

# Mitochondrial separation protein inhibitor inhibits cell apoptosis in rat lungs during intermittent hypoxia

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**Abstract.** Obstructive sleep apnoea (OSA) is a very common sleep and breathing disorder that occurs in worldwide. It is important to develop a more effective treatment for OSA to overcome lung cell apoptosis during intermittent hypoxia (IH). A mitochondrial separation protein inhibitor (Mdivi-1) has been demonstrated to be a powerful tool for inhibiting apoptosis. In the present study, the protective effect and possible mechanism of apoptosis in lung cells during IH was investigated using *in vivo* and *in vitro* experiments. Following IH exposure for 4 weeks, the lung tissues of Sprague Dawley rats exhibited interstitial lesions, while Mdivi-1 reduced these pulmonary interstitial lesions. B-cell lymphoma (Bcl)-2 mRNA and protein expression levels were decreased however caspase-3, caspase-9 and dynamin-related protein 1 (Drp-1) mRNA and protein expression levels were increased. Following Mdivi-1 intervention, Bcl-2 mRNA and protein expression levels were increased while caspase-3, caspase-9 and Drp-1 mRNA and protein expression levels were decreased ( $P<0.05$ ). After exposure to IH for 12 h, the apoptosis rate of WTRL1 cells in rats increased gradually with the IH time ( $P<0.05$ ). Bcl-2 mRNA and protein expression levels were decreased, whereas caspase-3, caspase-9, cytochrome C (Cyt-C) and Drp-1 mRNA levels were increased, and caspase-3, caspase-9 and Drp-1 protein expression levels were increased. After Mdivi-1 intervention, Bcl-2 mRNA and protein expression levels were increased but caspase-3, caspase-9, Cyt-C and Drp-1 mRNA levels were decreased

along with caspase-9, Cyt-C and Drp-1 protein expression levels which were decreased ( $P<0.05$ ). The results of the present study suggest that Mdivi-1 may be a potential agent for treating OSA because it inhibits the mitochondrial pathway and reduces apoptosis.

## Introduction

Obstructive sleep apnoea (OSA) is a very common sleep and breathing disorder that is characterized by the repetitive collapse of the upper airway during sleep (1); in addition, OSA has high morbidity in paediatric and adult populations. In OSA, upper airway obstruction is usually associated with recurrent hypoxemia interspersed with periods of reoxygenation and is typically terminated by brief arousals, resulting in intermittent hypoxia (IH) and sleep fragmentation (2). The oscillatory nature of IH, especially chronic IH, may mimic the paradigm of ischaemia-reperfusion in that tissues and cells are exposed to periods of low and high  $O_2$ , which may lead to oxidative stress and induces lipid peroxidation, apoptosis, stress-responsive proteins, cell death and inflammation (3-5). Studies have confirmed that IH via different signalling pathways induces cell apoptosis (6,7). Mitochondrial separation protein inhibitor (Mdivi-1) is a selective dynamin-related protein 1 (Drp-1) inhibitor that has been reported to be a potential therapeutic target for brain, cardiovascular, liver and tumour angiogenesis (8,9). How Mdivi-1 inhibits lung apoptosis during IH remains unclear. The aim of the present study was to investigate the protective effect and possible mechanism of Mdivi-1 in lung cell apoptosis during IH.

## Materials and methods

**Animals.** A total of 32 specific pathogen free 6-8-week-old male Sprague Dawley (SD) rats (body weight, 180-220 g) were purchased from the Animal Care and Use Committee of the Army Medical University (Chongqing, China). The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (10). All experimental procedures were approved by the Ethics Committee of Guizhou Provincial People's Hospital (Guiyang, China). All

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efforts were made to minimize animal suffering and to reduce the number of animals used.

***In vivo experimental design.*** All animals were maintained in a controlled environment (22–25°C, 40–60% relative humidity, with a 12-h light-dark cycle with the lights on at 08:00 am) with food and water available. Animals were allowed to acclimatize for 1 week to the experimental chamber. The rats were allocated randomly to four groups: Control group (n=8), IH group (n=8), IH+25 mg/kg Mdivi-1 group (n=8) and IH+50 mg/kg Mdivi-1 group (n=8). The rats in the IH group, IH+25 mg/kg Mdivi-1 group and IH+50 mg/kg Mdivi-1 group were exposed to IH conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub> and balanced N<sub>2</sub>) for 90 sec and hypoxia (8% O<sub>2</sub>, 5% CO<sub>2</sub> and balanced N<sub>2</sub> for 30 sec) from 08:00 am to 04:00 pm daily for 4 consecutive weeks (11–13). The chambers had a rear N<sub>2</sub> inlet and a front air extractor, which enabled recovery to normoxia. A computerized system controlled the valve inlets and the alternating cycles of the extractors. The CO<sub>2</sub> in the chamber was maintained at a low level by continuous air extraction (14). Rats in the control group were maintained under normoxic conditions during the experiment. The treatment groups were injected intraperitoneally with Mdivi-1 (MedChemExpress, Monmouth Junction, NJ, USA) prior to oxygen treatment every day (15). A general clinical pain score for scoring cyanosis condition for decisions about endpoint was used as presented in Table I (16).

***Sample collection and tissue preparation.*** Trained personnel euthanized the experimental animals via CO<sub>2</sub> inhalation following IH exposure. Rats were left in the chamber until clinical death could be ensured. The flow rate of CO<sub>2</sub> displaced 20% of the volume chamber per minute. If no breath was seen for 60 sec the rodent was removed from the chamber. To ensure death, the rat's chest was felt to determine that there is no longer a heartbeat. Death was verified following euthanasia and prior to disposal. The lungs were dissected out and the right lower lobes were fixed in 4% paraformaldehyde at room temperature for 48 h. The remaining lung tissues were frozen in liquid nitrogen and then stored at -80°C until further analysis.

***Histological examination.*** The right lower lobes were fixed in 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin blocks and sliced into 6- $\mu$ m-thick sections. The sections were then stained with haematoxylin for 5 min and eosin for 90 sec at room temperature, and observed by light microscopy. Damage to the lung tissue was scored by the pathologist on a scale of 1 (no injury) to 4 (worst), as described previously (17).

***In vitro experimental design.*** WTRL1 cells (a rat pulmonary alveolar epithelial cell line; <http://www.cellresource.cn/fdetail.aspx?id=1349>) were purchased from the National Infrastructure of Cell Line Resource, (Beijing, China). The cells were grown in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml) and were cultured at 37°C in 5% CO<sub>2</sub> in a humidified incubator. WTRL1 cells in the

logarithmic growth phase were divided randomly into five groups (3 $\times$ 10<sup>6</sup> cells/well) in 6-well plates: Control, IH, IH+10  $\mu$ M Mdivi-1, IH+50  $\mu$ M Mdivi-1 and IH+100  $\mu$ M Mdivi-1. The control group was exposed to air, while the IH group, IH+10  $\mu$ M Mdivi-1 group, IH+50  $\mu$ M Mdivi-1 group and IH+100  $\mu$ M Mdivi-1 group were placed in a low oxygen culture box containing 5% O<sub>2</sub> for 1 h. The box was then reoxygenated with 21% O<sub>2</sub> for 30 min and this process was repeated for 12 h. The treatment groups were incubated with Mdivi-1 for 30 min prior to IH culture.

***Flow cytometry.*** WTRL1 cells were grown logarithmically and placed on 60-mm Petri dishes in a low oxygen culture box containing 5% O<sub>2</sub> for 1 h. The box was reoxygenated with 21% O<sub>2</sub> for 30 min and this process was repeated for 6, 12, 24 and 48 h. Next, the cells were resuspended in Annexin V-PI from a dual-staining assay kit (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) for 10 min at room temperature. The fluorescence signals from Annexin V and PI were measured by flow cytometry with a FACSCalibur™ flow cytometer (BD Bioscience), and the data were analysed using Cell Quest Pro software (version 5.0; BD Bioscience). The flow cytometry results were measured in 1 h.

***Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).*** Total RNA was extracted from lung tissues or cells with TRIzol (Thermo Fisher Scientific, Inc.). The D260/D280 ratio was measured and samples with a ratio between 1.8 and 2.0 were used for reverse transcription with a High Capacity cDNA Archive kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reverse transcription protocol were as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. qPCR was applied to analyze the cDNAs by using the 2X Maxima SYBR-Green/ROX qPCR Master Mix kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The amplification conditions were as follows: 95°C for 10 min for denaturation, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 60 sec. qPCR for Bcl-2, caspase-3, caspase-9, Cyt-C and Drp-1 was performed using TaqMan gene expression assays and the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method with GAPDH as the endogenous control (18). The primers were synthesized by Sheng Gong (Shanghai) Co., Ltd. (Shanghai, China) and the sequences are presented in Table II.

***Western blot analysis.*** Total protein cell lysates were isolated from rat lungs or from cells as described previously (19). Protein quantification was assessed using a bicinchoninic acid protein quantification assay and absorbance read at 562 nm was used to determine sample volumes for further analysis. A total of 50  $\mu$ g total protein from each tissue homogenate was separated by 30% SDS-PAGE and then transferred to polyvinylidene difluoride membranes, which were blocked with 5% skim milk at room temperature for 1 h. Membranes were washed twice with TBST and incubated with primary antibodies at 4°C overnight against Bcl-2 polyclonal antibody (cat. no. YT0470), caspase-3 polyclonal antibody (cat. no. YT0656), caspase-9 polyclonal antibody (cat. no. YT0662), Drp1 Polyclonal antibody (cat. no. YT1414; all 1:1,000; ImmunoWay Biotechnology Company, Plano, TX, USA), Cyt-C antibody

Table I. Assessing pain and distress in animal.

1) No indication of pain and distress	Normal; well groomed; alert; active; good condition; asleep or calm; normal appetite; BCS=3, 4 or 5
2) Mild or anticipated pain and distress	Not well groomed; awkward gait; slightly hunched; looks at wound or pulls away when area touched; mildly agitated; BCS=2
3) Moderate pain and distress	Rough hair coat; dirty incision; squinted eyes; moves slowly; walks hunched and/or slowly; depressed or moderately agitated; slight dehydration; pruritic; restless; uncomfortable; not eating or drinking; BCS=2.
4) Severe pain and distress	Very rough hair coat; eyes sunken (severe dehydration); slow to move or non-responsive when coaxed; hunched; large abdominal mass; dyspnea; self mutilating; violent reaction to stimuli or when approached; BCS=1

BCS, body condition score.

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Primer name	Primer sequence (5'-3')
GAPDH	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAATCC
B-cell lymphoma 2	F: TCCTTCCAGCCTGAGAGCAACC R: TCACGACGGTAGCGACGAGAG
Caspase-3	F: GTAGCAGTGTGCCGCTGTGTC R: ACTCTTGTCATCGCCTCTCCTC
Caspase-9	F: ATGGAGGAGGCTGACCGGCAACTCCTG R: TCATGAAGTTTTAAAGAACAGCTTCTTC
Cytochrome C	F: AGGAGGCAAGCATAAGAC R: ATTAGGTCTGCCCTTTCT
Dynamin-related protein 1	F: TTCGCCGCCTGGAGGACC R: CACTTCGTTGCCACAATGCTGAA

F, forward; R, reverse.

(cat. no. 10993-1-AP; 1:1,000; ProteinTech, Group, Inc., Chicago, IL, USA), mouse monoclonal  $\beta$ -actin antibody (1:4,000; cat. no. YM3138; ImmunoWay Biotechnology Company). Membranes were washed twice and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:6,000; cat. no. PMK-014-091; Bioprimary, Wuhan, China) and goat anti-rabbit IgG (1:8,000; cat. no. PMK-014-090; Bioprimary, Wuhan, China) for 2 h at room temperature. The blots were visualized with eECL western blot kit (Beyotime Institute of Biotechnology, Jiangsu, China). The intensities of the bands were measured using ImageLab 5.1 Software (Bio-Rad Laboratories, Inc.). Rat  $\beta$ -actin was used as the loading control in western blotting.

**Statistical analysis.** All experiments were repeated three times. All data are presented as mean  $\pm$  standard deviation. Comparisons of two or three experimental conditions were evaluated using an unpaired Student's t-test or one-way analysis of variance and the Bonferroni t-test followed by the Bonferroni post-test using GraphPad Prism 5.0 software (GraphPad Software Corp, San Diego, CA, USA).

$P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Lung histopathology.** Histopathological examinations were performed for the lungs of rats subjected to normoxia, IH, IH+25 mg/kg Mdivi-1 and IH+50 mg/kg Mdivi-1. IH caused severe interstitial pneumonia and a large number of inflammatory cells, while following Mdivi-1 intervention, interstitial pneumonia and inflammatory cells were reduced. The effect in the IH+50 mg/kg Mdivi-1 was more obvious compared with in the IH+25 mg/kg Mdivi-1 group (Fig. 1). Consistent with these histopathological observations, the lung injury score in the IH group was significantly increased compared with the control group ( $P < 0.005$ ) and the score in the IH+50 mg/kg Mdivi-1 group was significantly decreased compared with in the IH group ( $P < 0.005$ ).

**Detection of Bcl-2, caspase-3, caspase-9, Cyt-C and Drp-1 expression levels via RT-qPCR.** After SD rats were exposed

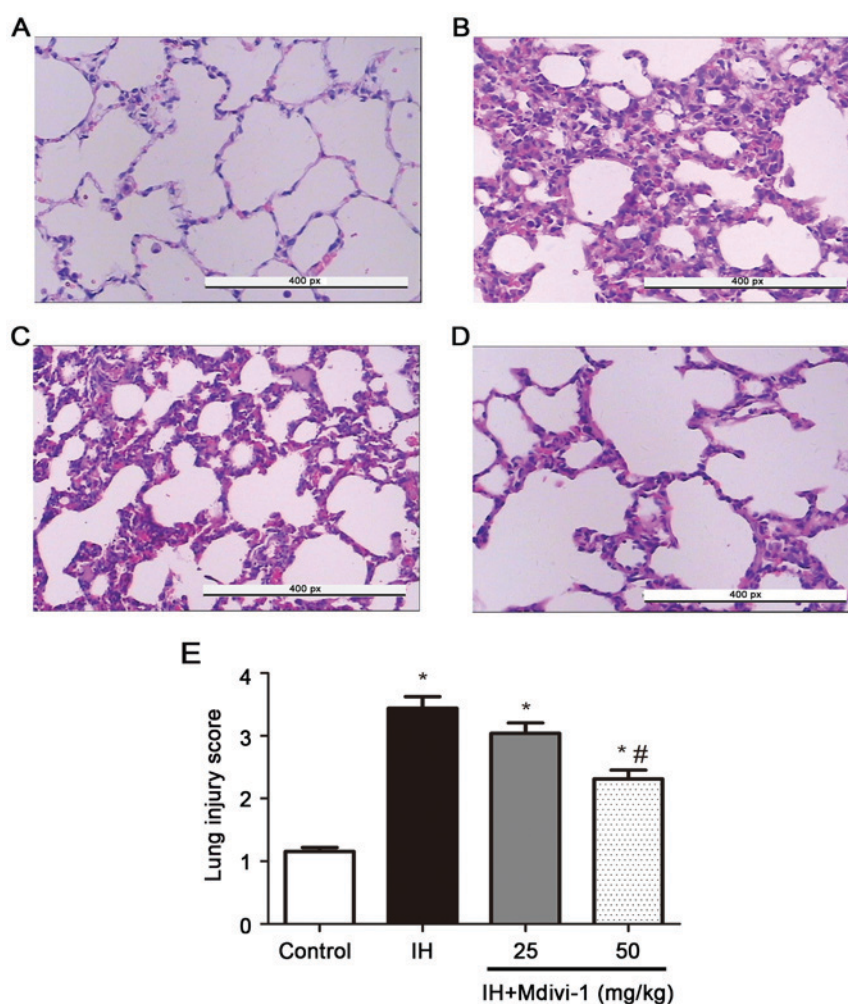


Figure 1. Detection of variations in the lung media of rats in each group using haematoxylin and eosin staining (magnification, 400; scale bar, 50  $\mu$ m). (A) The control group, n=8. (B) The IH group, n=8. (C) The IH+25 mg/kg Mdivi-1 group, n=8. (D) IH+50 mg/kg Mdivi-1 group, n=8. (E) Compared with normoxia, IH caused interstitial alterations in the lung tissues of SD rats, while Mdivi-1 significantly reduced the pulmonary interstitial lesions. The effects in the IH+50 mg/kg Mdivi-1 group were more obvious than those in the IH+25 mg/kg Mdivi-1 group. IH, intermittent hypoxia; SD, Sprague Dawley; Mdivi, mitochondrial division inhibitor-1. \* $P<0.05$  vs. group C; # $P<0.05$  vs. group IH.

to IH for 4 weeks and after WTRL1 cells were exposed to IH for 12 h in culture, the expression of apoptosis markers was increased at the gene level in the rat lung tissue cells and WTRL1 cells. In addition, Mdivi-1 treatment effectively reduced the apoptosis rate of the hypoxia-exposed cells. After SD rats were exposed to IH for 4 weeks, Bcl-2 mRNA levels were significantly decreased ( $P<0.05$ ; Fig. 2A), but caspase-3 and caspase-9 mRNA levels were significantly increased compared with the control group rats ( $P<0.05$ ; Fig. 2B and C). After Mdivi-1 intervention, Bcl-2 mRNA levels were significantly increased and caspase-3, and Drp-1 mRNA levels were significantly decreased in the IH+50 mg/kg Mdivi-1 group compared with the IH group ( $P<0.05$ ; Fig. 2A, B and D). However, no significant difference in Cyt C levels was identified between the groups (Fig. 2E). After WTRL1 cells were exposed to IH for 12 h, Bcl-2 mRNA levels were significantly decreased, but caspase-3, caspase-9, Cyt-C and Drp-1 mRNA levels were significantly increased compared with the control group ( $P<0.05$ ; Fig. 3A-E). After Mdivi-1 intervention, Bcl-2 mRNA levels were significantly increased and caspase-3, caspase-9, Cyt-C and Drp-1 mRNA levels were significantly decreased compared with in the IH group ( $P<0.05$ ).

*Western blotting results for Bcl-2, caspase-3, caspase-9, Drp-1 and Cyt-C.* After SD rats were exposed to IH for 4 weeks and after WTRL1 cells were exposed to IH for 12 h in culture, the expression of apoptosis markers was increased at the protein level in rat lung tissue cells and WTRL1 cells. In addition, Mdivi-1 treatment reduced the apoptosis rate of the hypoxia-exposed cells. Western blotting results for Bcl-2, caspase-3, caspase-9, Cyt-C and Drp-1 protein expression in rat lungs are presented in Fig. 4. After SD rats were exposed to IH for 4 weeks, the protein expression of Bcl-2 in the IH group was significantly decreased, but the protein expression of caspase-3, caspase-9 and Drp-1 was significantly increased compared with the control group ( $P<0.05$ ; Fig. 4A-E). After Mdivi-1 intervention, the protein expression of Bcl-2 was significantly increased and the protein expression of caspase-3, caspase-9, and Drp-1 was significantly decreased in the IH+50 mg/kg Mdivi-1 group compared with the IH group ( $P<0.05$ ). After WTRL1 cells were exposed to IH for 12 h, Bcl-2 protein expression was significantly decreased in the IH group and caspase-3, caspase-9 and Drp-1 protein expression was significantly increased ( $P<0.05$ ; Fig. 5A-E). After Mdivi-1 intervention, Bcl-2 protein expression was



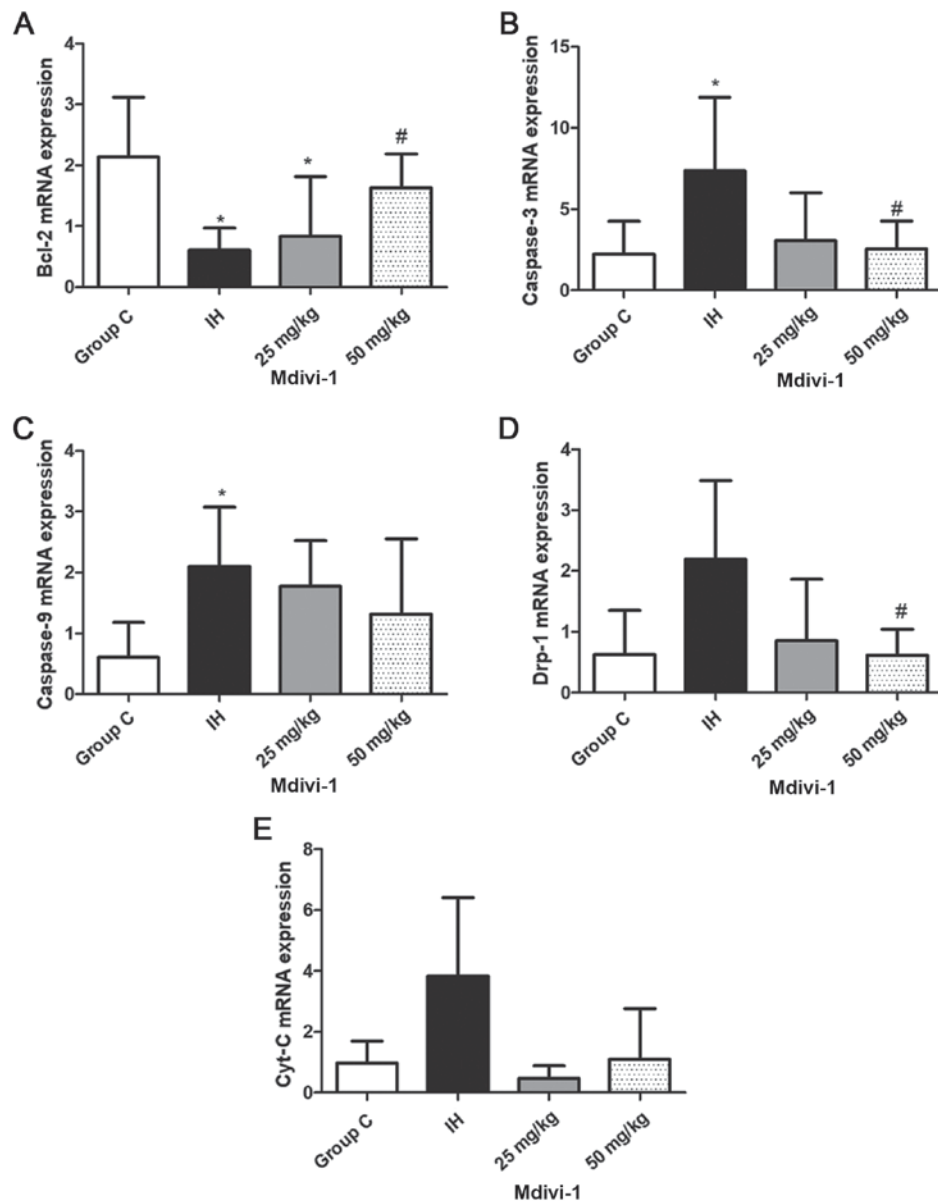


Figure 2. Detection of Bcl-2, caspase-3, caspase-9, Drp-1 and Cyt-C expression levels in rats lungs via reverse transcription-quantitative polymerase chain reaction. (A) Bcl-2 levels. (B) Caspase-3 levels. (C) Caspase-9 levels. (D) Drp-1 levels. (E) Cyt-C levels. Compared with those of group C, Bcl-2 mRNA levels were decreased, but caspase-3 and caspase-9 mRNA levels were increased in the IH group, and these differences were statistically significant \* $P < 0.05$  vs. group C. Compared with those of the IH group, Bcl-2 mRNA levels were increased and caspase-3 and Drp-1 mRNA levels were decreased in the IH+50 mg/kg Mdivi-1 group, # $P < 0.05$  vs. the IH group. IH, intermittent hypoxia; Mdivi, mitochondrial division inhibitor-1; Bcl, B-cell lymphoma; Cyt-c, cytochrome C; group C, control group.

significantly increased and caspase-9, Cyt-C, and Drp-1 protein expression was significantly decreased in the IH+100  $\mu$ M Mdivi-1 group ( $P < 0.05$ ; Fig. 5 A, B and D-F).

**Detection of apoptosis levels.** The apoptosis rate of WTRL1 cells was positively correlated with the time of exposure to IH. After WTRL1 cell exposure to IH for 6, 12, 24 and 48 h, the rate of apoptosis in WTRL1 cells increased gradually with the IH exposure time, and the apoptosis rate was the highest at 48 h ( $P < 0.05$ ; Fig. 6).

## Discussion

OSA is characterized by intermittent airway obstruction and systemic hypoxia during sleep. OSA affects 5-25% of the

general population. However, previous studies have illustrated a rising prevalence of moderate-to-severe sleep disordered breathing in men (ranging from 10-49%, depending on age and cohort) and women (ranging from 10-23%) in western countries (20,21). OSA frequently goes unnoticed by people who are at highest risk for developing the disease (22-25). OSA is recognized as a low-grade systemic inflammatory disease; it induces inflammatory responses and can be an indication of metabolic deregulation, in addition to posing a great risk for apoptotic cell death (26). Apoptosis can be triggered by two distinct signalling pathways, namely, the intrinsic and extrinsic pathways (27,28). The intrinsic apoptotic pathway is elicited by a wide range of intracellular stress conditions, including cytokine deprivation, DNA damage, oxidative stress, cytosolic  $Ca^{2+}$  overload and endoplasmic reticulum

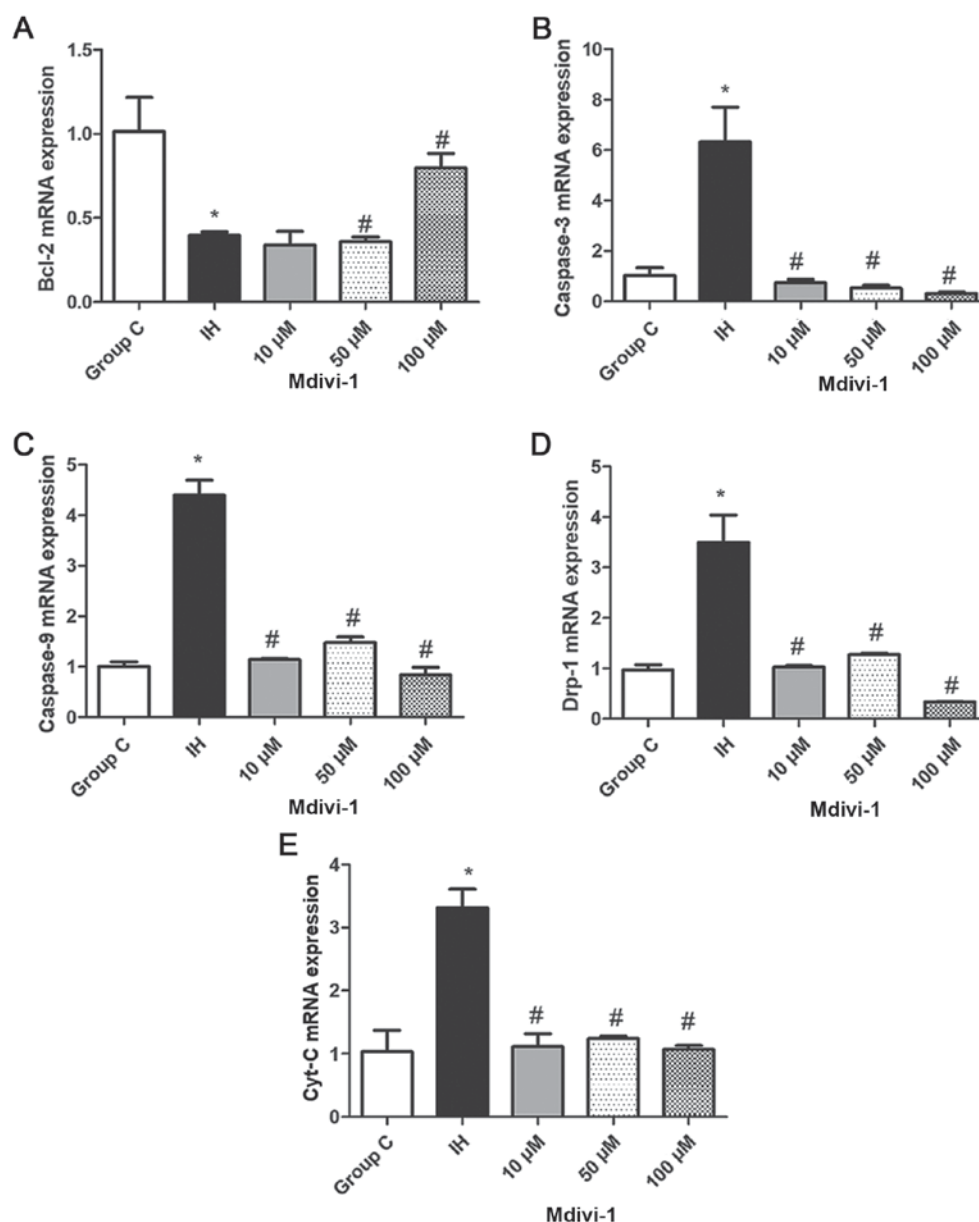


Figure 3. Detection of Bcl-2, caspase-3, caspase-9, Drp-1 and Cyt-C expression levels in WTRL1 cells via reverse transcription-quantitative polymerase chain reaction. (A) Bcl-2 levels. (B) Caspase-3 levels. (C) Caspase-9 levels. (D) Drp-1 levels. (E) Cyt-C levels. Compared with those in group C, Bcl-2 mRNA levels were lower, but caspase-3 and caspase-9 mRNA levels were higher in the IH group, and these differences were statistically significant \* $P < 0.05$  vs. group C. Compared with those in the IH group, Bcl-2 mRNA expression levels were increased and caspase-3, and Drp-1 mRNAs were decreased After Mdivi-1 intervention, # $P < 0.05$  vs. the IH group. IH, intermittent hypoxia; Mdivi, mitochondrial division inhibitor-1; Bcl, B-cell lymphoma; Cyt-c, cytochrome C; group C, control group.

stress. In the extrinsic pathway, apoptosis is triggered by death receptors, including FAS-associated death domain protein, therefore activating caspases-8 and 10 (the initiator caspases), which in turn activate executioner caspases-3, 6 and 7. In the intrinsic pathway, the mitochondrial permeability transition pore serves a pivotal role in regulating the release of pro-apoptotic proteins, including Cyt-C. The Cyt-C released from mitochondria initiates apoptosome assembly, activating factor 1 and caspase-9, an initiator caspase that cleaves and activates caspases-3 and 7 (29).

IH causes mitochondrial and endoplasmic reticulum system damage and produces inflammatory factors that cause lung injury. Sustained and IH-induced cell apoptosis mediates the mitochondrial apoptosis signalling pathway (30). A number

of hypoxia and inflammatory factors activate caspases via the mitochondrial apoptosis signalling pathway. Mitochondrial morphology is now recognized as an important determinant of the energetic state of mitochondria. IH/hypercapnia leads to oxidative stress due to the mitochondrial response. Mitochondria constantly undergo fusion and fission, which are essential for organelle fidelity. Lung cell apoptosis occurs through a highly regulated proteolytic process that eliminates unwanted, damaged, or altered cells. However, abnormal mitochondrial fission is involved in regulating apoptosis (31).

Mdivi-1 has emerged as a critical regulator of cellular function and differentiation under hypoxic conditions. However, the role of mitochondrial dynamics in hypoxia-induced angiogenesis remains to be elucidated. The aim of the present

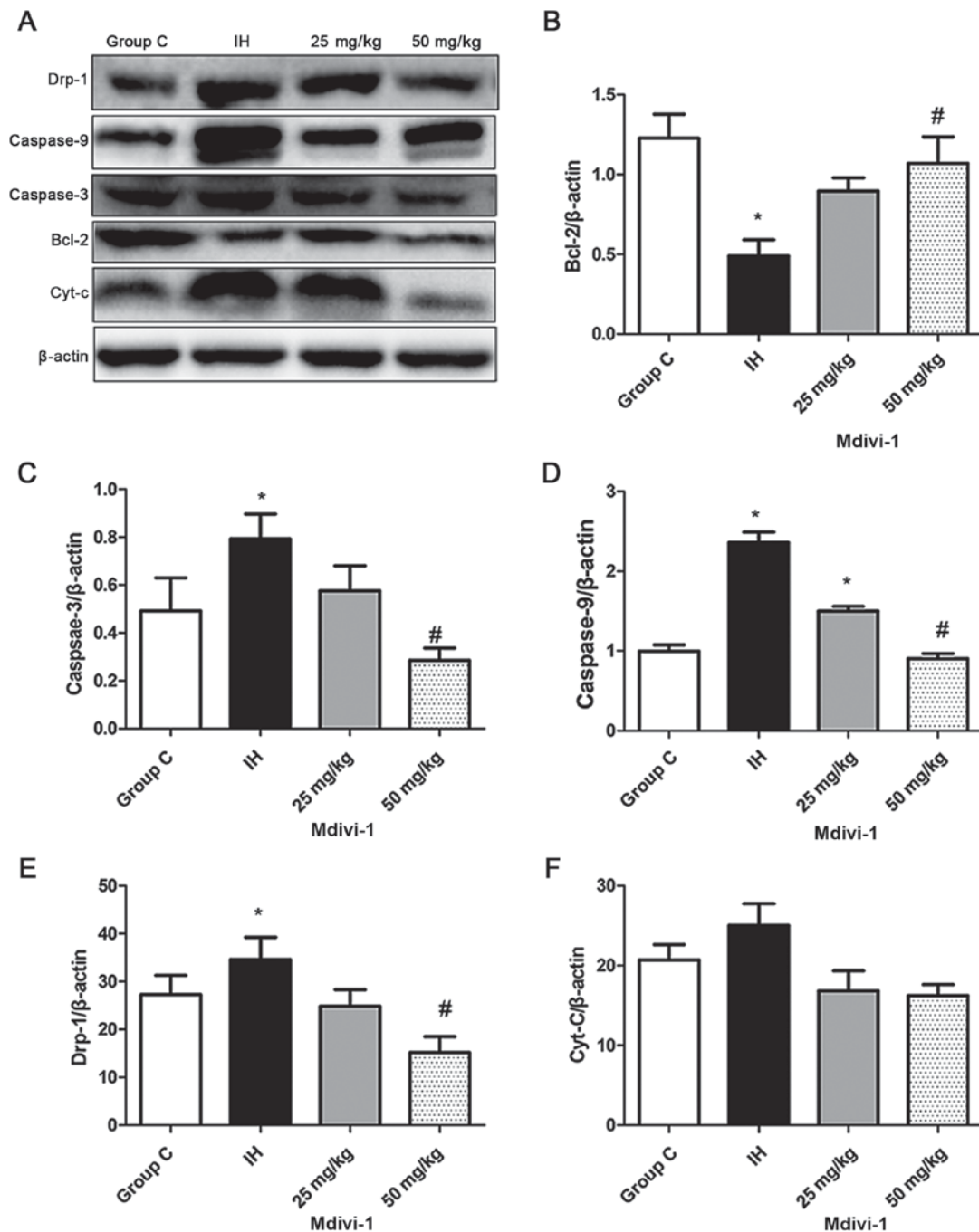


Figure 4. Detection of Bcl-2, caspase-3, caspase-9, Cyt-C and Drp-1 protein expression levels in rat lungs via western blotting assays. (A) (Lane 1) Group C, n=8. (Lane 2) IH group, n=8. (Lane 3) IH+25 mg/kg Mdivi-1 group, n=8. (Lane 4) IH+50 mg/kg Mdivi-1 group, n=8. Compared with that in the group C, the protein expression of (B) Bcl-2 in the IH group was decreased and that of (C) caspase-3, (D) caspase-9 and (E) Drp-1 was increased in the IH group. (F) The changes in protein expression of Cyt C were not significant. \* $P<0.05$  vs. group C. Compared with the IH group, the protein expression of Bcl-2 was increased and caspase-3, caspase-9, and Drp-1 was decreased in the IH+50 mg/kg Mdivi-1 group, # $P<0.05$  vs. IH. IH, intermittent hypoxia; Mdivi, mitochondrial division inhibitor-1; Bcl, B-cell lymphoma; Cyt-c, cytochrome C; group C, control group.

study was to investigate the protective effect and possible mechanism of Mdivi-1 in lung cell apoptosis induced by IH.

Several studies have revealed that Drp-1 participates in mitochondrial fission and the subsequent apoptosis induced by various stimuli (32,33). Drp-1 assembles from the cytosol onto mitochondria at focal sites of mitochondrial fission; emerging evidence indicates that it is concomitantly involved in apoptosis with mitochondrial outer membrane permeabilization and that Drp-1 inhibition prevents partially intrinsic apoptosis (34,35). The activity of the prototypical Bcl-2 protein

is usually considered anti-apoptotic (36). The Bcl-2 family is classified into three subgroups: Pro-apoptotic proteins (Bax, BAD, Bak and Bok); anti-apoptotic proteins (Bcl-2, Bcl-xL and Bcl-w) and BH3-only members [Bad, Bid, Bim, Noxa and PUMA (a p53-upregulated modulator of apoptosis)]. The caspase family of cysteine-aspartic proteases serves an important role in the initiation and completion of cell apoptosis and executes cellular apoptosis. For instance, caspase-3 is the primary activator of apoptotic DNA fragmentation. Certain reports have demonstrated that the pro-apoptotic signalling

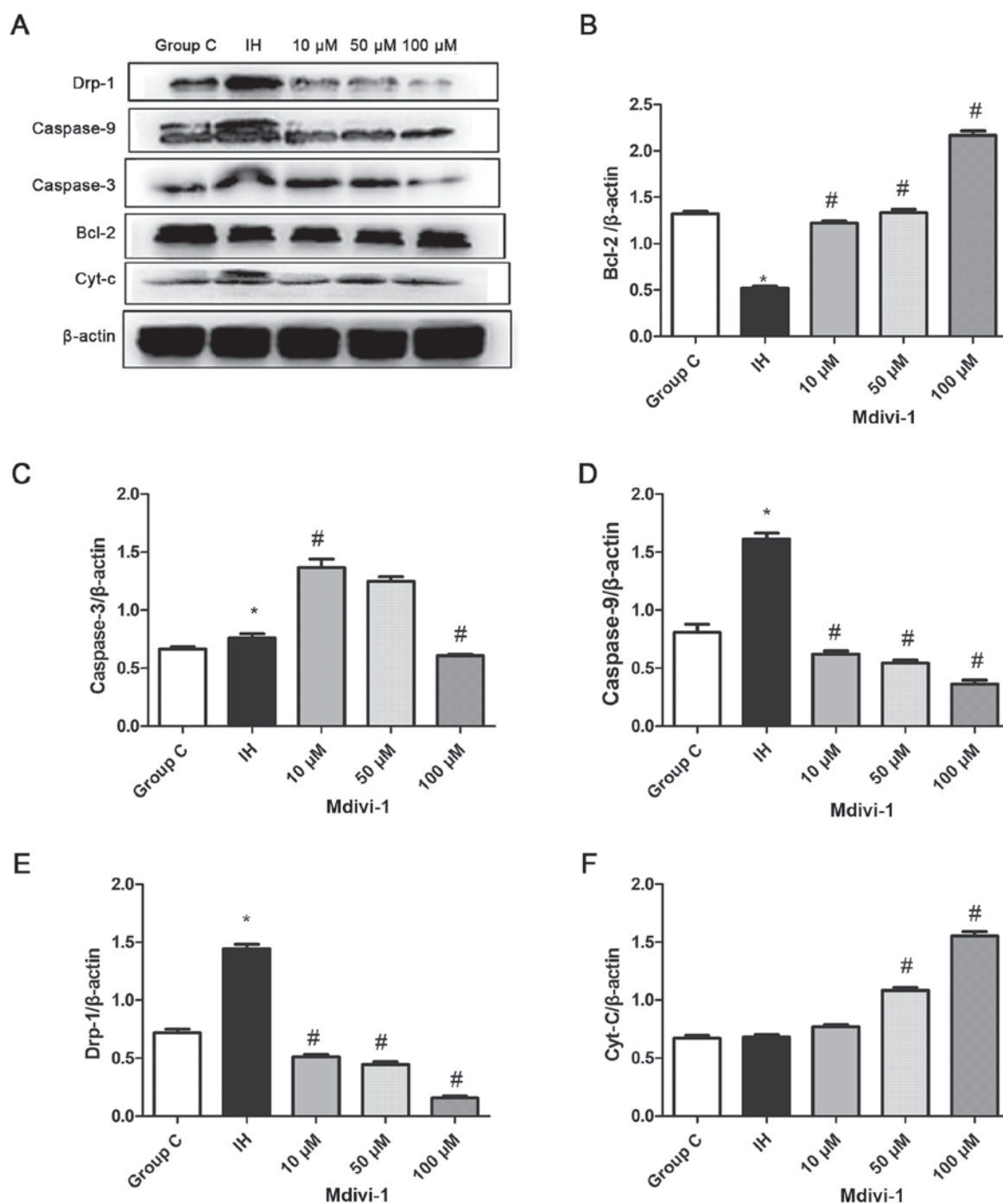


Figure 5. Detection of Bcl-2, caspase-3, caspase-9, Cyt-C and Drp-1 expression levels in WTRL1 cells via western blotting assays. (A) (Lane 1) Group C, n=8. (Lane 2) IH group, n=8. (Lane 3) IH+10  $\mu$ M Mdivi-1 group, n=8. (Lane 4) IH+50  $\mu$ M Mdivi-1 group, n=8. (Lane 5) IH+100  $\mu$ M Mdivi-1 group, n=8. Compared with the group C, (B) Bcl-2 protein expression in the IH group was decreased, but (C) caspase-3, (D) caspase-9 and (E) Drp-1 protein expression was increased, \* $P$ <0.05 vs. group C. Compared with the IH group, Bcl-2 protein expression was increased and caspase-9 Drp-1 and (F) Cyt-C protein expression was decreased in the IH+100  $\mu$ M Mdivi-1 group, # $P$ <0.05 vs. IH. Mdivi, mitochondrial division inhibitor-1; Drp-1, dynamin-related protein 1; IH, intermittent hypoxia; Bcl, B-cell lymphoma; group C, Control group.

of Cyt-C triggers the caspase cascade amplification reaction, which leads to the cleavage of a set of proteins (37,38).

Mitochondria are autonomous and morphologically dynamic organelles that structurally reflect a precise balance of ongoing fission and fusion within a cell. In the two models, *in vitro* and *in vivo*, it was observed that Mdivi-1 decreases mitochondrial fission and inhibits cell apoptosis in the lungs. These results agree with evidence from previous studies (39,40). In this study, it was demonstrated that inhibiting mitochondrial

fission by Mdivi-1 prevents lung apoptosis during IH and it was demonstrated that IH induced mitochondrial fission and apoptosis via a mitochondrial mechanism involving the transcriptional activation of caspase-3, caspase-9 and Drp-1. The results of the present study provide a well-characterized mechanism explaining how IH stimulates mitochondrial fission and consequent apoptosis, which may offer a novel therapeutic strategy for treating OSA via the mitochondrial apoptosis pathway.



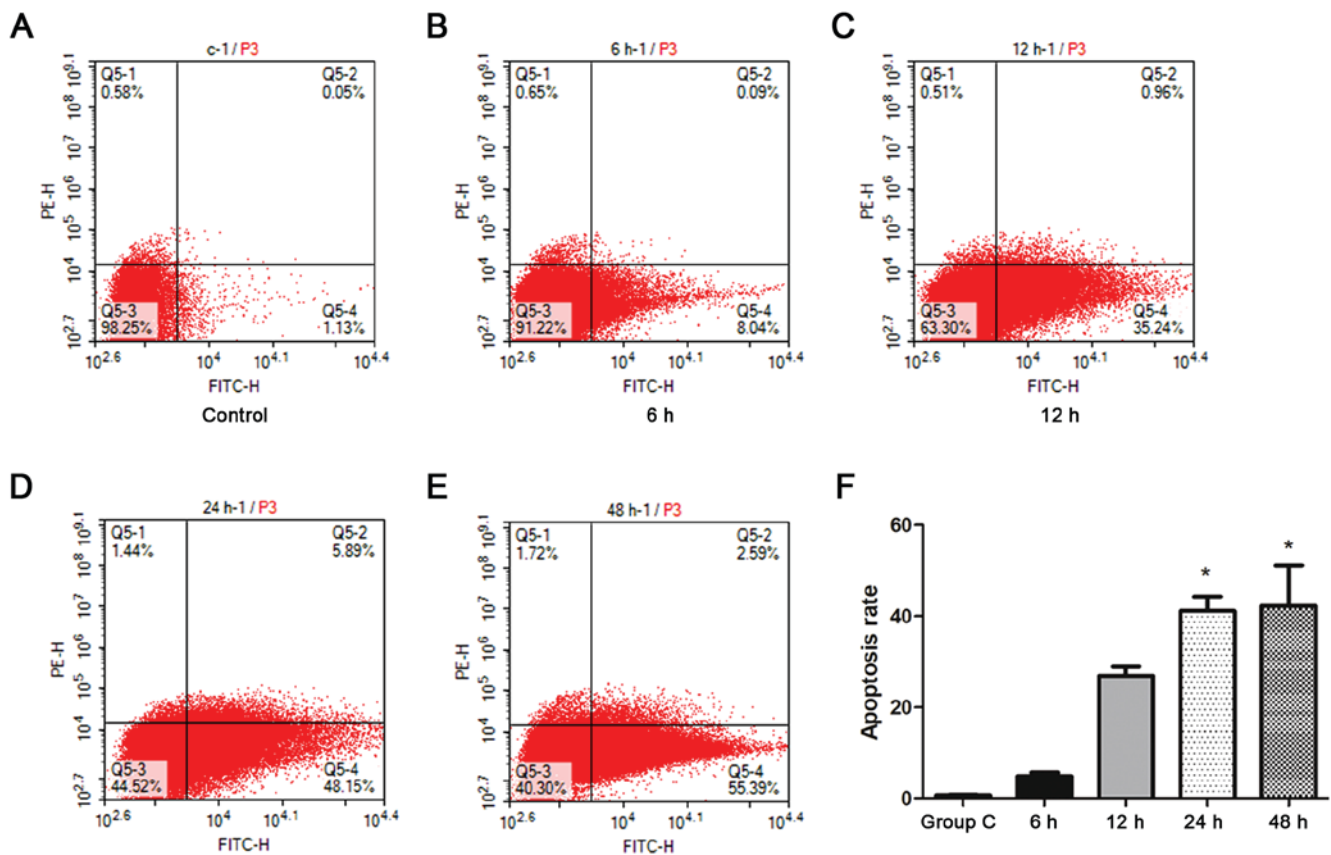


Figure 6. Detection of the apoptosis rate via flow cytometry. (A) Group C, n=8. (B) IH for 6 h, n=8. (C) IH for 12 h, n=8. (D) IH for 24 h, n=8. (E) IH for 48 h, n=8. (F) Detection of the apoptosis rate via flow cytometry. The apoptosis rate of WTRL1 cells increased gradually with the time exposed to IH and the apoptosis rate was the highest at 48 h. \*P<0.05 vs. group C. IH, intermittent hypoxia; group C, control group.

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## Availability of data and materials

The datasets used and/or analysed in the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

DZ and XY contributed to the conception and design of the study. CY and ZW collected the data and revised the manuscript for important intellectual content. DZ and XZ analysed and interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The study was approved by the Ethics Committee of Guizhou Provincial People's Hospital.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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