

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

Peng-Fei Li

Zhejiang University School of Medicine

Fei Xiong

Zhejiang University School of Medicine

Ying Yin

Zhejiang University School of Medicine

Hong-Yuan Xing

Zhejiang University School of Medicine

Shao-Jun Hu

Zhejiang University School of Medicine

Ning Zhang (Z zhangning@zju.edu.cn)

Zhejiang University School of Medicine

Research

Keywords: Intervertebral disc degeneration, pyroptosis, retinoic acid, Sirt1-SOD2 signaling, ROS/TXNIP/NLRP3 signaling

Posted Date: August 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-786450/v1

License: (c) This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract Background:

Intervertebral disc (IVD) degeneration is a common disease and initiated by the degeneration of nucleus pulposus (NP). The pyroptosis of degenerated NP cells (dNPCs) plays an important role in NP degeneration and may be a potential target in the treatment of IVD 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.

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 such as cleaved caspase-1, NT-GSDMD, IL-1 β , IL-18, and the pyroptosis rate of dNPCs was significantly decreased by RA. We also found that Sirt1-SOD2 signaling was activated, while ROS generation and TXNIP/NLRP3 signaling in dNPCs was inhibited after the addition of RA. Furthermore, RA also recovered the structure of NP and increased the contents of sGAG and collagen *in vivo*.

Conclusion

Our study demonstrated that RA can inhibit the pyroptosis and increase the ECM synthesis function of dNPCs and verified that RA has a protective effect in IVD degeneration.

Introduction

Intervertebral disc (IVD) degeneration is a common disease characterized by low back pain, and cause a large economic burden (1, 2). The degeneration of nucleus pulposus (NP) which is surrounded by annulus fibrosus and cartilaginous endplates is thought to be the origin of IVD degeneration (3). NP cells (NPCs) located in NP and can synthesis glycosaminoglycan (GAG) and collagen II in normal condition. Once the viability and function of NPCs are disturbed, the extracellular matrix (ECM) produced by NPCs would alter and lead to NP degeneration (4).

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 (5). Many growth factors such as growth differentiation factor 5 (6), bone morphogenetic protein-2 (BMP-2) (7), and fibroblast growth factor (FGF) (8) 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 (9). Liraglutide can protects NPCs against high-glucose induced apoptosis (10). 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 inflammatory response and regulate cell activities in many diseases, such as cardiac injury (11), macular degeneration (12) and even osteoarthritis (13). 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 nutrient, aging, and oxygen concentration (14). In addition, inflammation also plays an important role in inducing NP degeneration (15). Apoptosis is the most studied form of inflammatory death in NPCs. Apoptosis of NPCs leads to a down regulation of ECM synthesis and cell number, then furtherly aggravate NP degeneration (16). 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 cell and attract more and more attentions.

Pyroptosis is an inflammatory form of regulated cell death, and unlike apoptosis or necrosis, is always driven by caspase-1 (17). Canonical pyroptosis is initiated after the liberation of 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-1 β and IL-18, and then generated an inflammasome-associated inflammatory response (18). Pyroptosis is related to different diseases, such as cardiac injury and diabetes mellitus (19, 20). Pyroptosis can also influence the ECM synthesis function of chondrocytes (21). Resent study have demonstrated that pyroptosis also exists in NP, and play an important role in decreasing cell viability and ECM synthesis function of NPCs (22). Therefore, discovering new approach in inhibiting pyroptosis of NPCs may be benefit for the treatment of IVD degeneration.

Our study aimed to clarify the effects of RA in inhibiting pyroptosis and promoting 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 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 was 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 was bought from Jiancheng Bioengineering 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 RNAiso 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 IVDD according to the results of magnetic resonance imaging donated degenerative NP samples. The Pfirrmann classification of the degenerative NP samples were at most grade III (23). 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 (24). 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 hyaluronidase for 4 hours at 37 °C with gentle shaking. Next, the tissues were centrifuged at 1000 rpm for 5 min to isolated cells. Human healthy NPCs (dNPCs) were bought from Procell Life Science&Technology (Hubei, China). 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% CO₂. 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, and after treatment, were 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 a 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 were fixed with 4% paraformaldehyde for 10 min at room temperature. Then, the cells were incubated with 3% H_2O_2 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 stained 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)

Total RNA of each group was extracted using an RNAiso reagent, and quantified by measuring the absorbance at 260 nm/280 nm. PrimeScriptTM 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 was analyzed using the $2^{(-\Delta\Delta CT)}$ 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

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, miRNAs that at least four out of the eight samples have flags in Detected were chosen for further data analysis. Differentially expressed miRNAs were screened by a statistical significance of p < 0.01. The biological function of the differentially expressed miRNAs were indicated by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.

Measurement of cellular ROS

Before the measurement of cellular ROS, each group of cells was cultured in 24-well plates. During the measurement, cells was rinsed and incubated with 5 μ M 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 (SD) 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 degeneration model were fabricated by needle puncture of a 20-G sterile needle in the disc of coccygeal vertebrae (Co)7/Co8 and Co8/Co9 (25). After that, 3 µL of PBS or RA 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 needle puncture and 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 Safranine O-fast green. Cellularity and morphology of the IVDs were then assessed and represented by histological score according to a previously described grading scale (26). 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.

Results

RA increased the viability of dNPCs

Different concentrations of RA were added to dNPCs. After 72 hours treatment, 1 and 10 μ M RA significantly increased the viability of dNPCs, and 0.1 μ M 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 μ M RA had no cytotoxicity on dNPCs on day 0 and 7. However, 10 μ M RA significantly increased the LDH release of dNPCs after 14 days of treatment (Figure 1B). Therefore, we chosen 1 μ M 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 RA significantly decreased the death of dNPCs (Figure 2A and B). In addition, the proliferation of dNPCs were significantly increased by RA on days 7 and 14 (Figure 2C). ECM synthesis related gene markers were measured after 7 days of treatment. *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 (Figure 2D and E). The protein expression levels of sox9, aggrecan, and collagen II in dNPCs were also significantly increased by RA (Figure 2F and G).

Pyroptosis of dNPCs was inhibited by RA

The protein expression levels of cleaved Caspase-1, NT-GSDMD, IL-1 β , and IL-18 reflected the pyroptosis of NPCs. Our results showed that the protein expression levels of cleaved Caspase-1, NT-GSDMD, IL-1 β , 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-1 β , and IL-18 in hNPCs were not significantly changed. However, the expression of cleaved Caspase-1, NT-GSDMD, IL-1 β , and IL-18 in dNPCs were significantly decreased after the addition of RA (Figure 3A and 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 pyroptosis rate of hNPCs, while RA significantly decreased the pyroptosis rate and caspase-1 activity of dNPCs (Figure 3C and D).

Sirt1 was involved in RA mediated pyroptosis

miRNA microarray analysis was performed to search differentially expressed miRNA and signaling pathways between dNPCs and RA treated dNPCs. Only miRNAs with a mean fold change >5 or <0.2 and a p value <0.01 were selected for further analysis. Differentially expressed miRNA between the two groups were presented by heatmap (Figure 4A). Col2A1, Sirt1 and other 10 miRNAs were significantly increased in the RA group, and 17 miRNAs 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 and 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 and B). The pyroptosis rate in the dNPC group was significant 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 in the four groups (Figure 5C and 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 prevented the degeneration of 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 irregularly shaped. The cells and ECM of the NP in the NC group was severe decreased. The morphology and cellularity of AF and NP in the RA group was better compared with those in the NC group, but worse than 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 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 and D).

Discussion

The dysfunction and death of NPCs lead to NP degeneration, and subsequently IVD degeneration (27). 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 (28). RA can inhibit inflammatory response and regulate cell viability, so it had been used in treating many diseases (29). However, the effectiveness of RA in treating IVD degeneration has not been studied, and the effects of RA in the pyroptosis of NPCs was 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 μ M of RA could promote the viability of dNPCs, but 10 μ M of RA also showed cytotocixity on dNPCs after 14 days of cultivation. Therefore, we think 1 μ M 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 induce matrix loss in chondrocytes by the activation of Wnt/ β -catenin pathways (30). However, other studies also reported the activation of RA receptors enhanced aggrecan production in chondrocytes (31). We think the effects of RA on ECM synthesis are complicated, for RA both regulate the anabolism and catabolism of ECM (32, 33). 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 form death.

Both pyroptosis and apoptosis are existed in NPCs, and regulate the function and viability of NPCs (34, 35). Pyroptosis was involved in the degeneration process of NP (36). 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 -1 β 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 treated dNPCs. Sirt1 was important in regulating inflammatory reactions of cell and was positive for the activity of NPCs (37). Sirt1 can inhibit apoptosis in NPCs via ERK signaling pathway (38). Sirt1-overexpressed chondrocytes can also delay the degeneration of NPCs during co-culture (39). 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 inhibits the pyroptosis of dNPCs.

Sirt1 is an important mediator in FoxO signaling and regulates the mitochondrial oxidative stress of cells (40). Our results of 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 (41). 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 (42). Several studies have reported that ROS can activate TXNIP/NLRP3 signaling and induce pyroptosis (22, 43). We also found that RA inhibited the expression of TXNIP and NLRP3. In addition, 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 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 in 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 (44). We think that it is the complicated factors in IVD degeneration 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 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. However, further more studies should be performed before the clinical treatment of IVD degeneration by RA.

Conclusions

We demonstrated that RA has the ability to inhibit the 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 animal 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.

Declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Funding

This work is supported by the National Natural Science Foundation of China (no. 81603126, no. 81972514).

Authors' contributions

Peng-Fei Li, Fei Xiong, Ying Yin contributed equally to this work by conducting the experiment and collected and analyzed the data. Shaojun Hu and Hongyuan Xing 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

Acknowledgements

Not applicable

Availability of data and material

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

References

- Bjornsdottir G, Benonisdottir S, Sveinbjornsson G, Styrkarsdottir U, Thorleifsson G, Walters GB, et al. Sequence variant at 8q24.21 associates with sciatica caused by lumbar disc herniation. Nat Commun. 2017;8:14265.
- 2. Freemont AJ. The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. Rheumatology. 2009;48(1):5–10.
- 3. Neidlinger-Wilke C, Wurtz K, Urban JP, Borm W, Arand M, Ignatius A, et al. Regulation of gene expression in intervertebral disc cells by low and high hydrostatic pressure. Eur Spine J. 2006;15(Suppl 3):372-8.
- 4. Gopal D, Ho AL, Shah A, Chi JH. Molecular basis of intervertebral disc degeneration. Adv Exp Med Biol. 2012;760:114–33.
- 5. Longo UG, Petrillo S, Franceschetti E, Maffulli N, Denaro V. Growth factors and anticatabolic substances for prevention and management of intervertebral disc degeneration. Stem Cells Int. 2012;2012:897183.
- 6. Peng BG. Pathophysiology, diagnosis, and treatment of discogenic low back pain. World J Orthop. 2013;4(2):42–52.
- Kim DJ, Moon SH, Kim H, Kwon UH, Park MS, Han KJ, et al. Bone morphogenetic protein-2 facilitates expression of chondrogenic, not osteogenic, phenotype of human intervertebral disc cells. Spine (Phila Pa 1976). 2003;28(24):2679–84.
- 8. Thompson JP, Oegema TR Jr, Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. Spine (Phila Pa 1976). 1991;16(3):253–60.
- Fang W, Zhou X, Wang J, Xu L, Zhou L, Yu W, et al. Wogonin mitigates intervertebral disc degeneration through the Nrf2/ARE and MAPK signaling pathways. Int Immunopharmacol. 2018;65:539–49.

- Yao M, Zhang J, Li Z, Bai X, Ma J, Li Y. Liraglutide Protects Nucleus Pulposus Cells Against High-Glucose Induced Apoptosis by Activating PI3K/Akt/ mTOR/Caspase-3 and PI3K/Akt/GSK3beta/Caspase-3 Signaling Pathways. Front Med (Lausanne). 2021;8:630962.
- 11. Liu Y, Zhao J, Lu M, Wang H, Tang F. Retinoic acid attenuates cardiac injury induced by hyperglycemia in pre- and post-delivery mice. Can J Physiol Pharmacol. 2020;98(1):6–14.
- 12. Wu J, Cui D, Li H, Zeng J. Protective effects of NAC and salubrinal on apoptosis of retinal pigment epithelial cells induced by all-trans retinoic acid. Eur J Ophthalmol. 2021:11206721211000674.
- Takahata Y, Nakamura E, Hata K, Wakabayashi M, Murakami T, Wakamori K, et al. Sox4 is involved in osteoarthritic cartilage deterioration through induction of ADAMTS4 and ADAMTS5. FASEB J. 2019;33(1):619–30.
- 14. Barakat AH, Elwell VA, Lam KS. Stem cell therapy in discogenic back pain. J Spine Surg. 2019;5(4):561–83.
- 15. Molinos M, Almeida CR, Caldeira J, Cunha C, Goncalves RM, Barbosa MA. Inflammation in intervertebral disc degeneration and regeneration. J R Soc Interface. 2015;12(104):20141191.
- 16. Song Y, Li S, Geng W, Luo R, Liu W, Tu J, et al. Sirtuin 3-dependent mitochondrial redox homeostasis protects against AGEs-induced intervertebral disc degeneration. Redox Biol. 2018;19:339–53.
- 17. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016;535(7610):153–8.
- 18. Xue Y, Enosi Tuipulotu D, Tan WH, Kay C, Man SM. Emerging Activators and Regulators of Inflammasomes and Pyroptosis. Trends Immunol. 2019;40(11):1035–52.
- 19. Zheng X, Zhong T, Ma Y, Wan X, Qin A, Yao B, et al. Bnip3 mediates doxorubicin-induced cardiomyocyte pyroptosis via caspase-3/GSDME. Life Sci. 2020;242:117186.
- 20. Yu ZW, Zhang J, Li X, Wang Y, Fu YH, Gao XY. A new research hot spot: The role of NLRP3 inflammasome activation, a key step in pyroptosis, in diabetes and diabetic complications. Life Sci. 2020;240:117138.
- 21. Hu J, Zhou J, Wu J, Chen Q, Du W, Fu F, et al. Loganin ameliorates cartilage degeneration and osteoarthritis development in an osteoarthritis mouse model through inhibition of NF-kappaB activity and pyroptosis in chondrocytes. J Ethnopharmacol. 2020;247:112261.
- 22. Xu Q, Xing H, Wu J, Chen W, Zhang N. miRNA-141 Induced Pyroptosis in Intervertebral Disk Degeneration by Targeting ROS Generation and Activating TXNIP/NLRP3 Signaling in Nucleus Pulpous Cells. Front Cell Dev Biol. 2020;8:871.
- 23. Pfirrmann CW, Metzdorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine (Phila Pa 1976). 2001;26(17):1873–8.
- 24. Cheng X, Zhang G, Zhang L, Hu Y, Zhang K, Sun X, et al. Mesenchymal stem cells deliver exogenous miR-21 via exosomes to inhibit nucleus pulposus cell apoptosis and reduce intervertebral disc degeneration. J Cell Mol Med. 2018;22(1):261–76.

- 25. Liu Y, Zhou W, Chen FF, Xiao F, Zhu HY, Zhou Y, et al. Overexpression of LMP-1 Decreases Apoptosis in Human Nucleus Pulposus Cells via Suppressing the NF-kappaB Signaling Pathway. Oxid Med Cell Longev. 2020;2020:8189706.
- 26. Han B, Zhu K, Li FC, Xiao YX, Feng J, Shi ZL, et al. A simple disc degeneration model induced by percutaneous needle puncture in the rat tail. Spine (Phila Pa 1976). 2008;33(18):1925–34.
- 27. Croft AS, Illien-Junger S, Grad S, Guerrero J, Wangler S, Gantenbein B. The Application of Mesenchymal Stromal Cells and Their Homing Capabilities to Regenerate the Intervertebral Disc. Int J Mol Sci. 2021;22(7).
- 28. Zhao K, An R, Xiang Q, Li G, Wang K, Song Y, et al. Acid-sensing ion channels regulate nucleus pulposus cell inflammation and pyroptosis via the NLRP3 inflammasome in intervertebral disc degeneration. Cell Prolif. 2021;54(1):e12941.
- 29. Larange A, Cheroutre H. Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. Annu Rev Immunol. 2016;34:369–94.
- Yasuhara R, Yuasa T, Williams JA, Byers SW, Shah S, Pacifici M, et al. Wnt/beta-catenin and retinoic acid receptor signaling pathways interact to regulate chondrocyte function and matrix turnover. J Biol Chem. 2010;285(1):317–27.
- 31. Williams JA, Kondo N, Okabe T, Takeshita N, Pilchak DM, Koyama E, et al. Retinoic acid receptors are required for skeletal growth, matrix homeostasis and growth plate function in postnatal mouse. Dev Biol. 2009;328(2):315–27.
- 32. Shingleton WD, Jones D, Xu X, Cawston TE, Rowan AD. Retinoic acid and oncostatin M combine to promote cartilage degradation via matrix metalloproteinase-13 expression in bovine but not human chondrocytes. Rheumatology. 2006;45(8):958–65.
- 33. Abidin FZ, Gouveia RM, Connon CJ. Application of retinoic acid improves form and function of tissue engineered corneal construct. Organogenesis. 2015;11(3):122–36.
- 34. Bai Z, Liu W, He D, Wang Y, Yi W, Luo C, et al. Protective effects of autophagy and NFE2L2 on reactive oxygen species-induced pyroptosis of human nucleus pulposus cells. Aging. 2020;12(8):7534–48.
- 35. Chen D, Xia D, Pan Z, Xu D, Zhou Y, Wu Y, et al. Metformin protects against apoptosis and senescence in nucleus pulposus cells and ameliorates disc degeneration in vivo. Cell Death Dis. 2016;7(10):e2441.
- 36. He D, Zhou M, Bai Z, Wen Y, Shen J, Hu Z. Propionibacterium acnes induces intervertebral disc degeneration by promoting nucleus pulposus cell pyroptosis via NLRP3-dependent pathway. Biochem Biophys Res Commun. 2020;526(3):772–9.
- 37. Guo J, Shao M, Lu F, Jiang J, Xia X. Role of Sirt1 Plays in Nucleus Pulposus Cells and Intervertebral Disc Degeneration. Spine (Phila Pa 1976). 2017;42(13):E757-E66.
- 38. He F, Li Q, Sheng B, Yang H, Jiang W. SIRT1 Inhibits Apoptosis by Promoting Autophagic Flux in Human Nucleus Pulposus Cells in the Key Stage of Degeneration via ERK Signal Pathway. Biomed Res Int. 2021;2021:8818713.

- 39. Lei B, Wang K, Yang D, Liao L, Dong X, Huang Z. Co-culture with Sirt1-overexpressed chondrocytes delays the nucleus pulposus cells degeneration. Cell Tissue Bank. 2021.
- 40. Mouchiroud L, Houtkooper RH, Moullan N, Katsyuba E, Ryu D, Canto C, et al. The NAD(+)/Sirtuin Pathway Modulates Longevity through Activation of Mitochondrial UPR and FOXO Signaling. Cell. 2013;154(2):430–41.
- 41. Li K, Zhai M, Jiang L, Song F, Zhang B, Li J, et al. Tetrahydrocurcumin Ameliorates Diabetic Cardiomyopathy by Attenuating High Glucose-Induced Oxidative Stress and Fibrosis via Activating the SIRT1 Pathway. Oxid Med Cell Longev. 2019;2019:6746907.
- 42. Bharti V, Tan H, Zhou H, Wang JF. Txnip mediates glucocorticoid-activated NLRP3 inflammatory signaling in mouse microglia. Neurochem Int. 2019;131:104564.
- 43. Gu C, Draga D, Zhou C, Su T, Zou C, Gu Q, et al. miR-590-3p Inhibits Pyroptosis in Diabetic Retinopathy by Targeting NLRP1 and Inactivating the NOX4 Signaling Pathway. Invest Ophthalmol Vis Sci. 2019;60(13):4215–23.
- 44. Urban JP, Roberts S. Degeneration of the intervertebral disc. Arthritis Res Ther. 2003;5(3):120–30.

Tables

Table 1.

| | Gene | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|--|---------|---------------------------|---------------------------|
| | 18s | GAATTCCCAGTAAGTGCGGGTCATA | CGAGGGCCTCACTAAACCATC |
| | MMP1 | AAAATTACACGCCAGATTTGCC | GGTGTGACATTACTCCAGAGTTG |
| | MMP3 | TGATGGGCCTGGAATGGTC | TTCATGAGCAGCAACCAGGAATAG |
| | ADAMTS4 | CTCCTGCCTTTAGCCTGGTC | CCCAAAGGCTGGTAATCGGT |
| | ACAN | CTAGCTGCTTAGCAGGGATAACG | GATGACCCGCAGAGTCACAAAG |
| | SOX9 | AGGAAGCTGGCAGACCAGTACC | GGGTCTCTTCTCGCTCTCGTTCA |
| | COL2A1 | CTGGTGGAGCAGCAAGAGC | GTGGACAGTAGACGGAGGAAAG |
| | SIRT1 | TAGCCTTGTCAGATAAGGAAGGA | ACAGCTTCACAGTCAACTTTGT |

Figures



RA increased the viability of dNPCs. (A) Cell viability was detected at 24, 48, and 72 hours after treated with different concentrations of RA. (B) Cytotoxicity of different concentrations RA was measured on days 0, 7, and 14 by LDH release. Data represent mean \pm SD; **p < 0.01.



Appropriate concentration of RA decreased the death and increased the ECM synthesis function of dNPCs. (A) TUNEL method was performed to measure death of dNPCs (red) before and after the treatment of RA, and the results was observed by fluorescence. Nuclei (blue) were stained by DAPI. (B) Cell death was quantified according to the results of TUNEL. (C) Cell proliferation of dNPCs was measured on days 0, 7, and 14 by CCK8. (D) The gene expression levels of MMP-1, MMP-3, and ADAMTS4 in each group on day 7 were determined by RT-qPCR and normalized to 18s. (E) The gene expression levels of SOX9, ACAN, and COL2a1 in each group on day 7 were determined by RT-qPCR and normalized to 18s. (F) The protein expression levels of Sox9, aggrecan, and collagen II of dNPCs in each group was measured and (G) quantified on day 14. Data represent mean \pm SEM; **p < 0.01. Scale bar = 200 μ m.



Pyroptosis of dNPCs was inhibited by RA. (A) Protein expression levels of Cleaved caspase-1, NT-GSDMD, IL-1 β , and IL-18 in hNPCs and dNPCs before and after the treatment of RA were measured on day 3. (B) Quantification of the protein expression levels in each group. (C) Pyroptosis in hNPCs and dNPCs before and after the treatment of RA was assessed using FLICA/PI staining and flow cytometric analysis. Data represent mean ± SEM; **p < 0.01.



Sirt1 was involved in RA mediated pyroptosis. (A) The heatmap showed differentially expressed mRNAs in dNPCs before and after the treatment of RA. (B) GO terms with significant p values for biological processes, molecular function, cellular component. (C) KEGG terms with significant p values were analyzed. (D) The expression level of Sirt1 in dNPCs before and after the treatment of RA was verified by RT-PCR and normalized to 18s. Data represent mean ± SEM; **p < 0.01.



RA inhibited pyroptosis of dNPCs by activating Sirt1-SOD2 pathway. (A) Protein expression levels of SIRT1, SOD2, TXNIP and NLRP3 in dNPCs after the addition of RA, EX 527, and NS were measured on day 3 and (B) quantified. (C) Pyroptosis in dNPCs after the addition of RA, EX 527, and NS was assessed using FLICA/PI staining and flow cytometric analysis. (D) Quantification of pyroptosis rate in each group. (E) Intracellular ROS generation in dNPCs after using RA, EX 527, and NS was measured. (F) The gene expression levels of SOX9, ACAN, and COL2a1 in each group on day 7 were determined by RT-qPCR and normalized to 18s. Data represent mean ± SEM; **p < 0.01.



RA prevented the degeneration of 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. Data represent mean \pm SEM; **p < 0.01. Scale bar = 500 μ m.