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Parecoxib prevents nucleus pulposus cells apoptosis by suppressing endoplasmic reticulum stress

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Abstract. – OBJECTIVE: Intervertebral disc degeneration (IVDD) is the main cause of spine diseases, and apoptosis of nucleus pulposus (NP) cells is an important risk factor for the degeneration of intervertebral discs. Endoplasmic reticulum (ER) stress is involved in multiple apoptosis processes. This study investigated whether the specific COX-2 inhibitor parecoxib can inhibit NP cell apoptosis induced by ER stress.

PATIENTS AND METHODS: Human NP cells were isolated from the disc tissue collected from IVDD patients. We used IL-1β to establish an NP cell degenerated model. Degenerated levels were detected by the analysis of cell viability, collagen II, collagen X, aggrecan, TNF-α, IL-6, and MMP-13 expression. ER stress status was examined by GRP78 and CHOP expression. Apoptosis level was mainly indicated by the positive apoptotic cells and caspase-12 expression. CHOP-plasmid transfection was performed to overexpress the CHOP protein level.

RESULTS: IL-1β could induce the decrease of viability, collagen II, aggrecan, but an increase of collagen X, TNF-α, IL-6, and MMP-13 in NP cells, as well as the upregulation of GRP78/PERK/caspase-12 and apoptosis level, which could be inhibited by parecoxib. Parecoxib could also suppress CHOP caused by COX-2 upregulation and apoptosis in NP cells.

CONCLUSIONS: Parecoxib is a safe and efficient COX-2 inhibitor to NP cells, which could prevent NP cells apoptosis by suppressing ER stress.

Key Words:
Parecoxib, COX-2 inhibitor, Nucleus pulposus cells, Endoplasmic reticulum stress, Apoptosis.

Introduction

Intervertebral disc is a crucial structure for buffering vibration, dispersing the spinal stress, and maintaining the height of the vertebral body, which is also prone to degeneration of the human body. Trauma, mechanical stress, immune factors, ischemia, hypoxia, apoptosis, and inflammatory transmitters are factors causing premature intervertebral disc degeneration (IVDD)[1,2]. Apoptosis exists throughout the life of the body and participates in various physiological and pathological processes. Increased or decreased apoptosis is the basis of many diseases[3]. The endoplasmic reticulum (ER) is also involved in the pathways that constitute apoptosis, the primary mechanism of which is ER stress caused by various physical and chemical factors. ER is an essential organelle in the cell whose main function is to participate in the synthesis of membrane proteins, secreted proteins, glycogens, lipids, steroid hormones, the spatial folding and glycosylation modification of proteins[4]. Under normal physiological conditions, the ER maintains the balance of intracellular and extracellular protein metabolism. When cells exposed to various physical and chemical factors, the intracellular homeostasis will be broken, resulting in ER stress. Finally, the activation of the endoplasmic reticulum pathway leads to cell apoptosis. The nucleus pulposus (NP) cells are the main functional cells in the intervertebral disc and play an important role in the degeneration process of the intervertebral disc. Xu et al[5] eluci-

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dated hydrogen sulfide ameliorates IVDD against the suppression of ER stress in NP cells. Liao et al[6] discover the exosomes extracted from mesenchymal stem cells could prevent NP cell death by regulating ER stress.

Cyclooxygenase (COX) is an essential rate-limiting enzyme in the metabolism of arachidonic acid to prostaglandins. It is involved in many important physiological and pathological processes, especially inflammation and tumors, including two subtypes, namely COX-1 and COX-2[7,8]. COX-1 stably expressed in most normal tissues, synthesizing prostaglandins to regulate the regular physiological activity of cells and maintaining the stability of the internal environment. COX-2 is an inducible enzyme that is not expressed in most normal tissues but can rapidly activate after tissue damage, inflammation, or harmful stimuli, participating in the inflammatory process and the occurrence of pain and the development of tumors[9,10]. COX-2 participates in the pathogenesis of IVDD through the expression of PGE2, and its appearance gradually increases with the developed progression[11]. Excessive COX-2 results in ER stress and induces apoptosis[12-14]. Ou et al[15] found celecoxib could ameliorate osteoarthritis by suppressing chondrocyte apoptosis. Selective COX-2 inhibitors are primarily used for anti-inflammatory treatment of disc disease in the clinic, such as celecoxib and arcoxia[16,17]. However, whether the inhibitor of COX-2 plays a role in the apoptosis of NP cells by suppressing ER stress remains unknown.

In this present study, we isolated human NP cells from intervertebral disc tissues and used IL-1β to accelerate NP cell degeneration with an upregulation of ER stress. The specific COX-2 inhibitor parecoxib was applied to test its anti-apoptosis and anti-ER stress efficiency during the progress of NP cell degeneration. The effects and mechanism of parecoxib on ER stress and related to apoptosis were further investigated in NP cells transfected by C/EBP homologous protein (CHOP), the main components of the ER stress-mediated apoptosis pathway[18], *in vitro*.

Patients and Methods

Intervertebral Disc Tissue Collection

Intervertebral disc tissues were collected from 5 patients (3 male and 2 females, the average age is 42 years), who underwent lumbar disc herniation operations in Dongzhimen Hospital of Beijing University of Chinese Medicine from September to October 2018. Specimens were immediately taken from the NP tissue after cutting from patients and stored in DMEM culture medium for NP cell isolation. The project was supported by the Ethics Committee of Dongzhimen Hospital of Beijing University of Chinese Medicine, and all specimens were all obtained with patients' consent. This investigation was conducted in accordance with the Declaration of Helsinki.

NP Cells Isolation and Treatments

The NP cell isolation was performed by digestion with type II collagenase. After washing the specimens and cutting into small pieces. The fragments were incubated with 0.15% type II collagenase Dulbecco's Modified Eagle's Medium (DMEM) solution (Gibco, Rockville, MD, USA) at 37°C for 4-6 h until the digestive solution became cloudy and the fragments disappeared. Then, centrifuge the mixture at 1000 r/min for 10 min and discard the supernatant to get the NP cells. NP cells were seeded in 6-well plates at a dose of 1×10^5 per well and changed the medium every 3 days. The passage 1 to 2 NP cells were used in the following experiments. NP cells were treated with a ranged concentration of parecoxib (0-70 μM) and cultured with 50 μM parecoxib for ranged time (0-72 h). IL-1β (10 ng/mL) was used to degenerate CHs, and CHOP-plasmid was used to transfect NP cells for the activation of ER stress *in vitro*.

Cell Viability Assay

The viability of NP cells was determined by cell counting kit8 (CCK8) assay. NP cells were seeded at 1×10^4/well in 96-well plates were incubated with CCK8 kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The intensity of the product was detected by a microplate reader. Cell viability was shown as a percentage relative to non-treated value.

Flow Cytometry

We used flow cytometry to determine the cell apoptosis ratio. NP cells were cultured in 6-well plates and treated with different methods. NP cells were harvested with phosphate-buffered saline (PBS) and then incubated with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide Keygen (Nanjing, China) according to the manufacturer's instructions. Finally, cells were measured by a flow cytometer and analyzed with ImageJ software (NIH, Bethesda, MD, USA).

Real Time-PCR Analysis

The total RNA of NP cells in each group was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was measured by the NanoDrop protein-nucleic acid quantitation instrument. 1 μg of total RNA was used to prepare a complementary deoxyribose nucleic acid (cDNA) template using the Reverse Transcription Kit (ReverTra Ace, Tokyo, Japan). Construct a 10 μL reaction system refer to the RT-PCR Kit (Bio-Rad, Hercules, CA, USA), and 3 replicate wells were set in each treatment group. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative gene expression of COX-2, PGE-2, collagen II, collagen X, aggrecan, IL-6, TNF-α, and MMP-13 were calculated according to the $2^{-\Delta\Delta Ct}$ formula. Primers used in this experiment were referred to as the GenBank sequence (Table I).

Plasmid Transfection

Plasmid against human CHOP was constructed as previously described[19] and was synthesized by RiboBio (Guangzhou, China). NP cells were transiently transfected with 100 nM concentrations of plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The efficiency of CHOP overexpression was verified by Western blot.

Western Blot Analysis

The NP cells were washed 3 times with precooled PBS, lysed with radioimmunoprecipitation assay (RIPA) lysate (containing 10% phenylmethylsulfonyl fluoride (PMSF), Invitrogen, Carlsbad, CA, USA) for 30 min, and the cells were centrifuged to obtain the supernatant, and the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) was used for protein quantification. The supernatant was boiled at 100°C for 10 min and stored at -80°C until use. Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked with 5% skim milk (Tris Buffered Saline and Tween-20 (TBST) dissolved) for 3 h at room temperature, membranes were then incubated with primary antibody against GRP78 (ab21685, Abcam, Cambridge, MA, USA), CHOP (ab11419, Abcam, Cambridge, MA, USA), and caspase-12 (ab62484, Abcam, Cambridge, MA, USA) 4°C overnight. The appropriate secondary antibody was added for incubation for 1 h, and then the PVDF membrane was stained with the enhanced chemiluminescence (ECL) agent (Beyotime, Shanghai, China) for 1 min and exposed to X-ray films in a dark room.

Immunofluorescence

NP cells were seeded on coverslips. Before staining, coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X-100 (Solarbio, Beijing, China), and blocked with bovine serum albumin (BSA; 5% BSA in PBS). Then, the coverslips were incubated with the collagen II antibody (ab34712, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, goat anti-rabbit Dylight 488 fluorescent antibody (Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) were added to incubate for 1 h. The fluorescence intensity was observed under an inverted fluorescence microscope.

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

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
COX-2	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
PGE-2	CGCCTCAACCACTCCTACAC	GACACCGATCCGCAATCCTC
MMP-13	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
Collagen X	ATGCTGCCACAAATACCCTTT	GGTAGTGGGCCTTTTATGCCT
Aggrecan	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

Statistical Analysis

Data were expressed as mean ± standard deviation (Mean ± SD). We analyzed all the data by GraphPad Prism 8 software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. A comparison between multiple groups was done using One-way ANOVA test followed by a post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

The Safety and Efficiency of Parecoxib in the Treatment of NP Cells

To determine the safety and efficiency of parecoxib in the suppression of COX-2 of NP cells, CCK-8 assay was used to detect the viability of the cells, and RT-PCR was used to analyze the suppressed efficiency of COX-2. NP cells were treated with a ranged amount of parecoxib from 0 to 70 μM for 24 h, no significant difference was observed among the different concentrations to the viability of NP cells (Figure 1A). Besides, the mRNA expression of COX-2 was gradually decreased up to 50 μM, which was the same as 70 μM (Figure 1B). Therefore, we used 50 μM parecoxib to explore the optimized stimuli time. The result suggested parecoxib was safe to NP cells within 72 h (Figure 1C), and the expression of COX-2 was minimized at the time point of 24 h (Figure 1D). Above all, parecoxib is really safe to NP cells, and the most effectively treated in our experiment is 50 μM cultured for 24 h.

Parecoxib Prevents NP Cells from IL-1β Induced Degeneration

To determine whether parecoxib plays a role in the degenerated progress of CHs, IL-1β was used to induce the degeneration of NP cells. IL-1β significantly affected the viability of NP cells, and parecoxib partly resisted this damage (Figure 2A). As shown in Figure 2B, parecoxib was really powerful to suppress the COX-2 and PGE-2 expression, even at the presence of IL-1β. Collagen II and aggrecan are marked expressed in growth NP cells but typically decreased in degenerated NP cells, but collagen X is conversely different. Compared with the control, IL-1β efficiently suppressed collagen II and aggrecan but increased the level of collagen X, which were all objected by the

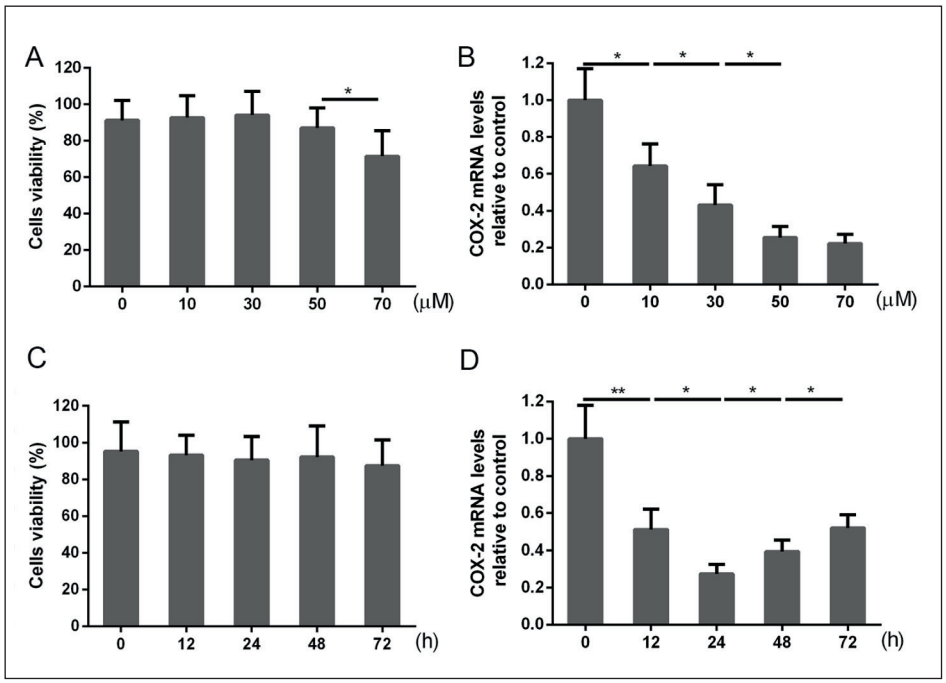

Figure 1. Parecoxib made less deleterious effects on CHs viability. CHs was cultured with different concentration of parecoxib (0-70 μM) for 24 h, or cultured with 50 μM parecoxib for 0-72 h. **A**, **C**, Cell viability was measured by CCK8 assay. **B**, **D**, mRNA levels of COX-2 were determined by RT-PCR. The values are mean ± SD of three independent experiments. (*$p<0.05$, **$p<0.01$ compared to control).

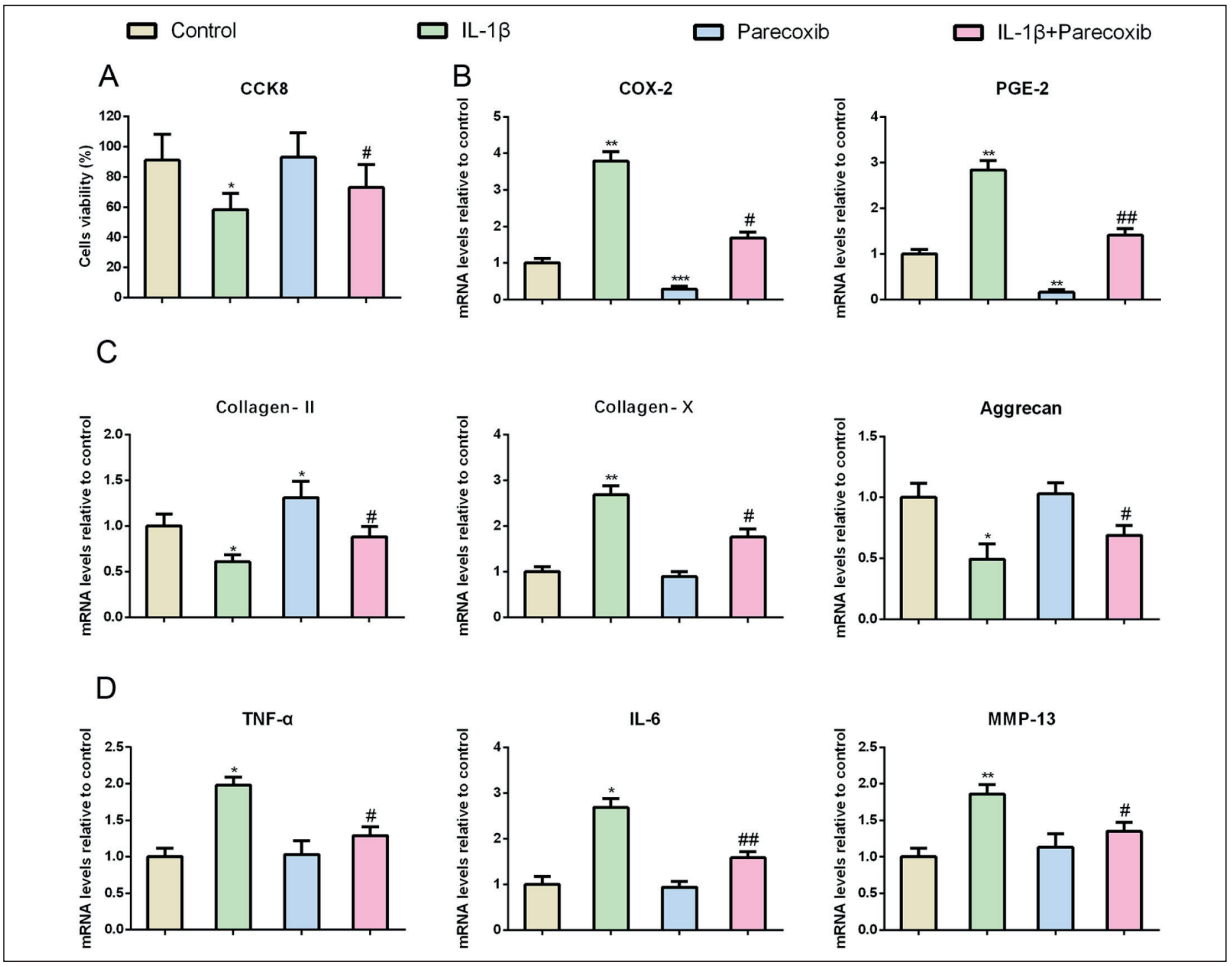

Figure 2. Parecoxib prevents NP cells from IL-1β induced degeneration. NP cells were pre-cultured with IL-1β (10 ng/mL) or parecoxib (50 μM) for 24 h. For co-treated group, NP cells were pre-cultured with IL-1β (10 ng/mL) 24 h, and then treated with parecoxib (50 μM) for another 24 h. **A**, Cell viability was measured by CCK8 assay. **B**-**D**, The mRNA expression levels of (**B**) COX-2, PGE-2, and (**C**) collagen II, collagen X, aggrecan, and (**D**) TNF-α, IL-6, MMP-13 were assayed by RT-PCR. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01 compared to control; $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared to IL-1β).

presence of parecoxib (Figure 2C). Also, as an anti-inflammation drug, parecoxib also significantly inhibited the mRNA expression of IL-6, TNF-α, and MMP-13 resulting from IL-1β treatment (Figure 2D). These results suggested that parecoxib efficiently inhibits COX-2 expression and prevents NP cells from IL-1β induced degeneration.

Parecoxib Suppresses IL-1β Induced ER Stress and Apoptosis in CHs

To determine whether parecoxib plays a role in suppression of ER stress and apoptosis caused by IL-1β, we measured the GRP78, CHOP, and caspase-12 protein expression and the apoptotic cell ratio under the stimulation of IL-1β with or without parecoxib. First, we detected the collagen II expression with immunofluorescence staining. As shown in Figure 3A and 3B, then the density of the collagen II was significantly suppressed by IL-1β but upregulated by parecoxib compared with the control group. The ER markers containing GRP78 and CHOP were activated by the stimuli of IL-1β, and parecoxib obviously resisted the expression of GRP78 and CHOP (Figure 3C, 3D). Furthermore, caspase-12 was also reduced, resulting from the treatment of parecoxib, as well as the apoptotic NP cell population was also decreased due to parecoxib (Figure 3C-3E). The data of these results indicated that the IL-1β could activate ER stress and apoptosis of NP cells, which was distinctly reversed by parecoxib.

Parecoxib Prevents NP Cells from Apoptosis Via the Suppression of ER Stress

To determine the relationship between ER stress and NP cell apoptosis, we used CHOP-plasmid to activate ER stress. The results of the COX-2 mRNA level indicated that parecoxib inhibited the COX-2 level, which upregulated by IL-1β, but this positive effect was canceled by CHOP-plasmid again. Additionally, overexpression of CHOP also had the ability to rise the COX-2 expression (Figure 4A). Furthermore, we observed that parecoxib protected IL-1β-induced collagen II lost. However, CHOP protein overexpression recalled the upregu-

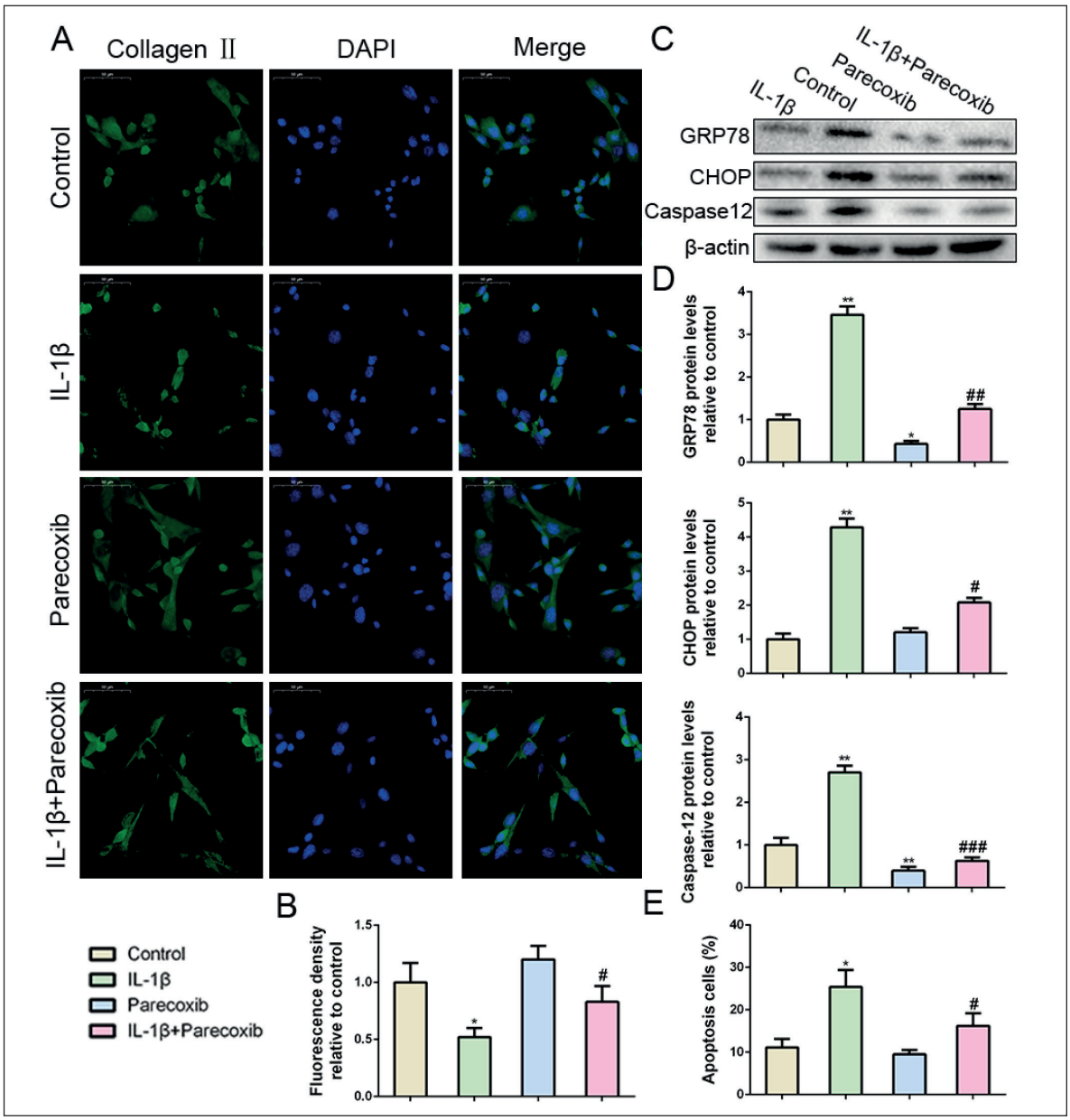

Figure 3. Parecoxib suppresses IL-1β induced ER stress and apoptosis of NP cells. NP cells were pre-cultured with IL-1β (10 ng/mL) or parecoxib (50 μM) for 24 h. For co-treated group, NP cells were pre-cultured with IL-1β (10 ng/mL) 24 h, and then treated with parecoxib (50 μM) for another 24 h. **A**, **B**, Protein expression level of collagen II was determined by (**A**) immunofluorescence (magnification: 200×) and (**B**) quantification analysis. **C**, **D**, Protein expression level of GRP78, CHOP, and caspase-12 were determined by (**C**) WB and (**D**) quantification analysis. (**E**) Apoptosis ratio of NP cells was assayed by flow cytometry. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01 compared to control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared to IL-1β).

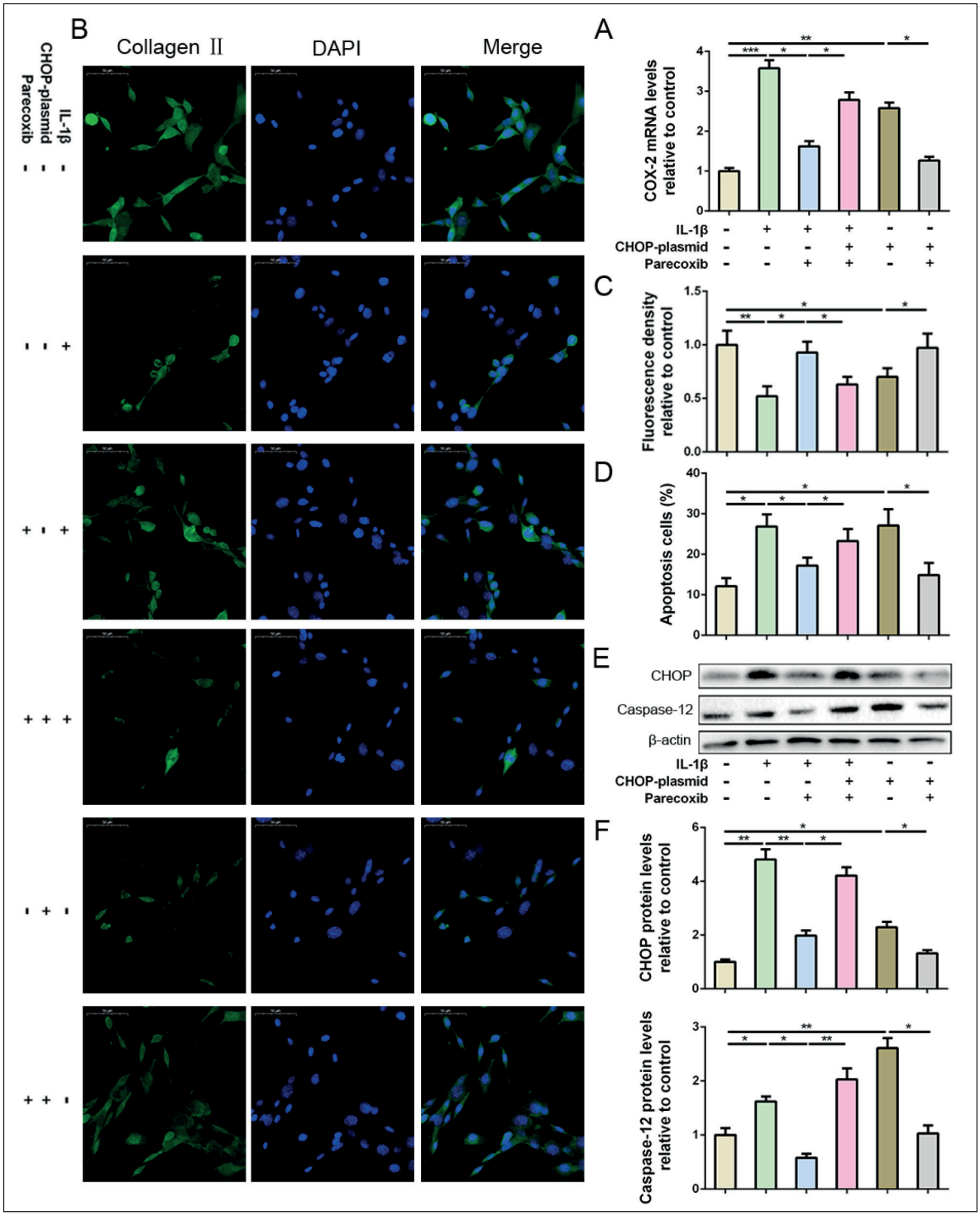

Figure 4. Parecoxib protects NP cells apoptosis by the inhibition of ER stress. NP cells were pre-cultured with IL-1β (10 ng/mL) or parecoxib (50 μM) for 24 h or transfected by CHOP-plasmid. For co-treated group, NP cells with or without CHOP-transfected were pre-cultured with IL-1β (10 ng/mL) 24 h, and then treated with parecoxib (50 μM) for another 24 h. **A**, The mRNA expression levels of COX-2 were assayed by RT-PCR. **B**, **C**, Protein expression level of collagen II was determined by (**B**) immunofluorescence (magnification: 200×) and (**C**) quantification analysis. **D**, Apoptosis ratio of NP cells was assayed by flow cytometry. **E**, **F**, Protein expression level of GRP78, CHOP, and caspase-12 were determined by (**E**) WB and (**F**) quantification analysis. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

lated collagen II expression caused by parecoxib. As IL-1β, CHOP overexpression also made NP cells a degenerated phenotype (Figure 4B, 4C). From the apoptotic population analysis, the enhanced CHOP expression increased the apoptosis of NP cells, and partly canceled the protected effect of parecoxib to IL-1β-treated NP cells, and parecoxib also can decrease the side effect of promoting apoptosis caused by CHOP (Figure 4D). Finally, we measured the caspase-12 protein expression, which was upregulated by IL-1β and CHOP but suppressed by parecoxib compared to the control (Figure 4E, 4F). From these data, it can be observed that CHOP overexpression led to a higher level of apoptosis, and parecoxib could reverse the apoptotic progress causing by IL-1β or CHOP, which means ER stress is positively related to the apoptosis of NP cells, and parecoxib could decrease COX-2 level and protect NP cells apoptosis by suppressing RE stress.

Discussion

At present, selective COX-2 inhibitors are commonly used in the treatment of chronic inflammatory diseases contains IVDD[20]. The content of PGE2 in degenerated intervertebral disc tissues is significantly higher than that in the normal[21], which has a role in regulating inflammation and direct pain. PGE2 has an inhibitory effect on aggrecan synthesis in the intervertebral disc. It can degrade the ECM by upregulating the expression and activity of MMPs. Meanwhile, PGE2 induces the generation of minute vessels in the disc, and further promotes MMP activation, macrophage infiltration, and ECM degradation. In addition, COX-2 is a key enzyme regulating PGE2 synthesis. COX-2 is a rate-limiting enzyme that up-regulates PGE2 synthesis during the arachidonic acid cascade reaction. Clinical researches have found that the administration of COX-2 inhibitors to IVDD patients can reduce the inflammatory response of the prominent issues[22]. The NP of the intervertebral disc is in a closed and lacking blood supply environment, which leads to NP tissue a strong inflammatory induction property[23]. In our study, IL-1β is an effective inducer that resulted in an upregulated COX-2 expression and a subsequent PGE-2 overexpression. The expression of TNF-α and IL-6 is increased after the degeneration of NP cells, which may cause the accumulation of damage in NP tissues with an autocrine or paracrine manner[24]. However, the selective COX-2 inhibitors used in this study have been widely applied in many diseases to anti-inflammation[25,26]. In addition to this, parecoxib is also showed to defend cell apoptosis progress through various ways[27-29]. Even so, whether the anti-apoptotic function of parecoxib covers ER stress is not clear.

In previous studies, COX-2 has been reported to be closely related to ER stress. Chen et al[30] stated that COX-2 induced ER stress and autophagy in DF-1 cells. Jin et al[31] found ER stress could activate COX-2 to regulate the autophagy of kidney podocyte. In our study, parecoxib could decrease the ER level by the inhibition of COX-2. However, CHOP overexpression also resulted in a higher level of COX-2, which conforms to the results in the previous references. The ER apoptosis pathway is one of the newly discovered apoptotic pathways in recent years, and its core content is ER stress[32]. ER apoptosis pathway mainly contains three pathways, namely: CHOP/GADD153 pathway, IREI/JNK pathway, and caspase-12 pathway, which induce apoptosis alone or synergistically crossed[33]. Caspase-12 is involved in cell growth, differentiation, and apoptosis regulation and is mainly distributed in the ER. Therefore, overexpression of Caspase-12 is thought to be the initiation of the apoptotic pathway of the EM[34]. Under normal conditions, intracellular GRP78 is combined with PERK (protein kinase R-like ER kinase), ATF6 (Activating transcription factor 6), and IRE1 (Inositol requiring enzyme 1) markers associated with ER stress, when ER stress-activated, GRP78 dissociates releasing from the three proteins. Therefore, the GRP78 overexpression is generally recognized as a sign of ER stress beginning[35]. After that, ER stress-specific apoptosis is mainly mediated by CHOP, which induces the expression of several pro-apoptotic factors, including TRB3, GADD34, and DR5[36]. NP cells are the only cells in the NP tissue that synthesize and secrete collagen II and aggrecan.

The number and activity of NP cells, as well as the synthesis of extracellular products, are essential for the maintenance of intervertebral disc function and structure[37]. Apoptosis of NP cells in the intervertebral disc is the basis for IVDD and leading to degenerative diseases of the spine[38]. Summarily, this study suggests that IL-1β can induce NP cells apoptosis and associated by activating the ER stress. COX-2 is perhaps related to the occurrence of ER stress-induced apoptotic progress, which can be suppressed by a specific COX-2 inhibitor parecoxib. Although the effica-

cy of parecoxib in the treatment of IVDD needs more observations, the results of our study provide a new perspective about parecoxib in the prevention of NP cell apoptosis, which is potentially helpful to the understanding of COX-2 and ER stress in the IVDD.

Conclusions

These results, briefly, indicated that parecoxib is a safe and efficient COX-2 inhibitor to NP cells, which could prevent NP cells apoptosis by suppressing ER stress.

Conflict of Interests

The authors declare that they have no conflict of interests.

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