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Original Research

Inflammatory Stimulation Mediates Nucleus Pulposus Cell Necroptosis Through Mitochondrial Function Disfunction and Oxidative Stress Pathway

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Abstract

Background: The mutual activation between nucleus pulposus (NP) cells death and inflammation is an important pathogenic factor of intervertebral disc degeneration. Whether inflammation mediates NP cells necroptosis, and its relationship with mitochondrial dysfunction and oxidative stress remains unclear. **Methods**: In this study, 50 ng/mL of TNF-α and 20 ng/mL of IL-1β were used to co-treatment with rat NP cells for 0, 24, 48, 72 hours, then Western blot and RT-PCR techniques were utilized to evaluate the expression level of necroptosis-associated target molecules, such as RIPK1, RIPK3 and MLKL. The results established that with prolongation of TNF-α and IL-1β treatment time, the expression level of necroptosis-associated molecules gradually increased. The 48 hours of TNF-α and IL-1β treatment was selected throughout the following experiments. The RIPK1 specific inhibitor necrostatin-1 (Nec-1), RIPK3 inhibitor GSK872, MLKL inhibitor necrosulfonamide (NSA) and small interfering RNA (siRNA) technology were employed. **Results**: Under the treatment of TNF-α or IL-1β, administration of Nec-1, GSK872 or NSA notably reduced NP cells death and up-regulated NP cells viability. Consistently, SiRNA-mediated knockdown of RIPK3 (SiRIPK3) or MLKL (SiMLKL) promoted the survival of NP cells. However, SiRIPK1 aggravated NP cells death. Furthermore, after 48 hours of TNF-α and IL-1β treatment, the mitochondrial membrane potential decreased, opening of mitochondrial permeability transition pore enhanced, and oxidative stress level notably elevated. The Nec-1, GSK872 or NSA treatment largely restored the normal mitochondrial function and down-regulated oxidative stress. **Conclusions**: In summary, RIPK1/RIPK3/MLKL-mediated necroptosis play an important role in NP cells death during inflammatory irritation, which might be closely related to mitochondrial dysfunction and up-regulation of oxidative stress.

Keywords: inflammation; nucleus pulposus cells; necroptosis; mitochondrial dysfunction; oxidative stress; intervertebral disc degeneration

1. Introduction

Low back pain (LBP), which has become a serious public health problem, ranking sixth in the global burden of disease, is a very common clinical symptom, and more than 80% of people have experienced LBP torture during their lifetime [1–3]. Intervertebral disc (IVD) degeneration is the main pathogenic factor of LBP [4]. IVD is a fibrocartilage disc located between two adjacent vertebral bodies. It is composed of three parts: the gel-like nucleus pulposus (NP), the inner and outer fibrous annulus (AF), and the upper and lower cartilage endplates (CEP). The NP degeneration is the primary factor of IVD degeneration and plays a central role in the degeneration process [5,6], which is closely related to NP cells death [7,8]. Among them, apoptosis and autophagic death are often reported in previous researches. However, no ideal regulatory target has been found to efficiently prevent NP cells death during multiple stages of disc degeneration.

Necroptosis is a type of death with necrosis-like morphological characteristics. Unlike apoptosis, necroptosis exhibits caspase-independent characteristics; unlike traditional necrosis, it is a precisely regulated form of death. Due to that it breaks the traditional view that necrosis cannot be controlled, it has been listed as a newest type of programmed death following apoptosis and autophagic cell death [9,10]. It has made important breakthroughs in tumors, brain injury, inflammatory diseases, etc., and is expected to become a new target for the treatment of many clinical diseases [11–13]. In most cases, the receptor-interacting protein kinase 1 (RIPK1)/receptor-interacting protein kinase 3 (RIPK3)/mixed series protein kinase-like domain (MLKL) axis is the classic signaling pathway to ini-

tiate and mediate necroptosis [14,15]. However, literature has reported that activating the RIPK3/MLKL signal axis can also effectively mediate necroptosis, which does not depend on RIPK1 activation [16]; in addition, study has even confirmed that RIPK1 can inhibit RIPK3/MLKL-mediated necroptosis [17]. In other words, the regulatory mechanism of necroptosis is a complex process involving the expression and regulation of a series of molecules. Chen *et al*. [18] reported for the first time that necroptosis mediated by the RIPK1/RIPK3/MLKL pathway is closely involved in compression-induced NP cells death. Then, in Jun 2018 they once again reported that RIPK1-mediated mitochondrial dysfunction is closely related to compression-induced NP cells necroptosis and apoptosis [19], which provides a new direction for the study of related mechanisms of IVD degeneration.

The IVD degeneration is accompanied by inflammation, and the inflammation further intensifies the degeneration, forming a cascade amplification effect similar to the "inflammation waterfall" [20,21]. The infiltration of the IVD mediated by inflammation is also an important pathological basis for the occurrence and development of disc degeneration and discogenic LBP [22,23]. Thus far, there is no report about inflammation-mediated necroptosis of NP cells. In view of the fact that inflammation plays a key role in the occurrence of necroptosis, necroptosis also closely regulate the occurrence and development of inflammation; therefore, further study whether inflammatory conditions can mediate necroptosis of NP cells and clarify the precise molecular regulation mechanism, is expected to open up a new idea for effectively inhibition of NP cells death.

Regarding the underlying mechanism of necroptosis, the mitochondrial signaling pathway is a hot research topic [24]. Mitochondrial division and fusion are not only pivotal for the maintenance of mitochondrial inheritance and its own functions, but also for energy metabolism, aging and cell death [25,26]. Mitochondrial is the regulatory center of apoptosis, autophagy and necroptosis. Literature reported that mitochondrial dysfunction caused by excessive mitochondrial division, such as increased opening of mitochondrial membrane pores (MPTP), decreased mitochondrial membrane potential (MMP), increased mitochondrial ROS production, and ATP depletion, are closely involved in necroptosis [27,28]. However, there is still study implying that necroptosis does not depend on mitochondrial function damage such as increased opening of MPTP and decreased MMP [29]. Mitochondrial signaling pathways and necroptosis are intricate, and more researches are needed to clarify.

In the present study, we systematically addressed whether inflammation-induced NP cells necroptosis. This study is also aimed at exploring the precise mechanism of its occurrence, such as mitochondrial dysfunction and oxidative stress, and ultimately providing a brand new and more efficient strategy to prevent NP cells death.

2. Materials and Methods

2.1 NP Cells Isolation and Culture

All experimental procedures were approved by the Animal Care and Ethics Committee of Zhengzhou University. The primary NP cells were were isolated and cultured as previously described [18,19]. The male Sprague-Dawley rats (3 months, 250–300 g) were purchased from Experimental Animal Center of Zhengzhou University. Briefly, the NP tissue of each IVD was obtained with ophthalmic forceps and cutted into 1 mm^3 fragments. The isolated fragments were digested with 0.25% type II collagenase (Sigma, USA) at 37 °C for 30 minutes and filtered through a 70 μm filter to remove debris. The obtained NP cells were cultured in complete culture medium Dulbecco's modified Eagle's medium/ham's F-12 (DMEM/F-12, Gibco, USA) and 20% fetal bovine serum (FBS, Gibco, USA) supplemented with 1% penicillin/streptomycin (Sigma, USA). When the cells reached 80–90% confluence, they were digested with 0.25% tripsinase (Beyotime, China). Due to the small amount of NP cells in the lumbar IVD, we extracted 8 rats each time for primary cell culture, and extracted NP cells 12 times. The total number of rats used in this article is about 96. The second generation of NP cells were used throughout the following experiments.

2.2 Inflammatory Stimulation was Applied to Rat NP Cells

TNF-α and IL-1β are the extremely broad studied inflammatory factors in IVD degeneration [30–33]. Referring to previous research, the intervention concentration of TNF-α or IL-1β is 50 ng/mL, 20 ng/mL respectively [30–33], the treatment time periods are 0, 24, 48, 72 hours, and choose the appropriate action time on this basis. The RIPK1 inhibitor necrostatin-1 (Nec-1, Sigma, USA), RIPK3 inhibitor GSK872 (Merck, Germany) and MLKL inhibitor necrosulfonamide (NSA, Sigma, USA) were applied to experimental groups, while the control groups were given isopyknic dimethylsulfoxide (DMSO, Sigma, USA).

2.3 Cell Viability Detection

To evaluate NP cells viability, the cell counting kit-8 (CCK-8, Dojindo, Japan) was employed. NP cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates and then incubated for 24 hours. At the appropriate inflammation treatment time periods, following themanufacturer's instructions, 10 μL of CCK-8 solution (Dojindo, Japan) and 100 μL of DMEM-F12 were added to each well and incubated for 2 hours at 37 °C. The cell viability was quantified by detecting the absorbance at 450 nm using a spectrophotometer (ELx808 Absorbance Microplate Reader, Bio-Tek, USA).

2.4 Propidium Iodide (PI) Positive Ratio Assay

The PI positive ratio (indicating necrotic cells) of NP cells were determined using PI single-staining (Nanjing Keygen Biotech, China). At each time point, the cells were

harvested, stained according to the manufacturer's instructions, and then analyzed by flow cytometry. The PI staining positive ratio allowed us to quantify the necrotic cells (PI positive).

2.5 Lactate Dehydrogenase (LDH) Release Ratio

According to the manufacturer's (Beyotime, China) instructions, the release of LDH in culture medium was utilized to detect NP cells cytotoxicity under inflammatory condition. In brief, the LDH release activity is presented as the release of LDH in the culture medium relative to the total cellular LDH.

2.6 The Morphological Changes of NP Cells

The NP cells were exposed to TNF-α or IL-1β for 48 hours. The Nec-1, GSK872 or NSA were applied to observe the effect of necroptosis on morphological changes of NP cells during inflammatory condition. At the given time points, cells were photographed using phase-contrast microscopy (Olympus, Tokyo, Japan).

2.7 Live and Dead Cell Staining

NP cells were seeded in 24-well culture plates and treated as described above. Following 48 hours treatment, the NP cells were washed twice with PBS, and then incubated with Calcein-AM (2 μM), a membrane permeable probe to label the live cells, at 37 ℃ in the dark for 20 minutes. After being gently rinsed three times in PBS, the cells were stained with 5 μmol/L PI according to manufacturer's instructions. Under the blue light excitation, the living cells appeared green, and nuclei of dead NP cells displayed red fluorescence. Finally, the stained NP cells were observed under a laser scanning confocal microscope (LSM, Heidelberg, Germany).

2.8 Mitochondrial Membrane Potential (MMP) Evaluation

To analyze the MMP changes in NP cells under inflammatory conditions, JC-1 fluorescent probes (Beyotime, China) was introduced into this study. Briefly, the NP cells were incubated with the JC-1 solution in the dark for 20 minutes, and then the MMP in NP cells was evaluated under flow cytometry. Finally, the evaluation of MMP is expressed as the ratio of red relative to green fluorescence intensity.

2.9 Measurement of Mitochondrial Permeability Transition Pore (MPTP) Opening

The MPTP of NP cells was measured by MPTP Fluorescence Assay Kit (Genmed, China). At each time point, cells were collected, then 500 μL preheated cleaning solution (Reagent A) and isopyknic working solution containing neutralization solution and staining solution (Reagent B) were added into cell suspension. Next they were mixed gently and incubated in the dark at 37 °C for 20 minutes. Finally, the sample was resuspended in Reagent A and analyzed using flow cytometry and LSM.

2.10 Detection of Reactive Oxygen Species (ROS)

ROS levels in the NP cells were analyzed using the ROS-specific fluorescent probe 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) (Sigma, USA). Briefly, at each time point, the culture medium was discarded, and the NP cells were harvested. Then, the cells were resuspended and incubated with 20 μM H_2DCF-DA at 37 °C in the dark for 20 minutes. Subsequently, serum-free medium was used to rinse the cells twice. Finally, the mean fluorescence intensity (MFI) was quantified by flow cytometry. Furthermore, in order to evaluate the intracellular ROS level in situ, the cells were examined under the LSM.

2.11 Transfection of Small Interfering RNA

The Rat SiRNA-RIPK1, SiRNA-RIPK3 and SiRNA-MLKL were designed and manufactured by Biomics (Biomics Biotechnologies Co. Ltd, China) according to current guidelines. The effective siRNA sequences for SiRNA-RIPK1, SiRNA-RIPK3 and SiRNA-MLKL were used as follows. SiRNA-RIPK1: 5′-GUCUUCGCUAACACCACUAdTdT-3′, 5′-UAGUGGUGUUAGCGAAGACdTdT-3′; SiRNA-RIPK3: 5′-CAUGUCAGUACAACCGAGAdTdT-3′, 5′-TCTCGGTTGTACTGACATGdTdT-3′; SiRNA-MLKL: 5′-CUGGAGGCUACCAAGUAAATTdTdT-3′, 5′-UUUACUUGGUAGCCUCCAGTTdTdT-3′. The NP cells were transfected with above effective sequence at a concentration of 100 pmol/10^5 cell using lipofectamine RNAi MAX (invitrogen). 24 hours later, the transfected cells were digested and recultivated for subsequent experiments.

2.12 Immunofluorescence Staining

Following 0, 24, 48, 72 hours TNF-α or IL-1β treatment, NP cells were washed three times in PBS and fixed in 4% paraformaldehyde at room temperature for 15 minutes. Next, the cells were blocked in 5% bovine serum albumin diluted with 0.3% Triton X-100 for 30 minutes. Then, cells were incubated with p-MLKL primary antibody (Abcam, USA) at a 1:100 dilution overnight at 4 °C in the dark. After washing, the NP cells were incubated with fluorophore-conjugated secondary antibody for 60 minutes. Finally, the stained samples were visualized and photographed under LSM.

2.13 Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

At each time point of inflammation treatment, the total RNA of NP cells was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and then transcribed into complementary DNA (cDNA). Quantitative RT-PCR was performed using a standard PCR kit and SYBR Green/Fluorescein qPCR Master

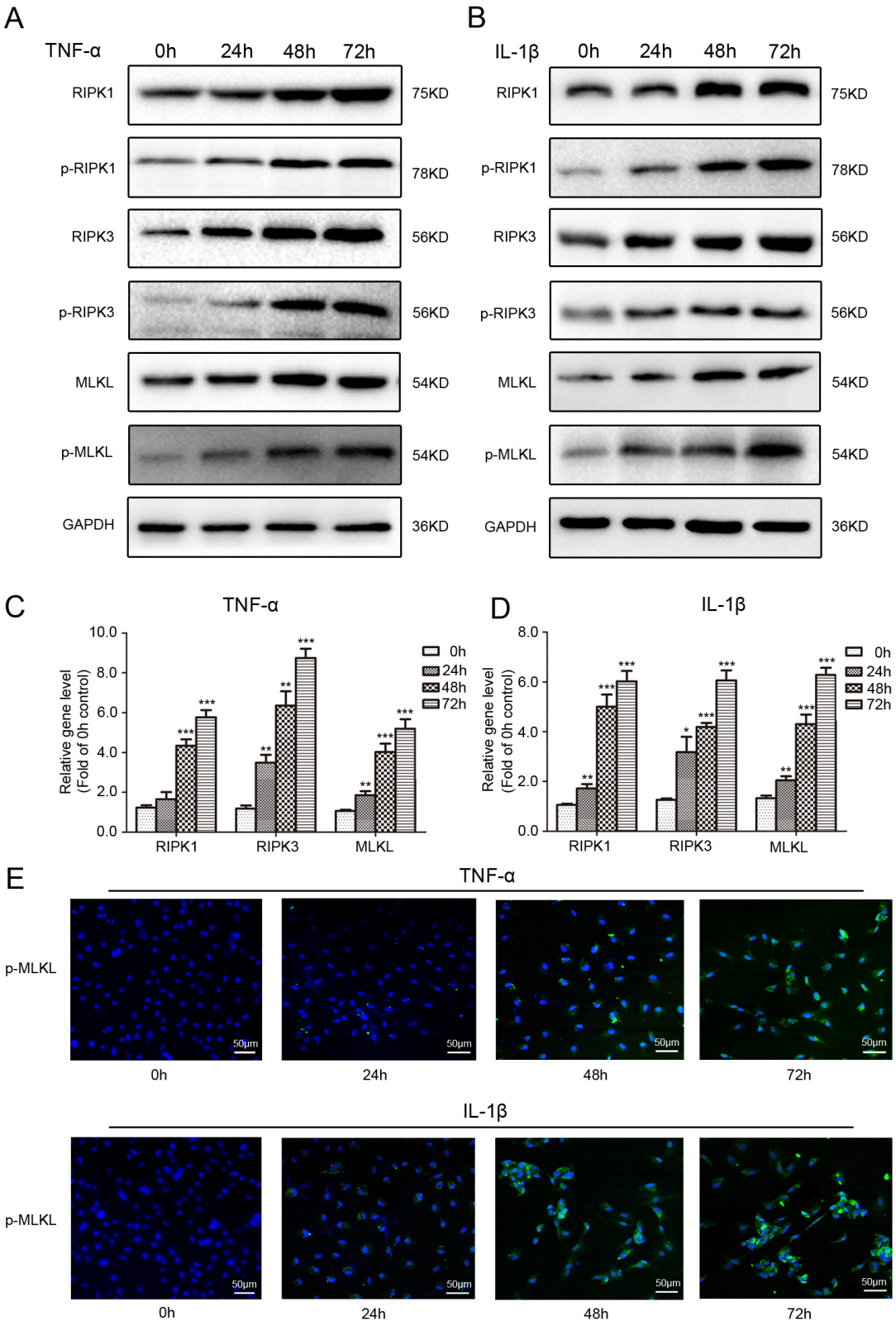

Fig. 1. Effects of inflammatory factor on protein and gene expression of necroptosis-associated target molecules in rat NP cells. (A,B) Representative western-blot graphs of RIPK1, p-RIPK1, RIPK3, p-RIPK3, MLKL, p-MLKL and GAPDH in NP cells subjected to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 0, 24, 48 and 72 hours. Data from treated groups have been normalized to GAPDH. (C,D) The mRNA level of RIPK1, RIPK3 and MLKL measured by RT-PCR in NP cells subjected to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 0, 24, 48 and 72 hours. (E) The fluorescence photomicrograph of p-MLKL expression detected by immunofluorescence staining. Scale bars = 50 μM. Values are expressed as mean $\pm$ SD from three independent experiments (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ vs. control).

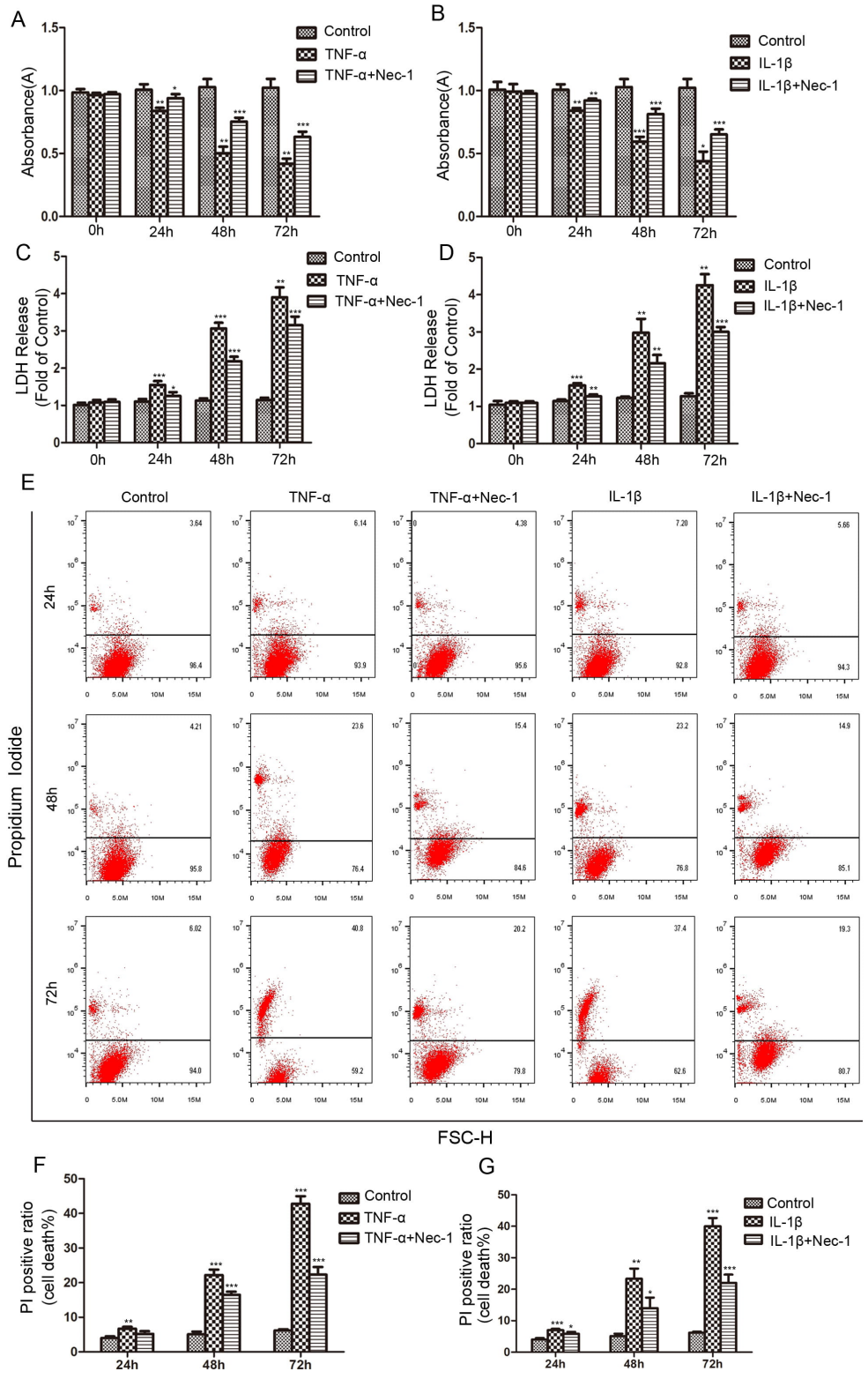

Fig. 2. The protective effects of Nec-1 (20 μM) against inflammatory factor-induced viability decreased and death in rat NP cells. (A,B) The cell viability of NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 0, 24, 48 and 72 hours was measured using the CCK-8 assay. (C,D) The cytotoxicity of NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 0, 24, 48 and 72 hours was determined by LDH release. (E–G) Representative graphs and statistical analysis of PI positive ratio by flow cytometry analysis after PI staining in NP cells. Values are expressed as mean $\pm$ SD from three independent experiments (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control).

Mix (2X) (Fermentas, Canada) on an ABI Prism 7900HT sequence detection system (Applied Biosystems, USA). The GAPDH was used as house-keeping gene (control), and relative mRNA expression levels of target genes were subjected to analysis of amplification curve, and the data were calculated using the $2^{-\triangle\triangle CT}$ method. The primer sequences used for RT-PCR were designed and synthesized as follows: RIPK1: 5′-AGGAGGAAAGGAAGCGAAGG-3′, 5′-GGTTGTGCTGGGATAAGGAAGA-3′; RIPK3: 5′-ATGTCTAAACTCTCAGCCGTA-3′, 5′-ATTGAGCCATAACTT GACAGA-3′; MLKL: 5′-TCTCCCAACATCCTGCGTAT-3′, 5′-TCCCGAGTGGTGTAACCTGTA-3′; GAPDH: 5′-CGCTAACATCAAATGGGGTG-3′, 5′-TTGCTGACAATCTTGAGGGAG-3′.

2.14 Western Blotting Analysis of the Protein Expression

Following 0, 24, 48, 72 hours treatment, the NP cells sample were lysed in a RIPA lysis buffer (Beyotime, China) containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime, China) at 4 °C for 20 minutes, sonicated for 1 minute, and centrifuged at 12000× g for 15 minutes, and then the supernatant was collected. The protein concentration was quantified using an enhanced BCA protein assay kit (Nanjing Keygen Biotech, China). Protein samples were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to the nitrocellulose membranes, which were incubated with primary antibodies overnight at 4 °C and then incubated with a horseradish peroxi-dase-conjugated secondary antibody for 2 hours according to the manufacturer's instruction. Finally, the protein was developed using the enhanced chemiluminescence (ECL) method as previously described. The primary antibodies were used as follows: RIPK1 (1:500, CST, USA), phospho-PKA substrate (1:1000, CST, USA), RIPK3 (1:500, Abcam, UK), pRIPK3 (phosphoS232, 1:1000, Abcam, UK), MLKL (1:500, Abcam, UK), GAPDH (1:5000, Abcam, UK).

2.15 Statistical Analysis

The data are expressed as the mean values ± standard deviation (SD) of at least three independent experiments. The data analysis was performed using SPSS 22.0 software package (Boao Yijie, Beijing Technology Co., Ltd, Beijing, China). Differences between groups were determined by Student's *t*-test or one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. The probability of $p < 0.05$ was considered statistically significant.

3. Results

3.1 The Activation of the RIPK1/RIPK3/MLK Pathway of NP Cells is Positively Correlated with the Inflammation Treatment Time

To investigate whether necroptosis was involved in inflammation-induced NP cells death, we first examined the expression of necroptosis-associated target molecules. The Western blot results demonstrated that the expression level of RIPK1, p-RIPK1, RIPK3, p-RIPK3, MLKL and p-MLKL, especially p-RIPK1, p-RIPK3 and p-MLKL, were all increased following 50 ng/mL TNF-α or 20 ng/mL IL-1β treatment for 24, 48 and 72 hours (Fig. 1A,B). Similarly, through the RT-PCR detection, the gene expression trend of RIPK1, RIPK3 and MLKL is highly consistent with the protein expression level following 50 ng/mL TNF-α or 20 ng/mL IL-1β treatment for 24, 48 and 72 hours (Fig. 1C,D). Also, through immunofluorescence detection, we intuitively observed that with the prolongation treatment of TNF-α or IL-1β, the expression level of necroptosis downstream core molecule p-MLKL gradually increased (Fig. 1E).

3.2 Necroptosis is Closely Involved in Inflammation-Induced NP Cells Death

To further confirm the involvement of necroptosis in inflammation-induced NP cells death, we used necroptosis specific inhibitor Nec-1 to treat NP cells under inflammatory conditions. The results of CCK-8 assays showed that Nec-1 markedly improved the decreased activity of NP cells caused by 50 ng/mL TNF-α treatment or 20 ng/mL IL-1β treatment for 24, 48 and 72 hours (Fig. 2A,B). The LDH release into the culture media from damaged cells is positively correlated with cell damage and cytotoxicity. The LDH release was increased in NP cells under 50 ng/mL TNF-α or 20 ng/mL IL-1β treatment for 24, 48 and 72 hours, which were effectively inhibited by 20 μM Nec-1 (Fig. 2C,D). Moreover, the PI positive (cell death) ratio was detected to synthetically evaluate the NP cells survival capacity. Following exposure to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 24, 48 and 72 hours, the positive rate of PI showed a clear trend of gradual increase (Fig. 2E–G). We then carried out inhibitor concentration gradient correlation detection to screen the optimal necroptosis inhibitor intervention concentration. On this basis, we selected Nec-1 (20 μM), GSK872 (5 μM) and NSA (5 μM) to perform the follow-up experiments. Supplementary material is the detailed screening process (**Supplementary Material**). As expected, Nec-1 (20 μM) can effectively inhibit the increased PI positive rate of NP cells induced by TNF-α and IL-1β (Fig. 2E–G). After systematic analysis and comparison, we chose 50 ng/mL TNF-α or 20 ng/mL IL-1β inflammatory factors treatment for 48 hours throughout the following experiments.

In order to further systematically verify that necroptosis is involved in NP cells death induced by inflammatory factors, we also introduced RIPK3 specific inhibitor GSK872 (5 μM) and MLKL specific inhibitor NSA (5 μM) into this experiment. The results displayed that Nec-1, GSK872 or NSA significantly reversed the decreased activity of NP cells under 48 hours TNF-α or IL-1β treatment, at the same time, Nec-1, GSK872 or NSA efficiently

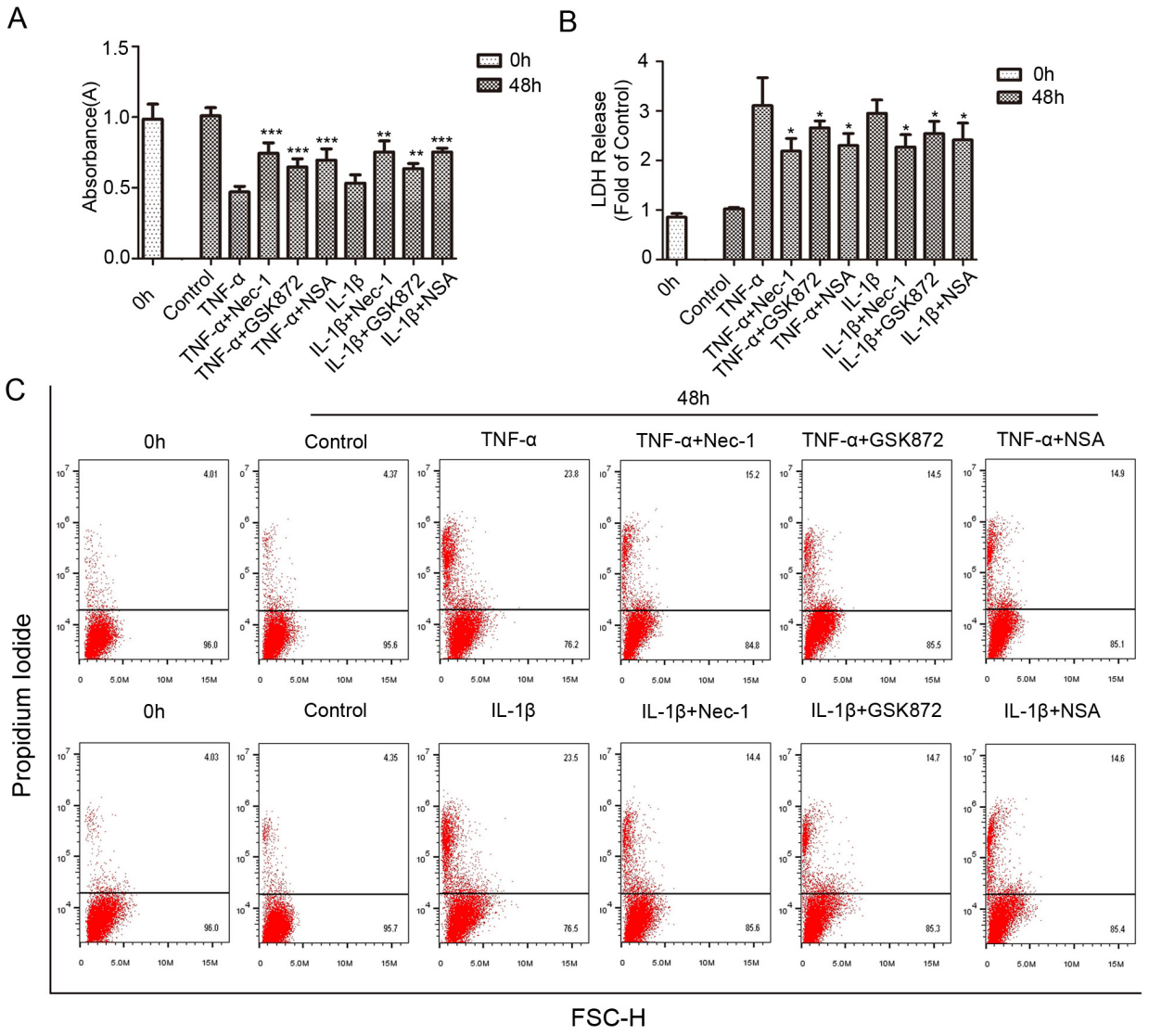

Fig. 3. The viability and death changes of rat NP cells treated with necroptosis specific inhibitors. (A) Following co-treatment with necroptosis specific inhibitor Nec-1 (20 μM), GSK872 (5 μM) or NSA (5 μM), the cell viability of NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours was measured using the CCK-8 assay. (B) The cytotoxicity of NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours was determined by LDH release. (C) Representative graphs of PI positive ratio by flow cytometry analysis after PI staining in NP cells. Values are expressed as mean $\pm$ SD from three independent experiments (*$p < 0.05$, ** $p < 0.01$, *** $p <$ 0.001 vs. control).

down-regulated the LDH release level and PI positive ratio (Fig. 3A–C). Collectively, these results indicated that RIPK1/RIPK3/MLKL-mediated necroptosis was involved in inflammation-induced NP cells death.

3.3 The Morphological Changes of NP Cells Treated with Necroptosis Specific Inhibitors

After 48 hours exposure of NP cells to TNF-α or IL-1β, the cells gradually lost their normal morphology, became round in shape, detached from the plates, and displayed morphological changes indicating of necrosis (Fig. 4A). We further observed the survival of NP cells using the Calcein-AM/PI (live/dead) cell staining. Consistently, live/dead cell staining showed that the number of dead cells (red fluorescence) markedly increased, while the number of live cells (green fluorescence) decreased after TNF-α or IL-1β treatment for 48 hours compared to that in control groups (Fig. 4B). The Nec-1, GSK872, or NSA are extremely effective in inhibiting the morphological changes of NP cells necrosis induced by inflammatory mediators (Fig. 4A,B).

3.4 The Inflammation-induced NP Cells Death is Inhibited by SiRIPK3 or SiMLKL, but Aggravated by SiRIPK1

To investigate the role of SiRIPK1, SiRIPK3 and SiMLKL in inflammation-induced NP cells death, NP cells

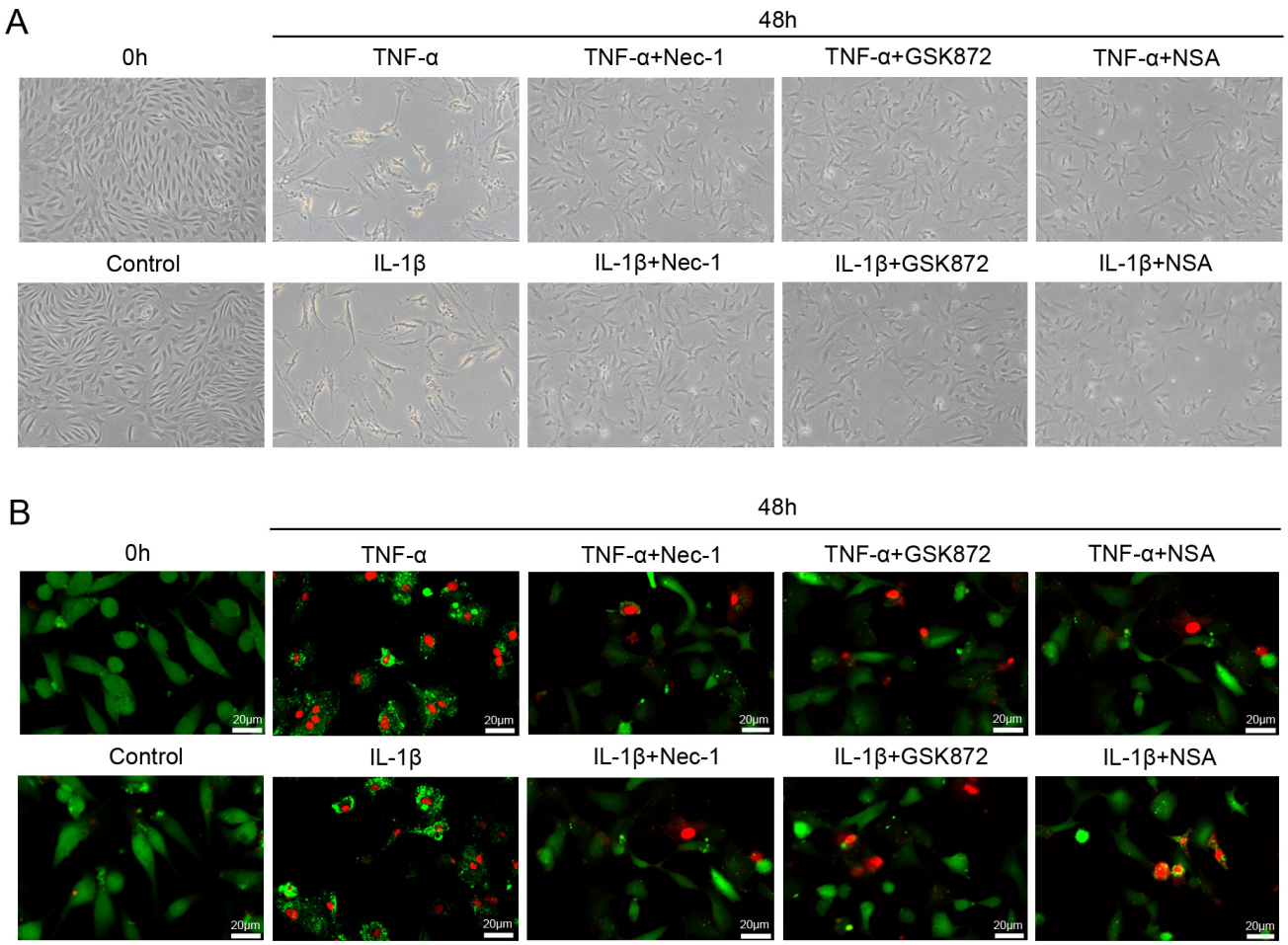

Fig. 4. The morphological changes of rat NP cells treated with necroptosis specific inhibitors. (A) The morphological changes of NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours was observed under the optical microscope. (B) Typical fluorescence photomicrograph of live/dead cell staining of NP cells. The green fluorescent signaling (Calcien-AM) indicates live cells and red fluorescent signaling (PI) indicates dead cells. Scale bars = 20 μM.

were treated with effective SiRIPK1, SiRIPK3 and SiMLKL sequences respectively according to our previous researches. The Western blot and RT-PCR results displayed that the transfected SiRNA sequences resulted in a marked decrease in protein and gene expression levels of constitutive RIPK1, RIPK3 and MLKL respectively (Fig. 5A,B,D,E,G,H). The NP cells were treated with these SiRNAs for 48 hours, prior to exposure to TNF-α or IL-1β for 48 hours. Contrary to the results presented by the aforementioned RIPK1 specific inhibitor Nec-1, SiRIPK1 down-regulated the activity of NP cells under inflammatory conditions and exacerbated NP cells death (Fig. 5C,J,K). As expected, both SiRIPK3 and SiMLKL remarkedly inhibited TNF-α or IL-1β induced NP cells death and notably upregulated cell activity (Fig. 5F,I,L,M,N,O). That is to say, the inflammation-induced NP cells death is inhibited by SiRIPK3 and SiMLKL, but aggravated by SiRIPK1. In follow-up research, we will further clarify its underlying mechanism.

3.5 The Inflammation-Induced MMP Loss and mPTP Opening in NP Cells Were Largely Reversed by Necroptosis Specific Inhibitors

The normal cells stained with JC-1 exhibited abundant red along with little green fluorescence. The JC-1 aggregates were dispersed to green fluorescence when the cells suffered from damages. Following 48 hours exposure of NP cells to TNF-α or IL-1β, MMP loss was notably observed, as indicated by the decrease in red and increase in green fluorescence, implying mitochondrial damage occured (Fig. 6A,B). When treated with Nec-1, GSK872, or NSA, the loss of MMP in NP cells induced by inflammatory factors were efficiently rescued (Fig. 6A,B). A key feature of necroptosis is the enhanced mPTP opening. The values of relative fluorescence intensity (RFI) detected by flow cytometry evidently decreased following TNF-α or IL-1β treated for 48 hours, which implyed the enhanced mPTP opening (Fig. 6C,D). In presence of Nec-1, GSK872, or NSA, the loss of fluorescence intensity was apparently alleviated, the flow cytometry and the fluorescence staining

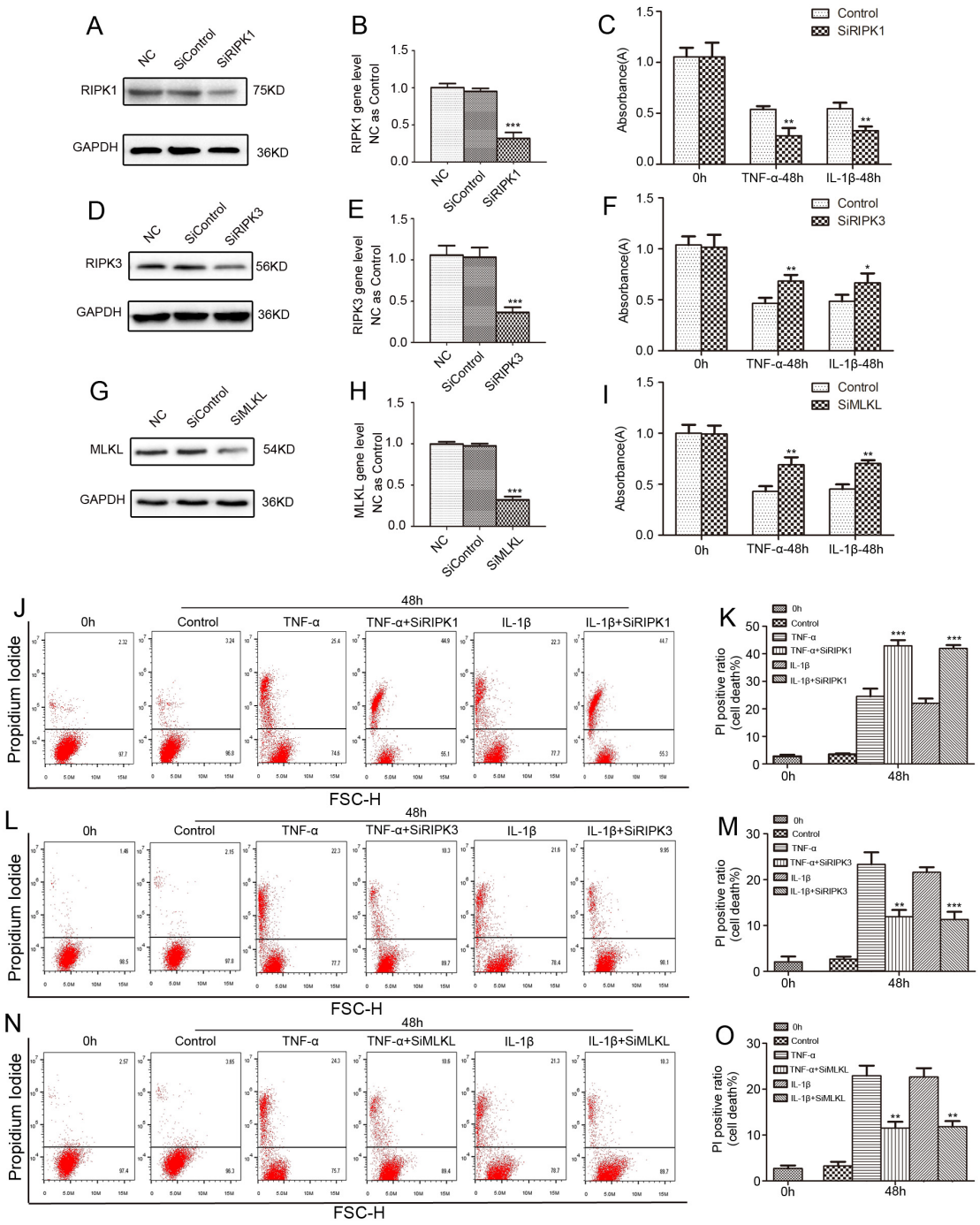

Fig. 5. The inflammation-induced rat NP cells death is inhibited by SiRIPK3 and SiMLKL, but aggravated by SiRIPK1. (A,B) The NP cells were treated with SiRIPK1 or nonspecific RNA (negative control, NC) for 48 hours, and total protein and gene expression levels were measured. (D,E) The NP cells were treated with SiRIPK3 or NC for 48 hours, and total protein and gene expression levels were measured. (G,H) The NP cells were treated with SiMLKL or NC for 48 hours, and total protein and gene expression levels were measured. The NP cells were pretreated with the selected siRNA sequence for 48 hours and then exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours. (C,F,I) Following pretreated with the selected SiRNA sequence for 48 hours, the NP cells were exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours, and cell viability of NP cells was measured using the CCK-8 assay. (J–O) Following pretreated with the selected SiRNA sequence for 48 hours, the NP cells were exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours, the representative graphs and statistical analysis of PI positive ratio by flow cytometry analysis after PI staining in NP cells. Values are expressed as mean $\pm$ SD from three independent experiments (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control).

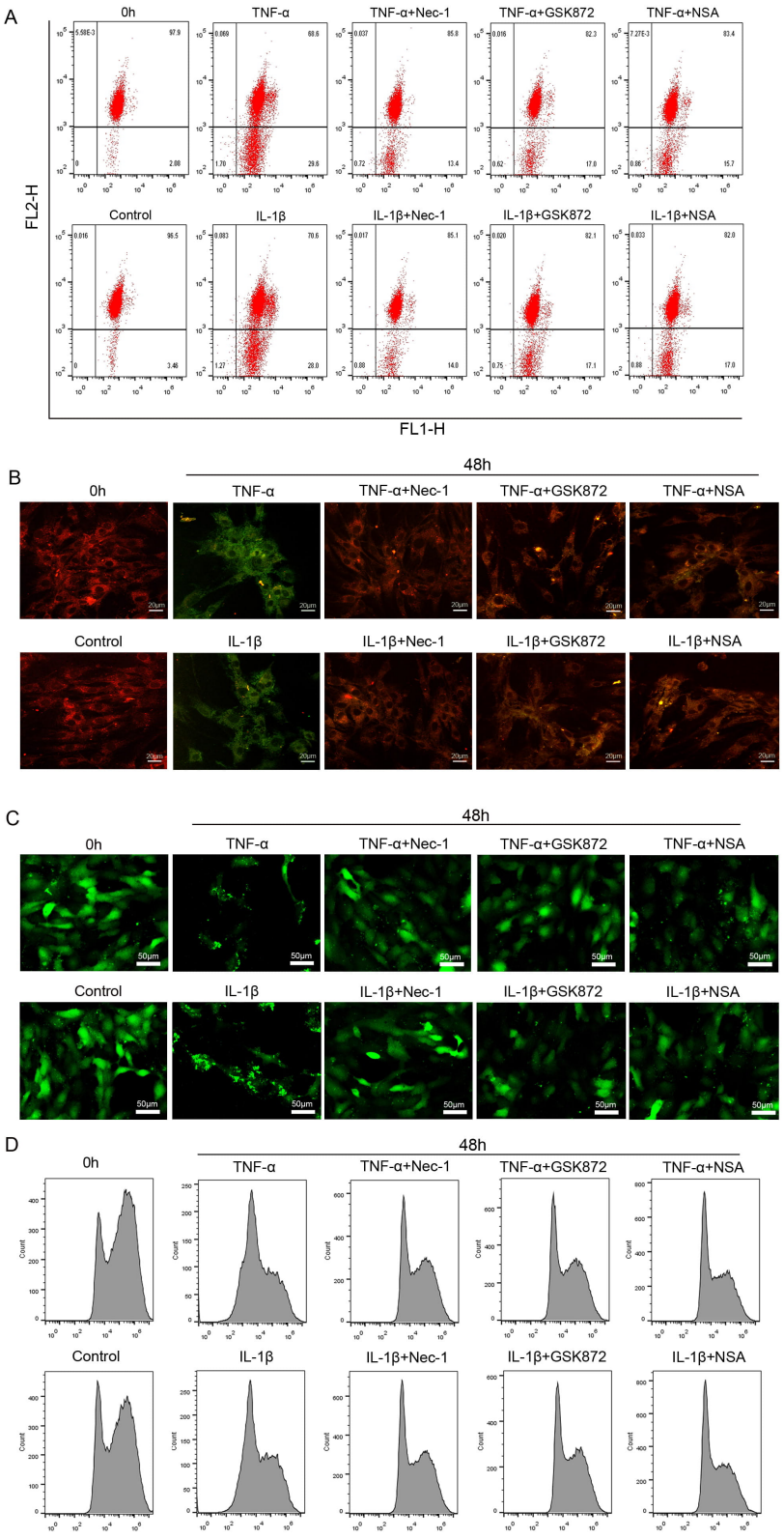

Fig. 6. The inflammation-induced MMP loss and mPTP opening in rat NP cells were largely reversed by necroptosis specific inhibitors. (A) Representative dot plot of MMP in NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours were detected by flow cytometry after JC-1 staining. (B) Typical fluorescence photomicrograph of MMP loss in NP cells by LSM. (C) Typical fluorescence photomicrograph of MPTP in NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours were observed under LSM. (D) The quantitative relative fluorescence intensity (RFI) of MPTP in NP cells by flow cytometry. Scale bars = 20 μM, 50 μM. Values are expressed as mean $\pm$ SD from four independent experiments (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control).

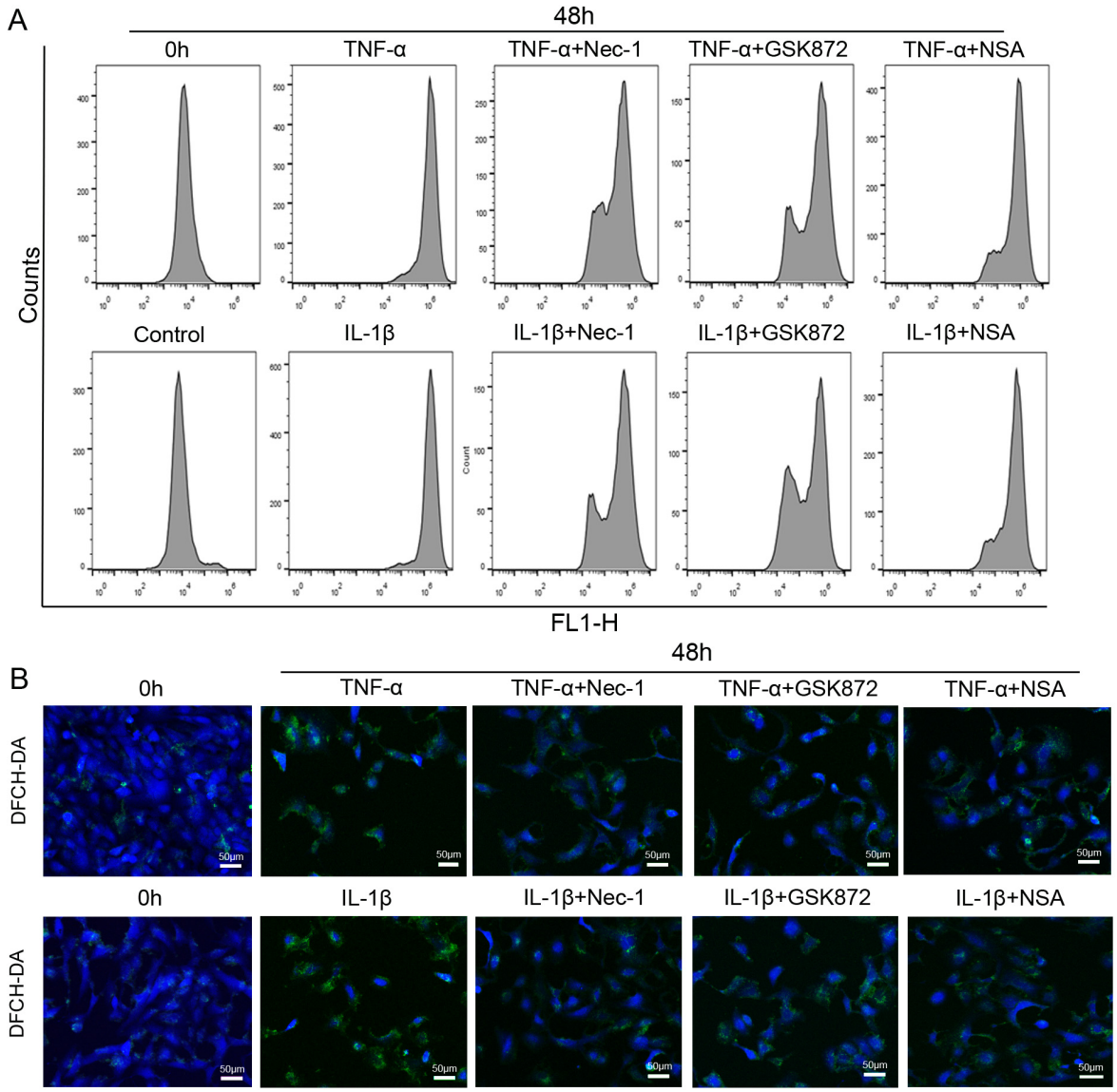

Fig. 7. The inflammation-induced oxidative stress in rat NP cells were largely reversed by necroptosis specific inhibitors. (A) Representative plots of ROS in NP cells exposed to 50 ng/mL TNF-α or 20 ng/mL IL-1β for 48 hours were detected by flow cytometry after the labeling of fluorescent probe DCFH-DA. (B) Visually observe the fluorescence intensity of ROS in NP cells through LSM. Scale bars = 50 μM.

clearly confirmed this point (Fig. 6C,D). Together, these results suggested that Nec-1, GSK872, or NSA may via inhibition mPTP opening and MMP loss to alleviate NP cells necroptosis.

3.6 The Inflammation-Induced Oxidative Stress in NP Cells Were Largely Reversed by Necroptosis Specific Inhibitors

To investigate ROS in inflammation-mediated NP cells necroptosis, ROS generation was measured after DCFH-DA staining. Compared with 0 hour, the DCF positive ratio was increased after 48 hours TNF-α or IL-1β treatment (Fig. 7A). Then we performed fluorescence detection, which was consistent with the result of flow cytometry. The fluorescence intensity of DFCH-DA markedly increased following 48 hours TNF-α or IL-1β treatment (Fig. 7B). Meanwhile, under inflammatory conditions, the ROS levels were notably attenuated when treated with Nec-1, GSK872, or NSA (Fig. 7A,B). These results suggest that the inflammation-induced oxidative stress in NP cells were largely reversed by necroptosis specific inhibitors.

4. Discussion

The IVD degeneration not only involve a large population of middle-aged and elderly people, but also show a rising trend among young people [34,35]. The conservative therapy and surgical therapy are currently used in clinical practice. Conservative therapy can alleviate clinical symptoms to a certain extent, but cannot reverse the biological

function of the degenerated IVD; while the surgical therapy represented by discectomy and fusion have problems such as the inability to restore the normal height and weight-bearing capacity of IVD, and even aggravate problems such as degeneration of adjacent segments [36,37]. Therefore, in-depth research on the precise molecular mechanism of IVD degeneration and exploring new ways to prevent and treat IVD degeneration is a major demand in the health field.

The interaction between NP cells death and inflammation plays a key role in the occurrence and development of IVD degeneration [38,39]. Necroptosis is generally considered to be a death mode that aggravates the development of inflammation. For example, in a study of TNF-α-mediated kidney inflammation and injury [40], inhibiting inflammation can restore kidney function to a large extent, which is closely related to the down-regulation inflammation can largely inhibit macrophage polarization and necroptosis of kidney tissue. In another study of cerebral ischemia-reperfusion model, microglioma cells can produce a certain concentration of TNF-α, which in turn mediates endothelial cell necroptosis and finally breaks the blood-brain barrier, aggravates brain damage, and further promotes the development of inflammation and brain tissue necrosis [41]. Contrary to the above effects, necroptosis can also limit and eliminate inflammation. For example, when inflammatory factors such as TNF-α and Fas mediate necroptosis [42], if cells do not undergo death, they can synthesize and release more inflammatory factors. At this time, inhibiting necroptosis can aggravate the synthesis and release of inflammatory factors [42]. In the process of skin damage caused by staphylococcus epidermidis infection [43], activation of RIPK1/RIPK3/MLKL signal axis-mediated necroptosis effectively inhibit the IL-1β expression in epidermal cell and excessive inflammation activation, while down-regulation of inflammation can in turn produce a certain degree of negative feedback on necroptosis, and the two work together to repair skin damage. To sum up: there is a close relationship between necroptosis and inflammation, but their regulatory effects are diversified, depending on different environments or different intervention methods.

In current study, following 50 ng/mL TNF-α treatment or 20 ng/mL IL-1β treatment for 24, 48 and 72 hours, the Western blot results demonstrated that the expression levels of RIPK1, pRIPK1, RIPK3, pRIPK3, MLKL, and pMLKL, especially pRIPK1, pRIPK3, and pMLKL, were all notably increased. The gene expression trend of RIPK1, RIPK3 and MLKL is highly consistent with the protein expression level. Besides, through immunofluorescence detection, we intuitively observed that with the prolongation treatment of TNF-α or IL-1β, the expression level of necroptosis downstream core molecule pMLKL gradually increased. In order to further systematically verify that necroptosis is involved in NP cells death induced by inflammatory factors, we also employed RIPK1 specific inhibitor Nec-1, RIPK3 specific inhibitor GSK872 and MLKL specific inhibitor NSA into current study. During the condition of TNF-α or IL-1β treatment for 48 hours, the results demonstrated that Nec-1, GSK872 or NSA could markedly reverse the decrease of NP cells activity, meanwhile, Nec-1, GSK872 or NSA efficiently reduced the inflammation-mediated LDH release and cell death ratio. Taken together, these results indicated that RIPK1/RIPK3/MLKL-mediated necroptosis was closely involved in inflammation-induced NP cells death. In view of the *in vitro* findings that inflammation-mediated necroptosis of NP cells plays a critical role in NP cells death. We speculate that in an *in vivo* animal model of inflammation-induced IVD degeneration, regulation of NP cells necroptosis is expected to largely inhibit disc degeneration process.

In order to further clarify the role of RIPK1/RIPK3/MLKL pathway in the process of inflammation-induced NP cells necroptosis, NP cells were treated with effective SiRIPK1, SiRIPK3 and SiMLKL sequences respectively. Contrary to Nec-1, SiRIPK1 treatment reduced the activity of NP cells and exacerbated NP cells death under inflammatory conditions. As expected, both SiRIPK3 and SiMLKL notably restrained TNF-α or IL-1β induced NP cells death and markedly up-regulated NP cells activity. That is to say, the inflammation-induced NP cells death is inhibited by SiRIPK3 and SiMLKL but aggravated by SiRIPK1. Due to that RIPK1 has various functions, and its different expression levels or the effects of phosphorylation at different sites vary greatly. After carefully analysis, we speculate that overexpression of RIPK1 significantly increase cell death, and moderate expression is extremely necessary to promote cell survival. We will intensify the exploration of the exact molecular mechanism of SiRIPK1 in promoting inflammation-induced NP cells death in follow-up studies.

Classically, necroptosis signaling was thought to involve mitochondrial dysfunction and increased oxidative stress level mainly originated from the mitochondria in the execution of cell death [44,45]. The close association between necroptosis and mitochondrial dysfunction is illustrated in many studies. For example, the latest literature report that SIRT3 deficiency aggravate hyperglycemia-induced mitochondrial damage, increased ROS accumulation, promote necroptosis, possibly activate the NLRP3 inflammasome, and ultimately exacerbate diabetic cardiomyopathy in the mice [46]. Mitochondrial dysfunction can greatly promote the occurrence of necroptosis involving a variety of mechanisms, including production of mitochondrial ROS [47], activation of mitochondrial phosphatase phosphoglycerate mutase family member 5 [48], or promotion the opening of MPTP [49]. However, there is still study showing that the occurrence of necroptosis does not depend on mitochondrial function damage such as increased opening of MPTP and decreased MMP [29]. The

current study demonstrates that, under inflammation treatment conditions, Nec-1, GSK872, or NSA may via inhibition mPTP opening and MMP loss to alleviate NP cells necroptosis; meanwhile, the results suggest that the TNF-α or IL-1β induced oxidative stress in NP cells were largely reversed by necroptosis specific inhibitors. The above results suggested that taken mitochondria as the target to improve mitochondrial function, which can effectively inhibit inflammation-mediated NP cells death by inhibiting mitochondrial dysfunction-mediated necroptosis.

At present, the efficacy of these inhibitors are mainly reflected by cell experiments, and there are few studies focus on *in vivo* animal experiments to verify the effects of these drugs. In animal model studies and clinical studies related to IVD degeneration, there is no report about that necroptosis inhibitors can directly improve IVD degeneration. This is the key content that our research group is currently working on. In our study, several limitations need to be pointed out. First, no *in vivo* experiments were conducted. The drug concentrations of related inhibitors screened by cell experiments are difficult to apply to animal models. Hence, we intend to investigate whether inflammatory stimulation mediates NP cells necroptosis through mitochondrial function disfunction and oxidative stress pathway *in vivo* in subsequent studies. Only in this way can we better translate to animal models and clinical research. Second, the disc microenvironment is intricate in nature. In addition to biomechanical loading, low nutrient levels, hypoxia, high aciditye, high osmolarity, etc., also play crucial role in NP cells death and IVD degeneration. We are currently considering a composite model to better model the actual microenvironment of IVD degeneration. Therefore, *in vivo* animal experiments need to be strengthened in future research.

5. Conclusions

In conclusion, this study confirmed that mitochondrial dysfunction and oxidative stress act as a crucial role in NP cells necroptosis under inflammation condition. This finding introduces a new perspective to inhibition NP cells death, and it is extremely expected to offer a more efficient strategy of delaying or even retarding IVD degeneration.

Author Contributions

CC, ZMS and JZ designed the research. CC, ZMS, ZDL and SFC performed the experiments. CC, MZ and ZYM acquired and analyzed the data. CC, YHG and SLC contributed to writing of the manuscript. Finally, all authors have reviewed and approved the final submitted manuscript. The integrity of this work is guaranteed by CC and JZ.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Animal Care and Ethics Committee of Zhengzhou University (Ethic approval code is AF/SC-08/04.0).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2704111.

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