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Berberine ameliorates oxidative stress-induced apoptosis by modulating ER stress and autophagy in human nucleus pulposus cells

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ABSTRACT

Aim: Nucleus pulposus (NP) cell apoptosis induced by oxidative stress is known to be closely involved in the pathogenesis of intervertebral disc (IVD) degeneration. Berberine, a small molecule derived from *Rhizoma coptidis*, has been found to exert antioxidative activity and preserve cell viability. The present study aims to investigate whether berberine can prevent NP cell apoptosis under oxidative damage and the potential underlying mechanisms.

Methods and materials: The effects of berberine on IVD degeneration were investigated both in vitro and in vivo.

Key findings: Our results showed that berberine significantly mitigated oxidative stress-decreased cell viability as well as apoptosis in human NP cells. Berberine treatment could attenuate oxidative stress-induced ER stress and autophagy in a concentration-dependent manner. With 4-PBA (ER stress specific inhibitor) and 3-MA (autophagy specific inhibitor) administration, we demonstrated that berberine inhibited oxidative stress-induced apoptosis by modulating the ER stress and autophagy pathway. We also found that the IRE1/JNK pathway was involved in the induction of ER stress-dependent autophagy. With Ca^{2+} chelator BAPTA-AM utilization, we revealed that oxidative stress-mediated ER stress and autophagy repressed by berberine could be restored by inducing intracellular Ca^{2+} dysregulation. Furthermore, in vivo study provided evidence that berberine treatment could retard the process of puncture-induced IVD degeneration in a rat model.

Significance: Our results indicate that berberine could prevent oxidative stress-induced apoptosis by modulating ER stress and autophagy, thus offering a novel potential pharmacological treatment strategy for IVD degeneration.

1. Introduction

Intervertebral disc (IVD) degeneration, characterized by excess degradation of extracellular matrix (ECM) and cell loss in the nucleus pulposus (NP), is the most prominent cause of lower back pain that affects millions of people worldwide and leads to enormous socioeconomic burden [1,2]. Despite great efforts and resources spent on the prevention and treatment of IVD degeneration, its etiology and pathogenesis still remain elusive and there are currently no effective therapeutic strategies to prevent this undesirable condition [3–5]. Thus, clarifying the potential mechanisms and finding available strategies to curb and/or treat the initiation and progression of IVD degeneration is an urgent requirement.

Oxidative stress, resulting from disturbance to the basal cellular redox state resulting in excessive production and/or reduced clearance of reactive oxygen species (ROS) under stress conditions has been identified as an important driver of pathogenesis in multiple diseases [6,7]. Increasing evidence demonstrates that excessive ROS and oxidative stress is generated in aged and degenerated intervertebral discs, and is tightly associated with the pathogenesis of IVD degeneration by regulating apoptosis, necrosis, senescence of discs cells, and impeding ECM metabolism [3,8–11]. Moreover, administration of antioxidants such as resveratrol and glutathione was found to efficiently prevent the deleterious effects of oxidative stress on disc cells in vitro [12,13]. Therefore, targeting oxidative stress is considered a promising therapeutic approach for IVD degeneration.

Endoplasmic reticulum (ER) is the largest intracellular organelle responsible for multiple crucial functions including protein and lipid synthesis and sorting, post-translational modification, and calcium (Ca^{2+}) storage and release. Protein folding in the ER is highly sensitive

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to extracellular stimuli and changes in intracellular homeostasis including ER Ca^{2+}, redox state, and energy stores. Perturbation of ER function results in the accumulation of unfolded/misfolded proteins within the ER lumen, a condition called ER stress, which in turn activates the adaptive unfolded protein response (UPR) characterized by triggering three protein sensors on the ER membrane: inositol-requiring kinase 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) to resolve this protein-folding defect. However, excessive and prolonged ER stress switches toward apoptotic cell death via activation of downstream signals like CCAAT/enhancer binding protein homologous protein (CHOP) and caspase12 [14–16]. Previous studies have also demonstrated that ER stress is closely involved in the pathological process of IVD degeneration and that pharmacological inhibition of ER stress response could suppress apoptosis and ECM degradation of discs cells [17–19].

Autophagy is a dynamic homeostatic process that recycles misfolded proteins and damaged cellular organelles for lysosomal degradation to provide energy and nutrients. Generally, autophagy is characterized as a cytoprotective mechanism defending from a wide variety of stress stimuli like starvation, hypoxia, ER stress, and oxidative stress, whereas excessive or persistent autophagy can also promote apoptosis [20–22]. In addition, autophagy is closely associated with apoptosis in the pathological process of multiple degenerative diseases including osteoarthritis [23], neurodegeneration [24], and IVD degeneration [25]. Although numerous studies have verified the essential role of autophagy in the pathogenesis of IVD degeneration [26–29], the exact mechanisms of autophagy mediated promotion or prevention of IVD degeneration are still controversial.

Berberine, an isoquinoline alkaloid isolated from *Coptidis rhizoma* and *Cortex phellodendri*, is extensively used to treat diarrhea and diabetes [30]. Recent studies have revealed that berberine exerts multiple pharmacological activities including anti-inflammation, anti-oxidation, and hypoglycemia by modulating oxidative stress, autophagy, and ER stress [31–34]. Given the effective and wide spectrum clinical application of berberine, we hypothesize that berberine may have therapeutic potential for IVD degeneration via modulating oxidative stress, ER stress, and autophagy.

In the current study, we sought to determine the beneficial effects of berberine on human NP cells under oxidative stress, and to delineate the role of ER stress and autophagy by which berberine exerts its effects. Furthermore, we evaluated the effects of berberine on a puncture-induced rat IVD degeneration animal model in vivo. Therefore, our data indicate that berberine might be a promising therapeutic agent for IVD degeneration.

2. Materials and methods

2.1. Ethics statement

All the experimental protocols involving human IVD specimens used in the present study were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (NO. S214) and were in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants in our study. The animal procedures followed in the present study were in accordance with the ethical standards of the Animal Experimentation Committee of Huazhong University of Science and Technology and complied with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Reagents and antibodies

Hydrogen peroxide (H_2O_2), 3-methyladenine (3-MA), 4-phenylbutyrate (4-PBA), and 3, 5-dibromosalicylaldehyde (DBSA) were obtained from Sigma Aldrich (St. Louis, MO, USA). JNK inhibitor SP600125 and BAPTA-AM were acquired from MedChemExpress (Monmouth Junction, NJ, USA). Berberine was purchased from TCI (Tokyo, Japan). Primary antibodies against CHOP (#2895S), Bax (#2772), Cleaved-caspase 3 antibody (#9661), and GAPDH (#5174) were purchased from Cell Signaling Technology (Danvers, MA, USA). Bcl-2 (ab32124), GRP78 (ab21685), Caspase12 (ab62463), IRE1-α (ab37073), phospho-IRE1-α (ab48417), JNK (ab112501), and phospho-JNK (ab4821) were obtained from Abcam (Cambridge, UK). The antibody for LC3 (AF5402) was obtained from Affinity Bioreagents (Rockford, IL). Antibodies against p62 (18420-1-AP), and Beclin1 (11306-1AP) were purchased from Sanying (Wuhan, China).

2.3. Cell culture and treatment

Human NP cells were isolated as described previously [35]. Briefly, NP samples were collected from patients (n = 5) with idiopathic scoliosis undergoing deformity correction surgery. The NP tissues evaluated as Grade I-II according to the modified Pfirrmann grading system [36] were generally regarded as healthy. NP tissues were washed three times with phosphate-buffered saline (PBS, Gibco, Grand Island, NY, USA), cut into 2–3 mm^3 fragments and enzymatically digested for 6–8 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) with 0.25 mg/ml type II collagenase (Invitrogen, Carlsbad, CA, USA). The filtered NP cells were then resuspended in DMEM/F12 containing 15% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Invitrogen) and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After identified using fluorescently labeled antibody for NP cell markers (CD24, KRT18, Abcam), the second-passage cells were used for subsequent experiments.

For in vitro experiments, NP cells were exposed to various concentrations of H_2O_2 (0, 100, 200, 300, 400, 500 μM) for 24 h to induce oxidative stress, or were pretreated with indicated concentrations of berberine alone or in combination with 4-PBA (200 μM), 3-MA (10 μM), or BAPTA-AM (10 μM) for 2 h prior to treatment with 300 μM H_2O_2 for a further 24 h.

2.4. Cell viability assay

Viability of NP cells was detected using the Cell Counting Kit-8 (CCK-8, CK04, Dojindo, Japan) assay according to the manufacturer's instructions. Briefly, 1×10^4 cells/well were seeded in 96-well plates and treated as described as above. Then, CCK-8 solution (10 μl) was added to each well, and the cells were further cultured for 4 h at 37 °C. The absorbance of each well was measured at 450 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

2.5. Lactate dehydrogenase (LDH) release assay

Release of lactate dehydrogenase (LDH) in culture medium was used to determine NP cell damage by H_2O_2 (300 μM) with different concentrations of berberine (0, 1, 2, 4, 8 μM). LDH release was measured using the lactate dehydrogenase assay kit (A020-1, Jiancheng Bioengineering institute, Nanjing, China) according to the manufacturer's instructions.

2.6. Cell apoptosis detection

NP cells from each treatment group were labeled by double staining using an AnnexinV-FITC/PI Apoptosis Detection Kit (KeyGEN, Nanjing, China) as described previously [35]. After labeling, all samples were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed. Early apoptotic cells were annexin V+/PI−, and late apoptotic cells were annexin V+/PI+. The apoptotic cells (early stage and late stage) were counted, and the results were expressed as a percentage of the total cell count.

Apoptotic cells were also identified by the terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL)

method. Briefly, NP cells were fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 25 min, incubated with 0.1% Triton X-100 for 10 min and washed thrice with PBS at every step. Cells were stained using an in situ cell death detection Kit (12156792910; Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Fluorescence images were finally obtained using a fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.7. Western blot analysis

After intervention, cells were harvested and lysed using modified RIPA buffer (Beyotime, Shanghai, China) supplemented with 1 mmol/l of PMSF. Protein concentrations were measured using the Enhanced BCA Protein Assay Kit (Beyotime). Proteins (40 μg) from each sample were electrophoresed on 4–20% precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated overnight with primary antibodies diluted with antibody dilution buffer (P0023A, Beyotime) at the indicated concentrations at 4 °C: cleaved-caspase 3 (1:1000), Bax (1:1000), Bcl-2 (1:1000), GRP78 (1:1000), CHOP (1:1000), caspase12 (1:1000), Beclin-1 (1:1000), LC3 (1:500), p62 (1:1000), and GAPDH (1:10,000). This was followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Abcam) for 2 h at 37 °C. Immunoreactivity was visualized using an ECL chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA). Target molecule protein expression was normalized to the GAPDH levels.

2.8. Immunofluorescence assay

Cells were mounted on coverslips, washed thrice with PBS, fixed with 4% formaldehyde for 15 min at 37 °C, permeabilized with 0.5% (v/v) Triton X-100 for 20 min, and blocked with 3% bovine serum albumin (BSA) for 30 min. The cells were then incubated overnight at 4 °C with a primary antibody against GRP78 (1:100) and CHOP (1:100) followed by Cy3-conjugated goat anti-rabbit IgG antibody (1:100; Abcam) for 2 h at room temperature, and were then labeled with diamidino-2-phenylindole (DAPI; Beyotime) for 5 min. Finally, three fields of each slide were randomly chosen for microscopic observation with a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Transmission electron microscopy (TEM)

To determine the formation of autophagosomes and autolysosomes, cells were fixed overnight in 2.5% (vol/vol) glutaraldehyde, post-fixed in 2% osmium tetroxide for 3 h at room temperature. After washing, specimens were dehydrated with graded ethanol series, followed by infiltrating and embedding in epoxy resin (SPI-Chem, #90529-77-4). Ultra-thin sections were obtained and stained with saturated uranyl acetate–lead citrate and observed using a transmission electron microscope (Jeol, Tokyo, Japan).

2.10. Cytosolic calcium measurement

Cytosolic Ca^{2+} concentration was determined using the specific Ca^{2+} sensitive dye Fluo3-AM (Beyotime) according to the manufacturer's instructions. Briefly, after the indicated treatment, Fluo3-AM (containing 0.02% Pluronic F-127) was added to the culture at the final concentration of 5 μM. The cells were then incubated in the dark for 30 min at 37 °C. After incubation, the cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS) (without Ca^{2+} and Mg^{2+}) by centrifugation. Finally, the cells were resuspended in D-PBS to a volume of 500 μl and analyzed by flow cytometry (BD Biosciences).

2.11. Surgical procedure

To further evaluate the protective role of berberine against IVD degeneration in vivo, a simple annulus fibrosus (AF) puncture rat model was developed as described previously [35,37]. Briefly, 24 female Sprague–Dawley rats (three months old) were obtained from the Laboratory Animal Center of Huazhong University of Science and Technology (Wuhan, China) and were randomly divided into three groups: sham-operated group (control group), puncture operated group, and combination of puncture operation and berberine injection groups. After the rats were weighed and anesthetized with 2% (w/v) pentobarbital (40 mg/kg), the experimental level (Co7/8) was located by digital palpation on the coccygeal vertebrae and confirmed by counting the vertebrae from the sacral region in a trial radiograph. The tail skin was sterilized and the tail vertebral disc of Co7/8 was punctured though the tail skin, parallel to the end plates with a 27-gauge sterile needle from the lateral side of the tail and was held in position for 30 s before extraction. The depth of the needle was about 4 mm from the skin according to a preliminary experiment. Berberine was diluted with normal saline and injected intraperitoneally after surgery at a dose of 150 mg/kg/d, whereas the degeneration group was administered an equal amount of normal saline daily until the rats were sacrificed. All animals were allowed free unrestricted weight bearing and activity.

2.12. Magnetic resonance imaging

At 8 weeks after surgery, the rats were anesthetized and analyzed using a BioSpec MRI (Bruker, 7.0 T/20 cm). T2-weighted parameters were set as follows: a fast-spin echo sequence with a time to repetition of 2000 ms and a time-to-echo of 36 ms; a 256 (h) × 256 (v) matrix; a field of view 6.00/3.00 cm; a flip angle of 180°. Midsagittal T2-weighted images were taken to evaluate the signal and structural changes of the discs according to the Pfirrmann grading system [36], 1 point = Grade I, 2 points = Grade II, 3 points = Grade III, and 4 points = Grade IV. The rats were then euthanized and the discs were harvested for histological analysis and immunofluorescence analysis.

2.13. Histological staining immunofluorescence staining

Rat IVD specimens were decalcified and fixed in formaldehyde, dehydrated, and embedded in paraffin. The tissues were then cut into 4 μm sections and handled as described previously [38], midsagittal sections of each disc were stained with hematoxylin-eosin (HE) and safranin O-fast green (SO). The cellularity and morphology of the nucleus pulposus was examined using a microscope, and evaluated using a grading scale, as described previously [37,39]. The histologic score was 5 for normal discs, 6–11 for moderately degenerated discs, and 12–14 for severely degenerated discs.

For immunofluorescence examination, sections embedded in paraffin were deparaffinized, rehydrated and blocked with endogenous peroxidase in hydrogen peroxide; the antigens were then retrieved in citrate buffer, and incubated overnight with primary antibody against CHOP (1:100) at 4 °C, washed thoroughly, and labeled for 30 min at 37 °C with secondary antibody (1:400; Invitrogen).

2.14. Statistical analysis

Statistical analyses were performed using IBM SPSS 25.0 version (IBM Cor., Armonk, NY). Quantitative data were presented as the mean ± standard deviation (SD) of at least three independent experiments. Statistical significance was determined using Student's *t*-test and/or one-way analysis of variance (ANOVA) followed by Tukey's test for comparison between experiment groups. Nonparametric data (Pfirrmann scores and histological scores) were analyzed by the Kruskal–Wallis test. p values < 0.05 were considered statistically significant.

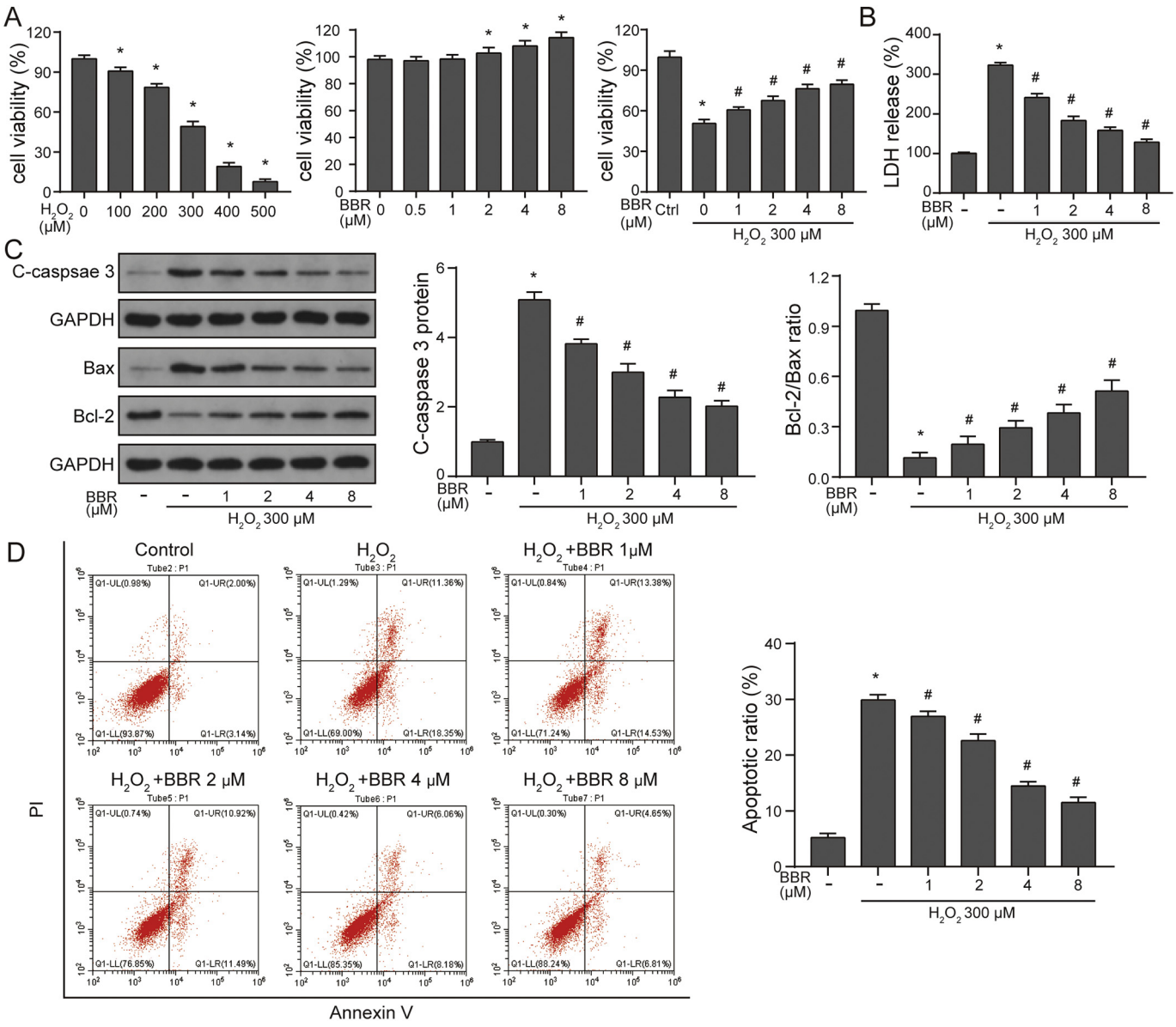

Fig. 1. The protective effect of berberine against H_2O_2-induced viability decline and apoptosis in NP cells. NP cells were treated with various concentrations of H_2O_2 or berberine alone, or exposed to H_2O_2 (300 μM) with various concentrations of berberine pretreatment for 24 h. (A) Cell Counting Kit-8 (CCK-8) results for cell viability after indicated treatments. (B) LDH release assay results for cell damage profiles. (C) Representative western blotting assay and quantitation of cleaved-caspase 3, Bax, and Bcl-2 levels; GAPDH expression was used as the protein loading control. (D) Representative dot plot and quantitation results of apoptosis evaluated by flow cytometric analysis after Annexin-V/PI double staining. The quantitative values are expressed as mean ± SD from at least three independent experiments, *$p < 0.05$ versus control, $^{\#}p < 0.05$ versus H_2O_2 treatment.

3. Results

3.1. Berberine protects against oxidative stress-induced apoptosis in NP cells

First, to evaluate the cytotoxicity of H_2O_2 and berberine in human NP cells, the CCK-8 assay was used to determine cell viability. As shown in Fig. 1A, the viable cells were significantly decreased after treatment with different concentrations of H_2O_2 for 24 h and the LC_{50} (50% lethal concentration) of H_2O_2 was approximately 300 μM, Thus, 300 μM of H_2O_2 was used for all the following experiments. Meanwhile, berberine showed no cytotoxicity in NP cells at concentrations < 8 μM and exhibited protective effects against H_2O_2-induced cellular mortality in a concentration-dependent manner (Fig. 1A). The LDH release assay also verified the protective effect of berberine against H_2O_2-induced cytotoxicity in NP cells.

Next, to investigate the effect of berberine against the apoptosis response of NP cells elicited by oxidative stress, apoptosis-related protein expression of cleaved-caspase 3, Bax, and Bcl-2 was determined by western blot. As shown in Fig. 1B, pre-treatment of NP cells with berberine, followed by co-incubation with H_2O_2 could reduce the amount of pro-apoptotic cleaved-caspase 3 and Bax, in a concentration-dependent manner; likewise, the amount of anti-apoptotic Bcl-2 was observed to be markedly increased. Furthermore, flow cytometric analysis using Annexin V-FITC/PI double staining also showed that pretreatment with berberine markedly inhibits H_2O_2-induced NP cell apoptosis in a concentration-dependent manner. Collectively, these data indicate that berberine could markedly attenuate H_2O_2-triggered apoptosis in NP cells.

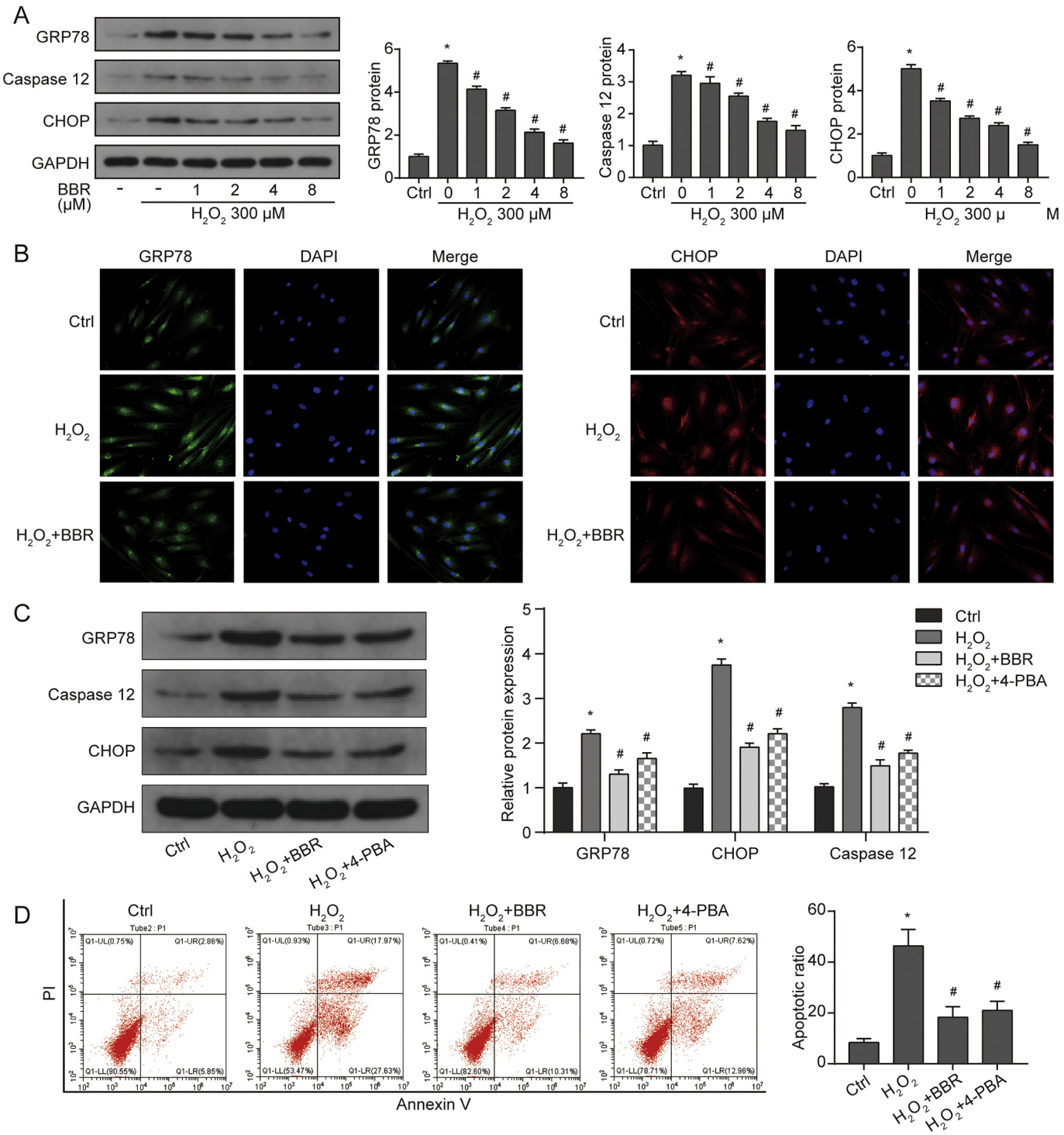

Fig. 2. Berberine downregulates ER stress triggered by H_2O_2 in NP cells. NP cells were exposed to H_2O_2 (300 μM) with various concentrations of berberine pretreatment for 24 h, or treated with H_2O_2 (300 μM) in the presence or absence of berberine or 4-PBA (ER stress specific inhibitor) for 24 h. (A) Representative western blotting images and quantitation of ER stress related molecules GRP78, caspase12, and CHOP; GAPDH expression was used as the protein loading control. (B) The representative GRP78 and CHOP expression was detected by the immunofluorescence combined with DAPI staining for nuclei (original magnification: ×400). (C) Representative western blotting images and quantitation of GRP78, caspase12, and CHOP. (D) Representative dot plot and quantitation results of apoptosis evaluated by flow cytometric analysis after Annexin-V/PI double staining. The quantitative values are expressed as mean ± SD from at least three independent experiments, *$p < 0.05$ versus control, #$p < 0.05$ versus H_2O_2 treatment.

3.2. Berberine attenuates oxidative stress-induced apoptosis through downregulation of ER stress in NP cells

ER stress activation has been implicated in oxidative stress-induced cell death and the process of intervertebral disc degeneration [14,17,18]. To investigate the underlying mechanisms of berberine against oxidative stress-induced apoptosis, NP cells were pretreated with different concentrations of berberine followed by coincubation with 300 μM H_2O_2 for 24 h. The ER stress sensor GRP78, and the ER-resident apoptotic related molecules caspase12 and CHOP were

detected by western blotting. The data showed that H_2O_2 treatment significantly elevated the protein levels of GRP78, caspase12, and CHOP, and that these effects of H_2O_2 were markedly attenuated by berberine pretreatment in a concentration-dependent manner (Fig. 2A). Additionally, immunofluorescence staining for GRP78 and CHOP was performed in NP cells treated with H_2O_2 for 24 h in the presence or absence of berberine, which confirmed that berberine could markedly attenuate ER stress elicited by H_2O_2 in NP cells (Fig. 2B).

To verify the assumption that berberine alleviates oxidative damage in cultured NP cells by inhibiting ER stress-mediated cell death, the ER stress specific inhibitor, 4-PBA was introduced. NP cells were treated with berberine or 4-PBA prior to H_2O_2 exposure. As shown in Fig. 2C, both berberine and 4-PBA markedly attenuated the protein levels of GRP78, caspase12, and CHOP. Consistently, the data of flow cytometric analysis for Annexin V-FITC/PI staining revealed that H_2O_2 induced-apoptosis was significantly deceased in the presence of berberine or 4-PBA (Fig. 2D). Taken together, these data indicate the possibility that suppression of ER stress may be involved in berberine -mediated NP cell protection upon oxidative stress.

3.3. Berberine attenuates oxidative stress-induced apoptosis through downregulation of autophagy in NP cells

It has been well established that oxidative stress can trigger autophagy response activation in NP cells, which may act as a protective or detrimental mechanism in cell survival [22,40]. To further understand the mechanisms of autophagy in berberine-mediated protective effects in NP cells upon oxidative stress elicited by H_2O_2, we assessed the levels of autophagy protein LC3, regulatory protein Beclin1, and adapter protein p62 by western blot. As shown in Fig. 3A, levels of LC3-I convert to LC3-II and Beclin1 were significantly upregulated upon H_2O_2 treatment, which was markedly decreased by berberine pretreatment in a concentration-dependent manner, along with increased levels of p62. Additionally, autophagosomes and autolysosomes were detected by TEM, which is a standard method to check autophagy activation, and the results revealed that the amount of autophagosomes and autolysosomes in the H_2O_2 + berberine group were markedly reduced compared to those in the H_2O_2-treated group (Fig. 3B). These results indicate that autophagy triggered by H_2O_2 was inhibited by berberine administration in NP cells.

Furthermore, to examine the role of autophagy in berberine-induced protective effects against apoptosis in NP cells, the cells were pretreated with the autophagy inhibitor 3-MA and berberine. Western blot results showed that the LC3-II/LC3-I ratio and Beclin-1 expression stimulated by H_2O_2 were significantly decreased, whereas the amount of p62 was significantly restored (Fig. 3C). Moreover, we also detected the expression of apoptosis-related proteins including cleaved-caspase 3, Bax, and Bcl-2. As expected, the expression of cleaved-caspase 3 and Bax was markedly declined, along with increased expression of Bcl-2 after 3-MA or berberine pretreatment in NP cells (Fig. 3D), indicating that suppression of autophagy could protect the cells from apoptosis. In addition, TUNEL assay results showed that H_2O_2-mediated apoptotic incidence was markedly attenuated in both berberine or and 3-MA pretreated cells (Fig. 3E). Therefore, these results indicate that berberine may protect NP cells from H_2O_2-induced cell death via downregulation of autophagy.

3.4. IRE1/JNK pathway is involved in ER stress-induced autophagy in NP cells

Having determined the involvement of ER stress and autophagy in oxidative stress-induced cell damage, we next sought to examine the correlation between ER stress and autophagy in H_2O_2 treated NP cells and focused on IRE1/JNK pathway, which was shown to be essential for ER stress-induced autophagy [41,42]. The protein expression levels of p-IRE1, IRE1, p-JNK, JNK were evaluated by western blotting. As shown in Fig. 4A, the protein levels p-IRE1/IRE1and p-JNK/JNK were obviously increased after H_2O_2 treatment compared with the control group, whereas they were decreased by berberine pretreatment, as well as 4-PBA, DBSA (specific inhibitor of IRE1) and SP600125 (specific inhibitor of JNK). To further investigate the role of IRE1/JNK pathway in ER stress-induced autophagy in NP cells, the cells were pretreated with the ER stress inhibitor 4-PBA, or DBSA and SP600125; western blot results showed that the elevated protein levels of LC3-II/LC3-I ratio and beclin1 were significantly attenuated in the presence of berberine, 4-PBA, DBSA and SP600125 (Fig. 4B). Meanwhile, the H_2O_2-induced reduction of p62 was also partially reversed in the berberine, 4-PBA, DBSA, and SP600125 groups (Fig. 4B). These results further suggest that the IRE1/JNK pathway may play an important role in the activation of ER stress dependent-autophagy.

3.5. Ca^{2+} deregulation correlates with oxidative stress-mediated ER stress and autophagy activation in NP cells

Having determined the involvement of ER stress and autophagy in oxidative stress-mediated apoptosis, we next sought to examine the link between ER stress and autophagy. Orchestrating intracellular Ca^{2+} homeostasis is the prerequisite for multiple cellular physiological activities and disturbance of intracellular Ca^{2+} homeostasis can induce ER stress, autophagy and apoptosis [43]. To further characterize the role of Ca^{2+} homeostasis in oxidative stress-mediated ER stress and autophagy, intracellular Ca^{2+} levels were evaluated using Fluo-3AM by flow cytometric analysis. As shown in Fig. 5A, the results demonstrated that cytosolic Ca^{2+} levels were obviously increased after H_2O_2 treatment compared with the control group, whereas it could be markedly blocked by berberine and BAPTA-AM (Ca^{2+} chelator) pretreatment, meanwhile, 4-PBA and 3MA were also found to partially ameliorate H_2O_2-mediated cytosolic Ca^{2+} elevation (Fig. 5A). Additionally, NP cells pretreatment with BAPTA-AM significantly attenuated the upregulation of GRP78, caspase 12, and CHOP elicited by H_2O_2 (Fig. 5B). Meanwhile, chelation of Ca^{2+} with BAPTA-AM also markedly decreased the autophagic activity characterized as enhanced LC3-I to LC3-II conversion, beclin1 expression and p62 degradation observed with H_2O_2 treatment (Fig. 5C). Altogether, these results indicate that the cytosolic Ca^{2+} deregulation mediated by H_2O_2 participates in the induction of ER stress and autophagy, and berberine prevents H_2O_2-mediated cell damage possibly through inhibiting the intracellular Ca^{2+} homeostasis perturbation.

3.6. Berberine treatment partially ameliorates IVD degeneration in vivo

To further investigate the therapeutic efficacy of berberine in vivo, we established a tail disc percutaneous needle puncture animal model in Sprague-Dawley rats as described previously. The degeneration level of rat IVD was determined at 8 weeks after puncture by MRI and was quantified by Pfirrmann MRI grade scores. As shown in Fig. 6A, stronger T2-weighted signal intensities were observed in the berberine-treated group than those in the IVD degeneration group, and Pfirrmann MRI grade scores were also found to be significantly lower in the berberine -treated group than those in the IVD degeneration group. Moreover, HE and SO staining was performed to assess the general histological structure of NP tissues, As shown in Fig. 6B and C, progressive degenerative changes including decreased size of the NP, annular layer disorganization, narrowed disc space, and inward bulging of the inner annulus was confirmed in the IVD degeneration group, as expected, which was markedly attenuated by berberine administration. The histologic score were assessed according to morphologic changes including morphology of NP and AF, cellularity of NP and AF, and border between NP and AF [37], and the results showed that the score of berberine group was significantly lower than that of the IVD degeneration group. Moreover, as the immunofluorescence data shown in Fig. 6D, CHOP was robustly up-regulated in IVD degeneration group,

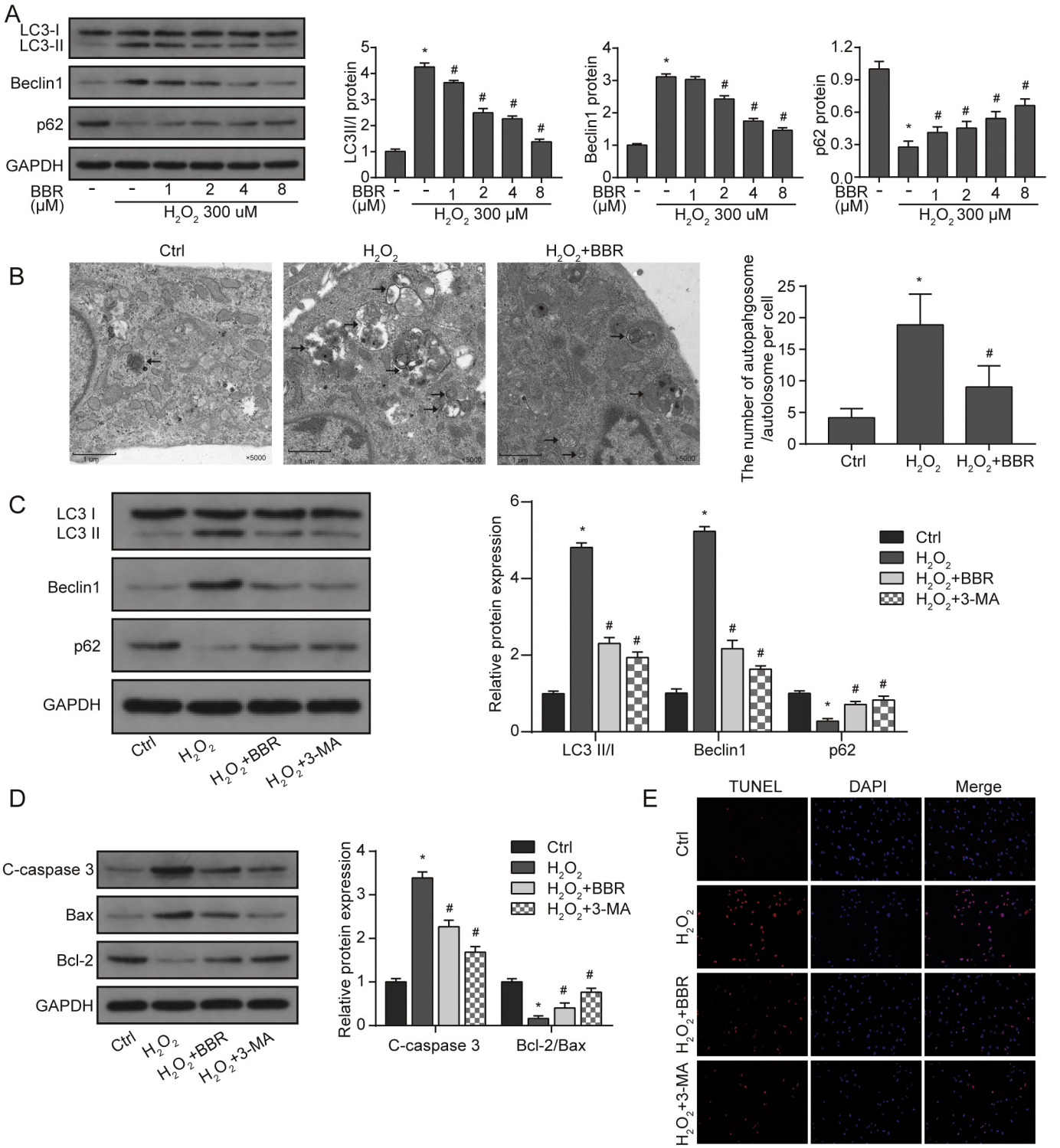

Fig. 3. Berberine attenuates autophagy triggered by H_2O_2 in NP cells. NP cells were exposed to H_2O_2 (300 μM) with various concentrations of berberine pretreatment for 24 h, or were treated with H_2O_2 (300 μM) in the presence or absence of berberine or 3-MA (autophagy specific inhibitor) for 24 h. (A) Representative western blotting images and quantitation of the level of autophagy related molecules such as LC3-I, LC3-II, Beclin1, and p62; GAPDH expression was used as the protein loading control. (B) Transmission electron microscopy results for autophagosomes and autolysosomes (as indicated by black arrow), magnification: ×5000, scale bar: 1 μm. (C) Representative western blotting images and quantitation of LC3-I, LC3-II, Beclin1, and p62 levels. (D) Representative western blotting images and quantitation of cleaved-caspase 3, Bax, and Bcl-2 levels. (E) Representative fluorescence images with TUNEL staining for apoptotic NP cells (original magnification: ×200). The quantitative values are expressed as mean ± SD from at least three independent experiments, *$p < 0.05$ versus control, $^{\#}p < 0.05$ versus H_2O_2 treatment.

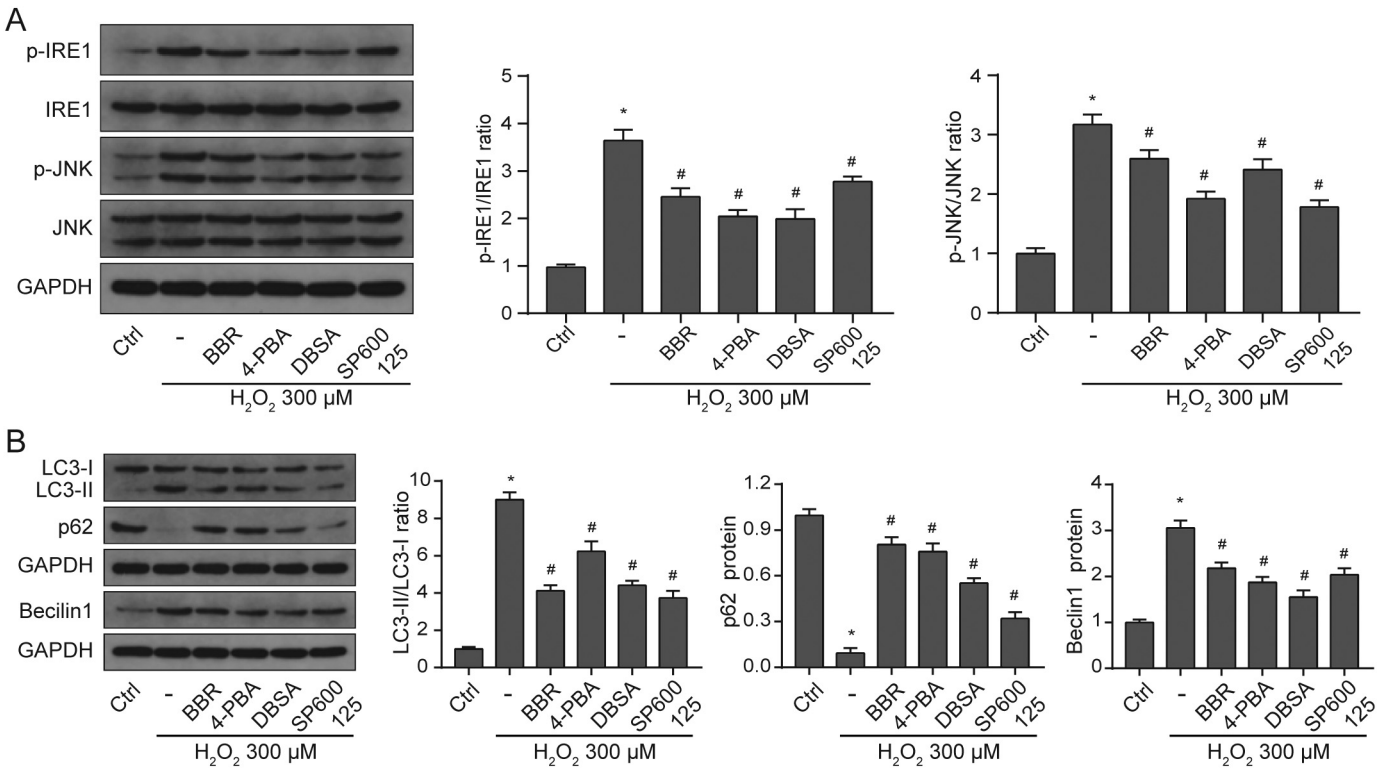

Fig. 4. The IRE1/JNK pathway is involved in the induction of ER stress-dependent autophagy in NP cells. NP cells were treated with H_2O_2 (300 μM) in the presence or absence of berberine, DBSA (specific IRE1 inhibitor), SP600125 (specific JNK inhibitor) or 4-PBA for 24 h. (A) Representative western blotting images of p-IRE1, IRE1, p-JNK and JNK; GAPDH expression was used as the protein loading control; the ratio of p-IRE1/IRE1 and p-JNK/JNK were quantified. (B) Representative western blotting images and quantitation of LC3-I, LC3-II, beclin1 and p62 levels. The quantitative values are expressed as the mean ± SD from at least three independent experiments, *$p < 0.05$ versus control, $^{\#}p < 0.05$ versus H_2O_2 treatment.

while BBR administration could alleviate these responses. We further performed TUNEL staining in rats IVD specimens, as shown in Fig. 6E, the positive ratio of TUNEL staining was markedly increased in IVD degeneration group compared to that of control group, which was attenuated by BBR administration. Taken together, these results demonstrate that berberine could effectively retard the degenerative process of IVD in rats.

4. Discussion

Oxidative stress resulting from the imbalance of ROS production and clearance has been implicated in the pathogenesis of IVD degeneration by promoting NP cell death and impeding ECM metabolism [8,10]. Given the potential effect of berberine against inflammation and oxidation [30,44], we aimed to investigate the role of berberine in NP cell apoptosis elicited by oxidative stress. Our results demonstrated that berberine treatment could concentration-dependently mitigate H_2O_2-mediated apoptosis by modulating ER stress and autophagy. Cytosolic Ca^{2+} deregulation triggered by oxidative stress was involved in the induction of ER stress and autophagy (Fig. 7). In addition, the in vivo study indicated that berberine may play a protective role in a puncture-induced IVD degeneration rat model.

The ER is a subcellular compartment that governs protein quality control in the secretory pathway to prevent protein aggregation and misfolding, which is highly sensitive to changes in intracellular homeostasis and extracellular stimuli including Ca^{2+} homeostasis, energy stores, and redox state. The accumulation of unfolded and misfolded proteins in the ER lumen activates ER stress attempting to strengthen the capacity for protein folding and modification, and attenuate global mRNA translation. However, excessive and/or prolonged ER stress switches toward apoptotic cell death rather than survival via activation of the downstream signals, CHOP and caspase 12 [14,45,46]. Many studies have shown that activation of the ER stress-induced apoptosis pathway contributes to various degenerative diseases such as neurodegenerative diseases [47], osteoarthritis [48], and IVD degeneration [18]. In addition, berberine was revealed to have a profound therapeutic potential for targeting ER stress in multiple diseases [34,49,50]. In the present study, we confirmed that berberine could markedly inhibit ER stress and subsequent ER stress-dependent apoptosis triggered by oxidative stress stimuli in NP cells.

Autophagy is an evolutionarily conserved lysosomal activity to degrade and turnover misfolded proteins and damaged cytoplasmic organelles upon encountering stress conditions such as hypoxia, nutrient deprivation, ER stress, and oxidative stress [20]. The role of autophagy in diseases is dual, and predominantly appears to be a cytoprotective process preventing the accumulation of toxic aggregates and damaged organelles and maintains cellular homeostasis. However, autophagy could also eventually change to a pro-apoptosis process resulting from excessive lysosomal degradation of cell constituents under severe and persistent stress conditions [51]. Livingston et al. verified the critical role of persistently activated autophagy in proximal renal tubular cell apoptosis [52]. Additionally, the interplay between autophagy and IVD degeneration is complex and controversial. Either higher or lower levels of autophagy were observed in degenerative IVD cells, depending on the cell type, environment, and stimulus [26,40]. Li et al. demonstrated that autophagy was increased in human degenerative discs and could attenuate compression-induced apoptosis of human NP cells via MEK/ERK/NRF1/Atg7 signaling pathways [28]. Chen et al. revealed that inhibition of ERK-dependent autophagy markedly reduced the mitochondria-mediated apoptosis in the NP cells under oxidative stress [25]. In the present study, we observed significant increased LC3-I to LC3-II conversion and p62 degradation under oxidative stress using H_2O_2 (300 μM), indicating the increase of autophagy activity. Meanwhile, down-regulation of autophagy with 3-MA or berberine markedly

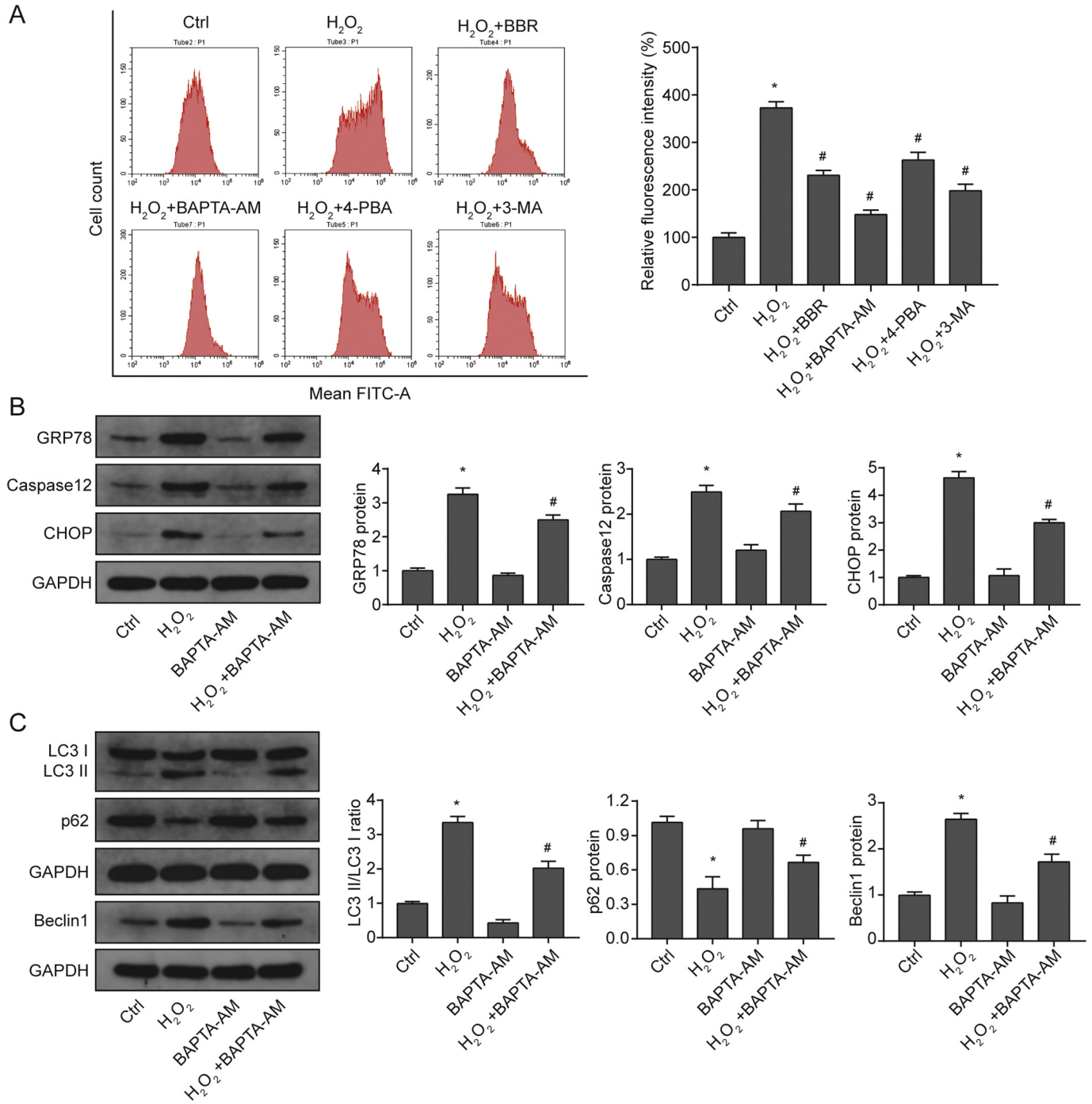

Fig. 5. Ca^{2+} deregulation is involved in induction of ER stress and autophagy elicited by H_2O_2 in NP cells. NP cells were treated with H_2O_2 (300 μM) in the presence or absence of berberine, BAPTA-AM (cytosolic Ca^{2+} chelator), 4-PBA or 3-MA for 24 h. (A) Variation of intracellular Ca^{2+} was detected using Fluo-3AM by flow cytometric analysis, results were normalized in ratio to the untreated control. (B) Representative western blotting images and quantitation of GRP78, caspase 12, and CHOP levels. (C) Representative western blotting images and quantitation of LC3-I, LC3-II, beclin1 and p62 levels. The quantitative values are expressed as the mean ± SD from at least three independent experiments, *$p < 0.05$ versus control, #$p < 0.05$ versus H_2O_2 treatment.

alleviated apoptosis elicited by oxidative stress in human NP cells. Thus, we suggested that berberine might be an effective strategy to reduce cell apoptosis and delay the process of disc degeneration via control and regulation of autophagy activity. However, the exact mechanisms that excessive autophagic activation leads to cell damage warrant further investigation.

It is well established that autophagy has a close functional relationship with ER stress, Induction of autophagy in response to ER stress acts as an alternative degradation mechanism for unfolded/misfolded proteins and attempts to maintain intracellular homeostasis. Inversely, in case prolonged ER stress can't be relieved, the protective effect of autophagy may switch to cell death [53,54]. Furthermore, the IRE1/JNK pathway has been implicated in ER stress-dependent autophagy activation. IRE1 is one of the transmembrane ER stress sensors that exerts powerful effects on cell fate via homo-oligomerization and autophosphorylation under stress conditions, and could activate autophagy in a JNK-dependent manner [54–56]. Imaizumi et al. revealed that thapsigargin/tunicamycin-induced accumulation of LC3-positive

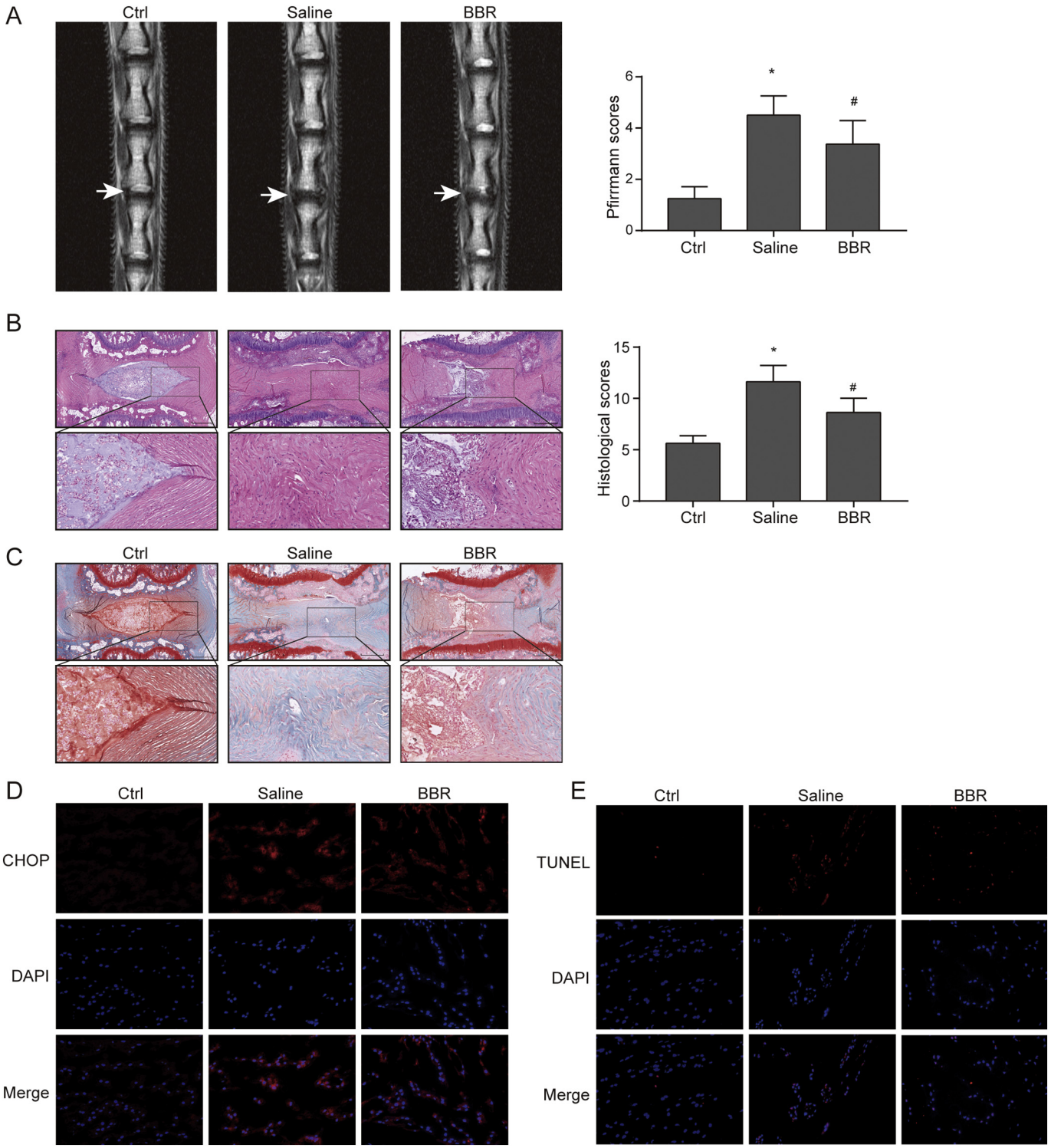

Fig. 6. Berberine ameliorates IVD degeneration in rats in vivo. (A) Representative T2-weighted MRI images of a rat tail with a needle-punctured disc at 8 weeks after surgery (white arrow: the operated level); degeneration levels were assessed using Pfirrmann MRI grade scores. (B and C) Representative HE and SO staining images of the midsagittal sections of IVD specimens and corresponding histological scores (original magnification ×25, scale bar: 500 μm). (D) The representative CHOP expression was detected by the immunofluorescence combined with DAPI staining for nuclei in rat IVD specimens (original magnification: ×400). (E) Representative fluorescence images with TUNEL staining for apoptotic NP cells (original magnification: ×400) in rat IVD specimens. The quantitative values are expressed as mean ± SD (n = 8), *$p < 0.05$ versus control, $^{\#}p < 0.05$ versus the saline group.

vesicles was significantly inhibited in IRE1-deficient MEF cells or upon treatment with JNK inhibitor [57]. Feng et al. demonstrated that suppression of the IRE1/JNK signaling pathway could efficiently counteract ER stress-dependent autophagic cell damage induced by ischemia reperfusion [58]. In the present study, the expression of p-IRE1/IRE1 and p-JNK/JNK was notably increased under oxidative stress, indicating activation of the IRE1/JNK pathway. Moreover, oxidative stress induced LC3 conversion and p62 degradation was markedly inhibited by pharmacological inhibitors of ER stress and IRE1/JNK pathway, suggesting that the IRE1/JNK pathway plays an essential role

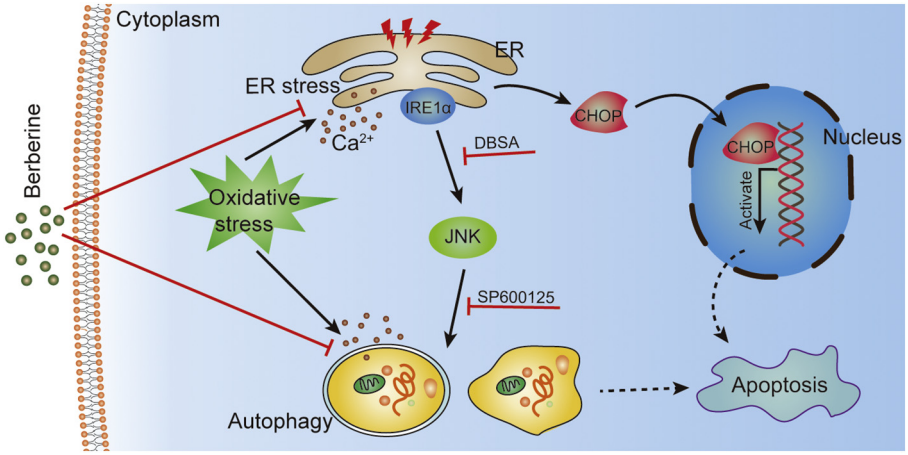

Fig. 7. Schematic illustration of the possible mechanisms of berberine-mediated prevention of oxidative stress-induced cell damage through modulating ER stress and autophagy in NP cells.

in inducing ER stress-dependent autophagy in NP cells challenged with oxidative stress.

Next, to further explore the potential mechanisms by which berberine inhibits ER stress response and autophagy elicited by oxidative stress in NP cells, we focused on Ca^{2+} signaling as Ca^{2+} is a ubiquitous intracellular signaling regulator and a key to the early step of elementary intracellular events including cell cycle, metabolism, proliferation, and apoptosis [43,59]. In turn, perturbation of intracellular Ca^{2+} homeostasis could lead to cell dysfunction including cell death. Previous studies have verified that Ca^{2+} dysregulation was tightly associated with ER stress and autophagy activation under stress conditions [60–62]. Indeed, our results revealed that sustained excessive elevation of cytosolic Ca^{2+} levels was triggered upon oxidative stress, and this effect was markedly inhibited by administration with berberine and the Ca^{2+} chelator, BAPTA-AM. In addition, the levels of ER stress and autophagy induced by oxidative stress were also markedly attenuated in the presence of BAPTA-AM, which support the involvement of Ca^{2+} in berberine's protective effects against ER stress and autophagic cell damage upon oxidative stress in NP cells. However, the upstream mechanisms and potential targets of berberine involved in Ca^{2+} deregulation still need further investigation.

However, this study has some limitations. First, since autophagy is generally considered as a cytoprotective process, the mechanisms underlying how oxidative stress-induced excessive autophagy promotes apoptosis are not fully elucidated. Meanwhile, whether other cell death pathways such as mitochondria-dependent apoptosis that are involved in oxidative stress-induced cell death were not investigated in the current study. Moreover, the potential upstream mechanisms and potential targets of berberine modulating oxidative stress mediated-Ca^{2+} deregulation still remain unclear.

In conclusion, our findings demonstrated a potent implication of ER stress and autophagy in apoptosis mediated by oxidative stress in NP cells, resulting from cytosolic Ca^{2+} dysregulation. Moreover, ER stress induced by oxidative stress was associated with autophagy activation via the IRE1/JNK pathway. Notably, our study provides evidence that treatment with berberine could prevent oxidative stress-mediated apoptosis by modulating Ca^{2+} deregulation, and consequently, ER stress and autophagy in NP cells, and could attenuate puncture-induced IVD degeneration in rats. Our findings provide preliminary evidences supporting berberine as a novel pharmacological treatment strategy for IVD degeneration.

Abbreviations

3-MA	3-methyladenine
DBSA	3, 5-dibromosalicylaldehyde
4-PBA	4-phenylbutyrate acid
AF	annulus fibrosus
ATF6	activating transcription factor 6
Ca^{2+}	calcium
CCK-8	Cell Counting Kit-8
CHOP	C/EBP homologous protein
JNK	c-Jun N-terminal kinase
DAPI	diamidino-2-phenylindole
ECM	extracellular matrix
ER	endoplasmic reticulum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRP78	glucose-regulated protein 78
H_2O_2	hydrogen peroxide
IVD	intervertebral disc
IRE1	inositol-requiring kinase 1
LDH	lactate dehydrogenase
NP	nucleus pulposus
PERK	pancreatic ER eIF2α kinase
PBS	phosphate-buffered saline
ROS	reactive oxygen species
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling
UPR	unfolded protein response

Conflicts of interest

All authors declare no conflicts of interest.

Author contributions

RJL and ZWL designed the research, performed experiments, and drafted the manuscript. YS, HPY, SFZ, and GCL helped with in vitro experiments and data analysis. LM, SDL, and SFZ participated in animal experiments and NP tissue collection. KW and SL assisted in revising the manuscript. YKZ and CY conducted the surgical operation and extensively reviewed and revised manuscript. All authors have read and approved the final manuscript.

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