Arch Biol Sci. 2022;74(2):135-145 https://doi.org/10.2298/ABS220310013W

Overexpression of BNIP3 in rat intervertebral disk cells triggers autophagy and apoptosis

Tingsheng Wu[1,2], Shaoyong Fan[2], Bin Zhang[2], Zhiqiang Tao[2], Hong Hua[2] and Min Dai[3,*]

[1]*School of Medicine, Nanchang University*, Nanchang, Jiangxi 330006, China
[2]*Department of Osteology, Nanchang Hongdu Hospital of Traditional Chinese Medicine*, Nanchang 330006, Jiangxi, China
[3]*Department of Osteology, the First Affiliated Hospital of Nanchang University*, Nanchang 330006, Jiangxi, China

***Corresponding author**: daiminqqq89@163.com

Preprint: The manuscript is available as a preprint at the Research Square with the following DOI: https://doi.org/10.21203/rs.3.rs-42040/v1.

Received: March 10, 2022; **Revised**: April 27, 2022; **Accepted**: April 29, 2022; **Published online**: May 4, 2022

Abstract: Excessive apoptosis of intervertebral disk cells and intervertebral disk degeneration (IDD) is the prime cause of low back pain. B-cell lymphoma 2 (Bcl-2) and adenovirus E1B 19 kDa interacting protein 3 (BNIP3), a member of the Bcl-2 family, are involved in cell autophagy and apoptosis. The roles and mechanisms of BNIP3 in intervertebral disk cell autophagy and apoptosis are unclear. In this study, primary rat intervertebral disk cells were prepared to study the effect of BNIP3 overexpression on their autophagy and apoptosis. The cell counting kit (CCK)-8 assay showed that BNIP3 overexpression decreased cell viability. Real-time PCR and Western blotting showed that BNIP3 overexpression significantly upregulated the expression of autophagy-related proteins and pro-apoptotic proteins, including hypoxia-inducible factor-1α, apoptotic protease activating factor 1, caspase 3 and cleaved caspase 3, microtubule-associated proteins 1A/1B light chain 3 (LC3) and Beclin-1 while downregulating the expression of anti-apoptotic protein Bcl-2. Cell staining detection of autophagy and apoptosis showed that BNIP3 overexpression increased the autophagy and apoptosis of rat intervertebral disk cells. BNIP3 RNA interference revealed that the effects of BNIP3 overexpression can be reversed. These findings suggested that BNIP3 enhanced the autophagy and apoptosis in the rat intervertebral disk cells *in vitro*, which might promote IDD development.

Keywords: apoptosis; autophagy; BNIP3; intervertebral disk cell; intervertebral disk degeneration

INTRODUCTION

A common spinal disease manifesting as low back pain has become a serious world public health problem that greatly affects patients' physical and mental health with a heavy economic burden to society [1]. Although the detailed pathological mechanism of low back pain is elusive, intervertebral disk degeneration (IDD) is currently regarded as an imaportant cause of low back pain [2,3]. The occurrence and development of IDD are related to many factors, such as genetic factors, lifestyle habits (such as smoking or alcohol consumption) and aging. In particular, apoptosis of intervertebral disk cells, degradation of the extracellular matrix and cell phenotype alterations are considered direct causes of IDD [4]. Autophagy is a lysosome-dependent cell degradation pathway that exclusively exists in eukaryotic cells [5]. Autophagy, a type II programmed cell death pathway, promotes or inhibits apoptosis in different ways; studies have reported that autophagy plays a crucial role in IDD development [6,7].

Bcl-2 and adenovirus E1B 19 kDa interacting protein 3 (BNIP3) is closely related to apoptosis triggered by ischemia and hypoxia [8-10]. Existing studies suggest that hypoxia-inducible factor-1α (HIF-1α) is the primary regulatory protein of BNIP3 [11], so the expression of BNIP3 is largely upregulated under hypoxic conditions. BNIP3 not only directly regulates cell apoptosis but also indirectly affects cell survival and apoptosis by regulating the level of autophagy or mitophagy [12] Therefore, BNIP3 plays a vital role in ischemia or hypoxia-related diseases [13-15].

Recent studies have also shown that BNIP3 expression is positively correlated with the severity of IDD, that is, patients with severe IDD frequently exhibit increased expression of BNIP3 [16,17], suggesting that BNIP3 has an important role in the development of IDD. However, the detailed molecular mechanism of BNIP3 in IDD development is unclear.

In this study, the methods of overexpression and RNA interference (RNAi) were used to study the effect of BNIP3 on the autophagy and apoptosis in the isolated rat intervertebral disk cells. The aim of the study was achieved, and the findings of this study may help in the development of a novel therapeutic approach for IDD.

MATERIALS AND METHODS

Ethics statement

All procedures, care and handling of animals were approved by the Ethics Committee of Nanchang Hongdu Hospital of Traditional Chinese Medicine and conducted in compliance with the standard guidelines.

Animals

Twenty healthy adult Sprague-Dawley (SD) rats weighing 200-250 g were used. The animals were purchased from the Beijing Charles River Laboratory Animal Co., Ltd. The rats were housed in individual cages under a constant temperature of 23±2°C and had access to food and water *ad libitum* throughout the study.

Antibodies and reagents

The SuperSignal™ West Pico PLUS chemiluminescent substrate (34580) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The polyvinylidene fluoride (PVDF) membrane with 0.45-μm pore size (IPVH00010) was purchased from Merck Millipore (Darmstadt, Germany). The bicinchoninic acid (BCA) protein assay kit (CW0014S), HiFiScript first strand cDNA synthesis kit (CW2569M), UltraSYBR mixture (CW0957M), Ultrapure RNA extraction kit (CW0581M) and TRIzol reagent (CW0580S) were purchased from Cwbio Inc (Beijing, China). The mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (TA-08), horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) heavy and light chains (H+L) secondary antibody (ZB-2305), and HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (ZB-2301) were purchased from ZSGB-Bio Technologies Inc (Beijing, China). The rabbit anti-hypoxia-inducible factor 1-alpha (HIF-1α) polyclonal antibody (ab2185), rabbit anti-B-cell lymphoma 2 (Bcl-2) polyclonal antibody (ab59348), rabbit anti-apoptotic protease activating factor 1 (APAF-1) monoclonal antibody (ab32372), rabbit anti-cleaved caspase 3 polyclonal antibody (ab2302), and rabbit anti-Beclin-1 polyclonal antibody (ab62557) were purchased from Abcam (Cambridge, UK). The rabbit anti-microtubule-associated proteins 1A/1B light chain 3 (LC3) polyclonal antibody (bs-8878R) was purchased from Biosynthesis Inc (Beijing, China). The cell autophagy staining test kit (G0170) and acridine orange staining solution (CA1143) were purchased from Solarbio Life Sciences (Beijing, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation and culture of the rat intervertebral disk cells

Twenty SD rats were used to isolate the intervertebral disk cells. In brief, the rats were anesthetized and euthanized by intraperitoneal injection of sodium pentobarbital (45 mg/kg), and the spine was isolated under aseptic conditions, following removal of ligaments and nucleus pulposus around the spine. The isolated spine was cut into small blocks with a size of 1 mm^3. After digestion with 0.25% trypsin for 20 min and 0.2% type II collagenase digestion for 2 h, the digested cells were collected by passing through a cell sieve and cultured in the Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (F12) medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin under an atmosphere of 5% CO_2 at 37 °C. The cultured medium was changed every 2-3 days. When the degree of cell fusion reached more than 80%, the cells were digested at a ratio of 1:2 for passage. Then, the second-generation cells were taken for detection by toluidine blue staining.

Toluidine blue staining

Cultured rat intervertebral disk cells were grown to the second generation and inoculated on 6-well plates. After the removal of the medium, the cells were washed with phosphate buffered saline (PBS) for 5 min. Then, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Thereafter, fixation solution was removed and the cells were stained with toluidine blue dye for 2 min. Subsequently, the staining solution was removed, and the cells were washed with ultrapure water for 3 min. The stained and washed cells were then air dried and observed under an optical microscope.

Experimental groups

The grouping was random as follows: (i) blank control (blank) group: Normal rat intervertebral disk cells; (ii) overexpression control (oeControl) group: Cells transfected with overexpression empty vector; (iii) BNIP3 overexpression (oeBNIP3) group: Cells transfected with BNIP3 overexpression vector; (iv) interference control (siControl) group: Cells transfected with RNAi empty vector; (v) BNIP3 interference (siBNIP3) group: Cells transfected with BNIP3 interference vector.

Overexpression and RNAi assay

To study the role of BNIP3 in the autophagy and apoptosis of the rat intervertebral disk cells, overexpression and RNAi were performed as previously described [18]. Briefly, the rat intervertebral disk cells were placed on 6-well tissue culture plates and grown to 60% confluence prior to transfection. The cells were transfected with 4 μg of plasmids (recombinant BNIP3-pcDNA 3.1 vector and pcDNA 3.1 empty vector) for 48 h. Lipofectamine 3000 transfection reagent was used. During the RNAi experiments, the cells were grown to 40%-50% confluence prior to transfection. Recombinant BNIP3 silenced plasmid sh-BNIP3-pLVshRNA (siBNIP3) and control vector (siControl) were transfected into cells using the Lipofectamine 3000 transfection reagent for 48 h. After BNIP3 overexpression and RNAi, the expression of BNIP3 mRNA in the rat intervertebral disk cells was detected by real-time polymerase chain reaction (qPCR).

Cell viability determination by the cell counting kit-8 (CCK-8) method

The CCK-8 method was employed to determine the viability of the rat intervertebral disk cells after transfection with the BNIP3 overexpression vector or the interference vector. Briefly, the transfected cells were placed in 96-well plates, and 150 μL of freshly prepared toxicity test solution containing 10 μL CCK-8 was added to each well and cultured for 4 h. The absorbance at 450 nm after 0, 12, 24 and 72 h was measured with a microplate reader. The cell viability assay was conducted three times, and data were presented as the means±SD. The cell growth inhibition rate was calculated as follows:

$$\%=(OD_{450}\text{Control}-OD_{450}\text{Experiment})/OD_{450}\text{Control}\times 100\%.$$

The cell growth inhibition rate curve was drawn. The cell groups were on the abscissa, and the cell growth inhibition rate (%) was the ordinate.

Cell apoptosis determination by acridine orange staining

The transfected cells were collected and prepared to a cell suspension at a concentration of 1×10^6 cells/mL. Every 100 μL of the cell suspension was mixed with an acridine orange staining solution to a final concentration of 15 μg/mL, stained at room temperature in the dark for 20 min, placed on a glass slide, and observed under a fluorescent microscope.

Cell autophagy determination by the monodansylcadaverine (MDC) method

The transfected cells collected by trypsin digestion were washed with PBS and suspended in 1× wash buffer at a concentration of 1×10^6 cells/mL. A 90-μL cell suspension was taken and transferred to an Eppendorf tube. Approximately 10 μL of MDC dye was added to the cell suspension, mixed, and stained at room temperature in the dark for 45 min. The stained cells were collected by centrifugation. The cells were washed twice with 1× wash buffer and resuspended in a 100-μL collection buffer. The cell suspension was loaded on the slide and observed under a fluorescent microscope. The normal cells had a uniform yellow-green

fluorescence, while the chromatin of autophagic cells appeared condensed. The nuclei were fragmented into dots, staining into uneven, dense, deep-green particles.

Cell membrane potential detection by flow cytometry

The membrane potential of the transfected cells was detected by flow cytometry. Briefly, the transfected cells collected by trypsin digestion were washed twice with PBS and suspended in a JC-1 working buffer at a concentration of 1×10^6 cells/mL. The cell suspensions were incubated at 37°C for 20 min, centrifuged, collected and resuspended in 1× incubation buffer. Finally, the cells were analyzed by a flow cytometer.

Real-time polymerase chain reaction (qPCR)

qPCR was used to detect the expression of HIF-1α, Bcl-2, APAF-1, caspase 3, LC3 and Beclin-1 mRNA of the transfected rat intervertebral disk cells. In brief, the total RNA of the transfected cells was extracted using a commercial RNA extraction kit, and the RNA purity was determined by the ratio of A260/A280 and agarose gel electrophoresis. The total RNA was synthesized on the first cDNA strand with a cDNA synthesis kit. With the synthesized first-strand cDNA as a template, the specific primers for HIF-1α, Bcl-2, APAF-1, caspase 3, LC3 and Beclin-1 were designed as described [19] and used for qPCR (Supplementary Table S1). The qPCR reaction was as follows: 95 °C for 10 min, 95°C for 5 min, and 60°C for 1 min, 40 cycles. The qPCR reaction system was as follows: 20 μL contained 2 μL cDNA template, 0.4 μL (10 μM) primer F and 0.4 μL (10 μM) primer R, 10 μL SYBR Green solution and 7.2 μL ddH_2O. The data obtained after qPCR were calculated and analyzed by using the $2^{-\Delta\Delta CT}$ method.

Western blotting

Western blotting was used to detect the expression level of HIF-1α, Bcl-2, APAF-1, cleaved caspase 3, LC3 and Beclin-1 proteins of the transfected rat intervertebral disk cells. Briefly, the cells were collected, washed, and lysed by adding a radioimmunoprecipitation assay (RIPA) buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 1 mM $NaVO_4$, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10% pepstatin A, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) in an ice-bath for 20 min. The cell lysate was centrifuged at 9391 ×*g* for 15 min, and the supernatant obtained after centrifugation was the total protein. The protein concentrations were determined by the BCA method. The total proteins were loaded and separated by 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electrotransferred onto PVDF membranes. Subsequently, the PVDF membranes were blocked with 3% bovine serum albumin and incubated with rabbit anti-HIF-1α (1:1000 dilution), anti-Bcl-2 (1:1000 dilution), anti-APAF-1 (1:1000 dilution), anti-cleaved caspase 3 (1:1000 dilution), anti-LC3 (1:500 dilution), anti-Beclin-1 (1:1000 dilution) and mouse anti-GAPDH (1:2000 dilution) primary antibodies overnight at 4°C. After incubation, the PVDF membranes were washed with a Tris-buffered saline buffer containing 1% Tween-20 three times and incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:5000 dilution). Finally, the protein bands were visualized with the SuperSignal™ West Pico PLUS chemiluminescent substrate.

Statistical analysis

All experimental data were presented as the mean±SD. Each experiment was conducted at least three times independently. The statistical analysis of the data was performed using SPSS 17.0 software. One-way ANOVA was used to analyze the differences between more than two groups, and a non-paired Student's t-test was used to analyze the difference between two groups. $P<0.05$ was considered statistically significant.

RESULTS

Identification of cultured rat intervertebral disk cells

Primary rat intervertebral disk cells were prepared to study the role of BNIP3 in the autophagy and apoptosis of rat intervertebral disk cells. After the cultured cells were stained with toluidine blue, the cytoplasm and nucleus were stained blue and bluish violet, respectively. The cells under the light microscope were

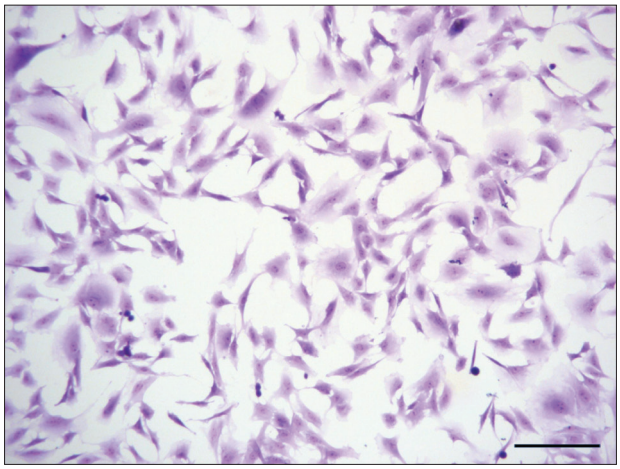

Fig. 1. Blue violet staining of the isolated rat intervertebral disk cells. Isolated cells were stained with a blue violet dye and observed under light microscopy (×100). The cells were dyed blue-purple and are presented as irregular polygons with some cells possessing long pseudopods. Scale bar=100 μm.

mostly spindly or irregular in shape, which met the morphological characteristics of the intervertebral disk cells (Fig. 1), suggesting that the isolated and cultured cells were the rat intervertebral disk cells.

BNIP3 overexpression decreased the viability of the rat intervertebral disk cells

The effect of BNIP3 on the viability of the rat intervertebral disk cells was examined by transfection. First, the transfection efficiency in the cells was estimated. The expression level of BNIP3 mRNA in the BNIP3 overexpression group (oeBNIP3) was significantly increased compared with the overexpression control group (oeControl) or blank group. Meanwhile, the expression level of BNIP3 mRNA in the BNIP3 interference group (siBNIP3) was significantly decreased compared with the interference control group (siControl) or blank group (Fig. 2A). Subsequently, the CCK-8 assay was used to detect the viability of the rat intervertebral disk cells. Our findings showed that the cell viability of the BNIP3 overexpression group was lower than in the overexpression control or blank group, even though the cell viability in all groups was increased with time, while the BNIP3 interference group had a higher cell viability than the interference control or blank group (Fig. 2B). These results suggested that BNIP3 negatively regulated the viability of the rat intervertebral disk cells.

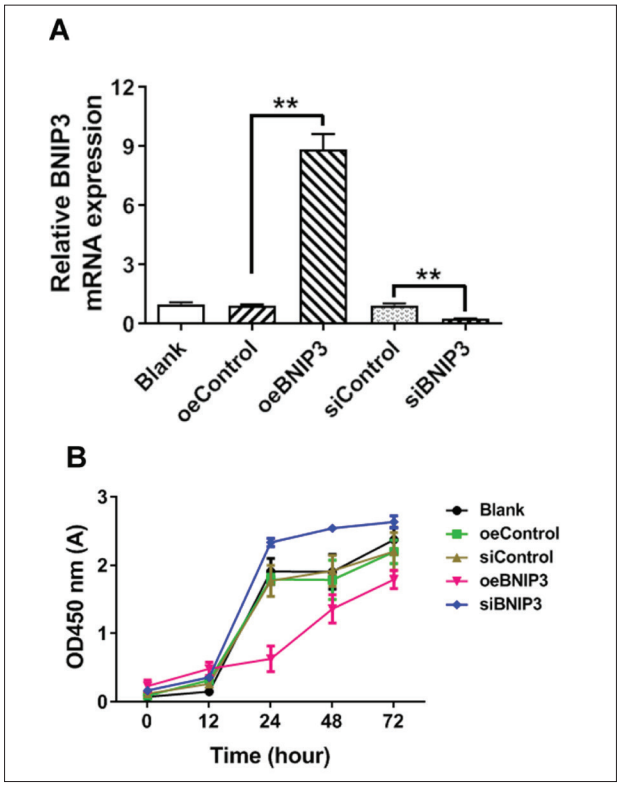

Fig. 2. Overexpression of BNIP3 decreased the viability of the rat intervertebral disk cells. **A** – Isolated rat intervertebral disk cells were transfected with BNIP3 overexpression and control vector, BNIP3 interference vector and control vector. The BNIP3 expression levels in different groups were detected by qPCR 48 h after transfection. The differences in BNIP3 mRNA expression were analyzed by the $2^{-\Delta\Delta CT}$ method. The BNIP3 expression in the samples normalized against the expression of GAPDH, and the gene expression in the untreated cells (blank group) normalized to one. **B** – Cells placed on 6-well plates and transfected with different vectors. The cell viability of the different groups was detected by CCK-8. The bars represent the mean±SD from three independent experiments. **P<0.01 versus the control using the unpaired two-tailed Student's t test.

BNIP3 overexpression enhanced the autophagy and apoptosis of the rat intervertebral disk cells

Although BNIP3 can induce autophagy and apoptosis in different types of cells, whether BNIP3 can trigger the autophagy and apoptosis of the rat intervertebral disk cells remains unknown. Apoptosis of the rat intervertebral disk cells was detected by acridine orange staining. We found an increase in apoptosis in the BNIP3 overexpressed rat intervertebral disk cells compared with the overexpression control or blank group, and a decrease in apoptosis in the BNIP3 interference group compared with the interference control

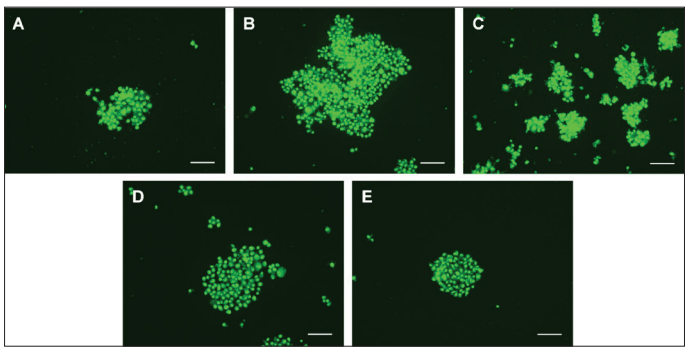

Fig. 3. Apoptosis of the rat intervertebral disk cells in different groups detected by acridine orange staining (×400). **A** – Normal rat intervertebral disk cells (blank group). **B** – Cells transfected with overexpression empty vector (oeControl group). **C** – Cells transfected with BNIP3 overexpression vector (oeBNIP3 group). **D** – Cells transfected with RNAi empty vector (siControl group). **E** – Cells transfected with BNIP3 interference vector (siBNIP3 group). The stained cells were observed by light microscopy. Scale bar=100 μm.

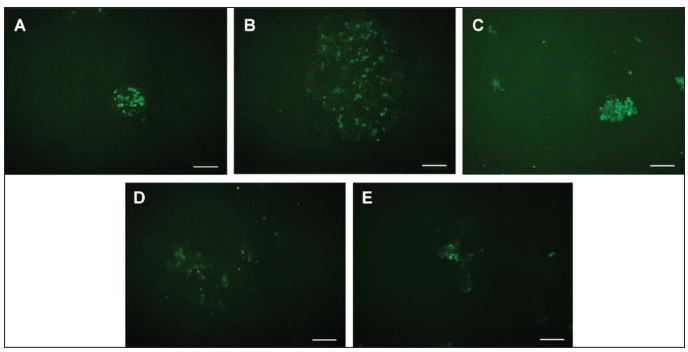

Fig. 4. Autophagy of rat intervertebral disk cells in different groups detected by MDC staining (×400). **A** – Normal rat intervertebral disk cells (blank group). **B** – Cells transfected with overexpression empty vector (overexpression control group). **C** – Cells transfected with BNIP3 overexpression vector (BNIP3 overexpression group). **D** – Cells transfected with RNAi empty vector (interference control group). **E** – Cells transfected with BNIP3 interference vector (BNIP3 interference group). The stained cells were observed by light microscopy. Scale bar=100 μm.

or blank group (Fig. 3). The results of the MDC method showed an increase in autophagy in the BNIP3 overexpressed rat intervertebral disk cells compared with the overexpression control or blank group, and a decrease in autophagy was observed in the BNIP3 interference group compared with the interference control or blank group. The rat intervertebral disk cells overexpressing BNIP3 were massed, and their nuclei were fragmented into dots; they stained into uneven, dense and deep-green staining particles under a light microscope compared with the control and interference group (Fig. 4), which is known to occur during autophagy. These findings suggested that BNIP3 overexpression triggered the autophagy and apoptosis of the rat intervertebral disk cells.

BNIP3 overexpression or interference disturbed the cell membrane potential

The cell membrane potential is closely related to a cell's state, such as autophagy and apoptosis. The cell membrane potential of the BNIP3 transfected cells was detected by flow cytometry. The membrane potential of the BNIP3 overexpression group was significantly decreased compared with the overexpression control or blank group, while the membrane potential of the BNIP3 interference group was significantly increased compared with the interference control or blank group (Fig. 5), suggesting that BNIP3 affected the cell membrane potential by triggering the autophagy and apoptosis of the rat intervertebral disk cells.

BNIP3 induced increased expression of autophagy and apoptosis-related molecules

We observed that BNIP3 overexpression activated the autophagy and apoptosis of the rat intervertebral disk cells. Accordingly, we examined whether BNIP3 can affect the expression of autophagy- and apoptosis-related proteins. The qPCR assay was employed to detect the different mRNA expression profiles of the autophagy- and apoptosis-related proteins, including HIF-1α, APAF-1, caspase 3, Bcl-2, LC3 and Beclin-1 in the rat intervertebral disk cells after transfection with BNIP3 overexpression or interference vectors. The qPCR results showed that the expression of HIF-1α, APAF-1, caspase 3, LC3 and Beclin-1 mRNAs was significantly increased when the rat intervertebral disk cells were transfected with the BNIP3 overexpression vector compared with the control or blank group. However, the expression of anti-apoptotic protein Bcl-2 was significantly decreased in the BNIP3 overexpression group compared with the control or blank group. Moreover, the expression of the autophagy- and apoptosis-related proteins in the rat intervertebral disk cells after BNIP3 RNAi was also detected by qPCR, demonstrating that the expression

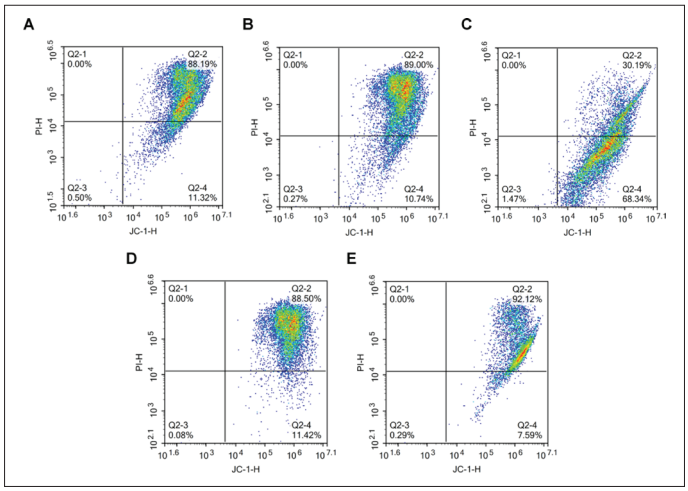

Fig. 5. Membrane potential of the rat intervertebral disk cells in different treatment groups detected by flow cytometry. **A** – Normal rat intervertebral disk cells (blank group). **B** – Cells transfected with overexpression empty vector (overexpression control group). **C** – Cells transfected with BNIP3 overexpression vector (BNIP3 overexpression group). **D** – Cells transfected with RNAi empty vector (interference control group). **E** – Cells transfected with BNIP3 interference vector (BNIP3 interference group).

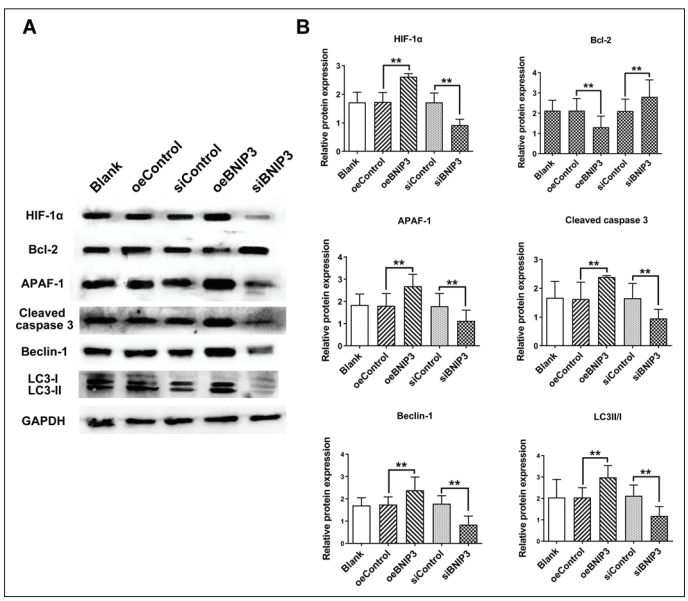

Fig. 7. Different expression of HIF-1α, Bcl-2, APAF-1, cleaved caspase 3, Beclin-1 and LC3 proteins of the rat intervertebral disk cells in different treatment groups detected by Western blotting (**A**). The expression of HIF-1α, Bcl-2, APAF-1, cleaved caspase 3, Beclin-1 proteins were detected by Western blotting after the cells were transfected with BNIP3 overexpression or BNIP3 interference vectors. **B** – Quantitative analysis of the band intensities according to their grayscale by Image J software. The bars represent the mean±SD from the three independent experiments. **P<0.01 versus the control using the unpaired two-tailed Student's t test.

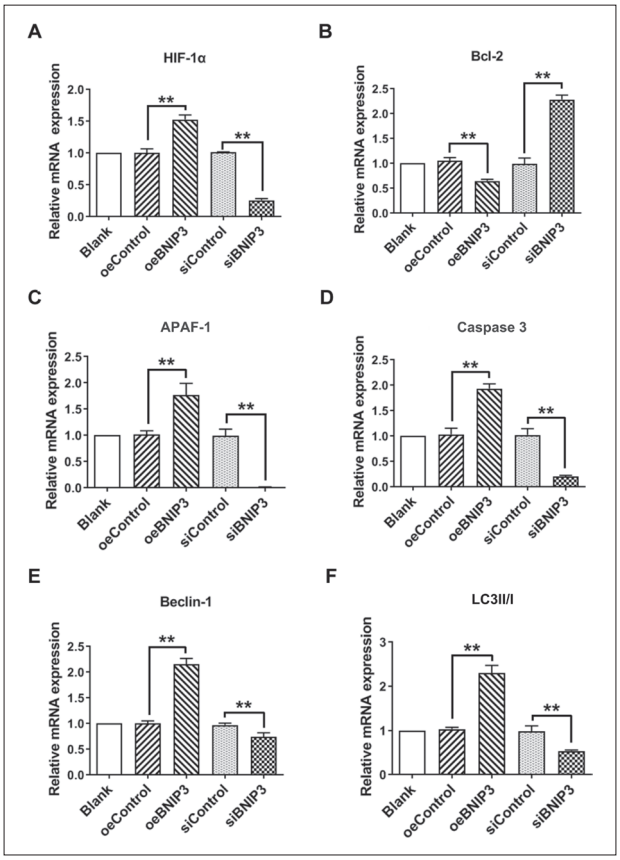

Fig. 6. Different expression of HIF-1α, Bcl-2, APAF-1, caspase 3, Beclin-1 and LC3 mRNAs of the rat intervertebral disk cells in different treatment groups detected by qPCR. HIF-1α (**A**), Bcl-2 (**B**), APAF-1 (**C**), caspase 3 (**D**), Beclin-1 (**E**) and LC3II/I (**F**) mRNAs analyzed by $2^{-\Delta\Delta CT}$. mRNA expression was normalized against the expression of GAPDH. The mRNA expression in the untreated cells (blank group) was normalized to one. The bars represent the mean±SD from the three independent experiments. **P<0.01 versus the control using the unpaired two-tailed Student's t test.

of HIF-1α, APAF-1, caspase 3, LC3 and Beclin-1 was significantly decreased when the intervertebral disk cells transfected with BNIP3 interference vector were compared with the control or blank group. The expression of anti-apoptotic protein Bcl-2 was significantly increased in the BNIP3 interference group compared with the control or blank group (Fig. 6).

Western blotting analysis of the autophagy- and apoptosis-related proteins revealed that the expression of HIF-1α, APAF-1, cleaved caspase 3, LC3 and Beclin-1 was increased when the intervertebral disk cells transfected with BNIP3

overexpression vector were compared with the matching overexpression control and blank group. In particular, the expression of anti-apoptotic protein Bcl-2 was decreased in the BNIP3 overexpression group compared with the control or blank group. In addition, the BNIP3 RNAi assay revealed that the expression of HIF-1α, APAF-1, cleaved caspase 3, LC3 and Beclin-1 was decreased when the intervertebral disk cells transfected with BNIP3 interference vector were compared with the control or blank group. The expression of anti-apoptotic protein Bcl-2 was increased in the BNIP3 interference group when compared with the control or blank group (Fig. 7A). To better display the expression differences of HIF-1α, APAF-1, cleaved caspase 3, Bcl-2, LC3 and Beclin-1 in the different groups, grayscale analysis of the Western blotting bands was performed, and similar results were obtained (Fig. 7B). These findings indicated that BNIP3 activated the expression of the autophagy- and apoptosis-related proteins and caused the autophagy and apoptosis of the rat intervertebral disk cells.

In summary, our findings showed that BNIP3 triggered the autophagy and apoptosis of the rat intervertebral disk cells by stimulating the expression of the autophagy- and apoptosis-related proteins. Augmentation of autophagy and apoptosis of the rat intervertebral disk cells may result in the development of IDD and exacerbation of low back pain.

DISCUSSION

Apoptosis of the lumbar intervertebral disk cells is a contributor to IDD [4,18]. Studies have shown that the apoptosis rate of the nucleus pulposus cells in IDD patients and IDD model animals is significantly higher than in the nucleus pulposus cells of normal humans or animals [4]. In addition, many factors that can induce IDD, such as ischemia and hypoxia, nutritional deficiencies, stress load, high glucose and hypertonicity, not only activate autophagy but also induce apoptosis [19-21]. Autophagy can inhibit apoptosis by clearing damaged cells, but excessive autophagy can also induce autophagic apoptosis [22,23]. Moreover, autophagy is a lysosome-dependent catabolic pathway that exclusively exists in eukaryotic cells and is composed of a series of autophagy-related genes that can degrade and recycle damaged, senescent and denatured organelles or macromolecular constituents in cells [24]. Furthermore, autophagy provides sufficient energy for cell survival. Autophagy mainly includes macrophage, microautophagy and molecular chaperone-mediated autophagy. The process of autophagy is divided into five stages: autophagy induction, autophagy vesicle nucleation, extension, autophagosome formation and autophagy lysosome formation [25].

Autophagy plays many different important roles in IDD development. Ye et al. [26] found that the number of autophagosomes and the ratio of autophagy-related proteins LC3-II/LC3-I in rat intervertebral disk nucleus pulposus cells increases with the age of rats. The level of autophagy in endplate cells in IDD patients is lower than that in normal humans [27], and the autophagy level of the annulus fibrosus in patients with IDD was higher than that in normal humans [28]. According to the abovementioned studies, the exact roles of autophagy in IDD are difficult to determine. The level of autophagy substantially varies in the course of different degrees of IDD or due to the difference in the age of rats, which needs to be judged according to the actual situation. The possible reasons are that the patients exhibited IDD of different severity, and the tissues were obtained from different sites. However, the detailed role and molecular mechanisms of autophagy in the development of IDD are unclear. Therefore, this study aimed to explore the role and mechanisms of the autophagy and apoptosis of the rat intervertebral disk cells.

The *BNIP3* gene encodes for a protein that was discovered in 1994 by yeast hybridization technology that can interact with the adenovirus E1B19kD protein and encodes for 194 amino acids. The ability to interact with adenovirus E1B19kD protein is determined by its own properties. The full-length *BNIP3* gene sequence is 585 bp. The encoded protein has two dimers. Subsequent studies confirmed that BNIP3 is a pro-apoptotic protein of the Bcl-2 family [29]. The C-terminus can bind to the mitochondrial membrane, which increases the permeability and reduces the potential of the mitochondrial membrane, thereby promoting apoptosis [30]. The biological role of BNIP3 under hypoxic conditions mainly depends on the regulation of HIF-1α, which can bind to the hypoxia response element of the BNIP3 promoter, thereby regulating the transcription of BNIP3. Accordingly,

the hypoxic environment can cause a significant induction of the expression of BNIP3; moderate expression of BNIP3 can activate autophagy and maintain the survival of cells, while excessive autophagy causes apoptosis [13-15]. Current research has shown that the positive expression rate of BNIP3 gradually increases with the severity of IDD, and that the expression of BNIP3 was closely related to the apoptosis of intervertebral disk cells during IDD development [16].

In this study, BNIP3 overexpression and RNAi were employed to explore the role and mechanisms of BNIP3 in IDD. First, the success of transfection was verified by qPCR. Second, the CCK-8 method was used to the assess the cell viability of the rat intervertebral disk cells after transfection with different vectors, demonstrating that BNIP3 overexpression resulted in a remarkable decrease in cell viability, and BNIP3 interference produced an increase in cell viability. These findings were consistent with the evidence that BNIP3 was closely related to the apoptosis of the intervertebral disk cells in IDD development [31], suggesting that BNIP3 expression affected the viability of the rat intervertebral disk cells. Subsequently, the apoptosis and autophagy of the rat intervertebral disk cells detected by acridine orange staining and MDC staining, respectively, showed that BNIP3 overexpression increased apoptosis and autophagy and that BNIP3 interference decreased the apoptosis and autophagy of the rat intervertebral disk cells. Detection of the cell membrane potential by flow cytometry revealed that BNIP3 overexpression significantly decreased the membrane potential, while BNIP3 interference substantially increased the membrane potential of the rat intervertebral disk cells, suggesting that BNIP3 activated the autophagy and apoptosis, and induced a decrease in the number of intervertebral disk cells, which might eventually result in IDD.

Cell apoptosis and autophagy are closely associated with the apoptosis- and autophagy-related proteins and pathways. Bcl-2 is one of the most important anti-apoptotic proteins that can form a dimer with the pro-apoptotic protein Bax, inhibiting apoptosis signaling and promoting cell survival [32]. *Beclin-1* is an autophagy-related gene that is homologous with yeast autophagy gene 6. Beclin-1 can recruit other autophagy-related proteins to localize on the membrane of the autophagosome, which plays a positive regulatory role in the formation of autophagosomes [33]. Furthermore, Beclin-1 interacts with Bcl-2 through its BH3 domain to maintain the dynamic equilibrium of cell autophagy and apoptosis [34]. LC3 is the homolog of mammalian autophagy-related gene 8 that contains two forms (LC3-I and LC3-II). BNIP3 can competitively bind to Bcl-2 with Beclin-1 because both proteins contain the BH3 domain. The overexpression of BNIP3 causes the release of Beclin-1 from the Beclin-1/Bcl-2 complex. The free Beclin-1 then forms a complex with phosphoinositide 3-kinase (PI3K) and further regulates the expression of different autophagy-related genes downstream of the PI3K/AKT pathway, eventually promoting the occurrence of mitochondrial autophagy [35,36]. In addition, BNIP3 overexpression increases mitochondrial membrane permeability and affects the membrane potential, leading to the release of cytochrome C. The binding of cytochrome C with APAF-1 can activate the caspase pathway, induce a series of cascades and trigger apoptosis. In this study, qPCR and Western blotting were employed to study the differences in the expression profiles of the autophagy- and apoptosis-related proteins of the rat intervertebral disk cells after transfection with BNIP3 overexpression or interference vectors. Our findings showed that the expression of pro-apoptotic or autophagy- proteins, such as HIF-1α, APAF-1, cleaved caspase 3, LC3 and Beclin-1, were significantly increased in cells overexpressing BNIP3, while the expression of anti-apoptotic protein, such as Bcl-2, was significantly decreased. The expression of pro-apoptotic proteins or autophagy-related proteins, such as HIF-1α, APAF-1, cleaved caspase 3, LC3 and Beclin-1, were significantly decreased after transfection of these cells with the BNIP3 interference vector. Meanwhile, the expression of anti-apoptotic protein, such as Bcl-2, was significantly increased. These findings suggested that BNIP3 overexpression increased the autophagy and apoptosis of the intervertebral disk cells by regulating the expression of the autophagy- and apoptosis-related proteins.

CONCLUSION

Primary rat intervertebral disk cells were prepared and used to study the effect of BNIP3 overexpression on their autophagy and apoptosis. BNIP3 overexpression in the rat intervertebral disk cells significantly

upregulated the expression of the autophagy-related and pro-apoptotic proteins, augmented the autophagy and apoptosis of the rat intervertebral disk cells and decreased the cell viability. Meanwhile, the effects of BNIP3 overexpression could be significantly reversed by BNIP3 interference. These findings indicated that BNIP3 enhanced the autophagy and apoptosis of the intervertebral disk cells and decreased the cell viability of the intervertebral disk cells, which may eventually result in IDD development. The findings of this study revealed the effect of BNIP3 on the autophagy and apoptosis of the intervertebral disk cells *in vitro*. In subsequent studies, we will explore the role of BNIP3 in the animal models of IDD for the evidence *in vivo*.

Funding: This study received no external funding.

Author contributions: Conceptualization: TW, SF and MD; methodology: TW, SF and MD; investigation: TW, SF, BZ, ZT and HH; data analysis: TW, SF, BZ, ZT, HH and MD; intellectual input: MD; writing, original draft: TW and SF; reviewing and editing: MD; supervision: MD; TW and SF contributed equally to this work.

Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Wu%20et%20al_7713_Data%20Report.pdf

REFERENCES

1. Edward J, Carreon LY, Williams MV, Glassman S, Li J. The importance and impact of patients' health literacy on low back pain management: a systematic review of literature. Spine J. 2018;18(2):370-6. https://doi.org/10.1016/j.spinee.2017.09.005
2. Le Maitre CL, Binch AL, Thorpe AA, Hughes SP. Degeneration of the intervertebral disc with new approaches for treating low back pain. J Neurosurg Sci. 2015;59(1):47-61.
3. Petit A, Roquelaure Y. Low back pain, intervertebral disc and occupational diseases. Int J Occup Saf Ergon. 2015;21(1):15-9. https://doi.org/10.1080/10803548.2015.1017940
4. Xu TT, Liao F, Jin HT, Tong PJ, Xiao LW, Wu CL. [Research advance on intervertebral disc degeneration and cell death]. Zhongguo Gu Shang. 2015;28(7):673-8. Chinese.
5. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741-52. https://doi.org/10.1038/nrm2239
6. Yurube T, Ito M, Kakiuchi Y, Kuroda R, Kakutani K. Autophagy and mTOR signaling during intervertebral disc aging and degeneration. JOR Spine. 2020;3(1):e1082. https://doi.org/10.1002/jsp2.1082
7. Zhang SJ, Yang W, Wang C, He WS, Deng HY, Yan YG, Zhang J, Xiang YX, Wang WJ. Autophagy: A double-edged sword in intervertebral disk degeneration. Clin Chim Acta. 2016;457:27-35. https://doi.org/10.1016/j.cca.2016.03.016
8. Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, Gibson SB. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. Autophagy. 2008;4(2):195-204. https://doi.org/10.4161/auto.5278
9. Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. Proc Natl Acad Sci U S A. 2002;99(20):12825-30. https://doi.org/10.1073/pnas.202474099
10. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, Gustafsson AB. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ. 2007;14(1):146-57. https://doi.org/10.1038/sj.cdd.4401936
11. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res. 2001;61(18):6669-73.
12. Zhang T, Xue L, Li L, Tang C, Wan Z, Wang R, Tan J, Tan Y, Han H, Tian R, Billiar TR, Tao WA, Zhang Z. BNIP3 Protein suppresses PINK1 kinase proteolytic cleavage to promote mitophagy. J Biol Chem. 2016;291(41):21616-29. https://doi.org/10.1074/jbc.M116.733410
13. Choi H, Merceron C, Mangiavini L, Seifert EL, Schipani E, Shapiro IM, Risbud MV. Hypoxia promotes noncanonical autophagy in nucleus pulposus cells independent of MTOR and HIF1A signaling. Autophagy. 2016;12(9):1631-46. https://doi.org/10.1080/15548627.2016.1192753
14. Vasagiri N, Kutala VK. Structure, function, and epigenetic regulation of BNIP3: a pathophysiological relevance. Mol Biol Rep. 2014;41(11):7705-14. https://doi.org/10.1007/s11033-014-3664-x
15. Ney PA. Mitochondrial autophagy: Origins, significance, and role of BNIP3 and NIX. Biochim Biophys Acta. 2015;1853(10 Pt B):2775-83. https://doi.org/10.1016/j.bbamcr.2015.02.022
16. Liu J, Peng N, Lu Y, Wang J. Preliminary research for correlation between BNIP3 expression and disc degeneration. Chin J Spine Spinal Cord. 2012;22(1):65-71. Chinese.
17. Liu J, Peng N, Wang J. Immunohistochemistry research on BNIP3 expressions of degenerated intervertebral disc in rabbit. J Reg Anat Oper Surg. 2012;21(1):3-5. Chinese.
18. Zhang F, Zhao X, Shen H, Zhang C. Molecular mechanisms of cell death in intervertebral disc degeneration (Review). Int J Mol Med. 2016 Jun;37(6):1439-48. https://doi.org/10.3892/ijmm.2016.2573
19. Shen X, Jiang H. Research progress on the relationship between autophagy and intervertebral disc degeneration. Chin J Trad Med Traum Orthop. 2017;25(8):77-82. Chinese.
20. Peng A, Yin C, Wu T, Huang W, Cheng X. Progress in association between autophagy and intervertebral disc degeneration. Chin J Cell Biol. 2016;38(11):1382-90. Chinese.
21. Chen D, Zhou K, Xu H. Research progress on the role of autophagy in intervertebral disc degeneration. J Med Res. 2016;45(12):9-12. Chinese.

22. Ding F, Shao ZW, Xiong LM. Cell death in intervertebral disc degeneration. Apoptosis. 2013;18(7):777-85. https://doi.org/10.1007/s10495-013-0839-1
23. Chen JW, Ni BB, Li B, Yang YH, Jiang SD, Jiang LS. The responses of autophagy and apoptosis to oxidative stress in nucleus pulposus cells: implications for disc degeneration. Cell Physiol Biochem. 2014;34(4):1175-89. https://doi.org/10.1159/000366330
24. Mizushima N. Autophagy: process and function. Genes Dev. 2007;21(22):2861-73. https://doi.org/10.1101/gad.1599207
25. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ. 2005;12 Suppl 2:1542-52. https://doi.org/10.1038/sj.cdd.4401765
26. Ye W, Xu K, Huang D, Liang A, Peng Y, Zhu W, Li C. Age-related increases of macroautophagy and chaperone-mediated autophagy in rat nucleus pulposus. Connect Tissue Res. 2011;52(6):472-8. https://doi.org/10.3109/03008207.2011.564336
27. Xu H, Xiong S, Wang H, Zhang M, Yu Y. The evidence and the possible significance of autophagy in degeneration model of human cervical end-plate cartilage. Exp Ther Med. 2014;7(3):537-42. https://doi.org/10.3892/etm.2013.1465
28. Gruber HE, Hoelscher GL, Ingram JA, Bethea S, Hanley EN Jr. Autophagy in the degenerating human intervertebral disc: in vivo molecular and morphological evidence, and induction of autophagy in cultured annulus cells exposed to pro-inflammatory cytokines-implications for disc degeneration. Spine (Phila Pa 1976). 2015;40(11):773-82. https://doi.org/10.1097/BRS.0000000000000865
29. Burton TR, Gibson SB. The role of Bcl-2 family member BNIP3 in cell death and disease: NIPping at the heels of cell death. Cell Death Differ. 2009;16(4):515-23. https://doi.org/10.1038/cdd.2008.185
30. Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ. 2009;16(7):939-46. https://doi.org/10.1038/cdd.2009.16
31. Liu J, Wang J, Zhou Y. Upregulation of BNIP3 and translocation to mitochondria in nutrition deprivation induced apoptosis in nucleus pulposus cells. Joint Bone Spine. 2012;79(2):186-91. https://doi.org/10.1016/j.jbspin.2011.04.011
32. Willis S, Day CL, Hinds MG, Huang DCS. The Bcl-2-regulated apoptotic pathway. J Cell Sci. 2003;116(20):4053-6. https://doi.org/10.1242/jcs.00754
33. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 2011;18(4):571-80. https://doi.org/10.1038/cdd.2010.191
34. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122(6):927-39. https://doi.org/10.1016/j.cell.2005.07.002
35. Decuypere JP, Parys JB, Bultynck G. Regulation of the autophagic bcl-2/beclin 1 interaction. Cells. 2012;1(3):284-312. https://doi.org/10.3390/cells1030284
36. Sheng R, Qin ZH. The divergent roles of autophagy in ischemia and preconditioning. Acta Pharmacol Sin. 2015;36(4):411-20. https://doi.org/10.1038/aps.2014.151

Supplementary Data

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Wu%20et%20al_7713_Supplementary%20Material.pdf