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Acidic Environment Induces Pyroptosis of NLRP3 Inflammatory Vesicle-Mediated Nucleus Pulposus Cells Through Up-Regulation of POSTN Expression

El Entorno Ácido Induce la Piroptosis de las Células del Núcleo Pulposo Mediadas por Vesículas Inflamatorias NLRP3 Mediante la Regulación Positiva de la Expresión de POSTN

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SUMMARY: Intervertebral disc degeneration (IVDD) is induced by nucleus pulposus (NP) dysfunction as a result of massive loss of NP cells. It has been reported that the acidic microenvironment of the intervertebral disc (IVD) can induce NP cell pyroptosis, and that up-regulation of periostin (POSTN) expression has a negative effect on NP cell survival. However, the relationship between the acidic environment, POSTN expression level and NP cell pyroptosis is unclear. Therefore, the aim of this study was to explore the relationship between acidic environment and POSTN expression level in NP cells, as well as the effect of POSTN in acidic environment on NP cell pyroptosis. NP cells were obtained from the lumbar vertebrae of Sprague Dawley (SD) male rats. These cells were divided into normal and acidic groups according to whether they were exposed to 6 mM lactic acid solution. And NP cells in the acidic group were additionally divided into three groups: (1) Blank group: no transfection; (2) NC group: cells transfected with empty vector plasmid; (3) sh-POSTN group: cells transfected with sh-POSTN plasmid to knock down the expression level of POSTN. Quantitative real-time PCR (qRT-PCR) and western blot was performed to assess the expression of POSTN at the mRNA and protein levels. CCK8 was used to evaluate cell survival. Western blot, in addition, was performed to examine acid-sensing ion channels (ASIC)-related proteins. And pyroptosis was detected by ELISA and western blot. The expression level of POSTN was significantly increased in NP cells in acidic environment. Knockdown of POSTN expression promoted the survival of NP cells in acidic environment and reduced the protein levels of ASIC3 and ASIC1a in NP cells. Moreover, knockdown of POSTN expression decreased the pyroptosis proportion of NP cells and the levels of pro-inflammatory cytokines interleukin (IL)-1β and IL-18. The levels of pyroptosis-related proteins NLRP3, ASC, cleaved-Caspase-1, and cleaved-GSDMD were also affected by the decreased POSTN expression. The extracellular acidic environment created by lactic acid solution activated NLRP3 inflammatory vesicle-induced caspase-1 to get involved in NP cell pyroptosis by up-regulating POSTN expression.

KEY WORDS: Intervertebral disc degeneration; Nucleus pulposus; Periostin; Acidic environment; Pyroptosis.

INTRODUCTION

The incidence and prevalence of intervertebral disc degeneration (IVDD) have exhibited a progressive increase with the aging of the population and the increase of sedentary lifestyle (Ohnishi *et al*., 2022). IVDD can result in a variety of symptoms, including lower back pain, restricted movement, numbness and weakness in the lower limbs, and even urinary and stool incontinence, even if it is not lethal. In the current population, IVDD is now the main contributor to low back pain (Kirnaz *et al*., 2022). Those symptoms produce varied degrees of subjective discomfort in IVDD patients and seriously affect their quality of life. More unfortunately, non-surgical treatment can often only provide palliative and temporary pain relief, but not a cure because of the different etiologies and complex pathogenesis of IVDD. While surgical treatment, although effective, is not suitable for large-scale clinical application due to its high

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surgical risks, long postoperative recovery time, and postoperative complications (Mohd *et al*., 2022). Therefore, a thorough investigation of the particular pathophysiology of IVDD will aid in the identification of new targets for intervention, which will enhance the efficacy of non-surgical therapy of IVDD as well as the quality of life of IVDD patients.

The intervertebral disc (IVD) consists of an external fibrous ring, a surrounding cartilage endplate, and an internal nucleus pulposus (NP) (Tomaszewski *et al*., 2015). The NP, a semi-fluid gel-like substance, plays a key role in cushioning, supporting, and absorbing pressure from the lumbar spine (Chen *et al*., 2017). The health status of the NP is critical for maintaining the normal morphology and function of the IVD. When pathological changes occur in the NP, they can lead to IVD compression as well as rupture and displacement of fibrous ring, all of which result in IVDD (Kos *et al*., 2019). NP cells and their secreted extracellular matrix are the main components of the NP, and maintaining adequate numbers of functional NP cells is crucial to ensure a healthy state of the NP (Le Maitre *et al*., 2007). Conversely, a sudden drop in the number of NP cells will promote the pathological progression of IVDD by impairing NP tissue function. Pyroptosis, a form of inflammatory cell death, has recently been identified as a significant contributor to massive NP cell loss. And the pyroptosis of massive NP cells is a key etiology of IVDD (Ge *et al*., 2022).

In addition, altered pH in the IVD microenvironment has also been found to be an important cause of IVDD. Under normal conditions, the extracellular environment of NP cells should be neutral to acidic. However, clinical studies have found that the disc tissues removed after discectomy from patients with IVDD mostly share a pH of 5.5-6.5 (Ohshima & Urban, 1992; Kitano *et al*., 1993). *In vitro* studies have similarly found that the artificially created acidic environment significantly contributed to NP cell pyroptosis and inhibited NP cell proliferation (Silagi *et al*., 2018; Zhao *et al*., 2021). This evidence suggest that the acidic environment of IVD can be involved in the pathological process of IVDD by promoting NP cell pyroptosis and suddenly decreasing functional NP cells. However, the specific pathways that affect NP cell pyroptosis have not been elucidated.

The periostin (POSTN) gene is localized on human chromosome 13, which can encode the protein POSTN with a molecular weight of approximately 93 kDa (Kudo, 2017). As an extracellular matrix protein, POSTN protein is widely expressed in several organs including heart, lung, liver, bone, and kidney. It is involved in a variety of important physiological processes such as cell signaling, extracellular matrix-cell interaction, and cell proliferation and differentiation (Dorafshan *et al*., 2022). According to a recent study, mechano-stress significantly increased the level of the protein POSTN in IVD tissues. This elevated POSTN protein caused NP tissue dysfunction by accelerating NP cell pyroptosis, which ultimately led to IVDD (Wu *et al*., 2022). This suggests that abnormal expression levels of POSTN genes are likely to have adverse effects on NP cells. But it is unknown what upstream factors that regulate POSTN gene expression during the development of IVDD.

Based on the findings of previous studies, we speculate that the acidic environment in IVD tissues may be involved in the pathological process of IVDD by inducing NP cell pyroptosis through up-regulation of POSTN gene expression. In this study, we used rat NP cells as study models and treated them in an artificial acidic environment through lactic acid intervention. To confirm our speculation, we observed the condition of NP cells and the level of pyroptosis through *in vitro* experiments. The results of this study will help to elucidate the specific pathogenesis of IVDD and provide new insights for future development of anti-IVDD drugs.

MATERIAL AND METHOD

Isolation and culture of nucleus pulposus cells in rats. Twenty SPF-grade Sprague Dawley (SD) male rats were purchased from Charles River (Beijing, China). All SD rats were 6 to 7 weeks old and weighed 220 to 240 g. SD rats were housed in the animal laboratory of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. The animal laboratory was set to standard 12 h light /12 h dark circadian rhythm, temperature 23±1 ºC, and humidity 55~60 %. The rats had free access to water and food. The feeding and subsequent experimental operations were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals, and this study has been reviewed and approved by the Ethics Committee for Animal Experimentation of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. After 1 week of acclimatization, the rats were put into deep anesthesia by inhaling anesthetic isoflurane (2~2.5 % volume). Referring to and adapting the method of previous studies (He *et al*., 2021), the spine and paravertebral tissue were exposed through a median longitudinal incision of 15-20 mm, centered on the line of the top iliac crest. After the exposure, the paravertebral muscles were carefully pushed away, and the entire lumbar vertebrae (L1-L5) was removed. The lumbar vertebrae were rinsed with PBS buffer (5 min/time, 3 times) on an ultra-clean bench. Next, soft tissues were gently and carefully separated with ophthalmic scissors under a microscope, and approximately 0.6-0.8 mg of NP tissues

per rat was obtained from the central part of each intervertebral disc. After the obtained NP tissue was centrifuged (1000 rpm/min, 4 ºC, 5 min), the supernatant was transferred to the EP tube (Shanghai Muchen Biotechnology Co., Ltd., China) and washed again with PBS buffer (5 min/time, 3 times). The supernatant was then digested with 0.25 % trypsin (MedChemExpress, USA) for 30 min at 37 °C. Later, an appropriate amount of Dulbecco's modified Eagle's medium (DMEM) medium (Sigma-Aldrich, USA) containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution was added to terminate the digestion. An incubation with 0.1 % collagenase type II (Sigma-Aldrich, USA) for 4 h at 37°C was performed subsequently. After completion of the digestion, centrifugation was performed again (1000 rpm / min, 4 °C, 5 min), and the supernatant was added directly to DMEM medium containing 10 % FBS and 1 % penicillin/ streptomycin solution for 3 weeks. During the culture process, the solution was renewed every 3 days. The cells passaged to the second generation were identified by toluidine blue staining, Alcian blue staining and immunofluorescence. And the morphological structure and molecular markers of typical NP cells were determined. The NP cells of the second generation were used for subsequent experiments.

NP cell transfection and treatment. NP cells were divided into two groups: normal group, with the cells continued to be cultured in DMEM medium at normal pH, and acid group, with the cells cultured in DMEM medium with an additional 6 mM lactic acid solution, which referred to a previous *in vitro* experiment using lactic acid to interfere with NP cells (Wu *et al*., 2014). Both groups of NP cells were cultured until the logarithmic growth phase when the confluence reached 70 % to 80 %.

In addition, NP cells cultured in DMEM medium supplemented with 6 mM lactic acid experienced further treatment. sh-POSTN plasmids (for knocking down the POSTN gene) as well as empty vector plasmids without carrying any specific gene (as blank controls) were designed, customized, and purchased from Shanghai GenePharma, China. NP cells were cultured in an acidic environment until confluence reached about 75 % The above plasmids were transiently transfected into cells using Lipofectamine 3000 (Invitrogen, USA), and cells were collected for subsequent experiments after continued incubation for 48 hours. NP cells cultured in the medium with 6 mM lactic acid were additionally divided into the following three groups: (1) Blank group: no transfection; (2) NC group: transfected with empty vector plasmid; (3) sh-POSTN group: transfected with sh-POSTN plasmid.

Table I. Primers for qRT-PCR of rat NP cells.

RNA	*Sequences (5' to 3')*
POSTN	*5'-AGGAGCCGTGTTTGAGACCAT-3' (forward)*
	5'-CGGTGAAAGTGGTTTGCTGTTT-3' (reverse)
GAPDH	*5'-GACATCAAGAAGGTGGTGAAGC-3' (forward)*
	5'-TGTCATTGAGAGCAATGCCAGC-3' (reverse)

Quantitative real-time fluorescence PCR (qRT-PCR). POSTN mRNA expression levels in NP cells were detected by quantitative real-time fluorescence PCR (qRT-PCR). Total RNA was extracted from NP cells using Trizol reagent (Aidlab, China), and then reversely transcribed into cDNA following the instructions of the All-in-One First-Strand cDNA Synthesis Kit (GeneCopoeia, USA). qRT-PCR was performed using the Hifair III One-Step RT-qPCR SYBR Green Kit (YEASEN, China) on Quant Studio 6 Flex System (Applied Biosystems, USA). qRT-PCR reaction conditions were 95 °C for 10 min, 95 °C for 15s, 60 ºC for 1 min, 95 ºC 15s, 60 ºC 15s, with a total of 40 cycles. GAPDH was used as an internal control to correct for POSTN mRNA expression levels, and quantitative calculations and analysis were performed using the $2^{-\Delta\Delta ct}$ method. qRT-PCR was repeated three times for each sample and the average value was taken as the final result. The primers used in the PCR are shown in Table I.

CCK-8 assay. The proliferation capacity of NP cells was assessed by CCK-8 assay. The NP cells in logarithmic growth phase were inoculated into 96-well plates at a density of 5×10^3 cells per well. After adding 10 μL of CCK-8 solution (MedChemExpress, USA) to each well, the NP cells were incubated for 2 h at 37 °C in an incubator with 5 % CO_2. Afterwards, the absorbance was measured at 450 nm using an enzyme marker (Thermo Fisher Scientific, USA) to assess the cell proliferation level.

ELISA assay. The levels of pro-inflammatory cytokines interleukin (IL)-1β and IL-18 in NP cells were measured by ELISA. Standards of different gradient concentrations prepared using dilutions were prepared in strict accordance with the instructions of commercial IL-1b (Invitrogen, # BMS630, USA) and IL-18 (Invitrogen, # KRC2341, USA) ELISA kits. RIPA buffer (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitors was added to the cell culture medium for sufficient contact of the cells with the lysate. After centrifugation (3000 rpm, 4 °C, 5 min), the supernatant was removed. In 96-well plates, 100 μL of diluted standard or cell supernatant sample and 50 μL of diluted detection antibody were added to each well and incubated at room temperature for about 1.5 h. After washing, 100 μL of diluted horseradish peroxidase (HPR)-labeled antibody was added to each well and incubated again for about 30 min at room temperature. Following that, the

supernatant was discarded, and each well was incubated with 100 mL of the chromogenic substrate TMB (3, 3', 5, 5' tetramethylbenzidine) for 30 minutes at room temperature in the dark. Finally, 100 μL of termination solution was added to each well, and the absorbance at 450 nm was measured using an enzyme marker (Thermo Fisher Scientific, USA). A standard curve of the results obtained from the standards was plotted. The absorbance was used to calculate the levels of IL-1β and IL-18 in the cell supernatant samples. The experiment was repeated three times for each sample and the average was taken as the final result.

Western blot analysis. The levels of POSTN and other proteins associated with pyroptosis were measured by western blot. Total proteins were extracted from the cells using RIPA buffer containing protease and phosphatase inhibitors, and protein quantification was performed using the bicinchoninic acid (BCA) kit (Abcam, UK). The proteins were separated using 10 % SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Abcam, UK). The PVDF membranes were blocked with 5 % skim milk for 1 h at ambient temperature. Then, PVDF membranes were incubated with primary antibody overnight at 4 ºC. The primary antibody used was as follows: POSTN (1:1,000, Abcam, #ab92460, UK); NLRP3 (1:1,000, Abcam, #ab263899, UK); pro-Caspase-1 (1:1,000, Abcam, #ab179515, UK); cleaved-Caspase-1 (1:1,000, Abcam, #ab286125, UK); pro-GSDMD (1:1,000, Abcam, #ab219800, UK); cleaved N-terminal GSDMD (1:1,000. Abcam, #ab215203, UK); ASC (1:1,000, Abcam, #ab307560, UK); ASIC3 (1:1,000, Abcam, #ab302776, UK); ASIC1a (1:1,000, Abcam, #ab300563, UK). The next day, PVDF membranes were washed (3 times for 5 min each) with Tris-buffered saline Tween solution (GuYan Biotechnology Co., Ltd., China) and then incubated with HRP-labeled Goat Anti-Rabbit IgG H&L secondary antibody (1:2,000, Abcam, #ab6721 , UK) at room temperature for 1 h. The membranes were imaged in a GEL imaging system (Bio-Rad, USA) and protein expression levels were quantified using Image J software. Relative expression levels of relevant target proteins were corrected by the expression level of the internal control GAPDH.

Statistictical analysis. Measured data were expressed as mean ± standard deviation. The Shapiro-Wilk test was used to determine whether the data conformed to normal distribution, and the results suggested that the data of each group basically satisfied the normal distribution characteristics. Therefore, the differences between the two groups were analyzed using the independent-samples t-test, and differences between multiple groups were analyzed using one-way ANOVA. If the one-way ANOVA results were significant, pairwise comparisons between groups were further made using the Tukey method. Statistical analysis was performed using SPSS 22.0 software (IBM Corp., NY, USA), and $p<0.05$ was used as the criterion for a statistically significant difference.

RESULTS

Acidic environment up-regulates POSTN expression in NP cells. To investigate the effect of acidic environment on POSTN expression, we first examined the mRNA expression levels and protein levels of POSTN in different culture environments. qRT-PCR results suggested that the mRNA expression level of POSTN in the acidic group was significantly higher than that in the normal group ($P<0.05$) (Fig. 1A). Similarly, the western blot results suggested that the protein level of POSTN in the acidic group was much higher than that in the normal group ($P<0.05$) (Fig. 1B). Overall, the acidic environment could significantly increase the mRNA expression level and protein level of POSTN in rat NP cells.

Knockdown of POSTN expression promotes NP cell survival in acidic environment. Decreased number of functional NP cells is an important factor in causing IVDD (Le Maitre *et al*., 2007). To investigate the relationship between the expression level of POSTN in acidic environment and the survival number of NP cells, we first transfected sh-POSTN plasmid into NP cells cultured in acidic conditions by liposome transfection to down-regulate POSTN expression. qRT-PCR results showed that there was

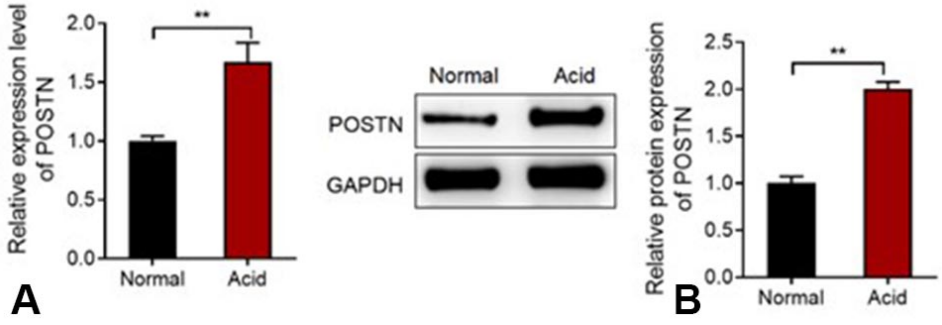

Fig. 1. Effect of acidic environment on the expression level of POSTN in NP cells. A, qRT-PCR detection of mRNA expression levels of POSTN in normal and acidic groups of NP cells. B, Western blot detection of POSTN protein levels in normal and acidic groups of NP cells. **P<0.01. POSTN: periostin; NP: nucleus pulposus.

no significant difference in the mRNA expression level of POSTN between Blank group and NC group (P> 0.05). After knockdown of POSTN expression, the mRNA expression level of POSTN in NP cells was obviously reduced compared with the Blank and NC groups (P<0.05) (Fig. 2A). Similarly, western blot results also suggested that there was no significant difference in POSTN protein level between Blank group and NC group (P>0.05), and knockdown of POSTN expression markedly lowered POSTN protein level in NP cells compared with Blank group and NC group (P<0.05) (Fig. 2B,C). It was evident that the liposome transfection successfully knocked down the expression of the target gene POSTN. Therefore, the role of POSTN can be explored in subsequent experiments by comparing the differences between the cells in the Blank group, NC group, and sh-POSTN group. Subsequently, we observed the difference in the number of surviving NP cells between the three groups by CCK8 assay. As for the CCK8 detection results, there was no significant difference in the number of surviving NP cells between the Blank and NC groups (P>0.05); compared with the Blank and NC groups, surviving NP cells in the sh-POSTN group mounted (P<0.05) (Fig. 2D). The above results indicated that knockdown of POSTN expression could effectively promote the survival of NP cells in acidic environment.

Knockdown of POSTN expression reduces protein levels of ASIC3 and ASIC1a in NP cells in an acidic environment. Acid-sensing ion channels (ASICs), a member of proton-gated channel family, play an important role in the inflammatory process associated with IVDD. Specifically, their protein levels are positively correlated with the severity of IVDD (Ran *et al*., 2022). Therefore, the relationship between POSTN expression levels and ASIC protein levels in NP cells was explored in our study through western blot. In terms of the western blot results, no significant difference existed in ASIC3 and ASIC1a protein levels between Blank and NC groups (P>0.05); and compared with the Blank group and the NC group, knockdown of POSTN expression significantly down-regulated protein levels of ASIC3 and ASIC1a in NP cells (P<0.05) (Fig. 3A-C).

Knockdown of POSTN expression inhibits NP cell pyroptosis in acidic environment. After that, we further explored the relationship between POSTN expression level and NP cell pyroptosis in acidic environment. During pyrogenesis, cells produce and release a large number of pro-inflammatory cytokines, the most prominent of which are IL-1β and IL-18 (Frank & Vince, 2019). Accordingly, we compared the differences in the levels of IL-1β and IL-18 between the three groups of cells. ELISA results revealed that the Blank and NC groups showed no significant difference in the levels of IL-1β and IL-18 (P>0.05). Following knockdown of POSTN expression, a notable decline was observed in the levels of IL-1β and IL-18 in NP cells compared with the Blank and NC groups (P < 0.05) (Fig. 4A-B).

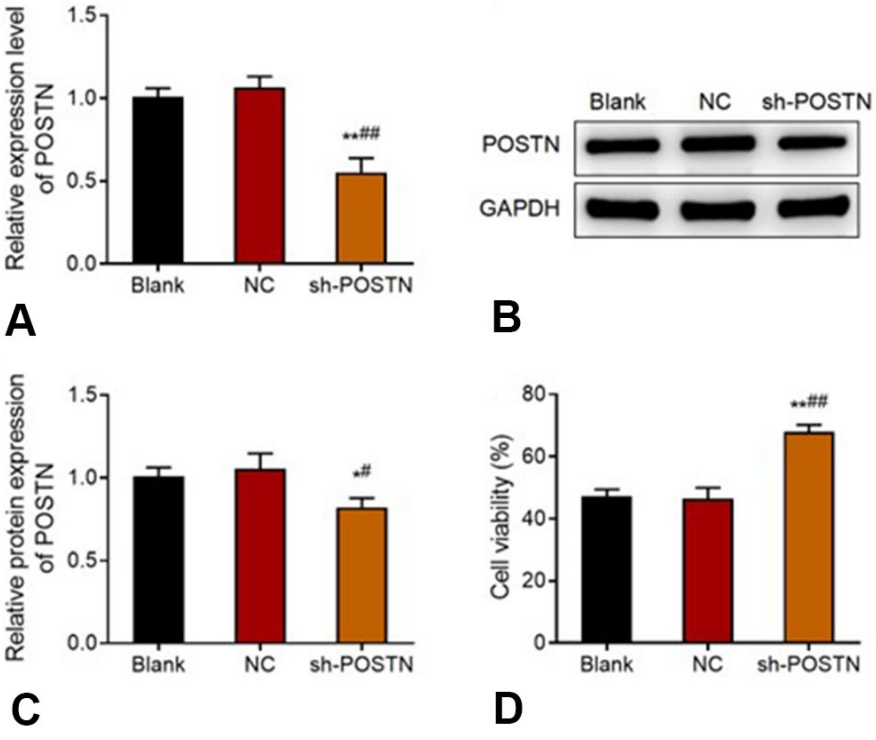

Fig. 2. Effect of knockdown of POSTN expression on the survival of NP cells in acidic environment. **A.** qRT-PCR assay to detect the mRNA expression level of POSTN in NP cells of Blank group, NC group and sh-POSTN group; **B**. Western blot to detect the protein expression level of POSTN in NP cells of Blank group, NC group and sh-POSTN group; **C.** Statistical analysis results of western blot; **D.** CCK8 detection of NP cell survival in Blank group, NC group, and sh-POSTN group. *P<0.05, **P<0.01, vs Blank group; #P<0.05, ##P<0.01 vs NC group. POSTN: periostin; NP: nucleus pulposus.

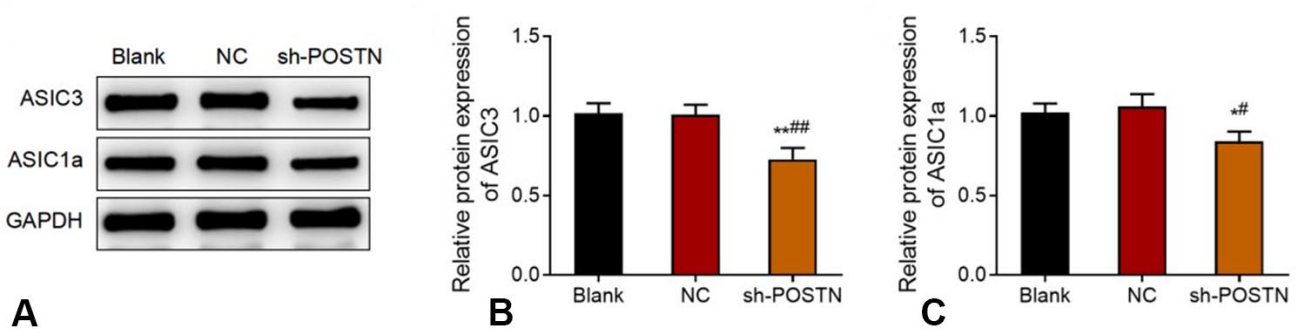

Fig. 3. Effect of knockdown of POSTN expression on protein levels of ASIC3 and ASIC1a in NP cells in acidic environment. **A.** Western blot detection of ASIC3 and ASIC1a protein expression levels in NP cells of Blank group, NC group and sh-POSTN group; **B.** Results of statistical analysis of ASIC3 protein level; **C.** Results of statistical analysis of ASIC1a protein level. *P<0.05, **P<0.01, vs Blank group; #P<0.05; ##P<0.01, vs NC group. POSTN: periostin; NP: nucleus pulposus.

The classical pathway of pyroptosis is a mode of cell death associated with NLRP3 inflammatory vesicle activation. Concretely speaking, activated caspase-1 drives GSDMD cleavage and release of the GSDMD-N-terminal domain; this domain targeted to cleave the cell membrane, which causes damage to the cell membrane and induces pyroptosis (Lucas-Ruiz *et al*., 2022). We therefore compared the levels of key proteins NLRP3, ASC, pro-Caspase-1, cleaved Caspase-1, pro-GSDMD, and cleaved GSDMD related with the classical pathway of pyroptosis among the three groups. According to western blot results, the protein levels of NLRP3, ASC, pro-Caspase-1, cleaved-Caspase-1, pro-GSDMD, and cleaved-GSDMD were not significantly different between Blank and NC groups (P>0.05); the protein levels of NLRP3, ASC, cleaved-Caspase-1, and cleaved-GSDMD were observably decreased after knockdown of POSTN expression in NP cells compared with those of Blank and NC groups (P <0.05). Notably, the protein levels of pro-GSDMD and pro-Caspase-1 were not significantly different (P>0.05) (Fig. 4C-I). Together, these results suggest that knockdown of POSTN expression significantly inhibits NP cell pyroptosis in an acidic environment.

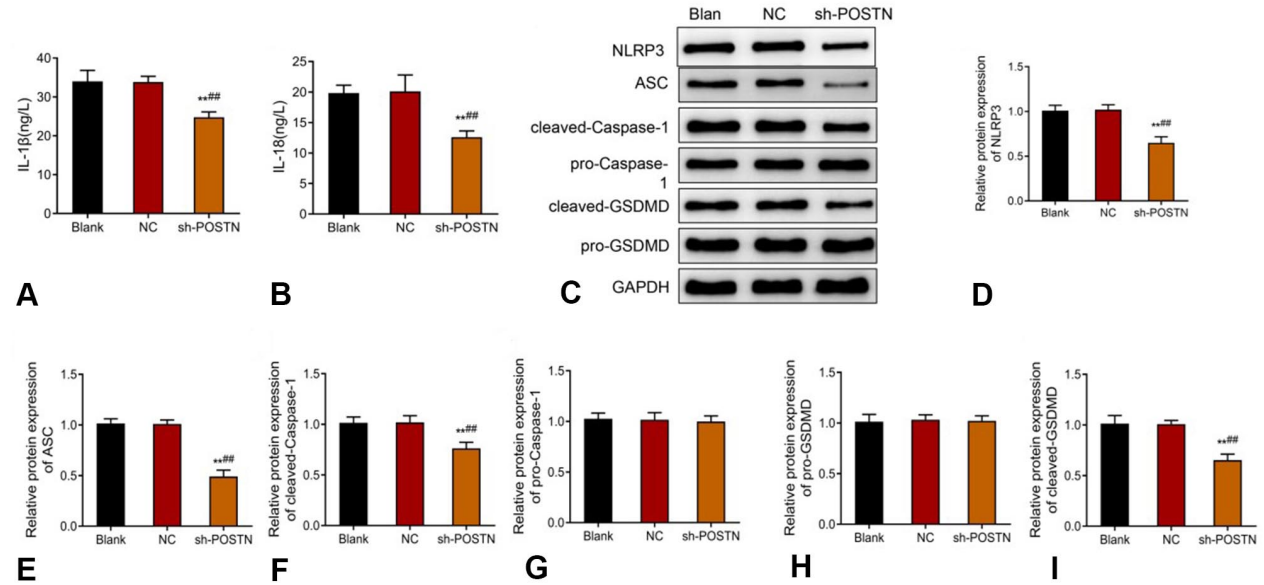

Fig. 4. Effect of knockdown of POSTN expression on the NP cell pyroptosis in acidic environment. **A-B.** ELISA detection of IL-1b (A) and IL-18 (B) levels in NP cells of Blank, NC and sh-POSTN groups; **C.** Western blot detection of protein expression levels of NLRP3, ASC, pro-Caspase-1, cleaved- Caspase-1, pro-GSDMD and cleaved-GSDMD; **D.** Results of statistical analysis of NLRP3 protein level; **E.** Results of statistical analysis of ASC protein level; **F.** Results of statistical analysis of pro-Caspase-1 protein level; **G.** Results of statistical analysis of cleaved-Caspase-1 protein level; **H.** Results of statistical analysis of pro-GSDMD protein level; **I.** Results of statistical analysis of cleaved-GSDMD protein level. **P<0.01, vs Blank group; ##P<0.01, vs NC group.

DISCUSSION

In this study, we discovered that the expression level of POSTN gene in NP cells was significantly up regulated by adding lactic acid solution to the medium to create an artificially acidic environment; while knocking down POSTN expression through shRNA promoted NP cell proliferation, attenuated NP cell inflammatory response and inhibited NP cell pyroptosis in an acidic environment. To the best of our knowledge, the present study was the first to reveal that the acidic environment induced NP cells to undergo pyroptosis by regulating the expression level of POSTN. Using POSTN as an intervention target, this study clarified one of the particular etiology of IVDD, laying the theoretical groundwork for the later development of effective anti-IVDD drugs.

High lactate concentration is not only an important feature of IVD, but also a significant risk factor for the development and progression of IVDD. In IVD, glycolysis provides the majority of the energy needed by cells. Acid hypertonic environment exists naturally in NP: 1) glycolysis generates lactate by consuming glucose; 2) IVD mainly relies on poorly permeable end-plate structures to excrete metabolites (Urban *et al*., 2004). In the findings of studies using *in vitro* simulated IVD-like organs as models and those using NP cells as models, the acidic environment created by elevated lactate concentrations accelerated NP cell death, prevented NP cells from secreting extracellular matrix and induced an inflammatory response in NP cells (Risbud & Shapiro, 2014; Wu *et al*., 2014; Wang *et al*., 2022). However, little research has been done on downstream molecules regulated by lactate in IVDD.

Clinical studies on multiple myeloma, non-small cell lung cancer, and atopic dermatitis (Kou *et al*., 2014; Terpos *et al*., 2016; Wang *et al*., 2019) found a positive correlation between POSTN protein levels and lactate dehydrogenase (LDH) concentrations in peripheral blood of patients. It is well known that the main function of LDH is to catalyze the production of lactate from pyruvate. The elevated concentration of LDH indicates an accelerated rate of lactate production and increased lactate accumulation in those patients (Khan *et al*., 2020). It also follows that increased lactate levels in patients with multiple myeloma, non-small cell lung cancer, and atopic dermatitis may be causally related to the up regulation of POSTN expression. Therefore, we hypothesized that there may also be a link between POSTN expression levels, and the acidic environment caused by high lactate formation in IVD during the development and progression of IVDD, and that POSTN may be a downstream molecule regulated by lactate. As shown by the results of qRT-PCR and western blot, both POSTN mRNA levels and protein levels were much higher in NP cells cultured in an acidic environment than those cultured in a normal environment. This confirms that an acidic environment can up-regulate POSTN gene expression in NP cells.

Subsequently, we explored the effect of POSTN expression level on NP cells. Through CCK-8 assay, we found that knocking down POSTN expression effectively increased the percentage of surviving NP cells in an acidic environment. Extracellular matrix, including type II collagen and proteoglycan released by active NP cells, has been said to be essential for maintaining the normal state of NP. When the number of surviving functional NP cells decreases, the content of type II collagen and proteoglycan in the IVD also decreases; as a consequence, NP dehydration, deformation and shrinkage occur in sequence, which speeds up the progression of IVDD (Kumar *et al*., 2022). Therefore, the results of this experiment confirm that POSTN in an acidic environment can be involved in the pathological process of IVDD by affecting NP cell survival.

We further explored the mechanisms underlying the negative effect of increased POSTN expression on NP cell survival. ASICs are widely distributed in various tissues and cells, including the nervous system, cancer cells, articular chondrocytes, and NP cells (Vullo & Kellenberger, 2020). ASICs were discovered to be very sensitive to reduced extracellular pH. Especially in the naturally acidic environment of the IVD, lactic acid buildup rapidly activates ASICs, which inhibit NP cell proliferation and accelerate NP cell death by exacerbating the inflammatory response of NP cells (Ran *et al*., 2023). We then compared the protein levels of ASIC3 and ASIC1a, two members of the ASIC family, among the three groups of cells by western blot. The western blot results demonstrated that knockdown of POSTN expression significantly reduced the two protein levels. Therefore, we can draw a conclusion that POSTN in acidic environment may exacerbate the inflammatory response of NP cells by raising ASIC3 and ASIC1a protein levels, which adversely affects the survival of NP cells.

After knowing that POSTN expression level was correlated with the inflammatory response of NP cells, we investigated the effect of POSTN on the cell death modality (i.e., pyroptosis) that is closely associated with inflammation. This point is also the main focus of this study. We assessed the differences in NP cell pyroptosis between the different groups on two aspects. On the one hand, previous studies have shown that IL-1β and IL-18 play an important role in cell pyroptosis. To be specific, caspase-1 generated during pyroptosis cleaves the cytoplasmic IL-1β and IL-18 precursors into active IL-1β and IL-18, which are then

released outside the cell through the broken cell membrane caused by pyroptosis. So, the levels of IL-1β and IL-18 are positively correlated with the severity of pyroptosis (Luo *et al*., 2022). We discovered that knockdown of POSTN expression significantly lowered IL-1β and IL-18 levels, indicating that the severity of NP cell pyroptosis was effectively attenuated after the knockdown. On the other hand, we looked at the concentrations of important proteins involved in the NP cell pyroptosis. NLRP3 is the initiator of the classical pathway of pyroptosis. Upon external stimulation, NLRP3 inflammatory vesicles are formed by binding NLRP3 with its adaptor protein ASC and Caspase-1 precursor. Thus, the elevated levels of NLRP3 and ASC proteins implies that NP cells experience pyroptosis. In addition, during pyroptosis, GSDMD is cleaved by cleaved-Caspase-1 to GSDMD-N-terminal domain that targetedly attacks the cell membrane and forms lacunae. Hence, decreased levels of GSDMD protein can suggest that NP cells undergo pyroptosis (Ge *et al*., 2022). In this study, we disclosed that the protein levels of NLRP3, ASC, cleaved-Caspase-1 and cleaved-GSDMD were obviously down-regulated after knockdown of POSTN expression, demonstrating that the classical pathway of NP cell pyroptosis was inhibited by the POSTN knockdown. Combining the above two results, it is logical to speculate that knockdown of POSTN expression in an acidic environment can effectively inhibit NP cell pyroptosis. Therefore, we can conclude that the expression level of POSTN in the acidic environment may affect the degree of NP cell pyroptosis. Additionally, a prior work (Zhao *et al*., 2021) showed that lactic acid contributes to NP cell pyroptosis by activating the NLRP3 inflammatory vesicle-mediated classical pathway of pyroptosis in cellular models or SD rat models, leading to the development and progression of IVDD. This result largely supports the findings of our investigation. The significant originality of this work in comparison to earlier studies was the identification of a crucial role for POSTN in the pyroptosis in the current study.

Acid-base homeostasis in the body is necessary to maintain normal physiological activity. The unique acidic microenvironment in IVD is responsible for IVD's fragility and susceptibility to degenerative changes (Zhang *et al*., 2016). Although it has been established that NP cell acidosis is a significant contributor to the pathophysiology of IVDD (Cai *et al*., 2016), the precise mechanisms at play are still up for debate. The unclear mechanisms have hindered the development of relevant drugs for IVDD. In this case, the present study attempts to clarify that high lactate concentration induces NLRP3 inflammatory vesicle-mediated NP cell pyroptosis through up-regulation of POSTN expression, thereby participating in the pathological process of IVDD. It is particularly challenging to reverse the high lactate environment of IVD because of the morphological structure and metabolite efflux pathways that are inherent to IVD (Urban *et al*., 2004). Contrarily, it is considerably simpler to control the expression levels of individual POSTN genes. As a result, this study not only elucidated the core role of POSTN in the high lactate concentration induced IVDD pathway, but also revealed the possibility of POSTN to be an intervention target in the future development of anti-IVDD drugs.

Of course, there are some shortcomings in this study. For one thing, in addition to the classical pathway, NLRP3 inflammatory vesicle-mediated caspase-1 activation, there are also non-classical pathways, including cytoplasmic lipopolysaccharide-mediated caspase-4, caspase-5 and caspase-11 activation (Mu *et al*., 2022). But this study did not explore whether NP cells go through non-classical pyroptosis. For another, the present study did not directly observe the changes in cell morphology and the formation of pyroptosis vesicles by transmission electron microscopy, which weakened the conclusion reliability of this study to some extent.

CONCLUSION

In summary, the acidic environment, through up-regulation of POSTN expression, activates NLRP3 inflammatory vesicle-induced caspase-1, which is implicated in NP cell pyroptosis in the presence of high lactate concentration. In short, this study elucidates one of the specific pathogenesis of IVDD and provides a new idea for future anti-IVDD drug development.

ZHANGL, L.; TAO, J.; LU, X.; YU, H.; CAI, F. & CHENL, X. El entorno ácido induce la piroptosis de las células del núcleo pulposo mediadas por vesículas inflamatorias NLRP3 mediante la regulación positiva de la expresión de POSTN. *Int. J. Morphol., 41(6)*:1734-1743, 2023.

RESUMEN: La degeneración del disco intervertebral (DDIV) es inducida por una disfunción del núcleo pulposo (NP) como resultado de una pérdida masiva de células NP. Se ha informado que el microambiente ácido del disco intervertebral (DIV) puede inducir la piroptosis de las células NP y que la regulación positiva de la expresión de periostina (POSTN) tiene un efecto negativo en la supervivencia de las células NP. Sin embargo, la relación entre el ambiente ácido, el nivel de expresión de POSTN y la piroptosis de las células NP es poco clara. Por lo tanto, el objetivo de este estudio fue explorar la relación entre el ambiente ácido y el nivel de expresión de POSTN en células NP, así como el efecto de POSTN en ambiente ácido sobre la piroptosis de las células NP. Las células NP se obtuvieron de las vértebras

lumbares de ratas macho Sprague Dawley (SD). Estas células se dividieron en grupos normales y ácidos según se expusieron a una solución de ácido láctico 6 mM. Las células NP en el grupo ácido se dividieron adicionalmente en tres grupos: (1) Grupo en blanco: sin transfección; (2) grupo NC: células transfectadas con plásmido vector vacío; (3) grupo sh-POSTN: células transfectadas con plásmido sh-POSTN para reducir el nivel de expresión de POSTN. Se realizó una PCR cuantitativa en tiempo real (qRT-PCR) y una transferencia Western para evaluar la expresión de POSTN en los niveles de ARNm y proteína. Se utilizó CCK8 para evaluar la supervivencia celular. Además, se realizó una transferencia Western para examinar las proteínas relacionadas con los canales iónicos sensibles al ácido (ASIC). La piroptosis se detectó mediante ELISA y Western blot. El nivel de expresión de POSTN aumentó significativamente en células NP en ambiente ácido. La eliminación de la expresión de POSTN promovió la supervivencia de las células NP en un ambiente ácido y redujo los niveles de proteína de ASIC3 y ASIC1a en las células NP. Además, la eliminación de la expresión de POSTN disminuyó la proporción de piroptosis de las células NP y los niveles de citocinas proinflamatorias interleucina (IL) - 1β e IL-18. Los niveles de proteínas relacionadas con la piroptosis NLRP3, ASC, Caspasa-1 escindida y GSDMD escindida también se vieron afectados por la disminución de la expresión de POSTN. El ambiente ácido extracelular creado por la solución de ácido láctico activó la caspasa-1 inducida por vesículas inflamatorias NLRP3 para involucrarse en la piroptosis de las células NP mediante la regulación positiva de la expresión de POSTN.

PALABRAS CLAVE: Degeneración del disco intervertebral; Núcleo pulposo; Periostina; Ambiente ácido; Piroptosis.

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