viable and functional disc cells due to apoptosis or cell death. On the other hand, senescent disc cells secrete various pro-inflammatory cytokines, ECM proteases and chemokines. Excessive matrix degradation enzymes reinforce ECM catabolism in discs (15). Pro-inflammatory cytokines and chemokines arouse inflammatory response, nociception and neovascularization in discs, which is strongly associated with low back pain (14,40,41). Thus, disc cell senescence is recognized as a new hallmark of IDD (22,23). However, the causes and molecular mechanisms of disc cell senescence are very complicated and involve telomere uncapping, aging, oxidative stress, nutrition deprivation and various molecular signaling pathways.

Herein, we investigated the effect of CMT on rat NP cell senescence using a Flexercell tension system. It showed that the percentage of SA-β-gal-positive cells in NP cells significantly increases after the application of CMT. CMT significantly suppressed the BrdU incorporation of NP cells, suggesting cell cycle arrest induced by CMT. The results indicate that CMT induces the premature senescence of NP cells. Notably, the pro-senescent effect of CMT was magnitude-dependent. CMT with 20% elongation caused strong morphological

changes of NP cells and induced premature senescence of NP cells starting at 12 h post-stimulation. However, CMT with 5% elongation induced premature senescence of NP cells starting at 48 h post-stimulation and showed little effect on the morphology of NP cells, suggesting that unphysiological mechanical stress accelerates the premature senescence of NP cells more prominently than physiological mechanical stress. Moreover, the pro-senescent effect of CMT was also duration-dependent. The number of senescent NP cells increased with the duration of CMT increasing. The results may explain the high risk of degeneration in discs with abnormal mechanical loadings. Prolonged mechanical stress with unphysiological magnitude induces the premature senescence of disc cells and consequently causes a decrease in the number of viable and functional disc cells, impairing the structural and functional homeostasis of IVDs to accelerate the initiation and progression of IDD. Therefore, keeping a healthy posture to reduce prolonged abnormal mechanical stress on IVDs is beneficial to retard disc cell senescence and to prevent or delay IDD.

CMT showed a direct genotoxic effect on NP cells. It markedly exacerbated the DNA damage of NP cells, which is revealed by the formation of γ-H2A.X foci in the nuclei of NP cells. The MID of γ-H2A.X foci in NP cell nuclei significantly increased in a duration-dependent manner starting at 12 h after 20% CMT application. DNA damage response activated by DNA damage is crucial to the cell cycle arrest of senescent cells, suggesting that DNA damage is involved in the CMT-induced premature senescence of NP cells. Moreover, ROS induce oxidative damage to biological macromolecules such as proteins, lipids and DNA. The DNA damage of senescent fibroblasts has been reported to be partially ROS-dependent (35). ROS are also an essential mediator of cell senescence and result in the stress-induced premature senescence (SIPS) (24,42). Notably, mechanical stress has been reported to induce SIPS of chondrocytes by increasing the intracellular ROS production (43). Therefore, we investigated the ROS production of NP cells subjected to CMT. CMT had little effect on the ROS production of NP cells, on the other hand, Msr is an enzyme repairing the oxidative damage of proteins through the reduction of methionine residues in proteins. The upregulation of Msr is recognized as a marker of oxidative stress in disc cells (36). However, the expression of MsrA, MsrB1 and MsrB2 in NP cells was not regulated by CMT.

In conclusion, the above results suggest that the DNA damage of NP cells induced by CMT is not associated with ROS overproduction. ROS are probably not involved in the CMT-induced premature senescence of NP cells. The findings remind us that preventing the excessive DNA damage of NP cells caused by CMT is a promising approach to suppress the premature senescence of NP cells. Antioxidant application may be ineffective to delay the CMT-induced premature senescence of NP cells. Furthermore, the mechanism underlying the CMT-induced DNA damage of NP cells needs to be elucidated further.

The p53-p21-Rb and p16-Rb are two central molecular pathways executing disc cell senescence. Generally, the p53-p21-Rb pathway is activated by telomere erosion, decreased telomerase activity and DNA damage to induce the replicative senescence of disc cells. The formation of γ-HAX foci at the site of DNA damage assembles DNA repair proteins, ATM and ATR and cell cycle checkpoint proteins Chk1 and Chk2, which leads to the activation of the p53-p21-Rb signaling cascade. Besides, the p16-Rb pathway is activated by various stimuli, including oxidative stress, pro-inflammatory cytokines and high glucose, to mediate the SIPS of disc cells (22,23). However, the molecular signaling pathways of cell senescence depend on differences in cause, cell type and species. Thus, we investigated the signaling pathways that mediate the CMT-induced premature senescence of NP cells. In contrast to our previous knowledge, CMT upregulated the expression of p53, p21 and Rb rather than p16 in NP cells, which was consistent with the enhanced DNA damage of NP cells induced by CMT. The p53-p21-p16 pathway mediated the pro-senescent effect of CMT on NP cells. Notably, p16 was prominently expressed in NP cells. It may be caused by stress resulting from the elastic silicone membrane coated with collagen type I. The external stresses induced the upregulation of p16 in NP cells. It also could explain why the percentage of BrdU-positive NP cells was relatively low even in the control group.

There are some limitations to the current study. First one is that CMT generated by the Flexercell tension system *in vitro* does not perfectly reflect the mechanical stress to which NP cells are subjected *in vivo*. Recent studies have used a custom-made external loading device to apply mechanical tension to the IVD of rabbit *in vivo* (44,45). Therefore, we can use an external loading device to apply mechanical tension to the discs of animals, and then investigate the effect of mechanical tension on senescence of NP cells *in vivo*, which will test our hypothesis further in depth. Secondly, the biological responses of disc cells to mechanical stress vary according to disc cell type as well as the magnitude, frequency and duration of mechanical stress (11). In the present study, we found that the effect of CMT on NP cell senescence is duration-dependent and magnitude-dependent. However, the effect of frequency remains unknown. Besides, taking the heterogeneity of NP cells (46-48) and the inherent biomechanical, biochemical and mechanobiological differences between rat and human NP cells into account (49,50), further studies based on human NP cells must be performed to translate the results from rats to humans.

In conclusion, prolonged exposure of unphysiological CMT dramatically induces the premature senescence of NP cells. CMT reinforces DNA damage of NP cells rather than disturbs the redox homeostasis of NP cells to result in oxidative stress in NP cells. Furthermore, the p53-p21-Rb pathway is activated to mediate the CMT-induced premature senescence of NP cells. The results are beneficial to understanding the mechanism of disc cell senescence and the mechanobiology of disc cells further. It suggests that prolonged mechanical stress with unphysiological magnitude induces the premature senescence of disc cells and consequently decreases the number of viable and functional cells in IVDs. Eventually, mechanical stress impairs the structural and functional homeostasis of IVDs and accelerates the process of IDD. It explains the high risk of IDD in persons with prolonged unphysiological mechanical loadings on the spine. Preventing the pro-senescent effects of mechanical stresses on disc cells is a promising approach to delay the process of IDD.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

The authors' contributions were as follows: CF, conception and design, acquisition of data, analysis and interpretation of data, and manuscript writing; MY, acquisition of data and provision of study material or patients; YZ, acquisition of data and analysis and interpretation of data; ML, acquisition of data and analysis and interpretation of data; BH, conception and design and final approval of the version to be published; HL, given final approval of the version to be published, conception and design, financial support, and administrative support; YZ, final approval of the version to be published, conception and design, financial support, and administrative support. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Xinqiao Hospital. All procedures described in this study were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (Washington, DC, USA).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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