Beneficial effect of the mitochondrial ATP-sensitive potassium channel-specific opener nicorandil on the collapsed lung via inhibition of apoptosis in clinical thoracic surgery

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Received October 14, 2020; Accepted October 20, 2022

DOI: 10.3892/mmr.2023.12948

Abstract. With the use of thoracoscopic surgery technology, one-lung ventilation (OLV) is becoming more crucial as a basic requirement for enhanced recovery after surgery; however, it can lead to severe pulmonary injury, which is an issue for anesthesiologists. Therefore, it is important to protect pulmonary function during thoracic surgery anesthesia, particularly to protect the function of the collapsed lung. Our previous study on rabbits reported that nicorandil, a US Food and Drug Administration-approved mitochondrial ATP-sensitive potassium channel-specific opener, can protect against lung injury in the collapsed lung. Therefore, the beneficial effect of nicorandil on OLV-induced pulmonary injury in clinical thoracic surgery was further evaluated in the present study. Nicorandil was infused at 2 mg/h for 2 h from induction to 1 h after OLV in the nicorandil group. Trends in arterial oxygen desaturation (SaO_2), arterial partial pressure for oxygen (PaO_2) and the lung microstructure were assessed. ELISA was used to assess the levels of TNF-α and malondialdehyde (MDA), and the activity of superoxide dismutase (SOD). A TUNEL assay was performed to evaluate apoptosis. Western blotting was used to analyze the relative expression levels of signaling proteins associated with apoptosis. Western blotting was performed to evaluate the protein expression levels of hypoxia-inducible factor 1α (HIF-1α), PI3K, Akt and NF-κB, and reverse transcription-quantitative PCR was used to detect HIF-1α mRNA expression levels in the lungs of patients infused with nicorandil and nitroglycerin. Nicorandil treatment was associated with higher SaO_2 and PaO_2 compared with nitroglycerin treatment in OLV. The levels of MDA and TNF-α in the operated lung of the nicorandil group were significantly lower compared with those in the control group. In addition, nicorandil was associated with higher SOD activity compared with nitroglycerin. The nicorandil-treated lung, similar to the sham group, exhibited improved microstructure and less apoptosis in the experimental group. The protein expression levels of PI3K, phosphorylated Akt and HIF-1α were significantly increased, whereas NF-κB was significantly decreased in the nicorandil-treated lung compared with the control group. Overall, nicorandil demonstrated beneficial effects by decreasing apoptosis in the operated lung, which was collapsed and then re-expanded during OLV in thoracic surgery anesthesia. Nicorandil may serve a vital role by decreasing the overloading of calcium in mitochondria, shutting off the mitochondrial membrane permeability transition pore, reducing the release of cytochrome *c*, simultaneously triggering activation of the PI3K/Akt signaling pathway around the cell membrane, downregulating NF-κB, upregulating HIF-1α, and then reducing Bax/Bcl-2, caspase-3 and apoptosis. The trial registration was ChiCTR-IOR-17014061 (registered on December 20, 2017).

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Abbreviations: ERAS, enhanced recovery after surgery; OLV, one-lung ventilation; TLV, two-lung ventilation; HR, heart rate; I/R, ischemia/reperfusion; mitoKATP, mitochondrial ATP-sensitive potassium channel; SaO2, arterial oxygen desaturation; SpO_2, pulse oxygen saturation; PaO_2, arterial partial pressure for oxygen; MDA, malondialdehyde; SOD, superoxide dismutase; HIF-1α, hypoxia-inducible factor 1α; RT-qPCR, reverse transcription-quantitative PCR; V/Q, ventilation/perfusion; mPTP, mitochondrial membrane permeability transition pore

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Key words: nicorandil, clinical thoracic surgery, OLV, OLV-induced pulmonary injury, apoptosis, PI3K/Akt, NF-κB, HIF-1α, Bax/Bcl-2, caspase-3

Introduction

One-lung ventilation (OLV) has been commonly applied in clinical settings (1); however, it can result in inhibition of hypoxemic pulmonary vasoconstriction, the imbalance of ventilation/perfusion (V/Q), the production of oxygen free radical products, inflammation and ischemia/reperfusion (I/R) injury, mainly in the surgical lung, which can lead to severe pulmonary injury and is an issue for anesthesiologists (2,3). Current methods of OLV do not satisfactorily improve the prognosis of patients. Previous studies have reported that the core problem of OLV-associated pulmonary injury is mitochondrial damage (4,5). According to previous studies, nicorandil, a US Food and Drug Administration-approved mitochondrial ATP-sensitive potassium channel (mitoKATP)-specific opener, has beneficial effects on the heart by opening the mitoKATP (6-8). To the best of our knowledge, only one study has previously reported its effect on pulmonary injury (9).

It is important to protect pulmonary function during thoracic surgery anesthesia, particularly that of the collapsed lung. This is very important for the perioperative safety of patients and guarantees the rapid recovery of patients after surgery, which is the core intention of enhanced recovery after surgery (ERAS) protocols (10). How to ensure the stability and clearance of the surgical field while maintaining the stability of vital signs during OLV, as well as the early recovery of postoperative pulmonary function, is an unsolved clinical issue.

Acute lung injury can cause changes in mitochondrial respiratory function and enzyme activity, the production of mitochondrial oxygen free radicals, mitochondrial calcium overload and mitochondrial permeability transition. The activation of PI3K can initiate the phosphorylation of various phosphatidylinositol intermediates and the generated phosphatidylinositol (3,4,5)-trisphosphate can be combined with Akt. After binding, Akt is transferred from the cytoplasm to the cell membrane with the help of 3-phosphoinositide-dependent protein kinase 1. The threonine phosphorylation site (Thr308) and serine phosphorylation site (Ser473) on Akt are phosphorylated to promote its activation. Phosphorylated (p)-Akt regulates cell functions, such as anti-apoptotic functions, by phosphorylating numerous enzymes and kinases (11). Moreover, p-Akt can phosphorylate Bcl-2 family member BAD so that it can bind to the effector protein instead of Bcl-XL, thereby inhibiting apoptosis. Furthermore, mitochondrial regulation of apoptosis is closely associated with the Bcl-2 protein. Pro-apoptotic members of this family, such as Bax, trigger the release of mitochondrial apoptotic factors into the cytoplasm by acting on the mitochondrial membrane permeability transition pore (mPTP), which leads to the activation of cysteine proteases. The anti-apoptotic protein Bcl-2 serves a role in preventing cell apoptosis. Oxidative stress products are closely associated with mitochondrial dysfunction and apoptosis (12). The mPTP is the main target of reactive oxygen species (ROS) (12). After the mPTP is opened, cytochrome *c* is released into the cytoplasm, which causes cell dysfunction.

Nicorandil can open the KATP channel on the vascular smooth muscle cell membrane, causing potassium ion outflow and leading to hyperpolarization of the cell membrane, which will inhibit the opening of voltage-dependent calcium ion channels, thus leading to reduced calcium ion influx (13). Whether nicorandil can act on mitoKATP to serve a protective role in the lungs requires elucidation.

Our previous study in rabbits demonstrated that nicorandil protected against lung injury in the collapsed lung during surgery (13). The present study assessed the beneficial effect of nicorandil on OLV-induced pulmonary injury in clinical thoracic surgery and evaluated its mechanisms in combination with the results of the rabbit OLV model (13).

Materials and methods

Patient information. The present study was approved by the Affiliated Hospital of Nantong University ethics committee (approval no. 2017-K031-D01; Nantong, China). A total of 60 American Society of Anesthesiologists class I-II (14) patients undergoing thoracoscopic radical resection for lung cancer at the Affiliated Hospital of Nantong University, Yangzhou Hongquan Hospital (Yangzhou, China) and Shuyang Hospital Affiliated of Xuzhou Medical University (Suqian, China) between January 2018 and December 2020 were enrolled in the present study. The patients volunteered and were randomly divided into the following groups: i) The sham group; ii) the control group; and iii) the nicorandil group [n=20 cases (10 male and 10 female cases)/group]. The 30 male and 30 female patients were 55-75 years old and weighed 50-70 kg. Their lung, brain, liver and kidney functions, and biochemical tests were normal prior to surgery. An electrocardiogram (EKG) was used to assess chronic myocardial ischemia, and changes to the ST-segment and T-wave. Nicorandil and nitroglycerin can be applied for myocardial protection. Patients with a history of digestive ulcers and the use of sulfa drugs were excluded. Subjects participated voluntarily and were able to withdraw; therefore, a potential exit rate was considered in the calculation of the sample size. After initial screening according to inclusion and exclusion criteria, informed consent was signed to collect basic information, disease history and anthropometric information of the patients in a face-to-face manner. The four enrolled cases in the control group and five enrolled cases in the nicorandil group that were benign were withdrawn from the study. Patients who were enrolled in the control and nicorandil groups underwent thoracoscopic radical resection of lung cancer after pathological biopsy. Patients who were enrolled in the sham group did not undergo thoracoscopic radical resection of lung cancer due to the mass being benign; their tissue samples were obtained from a pathological biopsy.

Instruments. The following instruments were used in the present study: Single double-sided clean bench (Suzhou Purification Engineering Installation Co., Ltd.), transmission electron microscope (Hitachi, Ltd.), ELISA plate reader (BioTek Instruments, Inc.), fluorescence quantitative (q)PCR instrument (Eppendorf), fluorescence microscope (Olympus Corporation), gel imaging analysis system (Beijing Yuanpinghao Biotech Co., Ltd.), SK-30 high-speed refrigerated centrifuge (Sigma-Aldrich; Merck KGaA), Cryostat CM1900-UV (Leica Microsystems GmbH), multi-function monitor (Spacelabs Healthcare; OSI Systems, Inc.), DNA thermal cycler PTC-200 (Eppendorf) and paraffin microtome RM2245 (Leica Microsystems GmbH).

Experimental methods

Anesthesia method. Atropine (0.5 mg) was injected intramuscularly before surgery. In the operation room, Ringer's solution was infused at 10 ml/kg/h. The signal lines of the EKG, the probe of pulse oxygen saturation (SpO_2) and the artery catheter were placed to continuously monitor the vital signs [heart rate (HR), SpO_2 and mean arterial pressure (MAP)] using a Spacelabs monitor. Midazolam (50 μg/kg), sufentanil (0.6 μg/kg), propofol (2 mg/kg) and vecuronium (0.2 mg/kg) were used for induction. A double lumen bronchial catheter was inserted ~3 min after induction and mechanical ventilation was performed with a tidal volume (VT) of 8 ml/kg and a respiratory rate (RR) of 12 beats/min. During OLV, the VT was adjusted to 5 ml/kg and the RR was adjusted to 16 beats/min. Anesthesia maintenance was performed by infusion of propofol (200 mg/h, remifentanil 0.3 (μg/kg/min) and atracurium (50 mg/h). OLV was performed when the double lumen bronchial catheter was inserted and two-lung ventilation (TLV) was restored before the incision was closed. Anesthesia was terminated after the operation. The patients were admitted to the post-anesthesia care unit and were then connected to a patient-controlled intravenous analgesia pump [sufentanil (4.5 μg/kg) and azasetron (20 mg); total volume, 150 ml]. Neostigmine (2 mg) and atropine (1 mg) antagonized residual muscle relaxant before consciousness, reflexes and breathing were completely restored. The patients were returned to the ward after extubation.

Patient treatment. Patients in the nicorandil group were treated according to the manufacturer's protocol for nicorandil (SHKB pharmaceutical), venous access was opened 1 h before induction and nicorandil (2 mg/h) was infused for 2 h until 1 h after OLV. OLV and TLV were performed and thoracoscopic radical resection of lung cancer was successfully completed. Patients in the control group were treated according to the same method as The nicorandil group, with the exception that nitroglycerin (0.3 μg/kg/min) was used instead of nicorandil. Patients in the sham group were treated with nitroglycerin (0.3 μg/kg/min) as was used in the control group without an OLV phase. Immediately after the operation, the target lung tissue that was removed by wedge resection was rapidly frozen and underwent pathological examination and was confirmed to be benign in the sham group. A schematic diagram of the experimental design was presented in the Fig. 1A.

Observation items. MAP, HR and SpO_2 were recorded at four time points, including before administration of nicorandil or nitroglycerin, 30 min after OLV, 30 min after TLV and 30 min after extubation. Arterial blood gas analysis was performed for arterial partial pressure for oxygen (PaO_2) and arterial oxygen desaturation (SaO_2) at the above four time points. Light microscopy and electron microscopy were used to evaluate the microstructure of the lung. A TUNEL assay was utilized to evaluate apoptosis and assess the proportion of apoptotic cells to total observed cells (AI). The levels of malondialdehyde (MDA) and TNF-α, and the activity of superoxide dismutase (SOD) were assessed using ELISA. The MDA (cat. no. S0131S), SOD (cat. no. S0103) and TNF-α (cat. no. PT518) ELISA kits were all obtained from Beyotime Institute of Biotechnology and were performed according to the manufacturer's protocols. The protein expression levels of PI3K, Akt, p-Akt, NF-κB and hypoxia-inducible factor 1α (HIF-1α) were assessed using western blotting. Reverse transcription (RT)-qPCR was used to assess the mRNA expression level of HIF-1α. The protein expression levels of apoptosis-related proteins, including Bax, Bcl-2 and caspase-3, were also semi-quantified using western blotting.

Specimen collection. A total of 1 ml arterial blood was collected at the four aforementioned time points for blood gas analysis, immediate assessment and ELISA. When the surgical specimen was removed, three pieces (~1 cm^3) of lung tissue around the mass were collected. One piece was fixed with 4% paraformaldehyde (PFA) at 4˚C overnight for use in subsequent H&E staining and TUNEL staining. Another piece was frozen in liquid nitrogen and then placed in an ultra-low temperature (-80˚C) experimental freezer for RT-qPCR and western blotting. The remaining piece of lung tissue was fixed using 2.5% glutaraldehyde and stored at 4˚C overnight for assessment using scanning electron microscopy.

Detection methods. All subsequent detection methods were previously reported for use in animal experiments (11).

Measurement of the pulmonary injury score (H&E staining). For histopathological analysis, lung tissue samples were fixed in 4% PFA at 4˚C overnight, and embedded in paraffin. Subsequently, sections (5 μm) were prepared, stained with hematoxylin at room temperature for 5 min and with eosin at room temperature for 3 min for pathological observation. Finally, the samples were examined using an OLYMPUS-CX23 light microscope (magnification, x400; Olympus Corp.). Each slide was assessed according to the following criteria: i) Alveolar septal congestion; ii) alveolar hemorrhage; iii) intra-alveolar cell infiltrates; and iv) intra-alveolar fibrin deposition (15).

Transmission electron microscopy. Lung tissues (1 mm^3) were fixed using 2.5% glutaraldehyde at 4˚C, overnight, followed by 1% osmium tetroxide and embedded in epoxy resin 618 at room temperature overnight. Subsequently, these samples were cut into ultrathin sections (0.1 μm) and examined under a Hitachi HT-7700 transmission electron microscope (magnification, x3000; HT-7700; Hitach, Ltd.).

TUNEL assay. Sections (10 μm) were dewaxed at room temperature for 30 min, rehydrated using ethyl alcohol according to kit instructions, protein was removed using proteinase K, the sample was immersed in equilibration buffer and incubated with terminal deoxynucleotidyl transferase buffer containing FITC-12-dUTP labeling mix at 37˚C for 1 h. The nuclei were stained with Hoechst (1 μg/ml) at room temperature for 5 min and slides were assessed using a fluorescence microscope (magnification, x200) after being covered with mounting media (Beyotime Institute of Biotechnology). Three fields of view were randomly selected for assessment. The apoptosis index (AI) was calculated as the percentage of TUNEL-positive nuclei among the total number of nuclei in a randomly selected area.

Western blotting. Lung tissues were homogenized using a RIPA lysis buffer (Beyotime Institute of Biotechnology Co., Ltd.), which contains protease, phosphatase and phosphorylase inhibitors. The total protein concentration in the supernatant was assessed using a BCA protein assay. Subsequently, an equal amount (30 μg) of proteins were separated by SDS-PAGE on 5

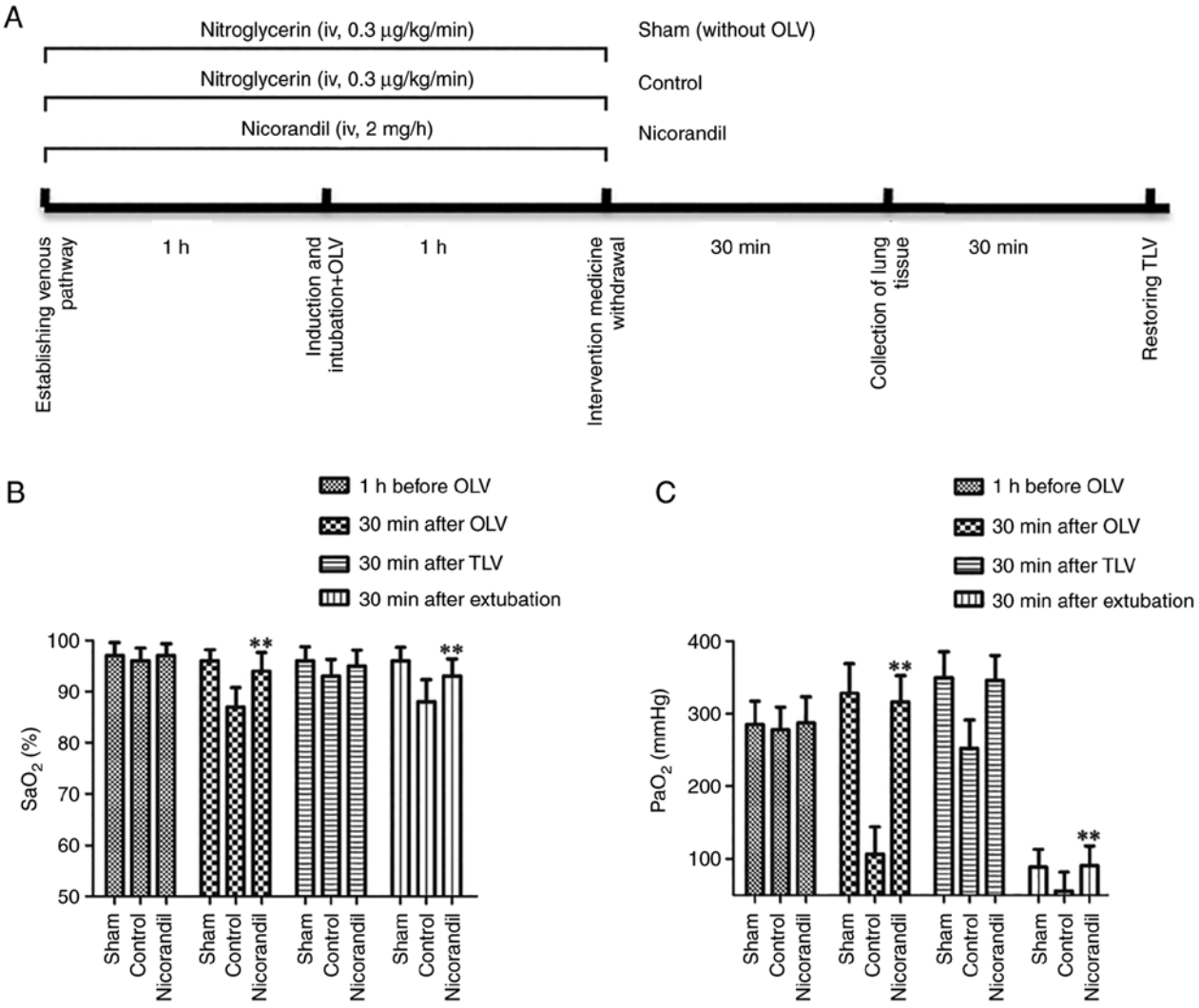

Figure 1. Flow chart of the clinical trial grouping and drug interventions in different groups, and the effect of nicorandil on oxygenation in OLV-induced pulmonary injury. (A) Clinical trial grouping and schematic diagram of the operation process. Effect of nicorandil on (B) SaO2 and (C) PaO_2 levels. $^{**}P<0.01$ vs. control. OLV, one-lung ventilation; TLV, two-lung ventilation; SaO_2, arterial oxygen desaturation; PaO_2, arterial partial pressure for oxygen; iv, intravenous.

or 10% gels and then transferred to a PVDF membrane. After blocking with 5% fat-free milk at room temperature for 30 min, the membranes were incubated with primary antibodies overnight at 4˚C. After washing three times with TBS-Tween (0.1%), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Protein bands were visualized using the ECL chemiluminescence detection kit (Vazyme Biotech Co., Ltd.) and analyzed using a EDAS120 gel imaging system (Kodak). The antibodies used were as follows: NF-κB (1:500; cat. no. MAB3026; MilliporeSigma), β-actin (1:1,000; cat. no. 3700; Cell Signaling Technology, Inc.), PI3K (1:1,000; cat. no. AF1966; Beyotime Institute of Biotechnology), Akt (1:1,000; cat. no. AF0045; Beyotime Institute of Biotechnology), p-Akt (1:1,000; cat. no. AF1546; Beyotime Institute of Biotechnology), HIF-1α (1:500; cat. no. NB100-105; Novus Biologicals, LLC), Bax (1:1,000; cat. no. AB026; Beyotime Institute of Biotechnology), Bcl-2 (1:1,000; cat. no. AF0060; Beyotime Institute of Biotechnology) and caspase-3 (1:1,000; cat. no. 9665; Cell Signaling Technology, Inc.), HRP-conjugated secondary goat anti-rabbit antibody (1:5,000; cat. no. AP156P; MilliporeSigma), and HRP-conjugated secondary goat anti-mouse antibody (1:5,000; cat. no. AP124P; MilliporeSigma).

RT-qPCR. Total RNA was extracted from lung tissue specimens using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, total RNA was reverse transcribed to cDNA at 42˚C for 60 min by using BeyoRT™ (cat. D7166; Beyotime Institute of Biotechnology) according to manufacturer's protocol. qPCR was performed on cDNA. Primer sequences used were as follows: HIF-1α (NM_001082782) forward (F), 5'-CAACATCACCACCATACA-3' and reverse (R), 5'-TCA GGAGCAGTAGTTCTTT-3'; and GAPDH (NM_001082253) F, 5'-AGAGCACCAGAGGAGGACG-3' and R, 5'-CTGGGA TGGAAACTGTGAAGA G-3'. The lengths of amplified products were 148 and 105 bp for HIF-1α and GAPDH, respectively. Amplification reaction conditions were as follows: HIF-1α, 94˚C pre-denaturation for 3 min, followed by 32 cycles of 94˚C denaturation for 30 sec, 56˚C annealing for 30 sec and 72˚C extension for 45 sec; and GAPDH, 94˚C pre-denaturation for 3 min, followed by 32 cycles of 94˚C denaturation for 30 sec,

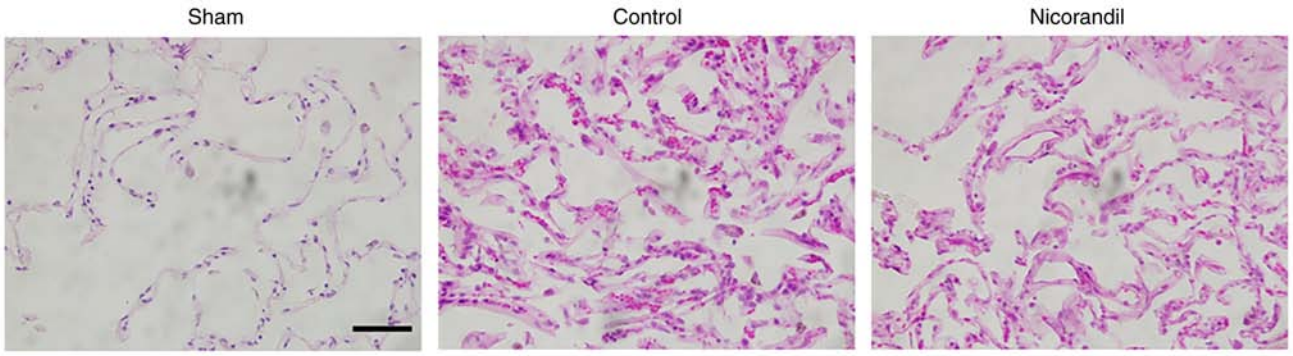

Figure 2. Beneficial effects of nicorandil on the operated lung in OLV were assessed using H&E staining. Scale bar, 50 μm. OLV, one-lung ventilation.

57˚C annealing for 30 sec and 72˚C extension for 45 sec. The SYBR Green PCR Master Mix kit [Roche Diagnostics (Shanghai) Co., Ltd.] was used for qPCR. Fluorescence intensity was assessed using a fluorescence qPCR instrument and the results were generated compared with the sham group. The relative expression of mRNA (normalized against GAPDH) was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. All data are presented as the mean ± standard deviation and each experiment was repeated at least three times. Statistical analysis was performed using GraphPad Prism (version 5; GraphPad Software, Inc.). Multiple comparisons were analyzed using one-way ANOVA followed by Holm-Sidak post hoc correction. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of nicorandil on patient oxygenation in OLV-induced pulmonary injury. The present study evaluated the oxygenation of patients by assessing their SaO_2 and PaO_2. For SaO_2 and PaO_2 (blood gas analysis), the levels in the nicorandil group were significantly improved compared with those in the control group at the 30 min after OLV and 30 min after TLV (P<0.01; Fig. 1B and C). The changes in the trend of SpO_2 (data not shown) were the same as those of SaO_2. These data suggested that nicorandil had beneficial effects on the pulmonary oxygenation of patients.

Effect of nicorandil on the microstructure of OLV-induced injured lungs. The microstructure, as assessed using H&E staining and electron microscopy, was markedly improved in the nicorandil group compared with that in the control group. The results of H&E staining demonstrated that the alveolar structure in the control group was severely damaged, partially collapsed and disappeared. A large amount of hyperemia was observed in the lung tissue and there were numerous red blood cells and inflammatory cells. In the control group, the alveolar wall was thick and edematous, whereas this was less observed in the nicorandil group, which appeared similar to the sham group with normal alveolar structure, including a thin alveolar wall, less infiltration of red blood cells and inflammatory cells, and no obvious exudation (Fig. 2). The results of electron microscopy demonstrated that the nuclear fractions of the three types of cells in the control group exhibited pyknosis and lobulation, and the perinuclear space was enlarged. The lamellar bodies of type II epithelial cells were emptying, and the microvilli on the cell membrane became thinner and smaller. The nicorandil group was similar to the sham group, which was close to the ultrastructure of normal lung cells, including full cell nucleus without pyknosis, lobulation and extension of the perinuclear space. The lamellar body of type II epithelial cells, which had more microvilli, was not empty (Fig. 3).

Effect of nicorandil on apoptosis in OLV-induced injured lungs. The results of the TUNEL assay demonstrated that the control group had a markedly increased proportion of apoptotic cells and a high AI, compared with in the sham and nicorandil groups. The nicorandil and sham groups demonstrated minimal apoptosis. There was a significant difference in AI (P<0.01) between nicorandil and control groups (Fig. 4).

Effect of nicorandil on OLV-induced pulmonary injury via downregulation of oxidative stress. Oxidative stress status was evaluated via assessment of MDA and TNF-α levels, and SOD activity. The MDA and TNF-α levels in the operated lung in the nicorandil group were significantly lower than those in the control group (P=0.0008 and P<0.01, respectively; Fig. 5A and C). The activity of SOD in the nicorandil group was significantly higher compared with that in the control group (P<0.01; Fig. 5B). These results suggested that nicorandil may have beneficial effects on the operated lung by decreasing oxidative stress.

Signaling pathways involved in the effect of nicorandil on OLV-induced pulmonary injury. The protein expression levels of p-Akt and PI3K in The nicorandil group were significantly higher compared with those in the control group (P<0.01; Fig. 6). The protein expression levels of NF-κB in the nicorandil group were significantly lower compared with those in the control group (P<0.01; Fig. 7A and C). Furthermore, HIF-1α mRNA and protein expression levels in the nicorandil group were significantly higher compared with those in the control group (P<0.01; Figs. 5D, 7A and C). Bax and Bcl-2 are apoptosis-related genes and caspase-3 is the main execution protein of apoptosis in pulmonary damage. The protein expression levels of Bax and caspase-3 in The nicorandil group were significantly decreased compared with those in the control group (P<0.01; Fig. 7A, D and F). By contrast, the

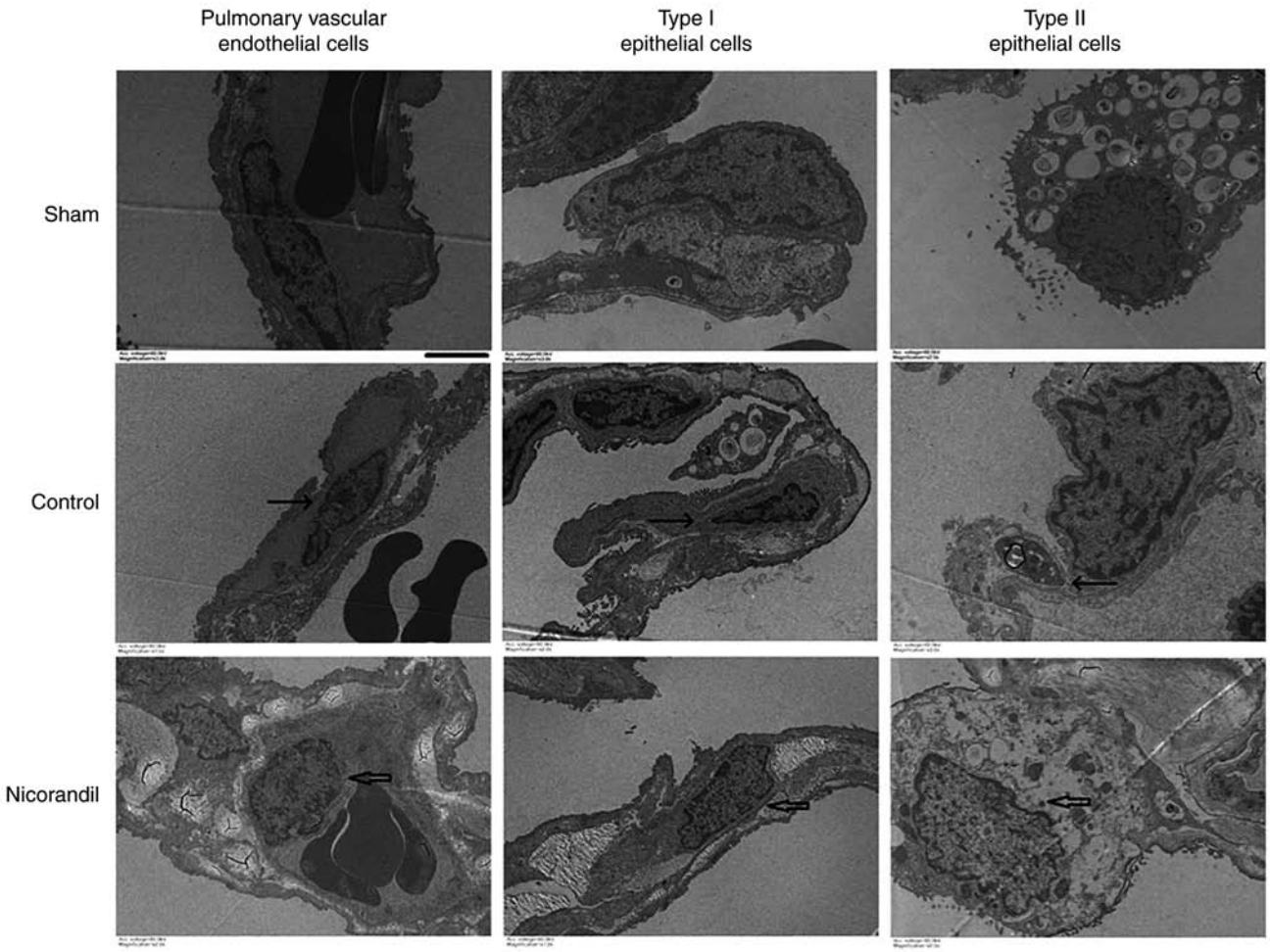

Figure 3. Beneficial effects of nicorandil on the operated lung in OLV were assessed using electron microscopy. The thin arrow indicates an abnormal nucleus and the thick arrow indicates a normal nucleus. Scale bar, 2 μm. OLV, one-lung ventilation.

protein expression levels of Bcl-2 in the nicorandil group were significantly increased compared with those in the control group ($P<0.01$; Fig. 7A and E). These results suggested that nicorandil acted on mitoKATP via the PI3K/Akt signaling pathway, which downregulated NF-κB expression and upregulated HIF-1α expression in nicorandil group in the process of inhibiting apoptosis.

Discussion

ERAS requires close multidisciplinary collaboration with the aim of minimizing perioperative injury, improving the prognosis of patients, accelerating recovery and reducing medical expenses. OLV is a basic requirement of ERAS in clinical settings. OLV technology, which can completely separate the operated lung from the contralateral lung and avoid secretions or exuded blood flowing to the healthy side, has become the favored core technology for anesthesiologists (1).

In thoracic surgery, an anesthesiologist must simultaneously address the violent fluctuations in the pathophysiology and mechanical stress in the lung tissue. These problems are caused by complete collapse of the surgical side and partial collapse of the contralateral side, resulting in hypoxemia due to the uncoordinated V/Q ratio. I/R injury, inflammatory reaction and mechanical stress can result in pulmonary injury, seriously affecting the recovery and prognosis of patients (2,3). Significant hypoxemia can occur in 6-20% of patients with OLV due to increased atelectasis (17-19). According to a previous report, an increase in airway pressure during OLV may result in acute pulmonary injury (20,21). Surgical patients should receive low VT, low to moderate positive end-expiratory pressure and a high fraction of inspired oxygen (6). Furthermore, Nieman *et al* (7) reported that the damage caused by OLV after I/R, accompanied by collapse and re-expansion of the surgical side of the lung, should be reduced as much as possible.

Szegedi *et al* (8) hypothesized that the oxidative stress in the surgical side was greater because gravity affects V/Q matching. The body has numerous different signaling pathways for producing ROS, including the xanthine oxidase signaling pathway (9,22). Molecular oxygen can become a superoxide radical (O^{2-}) in mitochondria (23). The presence of oxygen during reperfusion promotes the metabolism of hypoxanthine by xanthine oxidase, which forms ROS. Hydrogen peroxide produces a highly toxic hydroxyl via the Haber-Weiss reaction, which is promoted by the increase in free iron during ischemia. The aforementioned forms of ROS lead to major lung tissue damage. ROS, TNF-α and IL-6 are involved in pulmonary injury, which occurs during I/R, as they alter cellular proteins, lipids and ribonucleic acids, which leads to cell dysfunction, apoptosis or cell death (24).

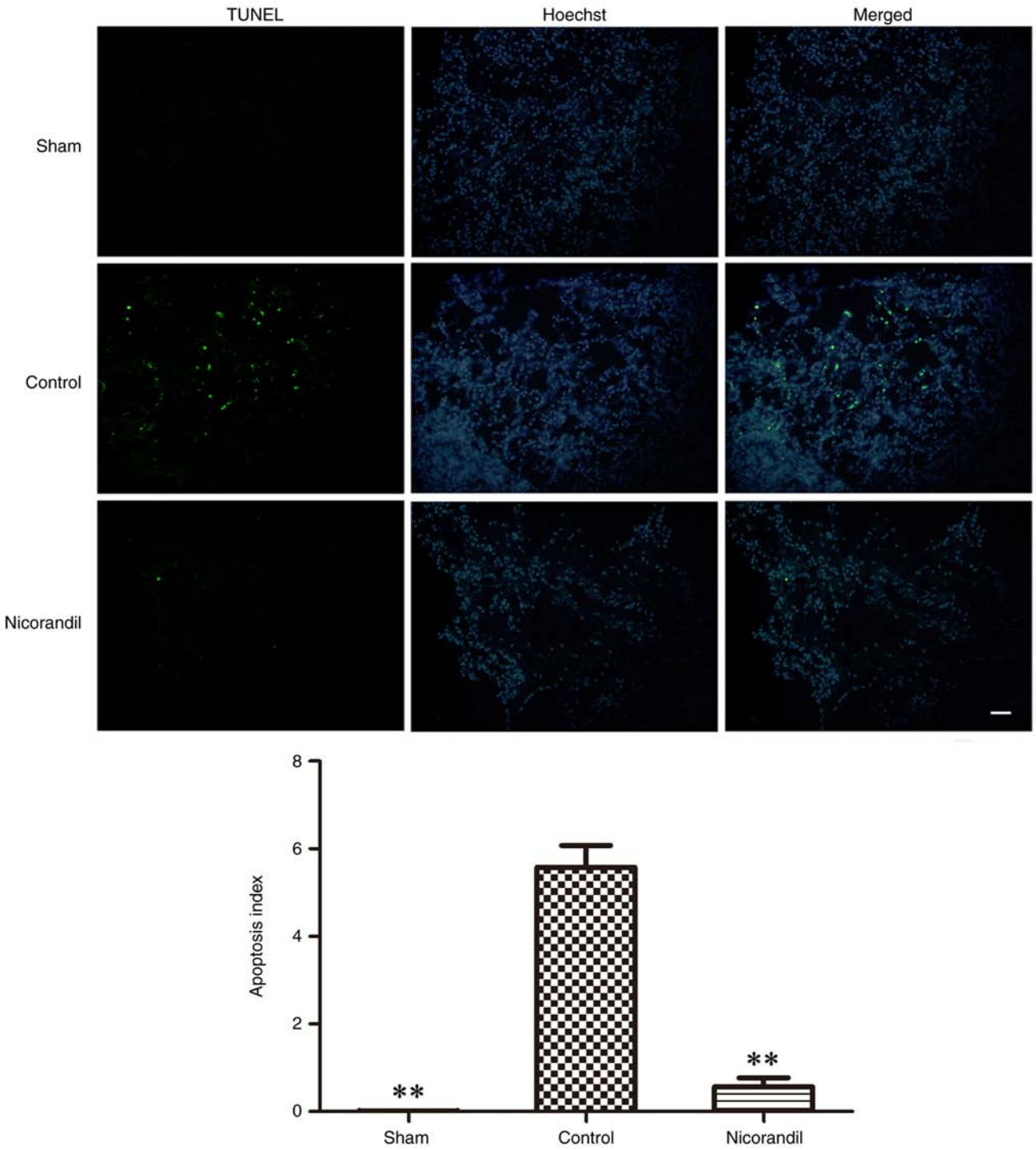

Figure 4. Beneficial effects of nicorandil on apoptosis in the collapsed lung were assessed using a TUNEL assay. Scale bar, 50 μm. The AI in the control, nicorandil and sham groups was 5.58±0.49, 0.56±0.19 and 0.02±0.01, respectively. **P<0.01 vs. control.

In the present study, pulmonary injury, which occurred in OLV, was decreased by nicorandil and this effect has been previously demonstrated in a rabbit model of OLV (13). The protective ventilation strategy (low VT, high frequency and occasional expansion of the lung) should be considered first in thoracic anesthesia; however, it cannot affect all the factors that cause pulmonary injury. A traditional drug (nicorandil, a mitoKATP opener), which has previously been used clinically for myocardial infarction, has been reported to be effective in prevention of pulmonary injury in an animal experiment. Based on the mechanism of action, nicorandil may serve protective roles in myocardial and pulmonary injury. Nicorandil may become another major method of treating pulmonary injury associated with OLV (25-27).

In the present study, nicorandil and low-dose nitroglycerin had different effects on certain lung indicators. Nicorandil was used at a conventional dose (close to the high dose for rabbits) for myocardial protection, based on animal experiments and the manufacturer's protocols. Nicorandil (2 mg/h) was infused before anesthetic induction and continued to be administered

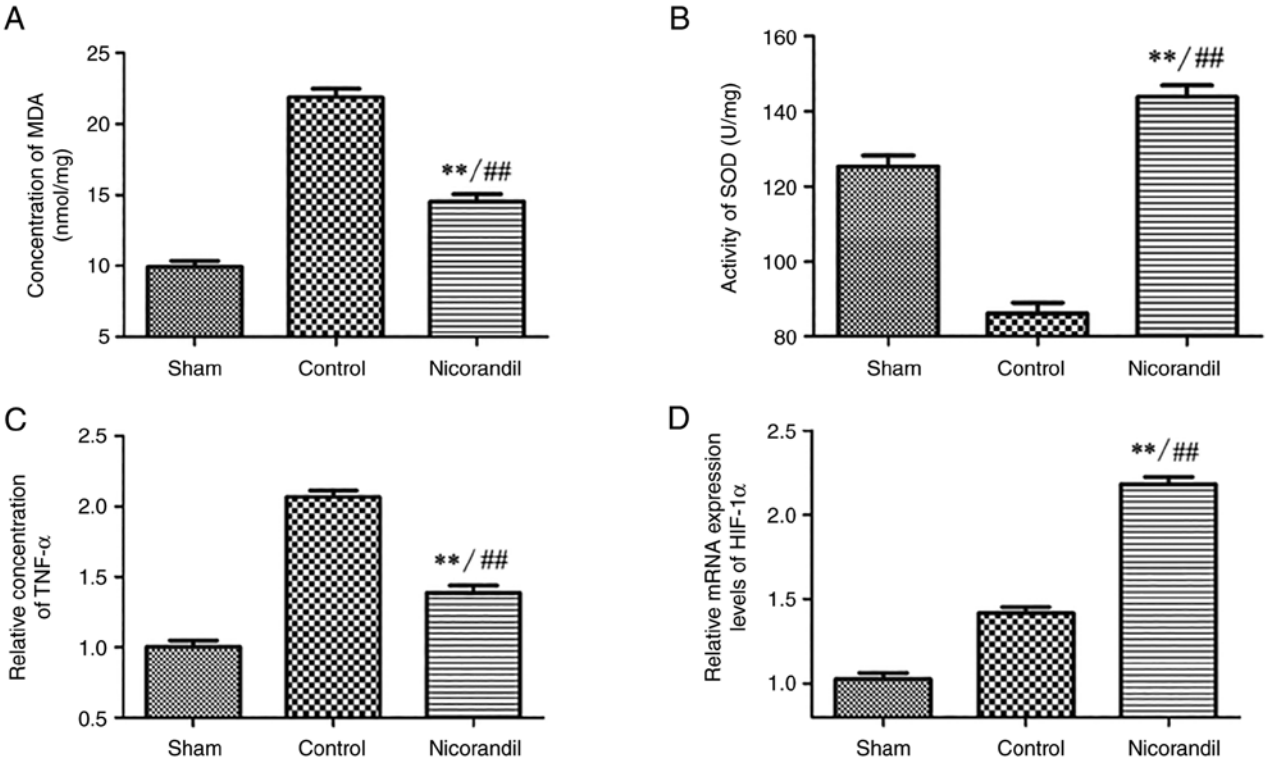

Figure 5. Effects of nicorandil on SOD activity, levels of MDA and TNF-α, and mRNA expression levels of HIF-1α. The effect of nicorandil on HIF-1α mRNA expression was examined by reverse transcription-quantitative PCR. Nicorandil decreased the levels of (A) MDA and (C) TNF-α in the operated lung, as assessed using ELISA. Furthermore, nicorandil upregulated the activity of (B) SOD and (D) mRNA expression levels of HIF-1α. **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. sham group. SOD, superoxide dismutase; MDA, malondialdehyde; HIF-1α, hypoxia-inducible factor 1α.

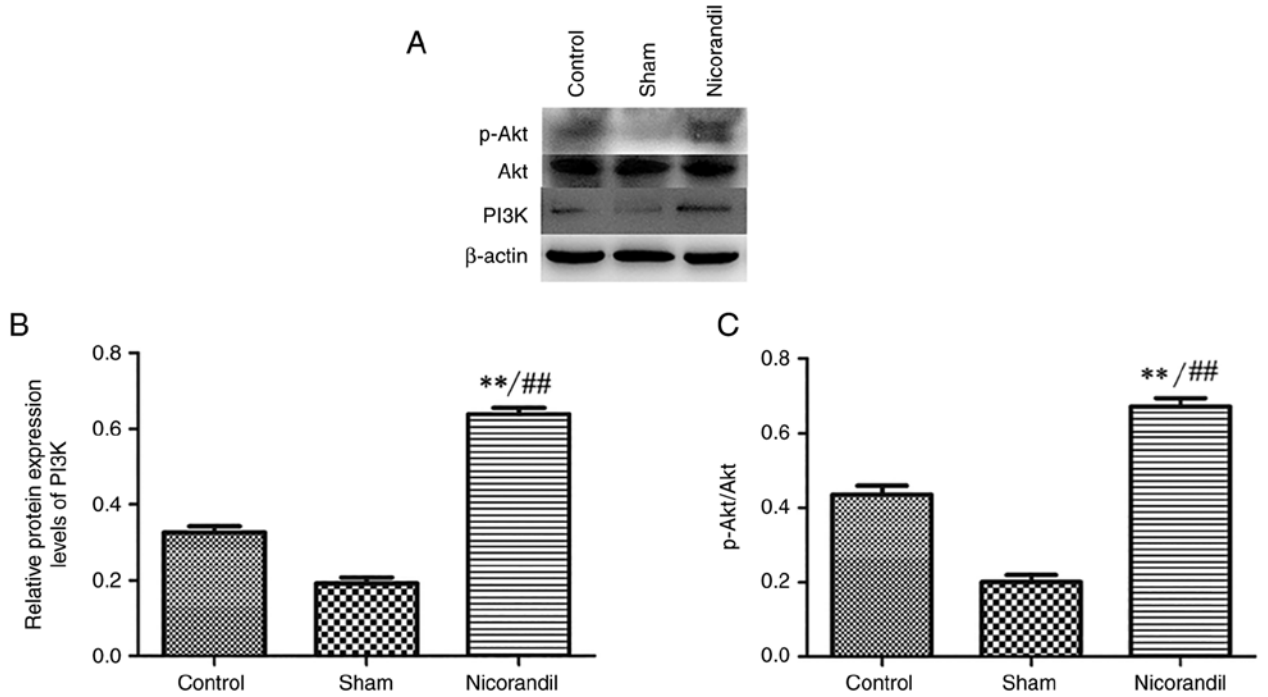

Figure 6. Effects of nicorandil on signaling pathways. (A) Effects of nicorandil on signaling pathways were assessed using western blotting. Nicorandil upregulated the protein expression levels of (B) PI3K and (C) p-Akt in the collapsed lung. β-actin was used as the internal standard for western blotting. **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. sham group. p, phosphorylated.

for 2 h during the operation. Nicorandil treatment was associated with significantly improved SaO_2 and PaO_2 after OLV and after extubation. It demonstrated a beneficial effect on lung function due to an improvement in oxygenation. According to the literatures, nicorandil has little effect on hemodynamics and airway pressure (28,29). Low-dose nitroglycerin also

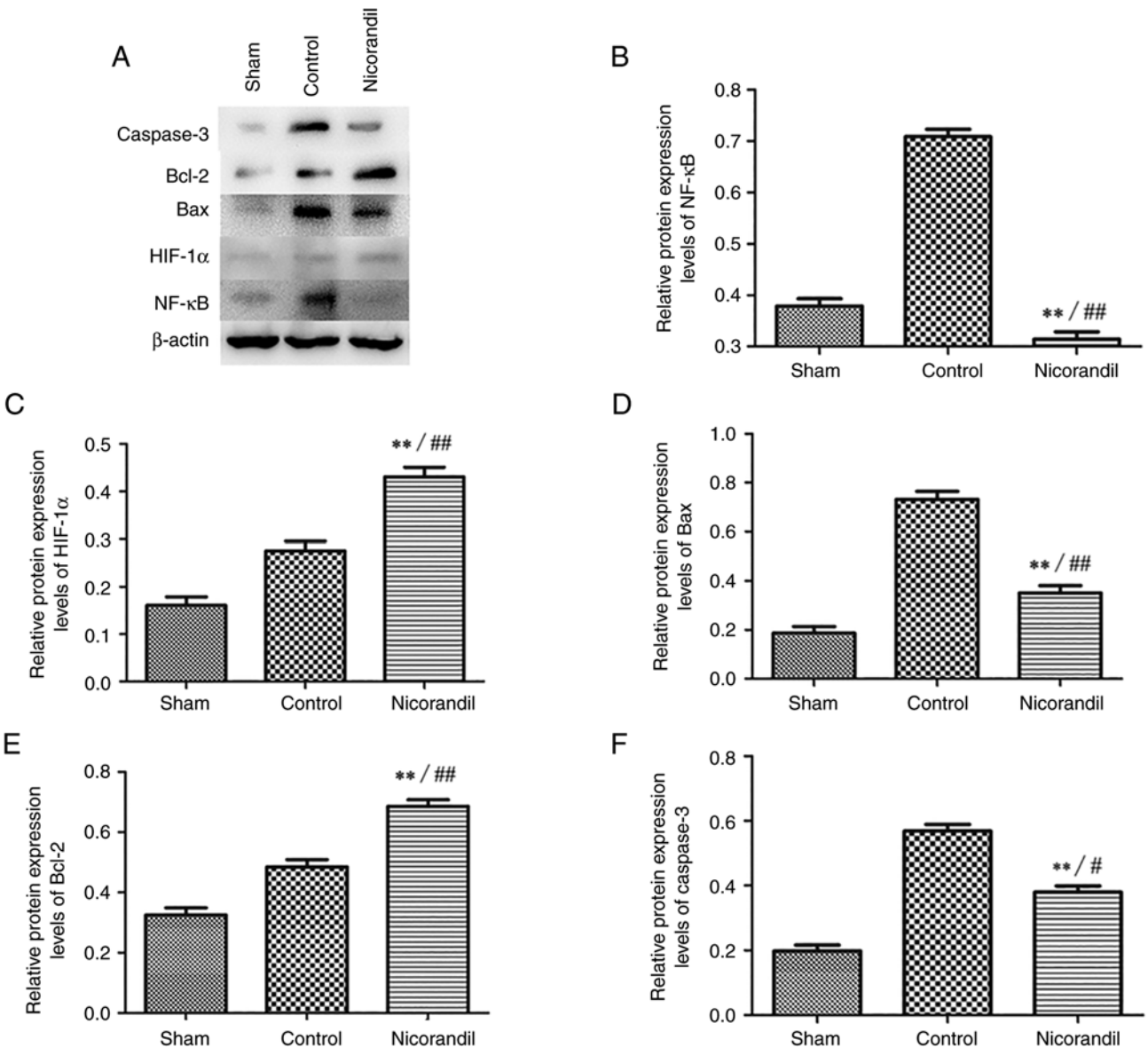

Figure 7. Effects of nicorandil on apoptosis-related molecules and signaling factors. (A) Effects of nicorandil on apoptosis-related molecules and signaling factors were assessed using western blotting. Nicorandil downregulated the protein expression levels of (B) NF-κB, (D) Bax and (F) caspase-3 in the operated lung. Furthermore, nicorandil increased the protein expression levels of (C) HIF-1α and (E) Bcl-2. β-actin was used as the internal standard for western blotting. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. sham group. HIF-1α, hypoxia-inducible factor 1α.

demonstrated a negligible effect in the normal physiological range on hemodynamics and lung indicators.

During OLV in thoracic surgery, nicorandil markedly influenced oxygenation and this was associated with a statistically significant increase in the SaO_2 and PaO_2 levels of patients, which was similar to those results previously reported in the rabbit OLV model (13). Therefore, the present study evaluated the material basis of the effect, which can be considered in terms of microstructure, inflammatory response and functional molecules. The present study demonstrated that the microstructure of the operated lung, which was improved due to nicorandil, was seriously damaged in the control group, including the cell nucleus, cell membrane and alveoli. H&E staining demonstrated that nicorandil, could serve protective roles in the alveoli, which exhibited little congestion and secretion. The alveolar wall exhibited minimal edema, thickening and exudation. Furthermore, electron microscopy demonstrated that nicorandil group had no pyknosis and lobulation of the nucleus, no marked emptying of lamellar bodies and no decrease in microvilli in type II epithelial cells. These results all demonstrated that nicorandil had a beneficial effect on the lung in the clinical setting.

In the present study, the selection of molecular indicators was based on the previously reported rabbit OLV experiment (13), and the consistency between the two experiments was evaluated. The present study demonstrated that the nicorandil group exhibited lower MDA and TNF-α levels compared with those in the control group, which demonstrated that nicorandil reduced oxidative stress and inflammation. SOD, which can scavenge ROS, serves a vital role in I/R injury (30). Furthermore, the present study demonstrated that nicorandil reduced I/R injury to protect the lung via SOD, the activity of which was significantly

increased in nicorandil group. Induction of apoptosis can occur in two ways. The intrinsic pathway is dependent on mitochondria and activated by ROS, whereas the extrinsic pathway is dependent on inflammatory molecules, such as TNF-α. Features of apoptosis include chromatin compression, cytoplasmic shrinkage, the appearance of apoptotic bodies and interruption of DNA (31). Apoptosis depends on energy. Forgiarini *et al* (32) reported that an increase in caspase-3 activity can result in the formation of more apoptotic cells after 45 min of ischemia. Therefore, the present study considered the mechanism of nicorandil and evaluated how the apoptosis of lung cells was regulated via a certain signaling pathway after the inflammatory response.

In the present study, the effect of nicorandil was regulated by apoptosis-related genes with opposite effects, including Bax and Bcl-2. Caspase-3, regulated by Bax/Bcl-2, is a vital terminal enzyme in apoptosis (15,33,34). Our previously reported rabbit study revealed that the nicorandil group had lower Bax expression and higher Bcl-2 expression, the ratio of which resulted in the decreased protein expression level of caspase-3 (13). Therefore, nicorandil treatment was demonstrated to be associated with decreased apoptosis. The results of the present study indicated important mechanisms, such as decreased protein expression levels of caspase-3 and Bax, and the upregulation of the Bcl-2, which resulted in less hypoxemia and oxidative damage, and improved the microstructure of the operated lung by decreasing apoptosis in the nicorandil group. The results were consistent with the observed TUNEL results. The results of the TUNEL assay indicated that the AI of nicorandil group was significantly less than that of the control group, whereas the control group is similar to the positive control group in the previous animal experiment (13). However, how nicorandil acts on apoptotic genes and the underlying mechanism require further investigation.

Nicorandil can activate the opening of mitoKATP on alveolar cells and a number of studies have demonstrated that nicorandil relies on PI3K/Akt, which is a signaling pathway involved in cell proliferation and apoptosis (27,35). In the present study, the protein expression levels of PI3K and the p-Akt/Akt ratio in the nicorandil group were significantly increased, which was similar to the previously reported rabbit experiment (13). These results indicated that nicorandil acted on mitoKATP via PI3K/Akt to reduce apoptosis in the operated lung. Due to its inhibitory effect on inflammation, the present study examined two transcription factors that were closely associated with nicorandil. NF-κB exists in almost all cells, and has an important physiological role in inflammation, stress, apoptosis and organ ischemia/reperfusion damage. In ventilatory lung injury, the NF-κB pathway is activated, and can produce a large number of chemokines and cytokines, subsequently triggering the aggregation of neutrophils, monocytes, macrophages and other inflammatory cells (36). The protein expression levels of NF-κB, which is related to inflammation, were significantly decreased in the nicorandil group, whereas the protein expression levels of HIF-1α, another nuclear transcription factor, were significantly increased. Zhao *et al* (37) reported that HIF-1α was one of the vital mechanisms for I/R pulmonary injury after the application of dimethyloxalylglycine.

In short, compared with previously reported animal experiments, consistent effects and mechanisms have been demonstrated in the clinical setting. Nicorandil had a beneficial effect by reducing apoptosis in the operated lung in clinical thoracic surgery. It may serve a beneficial role by inhibiting the overloading of calcium in mitochondria, shutting off mPTP (38), reducing the release of apoptosis-inducing factors and cytochrome *c*, simultaneously triggering activation of the PI3K/Akt signaling pathway around the cell membrane, downregulating NF-κB expression, upregulating HIF-1α expression and then reducing expression of Bax/Bcl-2, caspase-3 and apoptosis (32).

In conclusion, nicorandil demonstrated beneficial effects on the operated lung in clinical thoracic surgery via reduction of apoptosis. However, further research regarding the effects of nicorandil on cytochrome *c* and mPTP activity is required in the future.

Acknowledgements

The authors would like to thank Professor Maorong Jiang (Nantong University, Nantong, Jiangsu, China) for assistance with the design of experiments.

Funding

The present study was supported by the Science and Technology Plan Projects of Nantong City, Jiangsu Province, P.R. China (grant no. MSZ18094) and the General Topic Projects of Nantong Municipal Health Committee, Jiangsu, China (grant no. MA2021007).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW and ZWu designed the study. CW, ZWu, ZL, ZWa, HK and XH performed the research. CW, ZWu, ZL and ZWa analyzed the data and confirmed the authenticity of all the raw data. CW and ZWu wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were reviewed and approved by the Ethics Committee for Clinical Experimentation of The Affiliated Hospital of Nantong University (approval no. 2017-K031-D01). The enrolled patients provided written informed consent for participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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