

Aloperine attenuates hydrogen peroxide-induced injury via anti-apoptotic activity and suppression of the nuclear factor- κ B signaling pathway

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Abstract. Aloperine is an alkaloid that exerts significant inhibitive effects on acute inflammation and Type III and IV hypersensitivity caused by a variety of inflammatory agents. The aims of the present study were to investigate whether the protective effect of aloperine attenuates hydrogen peroxide (H_2O_2)-induced injury, and to identify the underlying mechanisms involved. Nucleus pulposus cells were extracted from adult male Sprague-Dawley rats, and incubated with fresh medium containing $200 \mu M H_2O_2$ for 24 h. In the study, treatment with aloperine significantly increased cell viability and suppressed apoptosis in H_2O_2 -treated nucleus pulposus cells in a dose-dependent manner. In addition, 10 and 100 nM aloperine significantly inhibited H_2O_2 -induced tumor necrosis factor- α and interleukin-6 activities, and significantly increased the H_2O_2 -reduced superoxide dismutase and glutathione peroxidase activities in nucleus pulposus cells (all $P < 0.01$). However, aloperine treatment (10 and 100 nM) significantly reduced the H_2O_2 -induced caspase-9 activity in nucleus pulposus cells. Furthermore, addition of 10 and 100 nM aloperine significantly suppressed nuclear factor- κ B (NF- κ B) and phosphorylated-protein kinase B expression levels in H_2O_2 -treated nucleus pulposus cells. In conclusion, the protective effect of aloperine attenuated H_2O_2 -induced injury via hyperproliferation, its anti-apoptotic activity and suppression of the NF- κ B signaling pathway.

Introduction

Intervertebral disc degeneration is characterized by a reduced number and impaired function of intervertebral disc cells,

and manifests as nucleus dehydration, decreased proteoglycan content, particularly in aggregation states, and changes in collagen type and distribution (1). These changes weaken or remove the tension and pressure on intervertebral discs, and the changes in histology eventually results in changes of intervertebral disc biomechanics (1). Therefore, it is evident that a decrease in the number of active cells in intervertebral discs will result in the reduction in the synthesis of extracellular matrix and alterations in the cell composition (2,3). This is the pathological basis of intervertebral disc degeneration, and excessive apoptosis is the direct cause of the decrease in intervertebral disc cells (2,3).

Apoptosis serves an important role in the development of the body, as well as in a number of physiological and pathological processes (3). It has been suggested that apoptosis may be involved in the pathophysiologic changes experienced during intervertebral disc tissue degeneration, and it is noted that apoptosis serves an important role in the intervertebral disc degeneration process (4). Excessive apoptosis of intervertebral disc cells results in the reduction of active cells, as well as changes of cell composition, which is the pathological basis of intervertebral disc degeneration (5). However, studies have demonstrated that oxidative stress resulting from reactive oxygen species is the primary cause of apoptosis (6).

Reactive oxygen species include superoxide anion, hydrogen peroxide and hydroxyl radicals. Various antioxidant defense mechanisms exist *in vivo*, including antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (3-5). When these defense mechanisms cannot prevent the generation of excessive reactive oxygen species, oxidative stress will occur, which causes the degeneration of cells or protein tissue, lipid oxidation, DNA damage and other physiological dysfunctional processes, leading to apoptosis (7). However, only a limited number of studies have focused on intervertebral disc degeneration and the apoptosis of nucleus pulposus cells due to oxidative stress (8-10).

Aloperine is a novel type of alkaloid drug with a significant inhibitory effect on acute inflammation, Type III and IV hypersensitivity, and adjuvant arthritis caused by multiple proinflammatory agents (11). The chemical structure of aloperine is presented in Fig. 1. Aloperine is a herb that has been found to have significant inhibitory effects on T cells and B cells, and on the production of interleukin (IL)-2 according to preliminary results (12).

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In the present study, hydrogen peroxide (H_2O_2) was used as a stimulus to study the effect of oxidative stress on nucleus pulposus cell injury and underlying mechanisms (8). The study determined that the protective effect of aloperine attenuates H_2O_2 -induced injury via its activation of AKT and suppression of the nuclear factor- κB (NF- κB) pathway.

Materials and methods

Compounds. Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12) and fetal bovine serum (FBS) were acquired from Hyclone (GE Healthcare Life Sciences, Little Chalfont, UK). Collagenase II (0.2%; C0014) was purchased from Sunshine Biotechnology (Nanjing) Co., Ltd. (Nanjing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; ST316) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Tumor necrosis factor- α (TNF- α ; R019), IL-6 (R016), SOD (A001-3) and glutathione peroxidase (GSH-Px; A005) commercial kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Aloperine (purity, >99%) was acquired from Yanchi Dushun Biological and Chemical Co., Ltd. (Ningxia, China).

Experimental animals and cell culture. Ethical approval for this study was provided by the Animal Ethical and Welfare Committee of Hebei Province (approval no. Hb14-3012). Adult male Sprague-Dawley rats ($n=24$, 9-10 weeks), weighing 250 ± 30 g, were obtained from the Animal Resource Center of the First Central Hospital of Baoding (Baoding, China). They were housed at $22-23^\circ C$ with 55-60% humidity and a 12:00/12:00 light/dark cycle. The rats were euthanized by an overdose of pentobarbital [100 mg/kg body weight; Sunshine Biotechnology (Nanjing) Co., Ltd.]. The spinal column was removed at the L1-L6 lumbar intervertebral discs, and then the gel-like nucleus pulposus was separated from the samples under aseptic conditions. The nucleus pulposus tissue samples were immediately placed into DMEM/F-12 and FBS, and were digested with 0.01% trypsin (Beyotime Institute of Biotechnology) at $37^\circ C$ for 0.5-1 h. The trypsin was absorbed and removed, and the nucleus pulposus tissue samples were washed with phosphate-buffered saline (PBS) and digested with 0.2% collagenase II at $37^\circ C$ for 4 h. Following digestion, nucleus pulposus cells were harvested using a $200\ \mu m$ mesh strainer. Next, the cells (1×10^7 cells) were seeded into a new culture bottle and incubated with DMEM/F-12 and 10% FBS containing 1% penicillin/streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a humidified atmosphere at $37^\circ C$ and 5% CO_2 . Subsequent to incubation, nucleus pulposus cells were washed with PBS.

Cell viability determined by MTT assay. Nucleus pulposus cells (5×10^3 cells/well) were seeded into a 96-well plate, and incubated with fresh medium containing $200\ \mu M$ H_2O_2 alone or with aloperine (0.1-1,000 μM) for 24 h. After incubation, 10 μl MTT was added into each well and incubated in a humidified atmosphere at $37^\circ C$ and 5% CO_2 . Subsequently, 150 μl dimethyl sulfoxide was added to each well and agitated for 20 min. The absorbance was measured using Labsystems Multiskan MS plate Reader (Synergy2; BioTek Instruments, Inc., Winooski, VT, USA) at 540 nm.

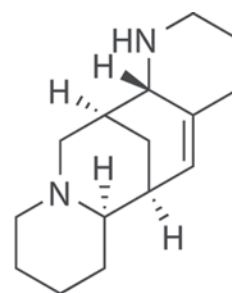


Figure 1. Chemical structure of aloperine.

Cell apoptosis determined by flow cytometry. Nucleus pulposus cells (2×10^6 cells/well) were seeded into a 6-well plate and incubated with fresh medium containing $200\ \mu M$ H_2O_2 alone or with aloperine (1, 10 and 100 nM) for 24 h. Following incubation, nucleus pulposus cells were washed twice with cold PBS and incubated with 500 μl binding buffer (BestBio, Shanghai, China). Subsequently, 5 μl Annexin V-FITC and 5 μl propidium iodide (BestBio) were added and the cells were incubated for 30 min at $4^\circ C$ in the dark. Cell apoptosis was analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) with CellQuest software (BD Biosciences).

Determination of inflammation and oxidation activity. Nucleus pulposus cells (5×10^3 cells/well) were seeded into a 96-well plate, and incubated with fresh medium containing $200\ \mu M$ H_2O_2 alone or plus aloperine (1, 10 and 100 nM) for 24 h. After incubation, TNF- α , IL-6, SOD and GSH-Px were measured using commercial ELISA kits according to the manufacturer's instructions and a Labsystems Multiskan MS Plate Reader was used to measure the absorbance at 450 nm.

Measurement of caspase-9 activity. Nucleus pulposus cells (2×10^6 cells/well) were seeded into a 6-well plate, and incubated with fresh medium containing $200\ \mu M$ H_2O_2 alone or with aloperine (1, 10 and 100 nM) for 24 h. Subsequently, nucleus pulposus cells were incubated with 100 μl tissue lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice. Homogenates were centrifuged at $12,000\times g$ for 10 min at $4^\circ C$ and the supernatant was collected to assess the protein concentration using a BCA assay kit, according to the manufacturer's protocol (KeyGen Biotech Co., Ltd., Nanjing, China). Equal protein (10 mg) was incubated with substrate (Ac-DEVD-pNA-caspase-9; BestBio) for 2 h in the dark at room temperature. The absorbance was then measured using a Labsystems Multiskan MS plate reader at 405 nm.

Western blot analysis of NF- κB and phosphorylated AKT (p-AKT) levels. Nucleus pulposus cells (2×10^6 cells/well) were seeded into a 6-well plate, and incubated with fresh medium containing $200\ \mu M$ H_2O_2 alone or with aloperine (1, 10 and 100 nM) for 24 h. Next, the cells were incubated with 100 μl tissue lysis buffer for 30 min on ice. Homogenates were centrifuged at $12,000\times g$ for 10 min at $4^\circ C$ and the supernatant was collected to assess the protein concentration using a BCA kit, according to the manufacturer's protocol. Equal protein (50-60 mg) was separated with 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred

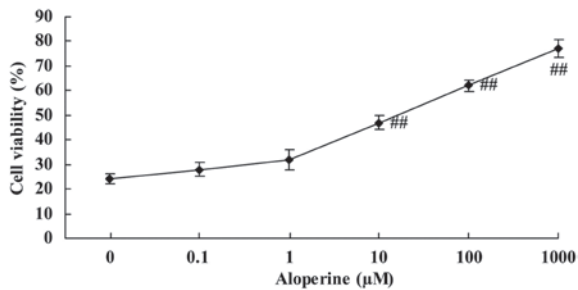


Figure 2. Cell viability of H₂O₂-induced cells following treatment with various concentrations of aloperine, as determined by MTT assay. ##P<0.01 vs. 0 nM aloperine.

onto a nitrocellulose membrane using an electroblotting apparatus. The nitrocellulose membrane was incubated with anti-NF-κB (sc-8008, 1:500), anti-p-AKT (sc-7985; 1:1,000) and anti-AKT (sc-8312; 1:1,000) antibodies all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) overnight at 4°C. Subsequently, the nitrocellulose membrane was incubated with the appropriate horseradish peroxidase (HRP)-conjugated IgG secondary antibody (sc-358915, sc-2008; 1:5000; Santa Cruz Biotechnology, Inc.) followed by incubation with an enhanced chemiluminescence substrate. The relative quantity of each protein was measured using AlphaEase FC (FluorChem FC2) software (ProteinSimple, Santa Clara, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA) and data are presented as the mean ± standard deviation from at least three experiments. Comparisons between two groups were performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Aloperine increases the viability of H₂O₂-treated nucleus pulposus cells. Treatment of nucleus pulposus cells with H₂O₂ alone (0 nM aloperine) resulted in low cell viability, as determined by MTT assay. However, aloperine (0.1-1,000 μM) promoted the cell viability of H₂O₂-treated nucleus pulposus cells in a concentration-dependent manner. In particular, aloperine significantly increased the cell viability of H₂O₂-treated nucleus pulposus cells at doses of 10, 100 and 1,000 μM (P<0.01; Fig. 2).

Aloperine suppresses H₂O₂-induced apoptosis. The effects of aloperine on H₂O₂-induced apoptosis in nucleus pulposus cells were determined using flow cytometry. Treatment with 10 and 100 nM aloperine significantly suppressed the H₂O₂-induced apoptosis (P<0.01; Fig. 3).

Aloperine decreases H₂O₂-induced caspase-9 activity. In nucleus pulposus cells, the caspase-9 activity in the H₂O₂ model group was significantly increased compared with that in the control group (P<0.01). Compared with the H₂O₂ model group, the caspase-9 activity was significantly decreased by 10 and 100 nM aloperine in H₂O₂-induced nucleus pulposus cells (P<0.01; Fig. 4).

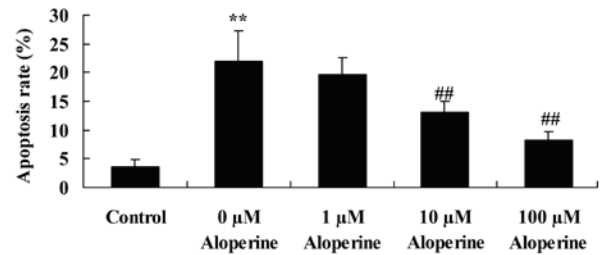


Figure 3. Apoptosis rate of H₂O₂-induced cells following treatment with various concentrations of aloperine, as determined by flow cytometric analysis. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine.

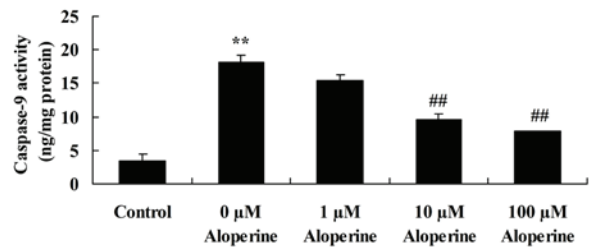


Figure 4. Caspase-9 activity levels in H₂O₂-induced cells following treatment with various concentrations of aloperine. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine.

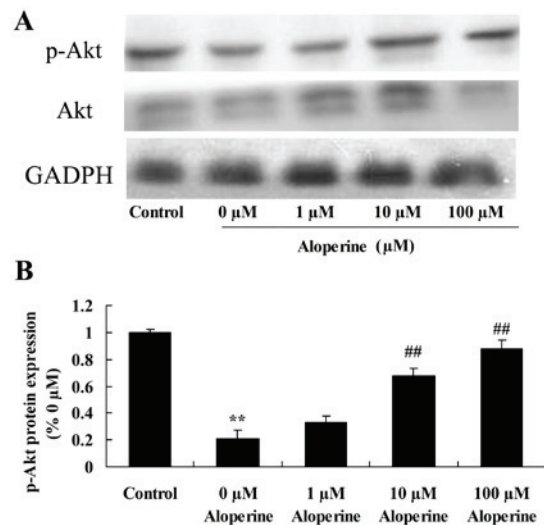


Figure 5. p-AKT protein expression in H₂O₂-induced cells following treatment with various concentrations of aloperine. The protective effect of aloperine on p-AKT protein expression by (A) western blotting assays and (B) statistical analysis of p-AKT protein expression. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine. p-AKT, phosphorylated-AKT.

Effect of aloperine on p-AKT expression in H₂O₂-treated nucleus pulposus cells. To examine the mechanism of action of aloperine on H₂O₂-induced cell apoptosis in nucleus pulposus cells, p-AKT expression was analyzed using western blotting. The p-Akt expression of the H₂O₂ model group was reduced compared with that of the control group (P<0.01). p-AKT was significantly upregulated upon treatment with 10 and 100 nM aloperine in H₂O₂-treated nucleus pulposus cells, compared with the H₂O₂ alone treatment group (P<0.01; Fig. 5).

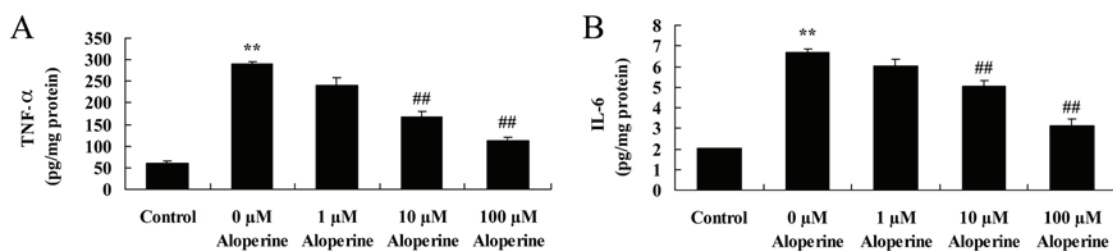


Figure 6. (A) TNF- α and (B) IL-6 protein expression levels in H₂O₂-induced cells treated with aloperine. A dose-dependent inhibitory effect of aloperine is observed. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine. TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

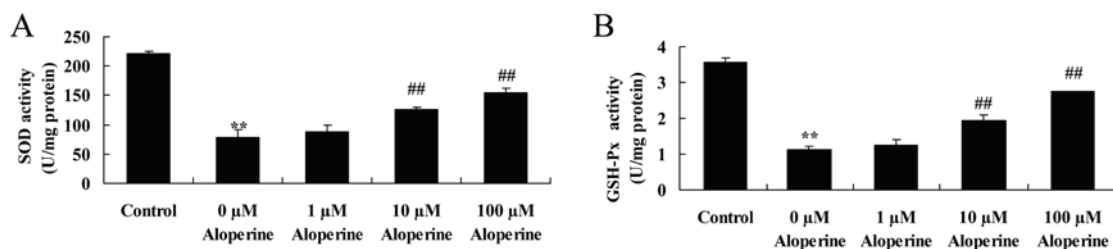


Figure 7. Oxidation activity in H₂O₂-induced cells treated with aloperine. A protective effect of aloperine on H₂O₂-inhibited (A) SOD and (B) GSH-Px activities is observed. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

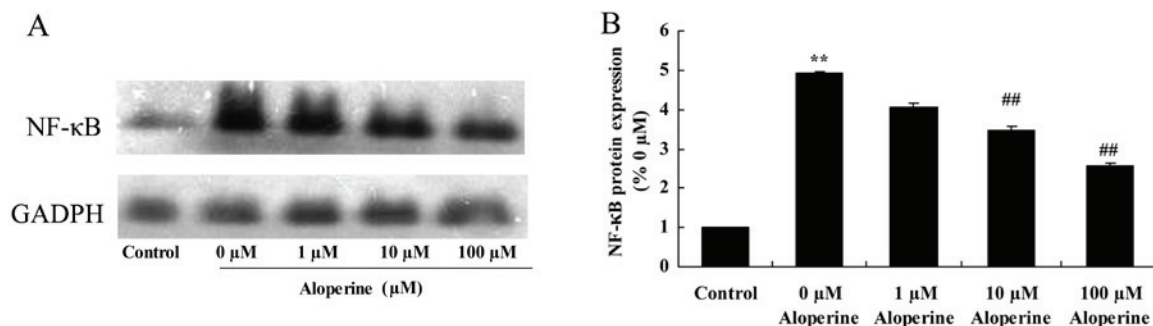


Figure 8. NF- κ B protein expression in H₂O₂-induced cells treated with aloperine, as shown in (A) western blots and (B) quantified levels. A dose-dependent inhibitory effect of aloperine on NF- κ B expression is observed. **P<0.01 vs. the control group; ##P<0.01 vs. 0 nM aloperine. NF- κ B, nuclear factor- κ B.

Aloperine reduces TNF- α and IL-6 expression levels in H₂O₂-treated cells. H₂O₂ significantly increased TNF- α and IL-6 levels in nucleus pulposus cells (P<0.01). Treatment with 10 and 100 nM aloperine was found to significantly inhibit the expression levels of TNF- α and IL-6 compared with those in the cells treated only with H₂O₂ (P<0.01; Fig. 6).

Effect of aloperine on H₂O₂-induced oxidation activity. The effect of aloperine on the SOD and GSH-Px activities in H₂O₂-treated nucleus pulposus cells is presented in Fig. 7. H₂O₂ significantly inhibited SOD and GSH-Px activities in the model group compared with the control group (P<0.01). Following treatment with 10 and 100 nM aloperine, there were significant increases in the SOD and GSH-Px activities in nucleus pulposus cells compared with those in the cells treated only with H₂O₂ (P<0.01; Fig. 7).

Effect of aloperine on H₂O₂-induced NF- κ B protein expression. To determine the protective mechanism of aloperine against H₂O₂-induced inflammation in nucleus pulposus cells,

NF- κ B protein expression levels were analyzed using western blotting. The results showed that NF- κ B protein expression of model group was higher than that of control group (Fig 8). Treatment with 10 and 100 nM aloperine significantly inhibited NF- κ B protein expression in H₂O₂-induced nucleus pulposus cells compared with the expression in cells treated with H₂O₂ alone (P<0.01; Fig. 8).

Discussion

Intervertebral disc degeneration is considered to be closely associated with apoptosis of nucleus pulposus cells (1). Although numerous studies have investigated apoptosis, few studies have examined how oxidative stress results in nucleus pulposus cell apoptosis and intervertebral disc degeneration (10,13,14). Therefore, the current study used H₂O₂ to establish an oxidative stress model in nucleus pulposus cells in order to identify the underlying mechanisms (15). In addition, the current study aimed to explore the role of apoptosis in intervertebral disc degeneration, and to clarify the signal transduction pathway

involved in intervertebral disc apoptosis, which may help to achieve the prevention of intervertebral disc degeneration by interfering with apoptosis (15). Following the primary culture of rat nucleus pulposus cells, 200 μ M H₂O₂ was used to stimulate the cells for 24 h. H₂O₂ treatment resulted in significant apoptosis, which confirms that oxidative stress can lead to apoptosis (16). In the present study, it was demonstrated that treatment with aloperine significantly increased cell viability and inhibited cell apoptosis of H₂O₂-treated nucleus pulposus cells in a dose-dependent manner.

Caspase exists in cells as an inactive zymogen form, and becomes an active fragment following proteolytic processing, and thus is used in the investigation of cell apoptosis (17). In neuronal apoptosis induced by various stimuli, caspase acts as a modulating agent (18). With increasing doses of H₂O₂, the expression of caspase-9 is increased (19). The caspase family serves an important role in the process of mediating apoptosis, in which caspase-9 is a critical executioner molecule, acting in numerous apoptosis signaling transduction pathways (20). In the current study, aloperine treatment significantly suppressed the H₂O₂-induced caspase-9 activity of nucleus pulposus cells via the upregulation of the AKT signaling pathway. Wang *et al* (21) suggested that aloperine exerts antitumor effects against multiple myeloma through the caspase-9/p-PTEN/p-AKT-dependent apoptotic pathways.

NF- κ B is a protein with multidirectional transcriptional regulation functions, which is widely distributed, adjusts the transcriptional regulation of numerous genes and is involved in a number of important physiological and pathological processes, such as inflammatory reactions, the immune response, cell proliferation, transformation and apoptosis (22). The most common form of NF- κ B is a heterodimer consisting of two protein subunits, P50 and RelA/P65. It has been demonstrated that an increase in H₂O₂ concentration, which is known to induce apoptosis, results in increased NF- κ B P65 expression and activity (23). The results of the present study suggest that treatment with aloperine significantly reduced TNF- α and IL-6 activities, and enhanced SOD and GSH-Px activities in H₂O₂-treated nucleus pulposus cells through the downregulation of the NF- κ B signaling pathway. Zhou *et al* (24) reported that the anti-inflammatory and anti-oxidative action of aloperine significantly inhibited the effects of allergic reactions. In addition, Xu *et al* (25) demonstrated that administration of aloperine attenuated neuropathic pain through its anti-oxidation activity and suppression of the NF- κ B signaling pathway.

In the present study, aloperine increased the viability and inhibited the apoptosis of H₂O₂-treated nucleus pulposus cells in a dose-dependent manner. In addition, aloperine exerted anti-inflammatory, anti-oxidative and anti-apoptotic effects, upregulated p-AKT expression and downregulated the NF- κ B signaling pathway in H₂O₂-treated nucleus pulposus cells. In particular, aloperine at concentrations of 10 and 100 nM exerted significant effects. In conclusion, aloperine attenuated H₂O₂-induced nucleus pulposus cell injury via anti-apoptotic activity and suppression of the NF- κ B signaling pathway.

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