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Necrosulfonamide (NSA) protects intervertebral disc degeneration *via* necroptosis and apoptosis inhibition

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Abstract. – OBJECTIVE: Previous studies have shown that nucleus pulposus (NP) cell death plays an extremely important role in the progress of intervertebral disc degeneration (IVDD). This research aimed to investigate the protective effect of the MLKL inhibitor necrosulfonamide (NSA) on human NP cells.

PATIENTS AND METHODS: We collected human NP tissues from the patients undergoing disc herniation operations and isolated NP cell from the samples. IL-1β (10 ng/ml) was used to establish a NP cells degenerated model. We analyzed the expression of caspase 3, caspase 8, RIPK1, RIPK 3, and MLKL in different degree of degenerate disc tissues. Cell viability was analyzed by the Cell Counting Kit-8 (CCK-8) assay. The expression levels of collagen II, β-galactosidase (β-gal), caspase 3, caspase 8, RIPK1, RIPK 3, and MLKL, several inflammatory and anti-oxidant enzymes of different NP cell treat groups were detected by Western blotting, immunofluorescence staining, or RT-PCR. Flow cytometry was used to measure the ROS level and cell apoptosis.

RESULTS: The data showed that expression of caspase 3, caspase 8, RIPK1, RIPK 3, and MLKL markedly increased in severely degenerated disc tissues. IL-1β promoted the cell death of NP cells, while NSA could reverse the effects of IL-1β. We found that NAS increased the antioxidant SOD1, SOD2, CAT, and GPX3 expression and suppressed oxidative stress in the disc. Moreover, MMP3, MMP10, IL-6, and TNF-α were significantly suppressed by the NSA.

CONCLUSIONS: These results suggest that NSA prevented NP degradation via inhibiting apoptosis and necroptosis of NP cells. Besides, the protective function of antagonizing cell death may owe to the inflammation and oxidative stress suppression.

Key Words:
Intervertebral disc degeneration, Nucleus pulposus cells, Necrosulfonamide, Necroptosis, Apoptosis.

Introduction

Low back pain, mainly results from intervertebral disc degeneration (IVDD), is widely known as one of the prevalent orthopedic ailments worldwide[1,2]. The prevalence of LBP is estimated to pervasively affect up to 80% of adults at a certain time point during their lives[3]. It not only brings persistent pain for the patients but also causes serious economic and social deficiency[4]. During degeneration, nucleus pulposus (NP) cells exhibit plenty of biochemical molecules change and result in extracellular matrix (ECM) metabolism disturbance[5]. As the pathogenesis of IVDD has yet been unclear, there is no effective treatment since now. Current treatment strategies include pharmacoconservative treatment to relieve pain, and operation to decompress sites of disc herniation in the spine. However, these treatments are only helpful to relieve clinical symptoms, they cannot address the cause of IVDD underlies[6]. NP cells are the most important type of cells in the intervertebral disc and they are responsible for synthesizing and maintaining the ECM. IVDD degeneration is characterized by loss of ECM and functional NP cells. The decline of NP cells is closely associated with cell death and contains three classical classes: apoptosis, autophagy, and necroptosis[7,8]. Thus, it is necessary to find an effective way to inhibit NP cells from death to protect IVDD[9,10].

The redundant apoptosis of NP cells contributes to the production of cartilage-specific ECM components, leading to a disc structural failure and biomechanical disorders[11]. Two well-characterized cell apoptosis pathways lead to the activation of caspase 8 and caspase 3, a pair of related executioner proteases that are responsible for the progress of cell death[12]. As important as caspase activity is for apoptosis, inhibiting caspases has become one of the key problems to solve the IVDD. Apart from these, the importance of

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necrotic cell death has been illustrated by several recent findings[13]. The determining factor for the necroptosis is the receptor-interacting serine-threonine kinase 3 (RIPK3). During the process of necroptosis, RIPK3 binds to the kinase RIP1 (RIPK1)[14] and mixed lineage kinase domain-like protein (MLKL) viewed as a critical substrate of RIPK3 has been reported to be critical in the occurrence of necroptosis in many documents[15,16]. Necroptosis activation requires the activity of RIPK1 first, mediates the activation of RIPK3 and MLKL, and then a molecular switch for two critical downstream mediators: necrosis and inflammation[17,18]. Necrosulfonamide (NSA) is a small-molecule specifically shown to block human MLKL and prevent plasma membrane rupture, which was identified as an inhibition on MLKL. NSA can reduce necroptosis of certain cell types, such as ovarian cells, dendritic cells, and acute myeloid leukemia cells[19-21] and it has been observed that this compound mediates necroptosis in compression-induced rat NP cells death via the RIPK1/RIPK3/MLKL pathway[22]. However, a limited study has elucidated the protective effect of NSA against human NP cells. We thus utilized NSA to investigate its protective effect against apoptosis and necroptosis using a classical IL-1β-induced NP cells degeneration *in vitro* model. As far as we know, this is the first work to report the effects of NSA on cell death in human NP cells.

Materials and Methods

Patient Tissue Collection and NP Cells Culture

This research was approved by the Ethics Committee of the Third Hospital of HeBei Medical University. All participants in our work have signed written informed consent before operations. This investigation was conducted in accordance with the Declaration of Helsinki. Degenerative lumbar NP samples were collected from 15 patients (7 women and 8 men; mean age, 47.8; range, 36-79) undergoing disc excision and spinal fusion surgery. Different degeneration degrees of IVD were assessed by the Pfirrmann grading system from magnetic resonance imaging (MRI) scans before the operation. The disc tissues of all patients with IDD were classified as Mild group (Grades II, III) and Severe group (IV, V).

Human NP cells used for cell culture were isolated from the discs of Mild group. Briefly, the tissues separated from the AF under a stereotaxic microscope were cut into small fragments and digested in cell culture medium (Dulbecco's Modified Eagle's Medium (DMEM)/F12, Gibco, Grand Island, NY, USA) with 0.2 mg/mL type II collagenase (Invitrogen, Carlsbad, CA, USA) overnight at 37°C. After that, the NP cells were resuspended in medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were added in six-well plates at 1×10^5 cells per well. After pretreatment (or not) with IL-1β (Invitrogen, Carlsbad, CA, USA) or Necrosulfonamide (NSA; Selleck Chemicals, Houston, TX, USA), the cells are cultured for 5 days for the next steps.

Protein Extraction and Western Blotting

The immunoblotting assay was used to measure the expression of correlative proteins. Briefly, NP tissues or cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Jiangsu, China). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Equal protein samples were transferred to methanol pre-activated polyvinylidene difluoride (PVDF) membranes. Then, membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with different primary antibodies overnight: anti- caspase-3/8(Abcam, Cambridge, MA, USA, 1:1000), anti-MLKL (Millipore, Billerica, MA, USA, 1:1000), RIPK1/3 (Abcam, Cambridge, USA, 1:1000) and anti-β-actin used as loading control (Cell Signaling Technology, Danvers, MA, USA, 1:2000). After washing with phosphate-buffered saline (PBS) three times, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL; Beyotime, Shanghai, China) was used to visualize the protein band development.

Immunofluorescence Staining

Cultured NP cells were washed three times in PBS, fixed with 4% formaldehyde for 15 min at 37°C, and blocked with 5% bovine serum albumin (BSA) for 1 h. Subsequently, the cells were incubated overnight at 4°C with a primary antibody against collagen II (Abcam, Cambridge, MA, USA, 1:200), β-gal (Abcam, Cambridge, MA, USA, 1:800), MMP3 (Abcam, Cambridge, MA, USA, 1:500), IL-6 (Abcam, Cambridge, MA, USA, 1:200) followed by Cy3-conjugated IgG antibody (Beyotime, Shanghai, China, 1:100) for 1 h at room temperature. Nuclei were counterstained with DAPI (Beyotime, Shanghai, China, 1:1000), and the cells were visualized using a fluorescence microscope (Olympus, Tokyo, Japan).

RNA Eextraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from NP tissue and cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After concentration determined with spectrophotometry, RNA was reverse transcribed to cDNA with PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) according to the manufacturer's instructions. qPCR was performed to quantify Caspase3/8, RIPK1/3, MLKL, MMP3/10, IL-6, TNF-α, SOD1/2, CAT, GPX3, and GAPDH expression levels using the One-Step SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China). GAPDH was used for normalization. The primers used for qRT-PCR are listed in Table I. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ methods.

Cell Proliferation Assays

Human NP cells proliferation was analyzed by Cell Counting Kit-8 assay (CCK-8, Dojindo Laboratories, Kumamoto, Japan). 5000 cells per well were seeded in 96-well plates, and CCK-8 was added to each well at specific time points. Cell viability was measured through absorbance detection at 450 nm using a spectrophotometer (ELx808 Absorbance Microplate Reader; Bio-Tek, Winooski, VT, USA).

Flow Cytometry

Intracellular ROS level and apoptosis incidence were detected by using the flow cytometry. DCFH-DA and Annexin V-FITC apoptosis detection kit (Kaiji, Nanjing, China) was used according to the manufacturer's instructions. Flow cytometry analysis in a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) was performed within 1 h: the apoptotic rate was calculated by the percentage of Annexin V+/PI- (early apoptotic) cells plus the percentage of Annexin v+/PI+ (late apoptotic) cells.

Statistical Analysis

All data are presented as mean ± standard deviation (SD) from at least three independent technical replicates. Data analysis was performed with SPSS software package 20.0 (IBM, Armonk, NY USA). Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Differences were considered statistically significant at $p< 0.05$.

Results

Necroptosis and Apoptosis Differences in Human NP Tissues with Pfirrmann Grades

We isolated total protein and RNA from patients' disc samples and Western blot was implemented to explore the caspase3 and MLKL,

Table I. Primer sequences of the genes for RT-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
aggrecan	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
RIPK1	GGGAAGGTGTCTCTGTGTTTC	CCTCGTTGTGCTCAATGCAG
RIPK3	ATGTCGTGCGTCAAGTTATGG	CGTAGCCCCACTTCCTATGTTG
MLKL	AGGAGGCTAATGGGGAGATAGA	TGGCTTGCTGTTAGAAACCTG
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
CAT	TGGAGCTGGTAACCCAGTAGG	CCTTTGCCTTGGAGTATTTGGTA
GSH	GGGAGCCTCTTGCAGGATAAA	GAATGGGGCATAGCTCACCAC
IL-6	TCCTGGCTAACGACAAATACGA	TTTCCCGGCCACCATAAAGG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP10	TGCTCTGCCTATCCTCTGAGT	TCACATCCTTTTCGAGGTTGTAG
Caspase3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC
Caspase8	TGCTTGGACTACATCCCACAC	TGCAGTCTAGGAAGTTGACCA
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction

classical markers of necroptosis and apoptosis, protein expression of both Mild and severe group according to different Pfirrmann grades. In addition, RT-PCR was carried out to measure the necroptosis and apoptosis relative gene expression in these disc samples. Each group contained 4 samples randomly. We found that the caspase3 and MLKL protein was significantly increased in Severe group in protein level (Figure 1A). Meanwhile, the caspase3, caspase8, RIPK1, RIPK3, and MLKL gene was markedly upregulated in Severe group compared with Mild group in mRNA level (Figure 1B-1F). The results showed that the cell necroptosis and apoptosis were activated significantly with the disc entering a severe degeneration.

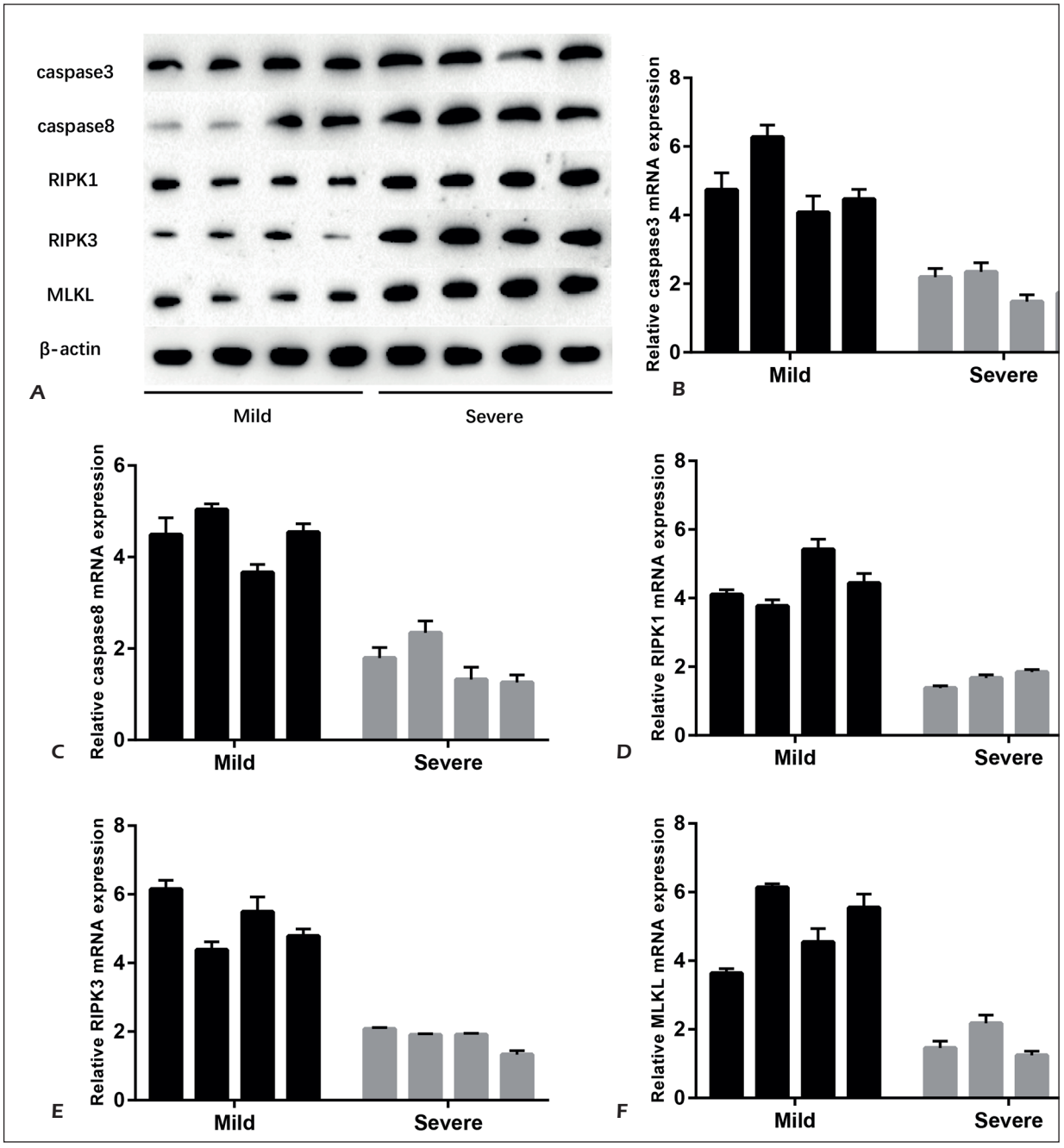

Figure 1. Necroptosis and apoptosis differences in human NP tissues with Pfirrmann grades. The protein expression of caspase3, caspase8, RIPK1, RIPK3, and MLKL in human disc nucleus pulposus tissue was determined by Western blot (**A**) and RT-PCR (**B-F**).

NSA Alleviates NP Cells Degeneration In Vitro

NP cells were isolated from the patients and cultured *in vitro*. We examined the cytotoxicity of NSA on NP cells using the CCK-8 assay to explore the optimal concentration for NP cells culture. As shown in Figure 2A, cell viabilities following stimulation of NSA at 0 μM, 5 μM, 10 μM, 20 μM for 48 h. Results showed that 10 μM contributed to the highest viability for NP cells. Thus, the concentrations of 10 μM were used for the following researches. We established NP cells degeneration model using IL-1β according to the previous method[23]. As a result of immunofluorescence, we found that IL-1β significantly reduced the collagen II expression and promoted the β-gal positive cells percentage, which meant IL-1β could degenerate human NP cells *in vitro*. After adding NSA, the degenerated phenotype of NP cells decreased as the collagen II expression upregulated, and the percentage of β-gal positive cells decreased compared to IL-1β group (Figure 2B, 2C). We also took the CCK-8 assay to analyze the proliferation of the three groups. As Figure 2D showed, IL-1β played a negative role in the progress of NP cells proliferation, and NSA reversed this situation successfully. The results demonstrated that NSA could protect degeneration and promote the proliferation of NP cells *in vitro*.

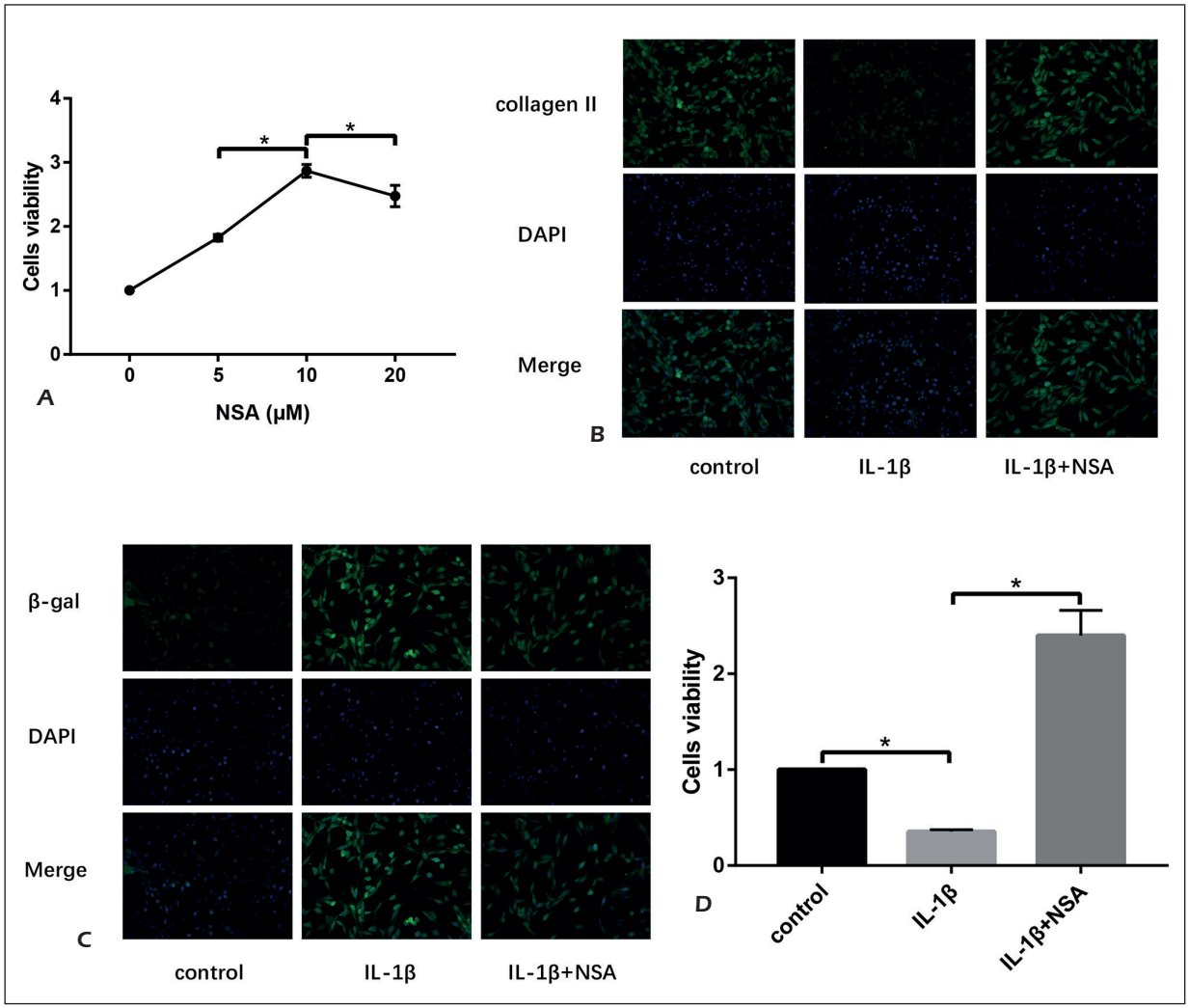

Figure 2. NSA alleviates NP cells degeneration *in vitro*. **A**, CCK8 assay for NP cells with treatment of 0, 5, 10, and 20 μM NSA. The expression of collagen II (**B**) and β-gal (**C**) was determined by immunofluorescence (magnification: 100×). **D**, CCK-8 assay for NP cells in three groups. ("*" means there is a statistical difference between two groups).

NSA Inhibits NP Cells Necroptosis and Apoptosis In vitro

Necroptosis and apoptosis often interact with each other and determine the final fate of the cells together. RIPK1, RIPK3, and its substrate MLKL are emerging as core molecules regulating necroptosis. Among the caspase superfamily factors, the crucial ones in the process of apoptosis, caspase3, and caspase8 are the center and frequently activated death proteases essential for apoptosis. Therefore, we checked these hallmarks for necroptosis and apoptosis evaluation. Results showed that IL-1β significantly increased the caspase3/8, RIPK1, RIPK3, and MLKL compared with control group. After treatment of NSA, all the expression of these proteins decreased compared with IL-1β group (Figure 3A). Also, this sequence was found in mRNA levels with RT-PCR method and flow cytometry analysis of apoptosis (Figure 3B, 3C). All these results indicated that the appropriate dose of NSA could inhibit the necroptosis and apoptosis of human NP cells, protecting the IVDD.

Effect of NSA in Anti-Oxidation and Anti-Inflammation in NP Cells

To further verify the potential mechanism of NSA in inhibiting NP cells degeneration, we investigated whether NSA had a role in anti-oxidation and anti-inflammation. Immunofluorescence staining results indicated that NP cells expressed a higher level of MMP3 and IL-6

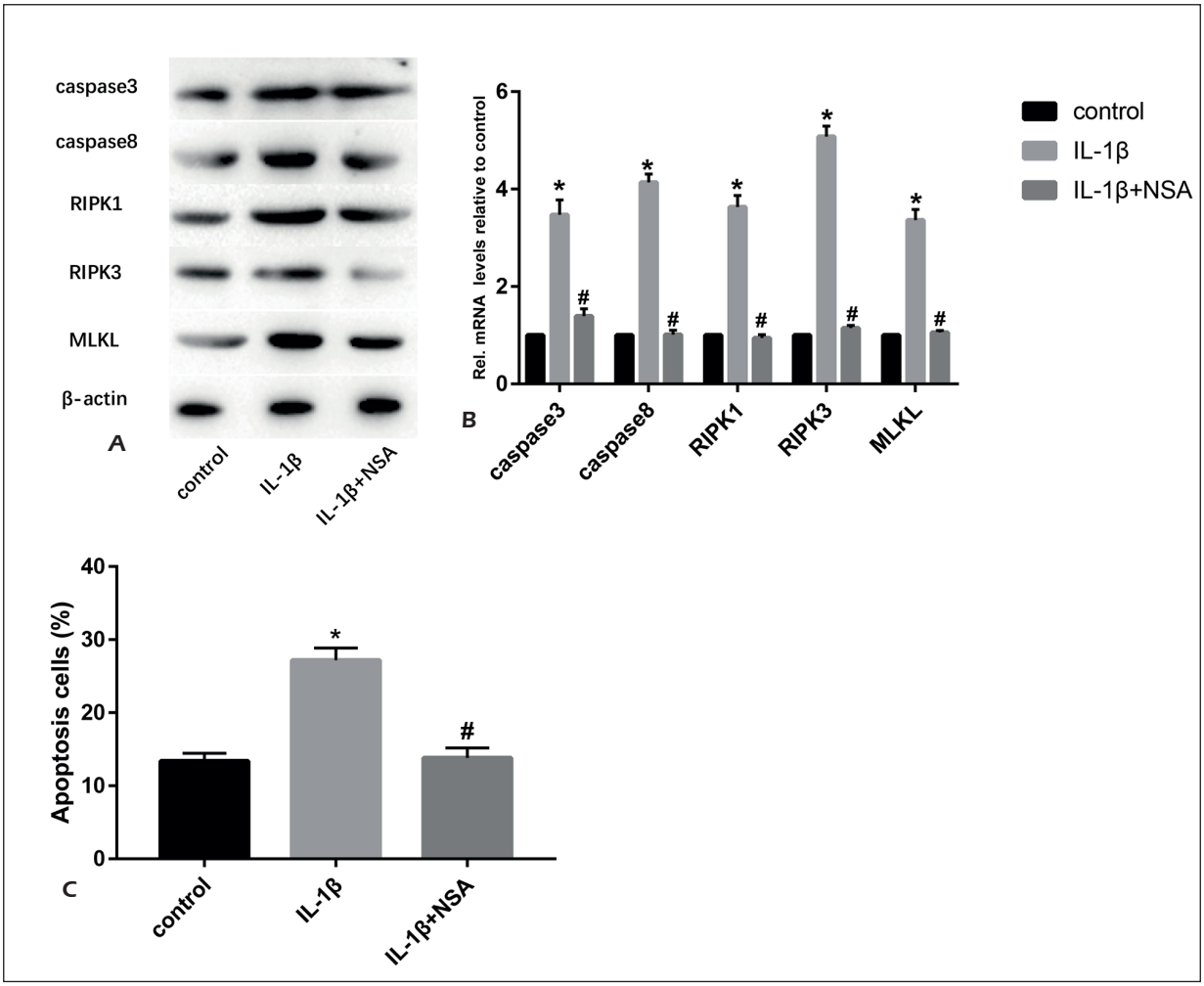

Figure 3. NSA inhibits NP cells necroptosis and apoptosis in vitro. The expression of caspase3, caspase8, RIPK1, RIPK3, and MLKL was determined by Western blot (**A**) and RT-PCR (**B**). **C**, Cell apoptosis level was detected by flow cytometry. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

gene compared with the control one. On the contrary, NSA decreased the MMP3 and IL-6 positive cells compared with IL-1β group (Figure 4A, 4B). The same results were proved by the RT-PCR analysis (Figure 4C). Flow cytometry analysis was used to measure the total ROS level of human NP cells (Figure 4D). We found that NSA+IL-1β group had a lower level of ROS compared with IL-1β group. Some relative anti-oxidative enzymes were analyzed by RT-PCR and the result suggested that SOD1, SOD2, CAT, and GPX3 were upregulated after the stimulation of NSA compared with IL-1β group. This indicated that NSA promoted the expression of anti-oxidative enzymes and significantly reduced the total ROS level in human NP cells. Results implied that NSA truly played a role in anti-oxidation and anti-inflammation in the protection of IVDD, which might be the reason for inhibiting the cells necroptosis and apoptosis.

Discussion

IVDD is a serious disease worldwide responsible for disc herniation, spinal canal stenosis, and degenerative scoliosis, which often results in low back pain in every aspect of life. It is generally acknowledged that IVDD is associated with the devitalization of NP cells, including increased cell apoptosis and necroptosis[24,25]. Caspase is a large family in apoptosis mediators and cascade reactions of caspases play a crucial role in apoptosis, among which caspase 3 serves critically[26]. Caspase3, interacting with caspase-8, is activated in the apoptotic cell by a death ligand and/or mitochondrial pathways post injury[27,28]. Necroptosis is a novel sight in the field of the IVD research. As we age, it is increasingly evident that NP cells appear to undergo necrosis, and this ratio reaches a staggering 80% of total cells[29]. In this present work, we elucidated the

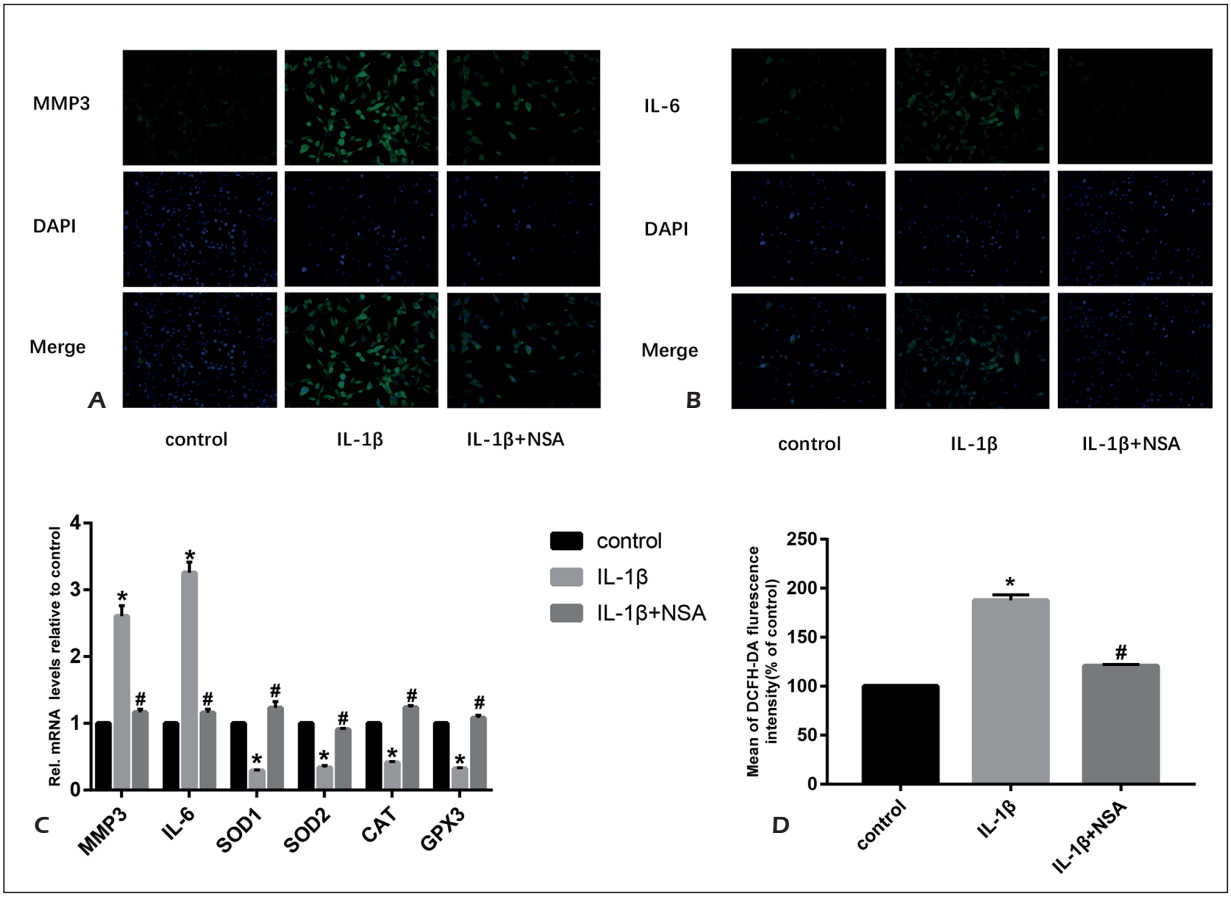

Figure 4. Effect of NSA in anti-oxidation and anti-inflammation in NP cells. The expression of MMP3 (**A**) and IL-6 (**B**) was determined by immunofluorescence (magnification: 100×). **C**, mRNA expression of MMP3, MMP9, IL-6, SOD1, SOD2, CAT, and GPX3 was determined by RT-PCR. **D**, ROS levels were detected by flow cytometry. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

protective function of NSA, a specific inhibitor on MLKL. The data suggested that the collagen II level increased, β-gal positive cell reduced, and NP cell proliferation enhanced under the simulation of NSA, indicating the protection of NAS in the IL-1β-induced human NP cells degeneration. Also, we found NSA could inhibit the expression of caspase 3 and caspase 8, as well as the RIPK1, RIPK3, and MLKL, which demonstrated that NSA played a protective role in the process of necroptosis and apoptosis of human NP cells. Several reports have shown that oxidative stress leads to the apoptosis of NP cells during the IVD degeneration. NP cells treated with IL-1β exhibited not only a degenerated cell phenotype, but also elevated the production of ROS levels, which triggered cell death. The data showed that the total ROS level decreased in NP cells treated with NSA, meanwhile also in apoptosis. Antioxidant enzymes, such as SOD1, SOD2, CAT, and GPX3, serve as a side reflection of oxidative stress in IVDD. We found significant changes in the activity of these antioxidant enzymes after the treatment with NSA. Furthermore, we measured MMPs, IL-6, TNF-α levels, and found these inflammatory factors decreased significantly with the NSA treatment. However, the anti-inflammatory ability of NSA needs to be deeply verified. In short, the results suggest NSA might play an important role in oxidative stress and inflammation regulation, which contributes a positive effect to inhibit NP cells apoptosis and necroptosis. As far as we know, this is the first research on human NP cells protection resulting from NSA treatment via necroptosis and apoptosis inhibition. Therefore, this present work suggests that NSA might be safe for usage to degenerated discs at an appropriate dose. These findings introduce a new plane in the field of IVDD and provide a new therapeutic treatment option.

Conclusions

We suggested that NSA prevented NP degradation via inhibiting apoptosis and necroptosis of NP cells. Besides, the protective function of antagonizing cell death may be due to the inflammation and oxidative stress suppression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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