Retinoic acid inhibits the pyroptosis of degenerated nucleus pulposus cells by activating Sirt1-SOD2 signaling

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ABSTRACT

Aim: Intervertebral disc (IVD) degeneration is a common disease initiated by the degeneration of the nucleus pulposus (NP). The pyroptosis of degenerated NP cells (dNPCs) plays an important role in NP degeneration. The purpose of this study is to identify a feasible solution that can inhibit NP cell pyroptosis to therapy the degeneration of the intervertebral disc.

Methods: Cell viability and proliferation were quantified by Cell Counting Kit-8 assay. The measurement of cellular reactive oxygen species (ROS) was detected by 2,7-Dichlorodihydrofluorescein diacetate. The death of cells was analyzed by the Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) method of fluorescence analysis. The pyroptosis of cells was assessed by flow cytometry analyses. The contents of sulfate glycosaminoglycans were detected by a blysacan assay kit.

Result: In this study, we determined the effects of retinoic acid (RA) on dNPCs and investigated the underlying mechanism of RA-mediated pyroptosis in dNPCs. We also verified the effects of RA on IVD degeneration in vivo. Our results demonstrated that RA significantly increased the proliferation and the protein expression of sox9, aggrecan, and collagen II of dNPCs. Pyroptosis-related proteins and the pyroptosis rate of dNPCs were significantly decreased by RA. We found that Sirt1-SOD2 signaling was activated, while ROS generation and TXNIP/NLRP3 signaling in dNPCs were inhibited after the addition of RA. Furthermore, RA also recovered the structure of NP and increased the contents of sulfated glycosaminoglycans and collagen in vivo.

Conclusion: Our study demonstrated that RA could inhibit the pyroptosis and increase the extracellular matrix synthesis function of dNPCs and verified that RA has a protective effect on IVD degeneration.

Introduction

Intervertebral disc (IVD) degeneration is a common disease characterized by low back pain, and causes a large economic burden. The degeneration of the nucleus pulposus (NP) which is surrounded by annulus fibrosus and cartilaginous endplates is thought to be the origin of IVD degeneration. NP cells (NPCs) are located in NP and can synthesize glycosaminoglycan (GAG) and collagen II in normal conditions. Once the viability and function of NPCs are disturbed, the extracellular matrix (ECM) produced by NPCs would alter and lead to NP degeneration.

Biological therapies targeted at NPCs can impact the future management of NP degeneration. Growth factors can control the proliferation and function of NPCs, and maintain NP homeostasis. Many growth factors such as growth differentiation factor 5, bone morphogenetic protein-2 (BMP-2), and fibroblast growth factor (FGF) have been used in enhancing the proliferation and ECM synthesis of NPCs. Drugs are also used in promoting the viability and function of NPCs. Wogonin can ameliorate the inflammation in NPCs and mitigates the progression of IVD. Liraglutide can protect NPCs against high-glucose-induced apoptosis. Small molecules are usually used to regulate the activity of signaling pathways and can also regulate the function and activities of NPCs. Because of their high efficiency and selectivity, they are now widely used in regulating cell activities. Retinoic acid (RA) is a potent immunomodulator and can inhibit the inflammatory response and regulate cell activities in many diseases, such as cardiac injury, macular degeneration, and even osteoarthritis. However, the effects of RA in the treatment of NP degeneration have never been studied.
Many factors have been found to regulate the viability and function of NPCs, such as nutrients, aging, and oxygen concentration\textsuperscript{15}. In addition, inflammation also plays an important role in inducing NP degeneration\textsuperscript{16}. Apoptosis is the most studied form of inflammatory death in NPCs. Apoptosis of NPCs leads to down-regulation of ECM synthesis and cell number, then furtherly aggravates NP degeneration\textsuperscript{17}. However, other forms of inflammatory death can also regulate the viability and function of NPCs and lead to NP degeneration. Pyroptosis is a novel form of inflammatory death of the cell and attracts more and more attention.

Pyroptosis is an inflammatory form of regulated cell death, and unlike apoptosis or necrosis, is always driven by caspase-1\textsuperscript{18}. Canonical pyroptosis is initiated after the liberation of the N-terminal domain of pore-forming protein gasdermin D (GSDMD). NT-GSDMD cleaved by active caspase-1 can also lead to the release of IL-1β and IL-18, and then generated an inflammasome-associated inflammatory response\textsuperscript{19}. Pyroptosis is related to different diseases, such as cardiac injury and diabetes mellitus\textsuperscript{20,21}. Pyroptosis can also influence the ECM synthesis function of chondrocytes\textsuperscript{22}. A recent study has demonstrated that pyroptosis also exists in NP, and plays an important role in decreasing cell viability and ECM synthesis function of NPCs\textsuperscript{23}. Therefore, discovering a new approach to inhibiting pyroptosis of NPCs may be a benefit for the treatment of IVD degeneration.

Our study aims to clarify the effects of RA in inhibiting pyroptosis and promoting the ECM synthesis function of NPCs. The underlying mechanisms of RA in mediating pyroptosis of NPCs were also been investigated. In addition, we verified the potential use of RA in preventing IVD degeneration in a rat injury-induced degeneration model. We hope our study will provide a new strategy for future IVD treatment.

Materials and methods

Reagents

RA, Ex 527, and Nigericin sodium salt (NS) were purchased from Selleck chem (Shanghai, China). Type II collagenase and hyaluronidase were purchased from Gibco (Shanghai, China). The cell counting kit-8 (CCK8) was bought from Dojindo (Dalian, China). The lactate dehydrogenase (LDH) assay kit and the hydroxyproline assay kit were bought from Jiancheng Biotechnology Institute (Nanjing, China). The in situ cell death detection kit was purchased from Roche Life Science (Shanghai, China). DCFH-DA used for the detection of cellular ROS was purchased from Sigma Aldrich (Shanghai, China). The FAM FLICA Caspase-1 kit was bought from Bio-rad (Shanghai, China). Primary antibodies against Sox9, aggrecan, collagen II, cleaved caspase-1, NT-GSDMD, IL-18, SIRT1, TXNIP, NLRP3 and GAPDH were purchased from Abcam (Shanghai, China). Primary antibodies against IL-1β, SOD2 were purchased from Cell Signaling Technology (Shanghai, China). The RNAsiso reagent, PrimeScript™ RT Master Mix and TB Green Premix Ex Taq were purchased from TaKaRa (Shanghai, China). The Blyscan assay kit was bought from Biocolor (Beijing, China).

Isolation and culture of NPCs

Fifteen 40- to 50-year-old patients suffer IVD degeneration according to the results of magnetic resonance imaging donated degenerative NP samples and nucleotomy and intervertebral fusion surgery were performed. The Pfirrmann classification of the degenerative NP samples was at most grade III\textsuperscript{24}. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine, and informed consent was obtained from all the patients involved in our study. The isolation of human NPCs was described previously\textsuperscript{25}. Firstly, the degenerative NP samples were washed three times with phosphate-buffered saline (PBS) and cut into small pieces. Then, the pieces were enzymatically dissociated by 0.2% collagenase II and 2 U/mL hyaluronidases for 4 hours at 37 °C with gentle shaking. Next, the tissues were centrifuged at 1000 rpm for 5 min to isolate cells. Human healthy NPCs (hNPCs) of passage 1 were bought from Procell Life Science&Technology (Hubei, China) and showed a fusiform phenotype. Human degenerative NPCs (dNPCs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine in a humidified incubator at 37 °C with 5% CO\textsubscript{2}. The complete medium was changed every two days. dNPCs of passage 2 were used for subsequent experiments.

Detection of cell viability and cytotoxicity

Cell viability and proliferation of each were measured by CCK8. Cells were firstly seeded into a 96-well plate, then treated with 10% CCK8 in 100 μL DMEM- High Glucose according to the manufacturer’s protocols. Absorbance values were taken at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The cytotoxicity of RA in each group was determined
after 0, 7, and 14 days of cultivation. The supernatant of each was collected and detected using an LDH assay kit following the manufacturer’s protocols. Absorbance values at 450 nm were detected using a microplate reader (Bio-Rad).

**Fluorescence analysis**

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used for measuring the death of NPCs. First, each group of NPCs was fixed with 4% paraformaldehyde for 10 min at room temperature. Then, the cells were incubated with 3% H2O2 and 0.1% Triton X-100 for 10 min at room temperature and washed three times with PBS. Next, an in situ cell death detection kit was used to stain the death of cells according to the manufacturer’s instructions. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei of cells. After staining, cells were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

**Total RNA isolation and real-time quantitative PCR (RT-qPCR)**

The total RNA of each group was extracted using an RNAiso reagent, and quantified by measuring the absorbance at 260 nm/280 nm. PrimeScript™ RT Master Mix was used in reverse transcription and TB Green Premix Ex Taq was used in RT-qPCR. The procedures of RT-qPCR were performed on an ABI StepOnePlus System (Applied Biosystems, Warrington, UK). 18s was used as a housekeeping gene, and the data were analyzed using the 2^(-ΔΔCT) method. All primer sequences were synthesized by Sangon Biotech (Table 1).

**Western blotting analyses**

Total protein was extracted from each group of cells by RIPA buffer supplemented with a proteasome inhibitor (Beyotime, Shanghai, China). Protein concentrations were tested by a BCA protein assay kit (Beyotime) and equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Shanghai, China) by electroblotting. All membrane was blocked for 2 hours at room temperature in 5% non-fat milk and incubated with primary antibodies overnight at 4 °C. Then, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (Beyotime) for 1 hour at room temperature. The blots were visualized using an enhanced chemiluminescence substrate (Millipore) and imaged using the Bio-Rad XRS chemiluminescence detection system (Bio-Rad).

**Flow cytometry analyses**

The pyroptosis of cells was assessed by flow cytometry analyses. The FAM FLICA™ Caspase-1 kit was used according to the manufacturer’s protocols and pyroptotic death of cells was detected by flow cytometry. PI (+) and caspase-1 FLICA (+) was defined as pyroptosis.

**Microarray analysis**

We performed ceRNA microarray and mRNA expression profile was included in our study. After the extraction and quantification of total RNA in NPCs, the total RNA was purified with a QIAGEN RNeasy Kit (QIAGEN, Shanghai, China), amplified and labeled with Cy-3. After RNA was hybridized at 65 °C for 17 h, array images were acquired using Agilent Scanner G5761A (Agilent Technologies) and analyzed using Agilent Feature Extraction software (version 12.0.1.1). Quantile normalization and subsequent data processing were performed by using the GeneSpring v14.8 software package (Agilent Technologies). After that, mRNAs that at least four out of the eight samples have flags in Detected were chosen for further data analysis. Differentially expressed mRNAs were screened by a statistical significance of \( p < 0.01 \). The biological functions of the differentially expressed mRNAs were indicated by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.

### Table 1. Primers used in quantitative RT-PCR.

| Gene   | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|--------|--------------------------|---------------------------|
| 18s    | GAATTCGCTAGATGCGGCTCTATA | CGAGGGGCTTCTACAACAACTC    |
| MMP1   | AAATTTACGGCGGATTTGCC     | GGTGTTGACATTACCTCGAGGTTG  |
| MMP3   | TGATGCGCTGGGAATGTC       | TCCATGAGCAACAGGAAGAAG     |
| ADAMTS4| CTGCTGCTTTAGGCTTGC      | CCACAGGGCTGATTCAG      |
| ACAN   | CTAGCTGCTTTAGCCAAGGAAGCG | AGATTGACGCGAGTACAGAAG     |
| SOX9   | AGGAGAAGCCTGACACCAGTACC  | GGGGCTCCTCTTCGCTCCCTCA    |
| COL2A1 | CTGGTGGGAGGAGCAAAGGC     | GGGGACAGTAGGGGAAAG        |
| SIRT1  | TAGCCCTTGTGATATAAGGAAGGA | ACAGCTTCAGCTCAACTTTTGA   |
Measurement of cellular ROS

Before the measurement of cellular ROS, each group of cells was cultured in 24-well plates. During the measurement, cells were rinsed and incubated with 5 μM DCFH-DA in the dark at 37 °C for 30 minutes. A fluorescence microscope (DM5500; Leica) was used to detect cellular fluorescence.

Animal experiments

Male Sprague-Dawley\textsuperscript{1} rats (200 g) were obtained from the Animal Center of the Academy of Medical Science of Zhejiang Province. All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the Institutional Animal Care and Use Committee of Zhejiang University. All SD rats were anesthetized with 1% sodium pentobarbital (Sigma Aldrich), and rat tail disc injury-induced degeneration model was fabricated by needle puncture of a 20-G sterile needle in the disc of coccygeal vertebrae\textsuperscript{26}\text/-Co8 and Co8/Co9\textsuperscript{27}. Two weeks after that, 3 μL of PBS or RA (1 μM) were injected using a microsyringe with a 31-G needle. The rats without needle puncture and injection were set as the normal group, the rats with needle puncture and PBS injection were set as the negative control (NC) group, and the rats with needle puncture and RA injection were set as the RA group. The follow-up experiments were conducted 12 weeks after injection.

Histological and biochemical analysis

The IVD tissues of SD rats were collected after sacrifice. Firstly, all tissues were fixed with 4% paraformaldehyde for 48 hours. Next, all tissues were immersed in a decalcifying solution and embedded in paraffin. The tissues were sectioned at a thickness of 5 μm using a microtome. For histological analysis, consecutive tissue sections were stained with hematoxylin and eosin (H&E) and Safranin O-fast green. Cellularity and morphology of the IVDs were then assessed and represented by histological scores according to a previously described grading scale\textsuperscript{28}. For biochemical analysis, the Blyscan assay kit was used to detect the contents of sulfate glycosaminoglycans (sGAG), and the hydroxyproline assay kit was used to detect the contents of collagen. The results were normalized to the dry weight of NP.

Statistical analysis

All experiments were repeated three times and data were presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA) and Statistical significance was determined using a two-tailed Student’s t-test and one-way ANOVA following Tukey’s post hoc test. A value of $p < 0.05$ was considered to indicate statistical significance.

Ethics approval and consent to participate

The study (No. 2016–124) was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine, and informed consent was obtained from all the patients involved in our study.

Results

RA increased the viability of dNpcs

Different concentrations of RA were added to dNPCs. After 72 hours of treatment, 1 and 10 μM RA significantly increased the viability of dNPCs, and 0.1 μM RA showed no significant influence on the viability of dNPCs (Figure 1A). The cytotoxicity of RA was also detected by measuring LDH release. 0.1, 1, and 10 μM RA had no cytotoxicity on dNPCs on days 0 and 7. However, 10 μM RA significantly increased the LDH release of dNPCs after 14 days of treatment (Figure 1B). Therefore, we have chosen 1 μM of RA to treat dNPCs in subsequent experiments.

Appropriate concentration of RA decreased the death and increased the ECM synthesis function of dNpcs

Fluorescence results showed that the death of hNPCs was the lowest, and RA significantly decreased the death of dNPCs (Figure 2A,B). The proliferation of hNPCs was significantly higher than that of the dNPCs on days 7 and 14. In addition, the proliferation of dNPCs was significantly increased by RA on days 7 and 14 (Figure 2C). ECM synthesis-related gene markers were measured after 7 days of treatment. hNPCs had lower expression of MMP1, MMP3, and ADAMTS4, and higher expression of SOX9, ACAN, and COL2a1 compared to dNPCs. MMP1 (0.31-fold), MMP3 (0.12-fold), and ADAMTS4 (0.09-fold) were significantly decreased by RA, while SOX9 (15.21-fold) , ACAN (10.34-fold), and COL2a1 (8.01-fold) were significantly increased by RA. However, RA showed no effects on hNPCs (Figure 2D,E). The protein expression levels of sox9, aggrecan, and collagen II in dNPCs were also significantly increased by RA (Figure 2F,G).
Pyroptosis of dNpcs was inhibited by RA

The protein expression levels of cleaved Caspase-1, NT-GSDMD, IL-1β, and IL-18 reflected the pyroptosis of NPCs. Our results showed that the protein expression levels of cleaved Caspase-1, NT-GSDMD, IL-1β, and IL-18 were significantly increased in dNpcs compared to those of the hNPCs. After the addition of RA, the expression of cleaved Caspase-1, NT-GSDMD, IL-1β, and IL-18 in hNPCs were not significantly changed. However, the expression of cleaved Caspase-1, NT-GSDMD, IL-1β, and IL-18 in dNpcs were significantly decreased after the addition of RA (Figure 3A,B). We also detected changes in caspase-1 in NPCs by flow cytometry to investigate the role of pyroptosis. The pyroptosis rate in the dNPC group was markedly higher and caspase-1 activity was markedly increased compared with the hNPC group. RA had no significant influence on the pyroptosis rate of hNPCs, while RA significantly decreased the pyroptosis rate and caspase-1 activity of dNpcs (Figure 3C,D).

Sirt1 was involved in RA mediated pyroptosis

mRNA microarray analysis was performed to search differentially expressed mRNA and signaling pathways between dNpcs and RA-treated dNpcs. Only mRNAs with a mean fold change>5 or<0.2 and a p-value<0.01 were selected for further analysis. 5972 genes were differentially regulated and differentially expressed 27 mRNAs with the smallest p value (p < 1 × 10⁻⁷) between the two groups were presented by heatmap (Figure 4A). Col2A1, Sirt1 and other 10 mRNAs were significantly increased in the RA group, and 17 mRNAs were significantly decreased in the RA group compared with the dNPC group. We also performed GO and KEGG enrichments and found that the GO terms with the most significant p values were related to ECM disassembly, collagen catabolic process, and positive regulation of NLRP3 inflammasome complex assembly, and the signaling pathways with the most significant p values were related to ECM-receptor interaction, Focal adhesion, and FoxO signaling pathway (Figure 4B,C). We further evaluated the expression levels of Sirt1 between the two groups by RT-qPCR. Our results showed that the expression level of Sirt1 in the RA group was almost 20.31-fold higher compared with that in the dNPC group (Figure 4D).

RA inhibited pyroptosis of dNpcs by activating Sirt1-SOD2 pathway

We further investigated the underlying mechanism of RA in inhibiting pyroptosis. We measured the protein expression levels of SIRT1, SOD2, TXNIP, and NLRP3. Our results showed that after the addition of RA, the expression levels of SIRT1 and SOD2 were increased while the expression levels of TXNIP and NLRP3 were decreased. Ex 527 is a specific inhibitor of SIRT1. After the addition of Ex527, the expression levels of SIRT1 and SOD2 were decreased, while the expression levels of TXNIP and NLRP3 were increased compared with the RA+EX-NS- group. NS is a specific inhibitor of NLRP3. The expression levels of SIRT1, SOD2, and TXNIP between the RA+EX-NS- and RA+EX-NS+ groups showed no significant difference, while the expression of NLRP3 was significantly higher in the RA+EX-NS+ group than in the RA+EX-NS- group (Figure 5A,B). The pyroptosis rate in the dNPC group was significantly higher compared with that in the dNPC+RA group. The pyroptosis rate of the dNPC+RA+EX group was higher than the dNPC+RA group.
and lower than the dNPC group. The dNPC+RA+NS group showed the highest pyroptosis rate and caspase-1 activity of the four groups (Figure 5C,D). The ROS generation of the RA-EX-NS- and RA+EX+NS- groups showed no significant difference, but both of these two groups had higher ROS generation than the RA+EX-NS- and RA+EX-NS+ groups (Figure 5E). The dNPC+RA group showed the highest mRNA expression levels of SOX9, ACAN, and COL2a1 in the four groups, while the dNPC+RA+EX group was the second highest. The dNPC+RA+NS group expressed the lowest mRNA levels of SOX9, ACAN, and COL2a1 in the four groups (Figure 5F).

**RA repair degenerated IVDs**

We performed H&E and Safranine O-fast green staining to show the morphology, cellularity, and ECM of the IVD. The normal group showed well-organized collagen lamellae in the AF and round NP with normal cellularity and rich ECM. The morphology of AF in the NC group was inward bulging and the NP was herniated. The cells and ECM of the NP in the NC group were severely decreased. The morphology and cellularity of AF and NP in the RA group were better compared with those in the NC group, but worse than in the normal group (Figure 6A). The histological score in the normal group was the lowest (5.2 ± 0.45). The
RA group (8.2 ± 1.3) had a lower histological score than the NC group (12.0 ± 0.71) (Figure 6B). The contents of sGAG and hydroxyproline in the normal group were significantly higher than those in the other groups. The RA group showed higher contents of sGAG and hydroxyproline than those of the NC group (Figure 6C,D).

Discussion
The dysfunction and death of NPCs lead to NP degeneration, and subsequently IVD degeneration. Pyroptosis is a new inflammatory form of programmed cell death and played an important role in regulating the ECM synthesis function and viability of NPCs. RA can inhibit the inflammatory response and regulate cell viability, so it had been used in treating many diseases. However, the effectiveness of RA in treating IVD degeneration has not been studied, and the effects of RA in the pyroptosis of NPCs were also unknown. In this study, we aimed to clarify the effects of RA in treating NP degeneration and inhibiting pyroptosis of NPCs. We also investigated the mechanisms of RA-regulated pyroptosis in NPCs.

We first evaluated the effects of different concentrations of RA on the viability and cytotoxicity of dNPCs. 1 and 10 μM of RA could promote the viability of dNPCs, but 10 μM of RA also showed cytotoxicity on dNPCs after 14 days of cultivation. Therefore, we think 1 μM of RA is appropriate in treating dNPCs, and this concentration was used in the subsequent experiments. The effects of RA on ECM synthesis are controversial. Some studies reported that RA induces matrix loss in chondrocytes by the activation of Wnt/β-catenin pathways. However, other studies also reported the
activation of RA receptors enhanced aggrecan production in chondrocytes. We think the effects of RA on ECM synthesis are complicated, for RA both regulates the anabolism and catabolism of ECM. Which role of RA play depends on the cells and environment. In our study, we found that RA can increase ECM synthesis in dNPCs. We think it is mainly because that RA inhibited the catabolism of dNPCs by downregulating the expression of MMP1, MMP3, and ADAMTS4, and increased cell number by promoting cell proliferation and protecting dNPCs from death.

Both pyroptosis and apoptosis are existed in NPCs and regulate the function and viability of NPCs. Pyroptosis was involved in the degeneration process of NP. So inhibiting pyroptosis is important in the treatment of IVD degeneration. In this study, we found that RA significantly inhibited the expression of cleaved caspase-1, NT-GSDMD, IL-1β and IL-18, and decreased the pyroptosis of dNPCs. It means that RA has the potential in treating IVD degeneration. We further discovered the mechanisms of RA in regulating pyroptosis of dNPCs. The results of microarray analysis showed that Sirt1 was differentially expressed between dNPCs and RA-treatment dNPCs. Sirt1 was important in regulating inflammatory reactions of the cell and was positive for the activity of NPCs. Sirt1 can inhibit apoptosis in NPCs via ERK signaling pathway. Sirt1-overexpressed chondrocytes can also delay the degeneration of NPCs during co-culture. In our study, we found that RA could increase the expression of Sirt1. After the addition of Sirt1 specific inhibitor, pyroptosis of dNPCs increased and the ECM synthesis function of...
NPCs was weakened. These results further demonstrated that Sirt1 induced by RA positively regulates the function of dNPCs. In addition, Sirt1 can also inhibit the pyroptosis of dNPCs.

Sirt1 is an important mediator in FoxO signaling and regulates the mitochondrial oxidative stress of cells. Our results of the KEGG enrichment analysis also demonstrated the FoxO signaling pathway involved in RA-regulated pyroptosis in dNPCs. SOD2 is also involved in FoxO signaling and is the downstream regulator of Sirt1. Sirt1-SOD2 signaling mediates oxidative stress and ROS generation in different cell types. We measured the expression of SOD2 and cellular generation of ROS. The results demonstrated that Sirt1 activates SOD2 and reduced ROS generation in dNPCs. TXNIP/NLRP3 inflammasome signaling is the key activator in pyroptosis. Several studies have reported that ROS can activate TXNIP/NLRP3 signaling and induce pyroptosis. We also found that RA inhibited the expression of TXNIP and NLRP3. In addition, The NLRP3 inhibitor reversed the inhibition effects of RA in pyroptosis. Therefore, we consider that RA inhibits pyroptosis in dNPCs by activating Sirt1-SOD2 signaling and thereby inhibiting ROS/TXNIP/NLRP3 signaling.
We also explored the in vivo effects of RA in a rat tail injury-induced disc degeneration model. The structure of IVD was abnormal, and the contents of sGAG and collagen were decreased in the degenerative NP. RA increased the contents of sGAG and collagen, and recovered the structure of NP, indicating that RA has protective effects of IVD degeneration. However, there were still differences between the normal and RA-treated IVD, so it is difficult to consider that RA has regenerative effects in degenerative NP. RA inhibits the pyroptosis and enhances the ECM synthesis function of dNPCs in degenerative NP. However, the degeneration of IVD was induced by nutrition, mechanical loading, inflammation and so on. We think that it is the complicated factors in IVD degeneration that leads to the limited effects of RA in degenerated IVD. There is also a limitation in our study, for we did not measure the pyroptosis in the injury-induced disc degeneration model. We demonstrated the effects of RA in inhibiting pyroptosis of dNPCs, and discovered the underlying mechanisms. Our in vivo experiments also demonstrated the protective effects of RA in degenerated NP.

Figure 6. RA repaired degenerated IVDs. (A) Representative H&E and Safranin O staining of discs at 12 weeks after injection were observed. (B) Histological grade of each group was evaluated. (C) the content of sGAG in each group at 12 weeks after injection were quantified by Blyscan assay. (D) the contents of collagen in each group at 12 weeks after injection were quantified by hydroxyproline assay. Normal: the rats without needle puncture and injection; NC: the rats with needle puncture and PBS injection; RA: the rats with needle puncture and RA injection. Data represent mean ± SEM; **p < 0.01.
However, furthermore, studies should be performed before the clinical treatment of IVD degeneration by RA.

Conclusions

We demonstrated that RA has the ability to inhibit pyroptosis and increase the ECM synthesis function of dNPCs. The inhibition of pyroptosis in dNPCs induced by RA was activated by Sirt1-SOD2 signaling. The activation of Sirt1-SOD2 signaling decreased the ROS generation and then inhibited the TXNIP/NLRP3 signaling, which led to the decrease in pyroptosis. We furtherly verified the protective effects of RA on degenerated IVD in an injury-induced degeneration model. Our findings identified pyroptosis in dNPCs as a novel target for NP degeneration and provided a candidate therapy for the treatment of IVD degeneration.

Disclaimers

The views expressed in the submitted article are our own and not an official position of the institution or funder

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Authors’ contributions

Peng-Fei Li, Fei Xiong, Ying Yin contributed equally to this work by conducting the experiment and were major contributors in writing the manuscript. Shaojun Hu and Hongyuan Xing collected and analyzed the data and arranged the article format. Zhang Ning, the corresponding author of the article, was responsible for the review of the article and the guidance of the experimental ideas

Ethics approval and consent to participate

The procedures used in this study adhere to the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine, and informed consent was obtained from individual participants involved in our study.

Consent for publication

All authors consent for publication

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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