

# High-pressure carbon dioxide pneumoperitoneum induces oxidative stress and mitochondria-associated apoptotic pathway in rabbit kidneys with severe hydronephrosis

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**Abstract.** The primary aim of the present study was to investigate the potential effect of high-pressure carbon dioxide (CO<sub>2</sub>) pneumoperitoneum on kidneys with severe hydronephrosis and to investigate the possible underlying mechanism. A total of 18 rabbits underwent a surgical procedure inducing severe hydronephrosis. Rabbits were then divided at random into three groups (n=6 each) and subjected to intraabdominal pressure of 0, 8 or 18 mmHg, respectively. CO<sub>2</sub> inflation lasted for 90 min in the pneumoperitoneum groups. Oxidative stress was assessed by measurements of reactive oxygen species (ROS). Activation of apoptosis was analyzed by western blot analysis of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated x protein (Bax), cytochrome c (Cyt c), caspase-3 and caspase-9 levels. In addition, TUNEL assay, hematoxylin and eosin (H&E) staining, measurement of mitochondrial membrane potential (MMP) and detection of changes to kidney ultrastructure were performed. In the 0 and 8 mmHg groups, all results were normal and similar. However, in the 18 mmHg group, the kidneys suffered oxidative damage and mitochondrial injuries, and increased ROS levels, lower MMP and mitochondrial vacuolization were observed. Furthermore, the mitochondrial/caspase-dependent pathway of apoptosis was activated, as indicated by the apoptotic index, and the expression levels and translocation of Bax, Bcl-2, Cyt c, caspase-3 and caspase-9. Therefore, it is concluded that high-pressure CO<sub>2</sub> pneumoperitoneum induces oxidative damage and

apoptosis in rabbit kidneys with severe hydronephrosis, which is associated with the mitochondrial apoptotic pathway.

## Introduction

Minimally invasive surgery using gas-induced pneumoperitoneum has become an important surgical approach. The reduced pain, shorter postoperative hospital stay and better cosmetic results also make this technique attractive for patients (1-3). Carbon dioxide (CO<sub>2</sub>) is the most commonly used gas for insufflation of the abdominal cavity to provide better exposure during laparoscopic surgery (4). However, an increasing numbers of studies have reported that several physiological complications are closely associated with high intraabdominal pressure caused by CO<sub>2</sub> (5-7).

Kidneys, as important splanchnic organs, are inevitably affected by intraabdominal pressure (8,9). Previous clinical and experimental studies have demonstrated that the laparoscopic procedure with pneumoperitoneum provides a typical model of ischemia/reperfusion (I/R) injury in the organs (10). Previous animal experiments have demonstrated that high and erratic elevations of intraabdominal pressure can decrease venous return, compress the renal vasculature and cause systemic hormonal changes, which eventually significantly decrease renal blood flow, urinary output and glomerular filtration rate (11). Further studies have observed increases in renal ischemia and oxidative stress response in the presence of increased intraabdominal pressure (12,13). Although abdominal deflation at the end of laparoscopic procedures reduces the intraabdominal pressure and increases renal perfusion, damage from the ischemic injury remains. Nevertheless, the majority of studies associated with pneumoperitoneum pressure damage involve normal kidneys (14); however, a number of patients who undergo laparoscopic surgery also exhibit a degree of kidney obstruction. To date, only a limited number of studies have reported how pneumoperitoneum affects kidneys with hydronephrosis (15).

In our previous study, the effect of pneumoperitoneum pressure on rabbit kidneys with no hydronephrosis, and with mild or severe hydronephrosis was investigated (16). The results indicated that severely obstructed kidneys have reduced cell

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tolerance to intraabdominal pressure, and that they are more likely to suffer oxidative damage and mitochondrial injuries when they were exposed to pneumoperitoneal pressure. It is generally accepted that oxidative damage and mitochondrial injuries are common ischemic pathological changes that can eventually cause apoptosis (17). Considering these facts, it can be speculated that with the increase of pneumoperitoneal pressure, apoptosis may occur as a consequence of severe oxidative damage and mitochondrial injuries in rabbit kidneys with severe hydronephrosis. Therefore, the present study aimed to examine the effects of high-pressure pneumoperitoneum with CO<sub>2</sub> on the kidneys of rabbits with severe hydronephrosis and to investigate the possible mechanism involved.

## Materials and methods

**Animals and groups.** In total, 18 adolescent male New Zealand rabbits were purchased from the Wuhan Institute of Biotechnology (Wuhan, China). The average weight of the rabbits was 2.2±0.3 kg, and the average age was 6±0.2 months. All experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (18) and were approved by the Ethics and Research Committee of the Wuhan University Medical School (Wuhan, China). The rabbits were housed in standard cages with free access to tap water and food in a room with a temperature of 18-25°C and relative humidity of 45-55%. The room was kept quiet to avoid any stress-inducing factors during the experimental period. All animals were determined to be healthy on the basis of clinical examinations, and were allowed to feed and drink freely 1 week before the experiment to adapt to environmental conditions.

The rabbits were randomly divided into three groups consisting of 6 rabbits each, including the 0, 8 and 18 mmHg groups. All these rabbits underwent surgical procedures to induce severe hydronephrosis. Following the surgery, the abdomens of the rabbits in these groups were insufflated with CO<sub>2</sub> to maintain an intraabdominal pressure of 0, 8 or 18 mmHg, respectively.

**Experimental protocol.** Following the method described by Wen *et al* (19), all the rabbits underwent surgery to establish a model of kidneys with hydronephrosis. Briefly, the rabbits were initially anesthetized by auricular vein injection of sodium pentobarbital at a dose of 30 mg/kg at room temperature. After 10 min, the left ureter and psoas muscle were exposed through a midline abdominal incision, and the proximal ureter was buried in a 2-cm notch within the psoas muscle. After 2 weeks, B-ultrasonography was used to confirm hydronephrosis. Pyelic distention levels of 1.69±0.34 cm and parenchymal thickness of 0.22±0.05 cm were observed. Next, the rabbits were randomly assigned to three groups, including the 0, 8 and 18 mmHg groups, and a second laparotomy was then performed. Following anesthetization, a Veress needle was introduced in the abdomen, and the incision was sutured to prevent CO<sub>2</sub> leakage from the abdomen. Intra-abdominal pressure was induced with an insufflator (Stryker, Kalamazoo, MI, USA) that delivered filtered medical CO<sub>2</sub> at body temperature with a flow rate of 0.5 l/min. The three groups of rabbits were subjected to intraabdominal pressures of 0, 8 or 18 mmHg,

respectively, for 90 min. Subsequent to the insufflation, the pneumoperitoneum was released, the psoas muscle obstruction was relieved, and the abdomen was sutured. The rabbits were sacrificed 2 days after the pneumoperitoneum with 150 mg/kg sodium pentobarbital (20%) through ear marginal vein injection, and the left kidneys were collected for biochemical and histological evaluations.

**Tissue processing.** Following sacrifice, the abdominal area of the rabbits was fully exposed, and the experimental kidneys were removed carefully with sharp scissors. The kidneys were then placed into cold 0.9% saline solution to wash away most of the blood. Next, the renal capsule and the adipose tissues around the kidney were carefully removed with tweezers, avoiding oppression of the renal tissue during the entire process. Finally, the unwanted renal pelvis was carefully removed with small scissors, appropriate sections of renal tissue were cut off with a sharp blade, and the renal tissue was washed with cold 0.9% saline solution for three times to clear the residual blood. The tissue samples were treated differently according to different detection methods.

**Detection of reactive oxygen species (ROS).** All of the rabbits were sacrificed 2 days after pneumoperitoneum. Kidneys were carefully removed and washed with cold 0.9% saline solution three times. The kidney tissue samples were homogenized (T25; IKA-Werke GmbH & Co. KG, Staufen, Germany) in 100 mmol/l phosphate buffer and centrifuged at 13,000 x g for 15 min at 4°C (Heraeus Biofuge Primo R; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following which the supernatants were collected. The bicinchoninic acid method was used to detect protein concentration. Then the homogenized supernatants were incubated with 1 mmol/l 2,7-dichlorodihydrofluorescein diacetate (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 30 min at 37°C. Subsequently, an automatic microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.) was used to detect the absorbance at 500 nm. The results are expressed as the arbitrary units per mg of protein.

**Measurement of mitochondrial membrane potential (MMP).** An MMP detection kit (Beyotime Institute of Biotechnology, Haimen, China) was used to determine changes in MMP, according to the manufacturer's protocol. Briefly, renal tissue was cleaned with 0.9% normal saline and digested in a trypsin solution (Beyotime Institute of Biotechnology) at 37°C for ~20 min, and the reaction was terminated by addition of 30% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China). Suspended cells were centrifuged at 2,000 x g for 4 min at 4°C and then washed three times with PBS. Subsequent to this step, JC-1 was added at a final concentration of 0.01 M. Following incubation for 30 min at 37°C, the cells (approximately 3x10<sup>5</sup> cells/ml) were washed three times with wash buffer and immediately analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and Flowjo software (version 7.6.1, BD Biosciences).

**Hematoxylin and eosin (H&E) staining, and TUNEL detection.** Rabbit kidney tissues in all three groups were

harvested and fixed in 10% formalin (pH 7.0) for a time period not exceeding 24 h. The tissues were processed routinely for paraffin embedding, and 5- $\mu$ m paraffin-embedded sections were cut for detection. The tissue sections were deparaffinized using xylene and rehydrated using a graded ethanol series prior to staining with H&E. In order to compare the kidney injury and renal cell apoptosis in these three groups, sections were screened per high-power field (HPF) under a microscope (Nikon 80i; Nikon Corporation, Tokyo, Japan). On each slide, 10 HPFs in the cortex and outer medulla were randomly selected, and 100 cells were randomly counted in each of these fields. Kidney injury was graded on the basis of H&E staining, as follows: 0, absence of necrosis; 1, mild necrosis; 3, moderate necrosis; and 5, severe necrosis.

Apoptotic scores were also determined with TUNEL staining using the *In Situ* Apoptosis Detection kit (Roche Applied Sciences, Basel, Switzerland). Apoptotic nuclei were stained brown, while negative nuclei were stained blue. The apoptotic index was calculated based on the percentage of positive nuclei. All quantifications were performed by two pathologists in a blinded manner.

**Kidney ultrastructure examination by electron microscopy.** Renal cortex tissues from the three groups were fixed for 24 h in 2.5% paraformaldehyde at 4°C and subsequently fixed at 4°C for 2 h with 2% osmium tetroxide. The kidney samples were then washed with PBS twice, dehydrated with a series of ethanol solutions and embedded in Spurr epoxy resin at 45°C for 12 h. Next, tissue specimens were cut into 50-nm sections on an ultra-microtome using diamond knives, followed by washing and staining with 2% aqueous uranyl acetate at 25°C for ~1 h. Finally, a minimum of 10 random fields of view from each section were visualized under a transmission electron microscope (H-600; Hitachi, Ltd., Tokyo, Japan).

**Western blot analysis.** To determine the possible mechanisms underlying the apoptosis induced by pneumoperitoneum, the expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated x protein (Bax), cytochrome c (Cyt c), caspase-3 and caspase-9 were detected in rabbit kidney tissues using western blot analysis. Briefly, 6 rabbits from each group were rapidly sacrificed 2 days after pneumoperitoneum, and the kidneys were removed. Next, tissues were washed three times with PBS (pH 7.2) and homogenized using an Ultra-Turrax (T25; IKA-Werke GmbH & Co. kg) in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). The samples were then centrifuged at 14,000 x g for 20 min at 4°C. For Cyt c testing, the cytosolic and mitochondrial extracts were obtained by the mitochondrial extraction kit (Beyotime Institute of Biotechnology) following the manufacturer's protocol. In addition, COX-IV and  $\beta$ -tubulin were used as controls for the detection of Cyt c in the mitochondria and cytoplasm, respectively. The concentration of the protein was detected using the bicinchoninic acid method. Proteins in each group were subjected to 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane for 1-2 h at 200 mA, according to their molecular weight. For each group, ~40  $\mu$ g proteins were added onto the gels per

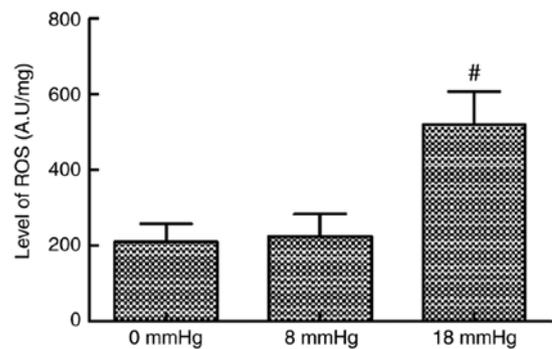


Figure 1. Levels of ROS in severely hydronephrotic rabbit kidneys perfused at different pneumoperitoneum pressures. #P<0.05 vs. the 0 mmHg group. ROS, reactive oxygen species.

lane for detection. Subsequent to blocking with Tris-buffered saline/Tween-20 containing 5% dried skim milk for 1 h at room temperature (approximately 25°C), the membranes were incubated with primary antibodies overnight at 4°C. The antibodies used for western blot analyses were as follows: Bax (PA5-70418; 1:3,000; Thermo Fisher Scientific, Inc.), Bcl-2 (PA5-68611; 1:2,000; Thermo Fisher Scientific, Inc.), Cyt c (NB100-56503; 1:5,000; Novus Biologicals, LLC, Littleton, CO, USA), caspase-3 (ab90437; 1:1,000; Abcam, Cambridge, UK), caspase-9 (ab115161; 1:3,000; Abcam),  $\beta$ -actin (ab28052; 1:5,000; Abcam), Cyt c oxidase (COX)-IV (ab66739; 1:5,000; Abcam) and  $\beta$ -tubulin (ab56676; 1:4,000; Abcam). Proteins were subsequently incubated for 1 h at room temperature with anti-mouse/rabbit secondary antibody (P/N 925-32210 or P/N 925-32211; 1:10,000; LI-COR Biosciences, Lincoln, NE, USA), which was conjugated to IRDye 800CW. Finally, the fluorescence signal emitted by the secondary antibody was quantified by a western blot detection system (Odyssey Infrared Imaging; LI-COR Biosciences), and semi-quantitative analysis was conducted to determine the corresponding protein expression levels (Image Studio version 5.2.5; LI-COR Biosciences).

**Statistical analysis.** All data are presented as the means  $\pm$  standard deviations, and all analyses were performed in duplicate. One-way analysis of variance and Turkey's test were performed for statistical comparison using IBM SPSS software, version 19 (IBM Corp., Armonk, NY, USA). P-values that were <0.05 were considered to denote statistically significant differences.

## Results

**ROS levels.** The ROS levels in kidney tissues were comparable between the 0 and 8 mmHg groups. However, when the intraabdominal pressure reached 18 mmHg, the ROS level significantly increased compared with the 0 and 8 mmHg groups (Fig. 1).

**Changes in MMP levels.** Disruption of MMP is one of the earliest events in apoptosis (20). JC-1, as a fluorescent probe, mainly exists in the mitochondrial matrix as a polymer, which can emit red fluorescence when MMP levels are high. However, when the MMP levels are low, JC-1 mainly exists

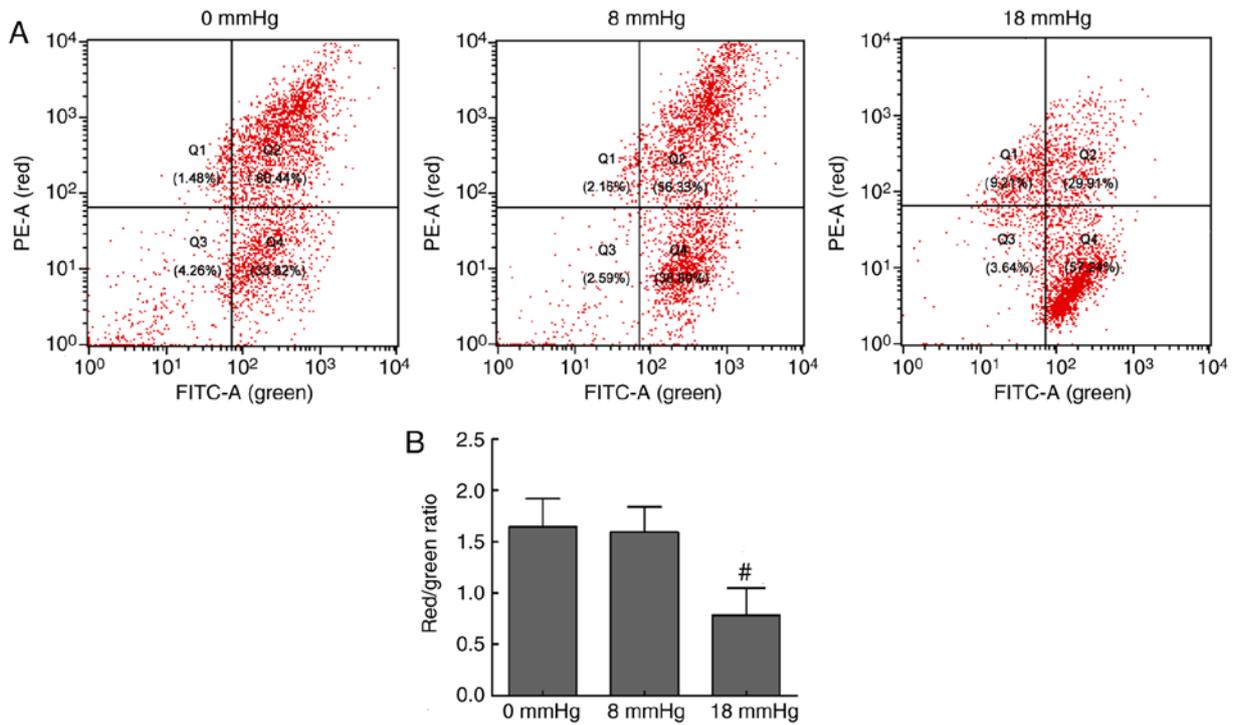


Figure 2. MMP of renal cells subjected to different pneumoperitoneum pressures in rabbit kidneys with severe hydronephrosis. (A) Flow cytometric analysis of MMP changes. PE-A represents the red fluorescence, while FITC-A represents the green fluorescence. MMP levels are expressed as the ratio of red to green fluorescence intensity, indicated in Q2 and Q4. (B) Ratio of red to green fluorescence intensity of renal cells in rabbits with severe hydronephrosis under different intraabdominal pressures. #P<0.05 vs. the 0 and 8 mmHg groups. MMP, mitochondrial membrane potential.

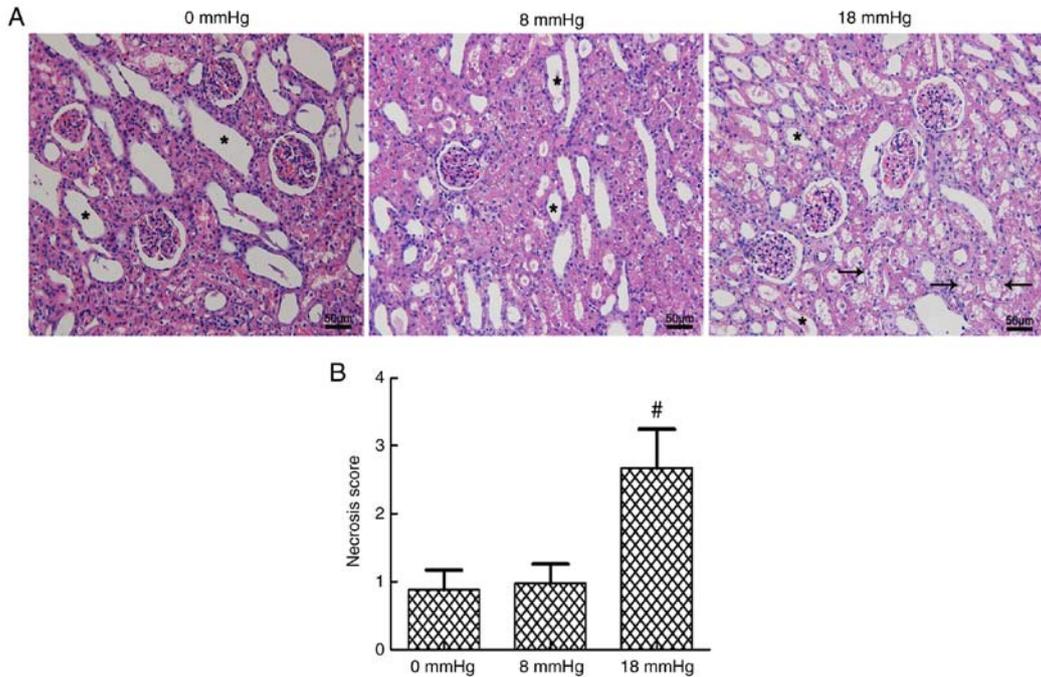


Figure 3. Histological evaluation of rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) Hematoxylin and eosin staining images of kidney sections (magnification, x200). Asterisks indicate examples of expansive kidney tubules caused by severe hydronephrosis, while the arrows display examples of necrotic tubular cells. (B) Tubular necrosis scores in each group. #P<0.05 vs. the 0 and 8 mmHg groups.

in the cytoplasm as monomers, which can emit green fluorescence. Thus, a decrease in the red/green fluorescence intensity ratio indicates MMP loss (21). In the present study, the flow cytometry scatter plots and the red/green ratios were obtained,

and are shown in Fig. 2. The renal cells exhibited strong red fluorescence and relatively weak green fluorescence in the 0 and 8 mmHg groups. However, when the pneumoperitoneum pressure was increased to 18 mmHg, the red fluorescence was

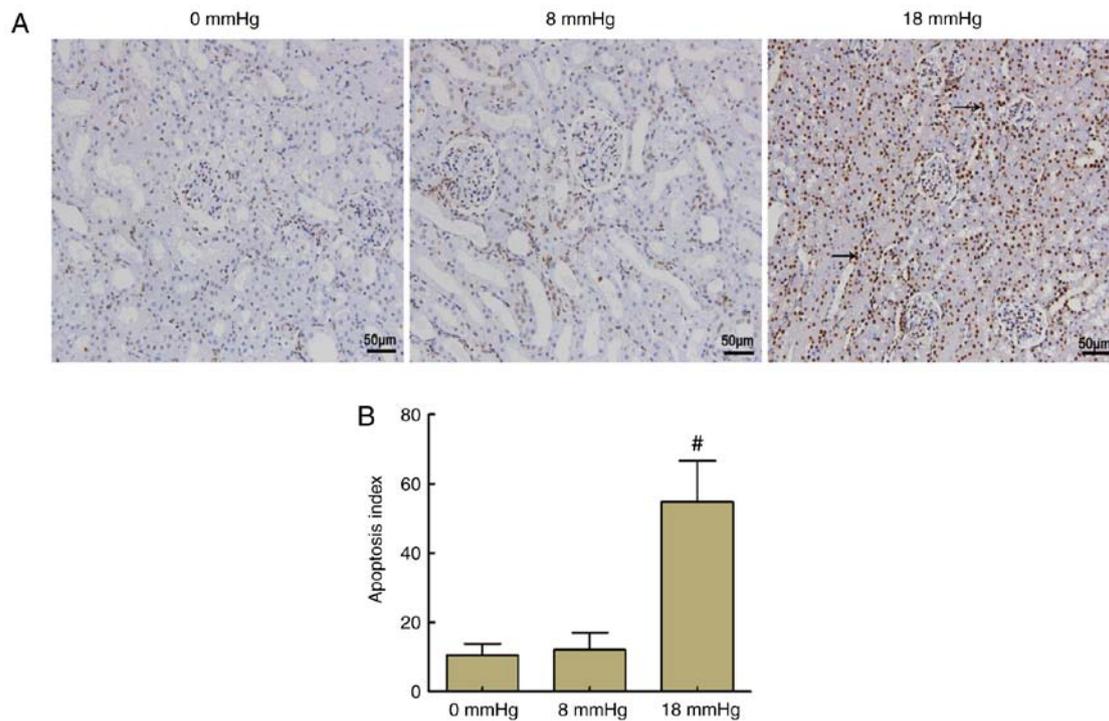


Figure 4. TUNEL detection of rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) TUNEL analysis images of kidney sections (magnification, x200). The arrows in the figure indicate examples of TUNEL-positive cells. (B) Apoptosis index of renal cells in each group. #P<0.05 vs. the 0 and 8 mmHg groups.

attenuated and green fluorescence was enhanced, which indicated a loss of MMP levels (Fig. 2). These changes suggested mitochondrial damage and early cell apoptosis associated with the mitochondrial pathway in the 18 mmHg group.

**Kidney necrosis.** Certain degrees of expansive kidney tubules were observed in all three groups due to the establishment of rabbit kidney models with severe hydronephrosis. The tubular necrosis scores were at a relatively low level when the intraabdominal pressure was only 0 or 8 mmHg, and no significant difference was observed between these two groups. However, a sudden significant increase in the tubular necrosis score was detected when the pneumoperitoneum pressure reached 18 mmHg (Fig. 3).

**Kidney apoptosis.** In the 0 and 8 mmHg groups, only a few nuclei were stained brown, and no significant difference was observed between these two groups. By contrast, >50% of the nuclei were stained brown in the 18 mmHg group, which indicated that the total number of apoptotic cells was markedly increased. These results indicated that apoptosis occurred in the kidneys of rabbits with severe hydronephrosis in the presence of increased pneumoperitoneum pressure (Fig. 4).

**Ultramicrostructure changes of tubular cells.** Mitochondrial-dependent apoptosis is one of the key apoptosis mechanisms (22). This mechanism involves ultramicrostructure changes in the cells. In the current study, transmission electron microscopy was performed to detect the apoptosis in renal cells by investigating changes to the mitochondria. In the 0 mmHg group, cells had an overall normal appearance,

with few swollen or vacuolar mitochondria observed. In the 8 mmHg group, similar results were observed. However, in the 18 mmHg group, the majority of the mitochondria were swollen and vacuolar (Fig. 5). These findings revealed that the mitochondria-mediated pathway was involved in apoptosis induced by high-pressure pneumoperitoneum in obstructed rabbit kidneys.

**Expression levels of Bax and Bcl-2.** An increase in the expression of the pro-apoptotic protein Bax and reduced expression of the anti-apoptotic protein Bcl-2 are considered important changes in early apoptosis (23). The results of the present study demonstrated that the expression of Bax was significantly increased and the expression of Bcl-2 was markedly decreased when the pneumoperitoneum pressure reached 18 mmHg. Notably, no significant changes were observed between the 0 and 8 mmHg groups (Fig. 6).

**Release of Cyt c.** Cyt c is an intermembrane space protein that is tightly associated with cardiolipin, a key lipid component of the inner mitochondrial membrane. The release of Cyt c from mitochondria to the cytosol is considered one of the key steps in the mitochondria-associated apoptosis (24). In the current study, the expression of Cyt c in mitochondria and cytosol were separately detected (Fig. 7). The results revealed that the majority of Cyt c existed in the mitochondria rather than the cytosol in the 0 and 8 mmHg groups, indicating that only limited amount of Cyt c was released. No significant difference was observed between these two groups. However, in the 18 mmHg group, the majority of the Cyt c existed in the cytosol rather than the mitochondria, indicating that rabbit kidneys with hydronephrosis subjected to 18 mmHg

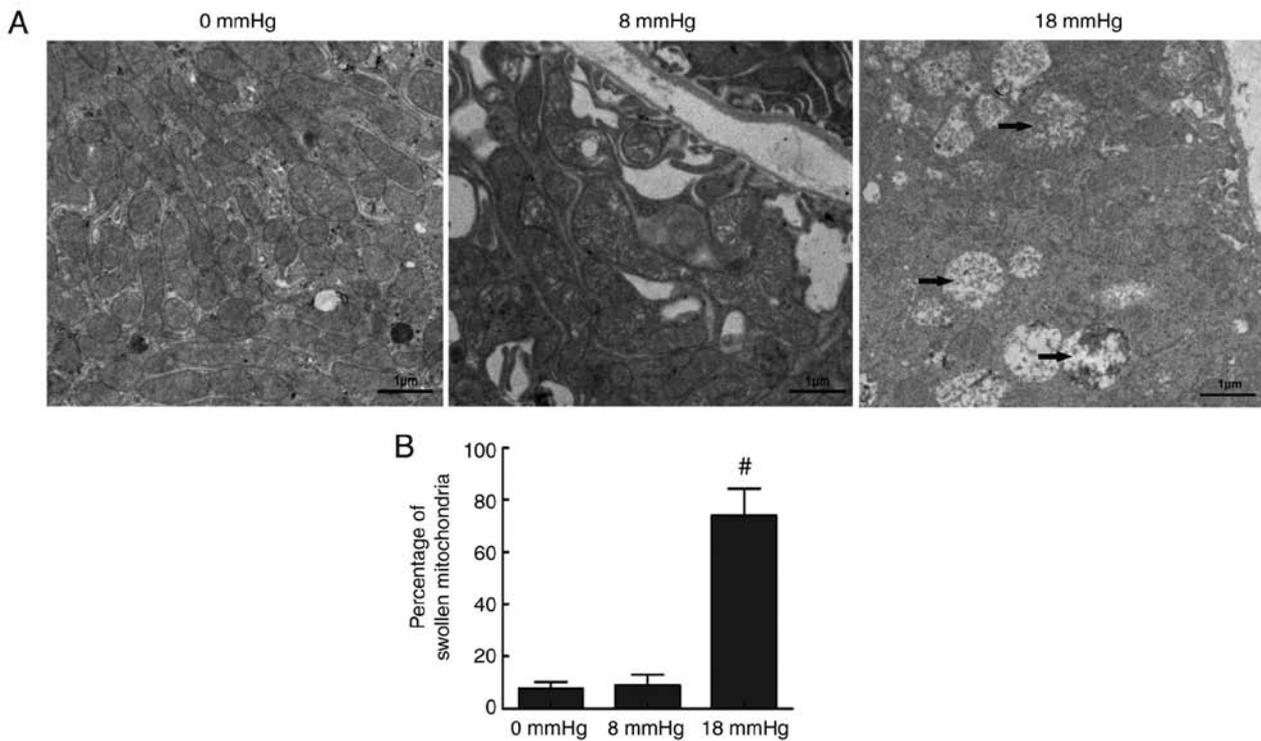


Figure 5. Ultramicrostructural changes of rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) Transmission electron microscopy images of ultramicrostructural changes in different groups (magnification, x3,000). The arrows indicate examples of swollen and vacuolar mitochondria. (B) Percentage of swollen mitochondria in each group, indicating apoptotic changes. <sup>#</sup> $P < 0.05$  vs. the 0 and 8 mmHg groups.

pneumoperitoneum were characterized by Cyt c release from the mitochondria to the cytosol (Fig. 7).

**Activity of caspase-3 and caspase-9.** The release of Cyt c activates caspase-9, a cysteine protease, and later cleaved caspase-9 activates caspase-3 and forms cleaved caspase-3, which is responsible for cell apoptosis (25). The current study detected the activities of caspase-3 and caspase-9 to assess whether the caspase-dependent pathway was involved in rabbit kidneys with severe hydronephrosis subjected to pneumoperitoneum. As shown in Fig. 8, cleaved caspase-9 and cleaved caspase-3, as well as the expression ratio of cleaved-caspase-3/pro-caspase-3 or cleaved-caspase-9/pro-caspase-9, were significantly increased when the intraabdominal pressure reached 18 mmHg. However, no significant changes were observed between the 0 and 8 mmHg groups (Fig. 8).

## Discussion

Pneumoperitoneum, although generally considered to be essential for adequate exposure in laparoscopic surgery, has adverse effects on renal physiology (26). A large number of trials have revealed the side effects of pneumoperitoneum; however, these trials have mainly focused on renal blood flow changes and renal function (27,28). A previous study revealed that the glomerular filtration rate decreased by 14 and 48% in rats when an intraabdominal pressure of 7 and 14 mmHg was applied, respectively. In parallel, the renal plasma flow decreased by 28 and 57% under these conditions (29). Certain studies have emphasized that high intraabdominal pressure can noticeably decrease renal blood flow, while other studies

reported that renal blood flow returned to the normal range following deflation (30,31). During the hypoperfusion and subsequent reperfusion periods, a typical I/R injury may occur. Several studies in animals and humans have investigated I/R injury following laparoscopic surgeries (32-34). Pneumoperitoneum may cause an increase in the oxidative stress, and active oxygen species (ROS) may cause damage to lipids and proteins during oxidative stress. A previous study has also demonstrated that oxidative stress response decrease and antioxidant defense systems strengthened with the administration of dexmedetomidine prior to pneumoperitoneum (35). Furthermore, a typical I/R injury is closely associated with oxidative damage and mitochondrial injuries (36), which can eventually cause cell apoptosis or death.

As mentioned earlier, hydronephrosis is a common urological disease that can be caused by a kidney stone, tumor or congenital anomalies. Kidneys with hydronephrosis have a thinner renal cortex and subnormal blood perfusion, while the hydronephrosis itself has adverse effects on renal tubule function, causing hydronephrotic kidneys to be more likely to suffer hypoxia problems (37). Severe hydronephrosis leads to prolonged operating time in laparoscopic surgery, which may consequently lead to increased oxidative stress (38). Based on these facts and the observations of our previous study (16), it can be inferred that high-pressure CO<sub>2</sub> pneumoperitoneum may cause severe oxidative stress, mitochondrial injuries and even cell apoptosis in rabbit kidneys with severe hydronephrosis. Therefore, the present study examined the effects of high-pressure CO<sub>2</sub> pneumoperitoneum on kidneys with severe hydronephrosis and investigated the possible underlying mechanism.

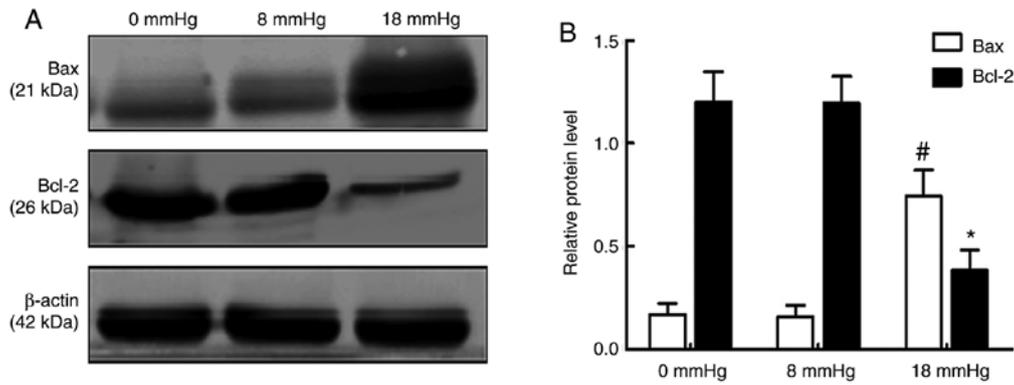


Figure 6. Expression levels of Bax and Bcl-2 in rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) Representative western blots and (B) relative protein expression levels of Bax, Bcl-2 and  $\beta$ -actin in rabbit kidneys from different groups. All data are expressed as the mean  $\pm$  standard deviation of three experiments, and each experiment included triplicate repeats. <sup>#</sup>P<0.05 vs. the 0 and 8 mmHg groups. <sup>\*</sup>P<0.05 vs. the 0 and 8 mmHg groups. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated x protein.

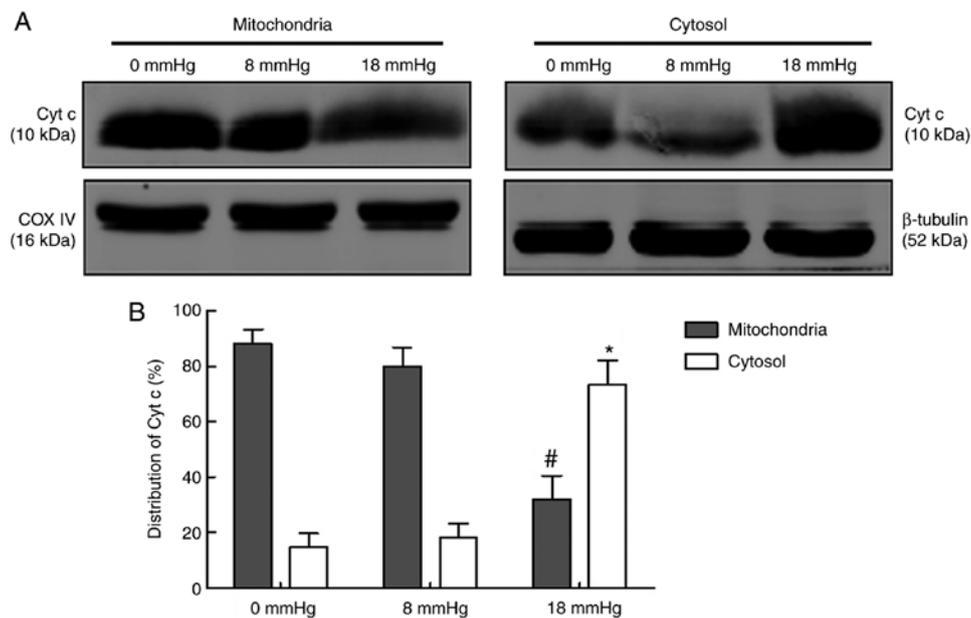


Figure 7. Expression of Cyt c in mitochondria and cytosol in rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) Representative western blots showing the protein expression levels of Cyt c, COX-IV and  $\beta$ -tubulin in mitochondria and cytosol in rabbit kidneys from different groups. (B) Distribution of Cyt c in mitochondria and cytosol in each group. All data are representative of three independent experiments. <sup>#</sup>P<0.05 vs. the 0 and 8 mmHg groups. <sup>\*</sup>P<0.05 vs. the 0 and 8 mmHg group. Cyt c, cytochrome C; COX-IV, Cyt c oxidase-IV.

In I/R injury, the generation of ROS appears to be an important source of tissue damage. To a certain extent, ROS content represents the degree of oxidative damage (39,40). In the experiments of the present study, the ROS levels, histological changes and apoptosis index were first detected to evaluate oxidative stress and tissue damage. As expected, higher oxidative stress, higher apoptosis index and more severe morphological changes were observed in the 18 mmHg group as compared with the 0 and 8 mmHg groups. Excessive ROS induces the peroxidation of mitochondrial membrane lipids, which promotes extensive release of ROS and spurs a vicious cycle, eventually leading to a loss of MMP (41,42). Accordingly, the MMP was tested by JC-1 staining in the current study, and the results identified a significant decrease in MMP in the 18 mmHg group. To confirm the changes to the mitochondria, the ultrastructure of kidneys was also observed

by electron microscopy. As expected, large swollen and vacuolar mitochondria were detected in the 18 mmHg group, while in the 0 and 8 mmHg groups, few abnormal changes were observed. The significant reduction of MMP, mitochondrial injuries and apoptosis largely suggested a mitochondrially mediated phenomenon, which is associated with the collapse of transmembrane potential, resulting in the expulsion of apoptogenic molecules.

The mitochondrial pathway is characterized by the release of apoptosis-promoting factors, such as Cyt c and apoptosis-inducing factor (AIF), from the mitochondria (43). The release of Cyt c from mitochondria to cytosol can activate the initiator caspase, namely caspase-9, and can thus trigger the activation of the executioner caspase, namely caspase-3, eventually causing caspase-dependent apoptosis (44). AIF stimulates caspase-independent apoptosis by moving into

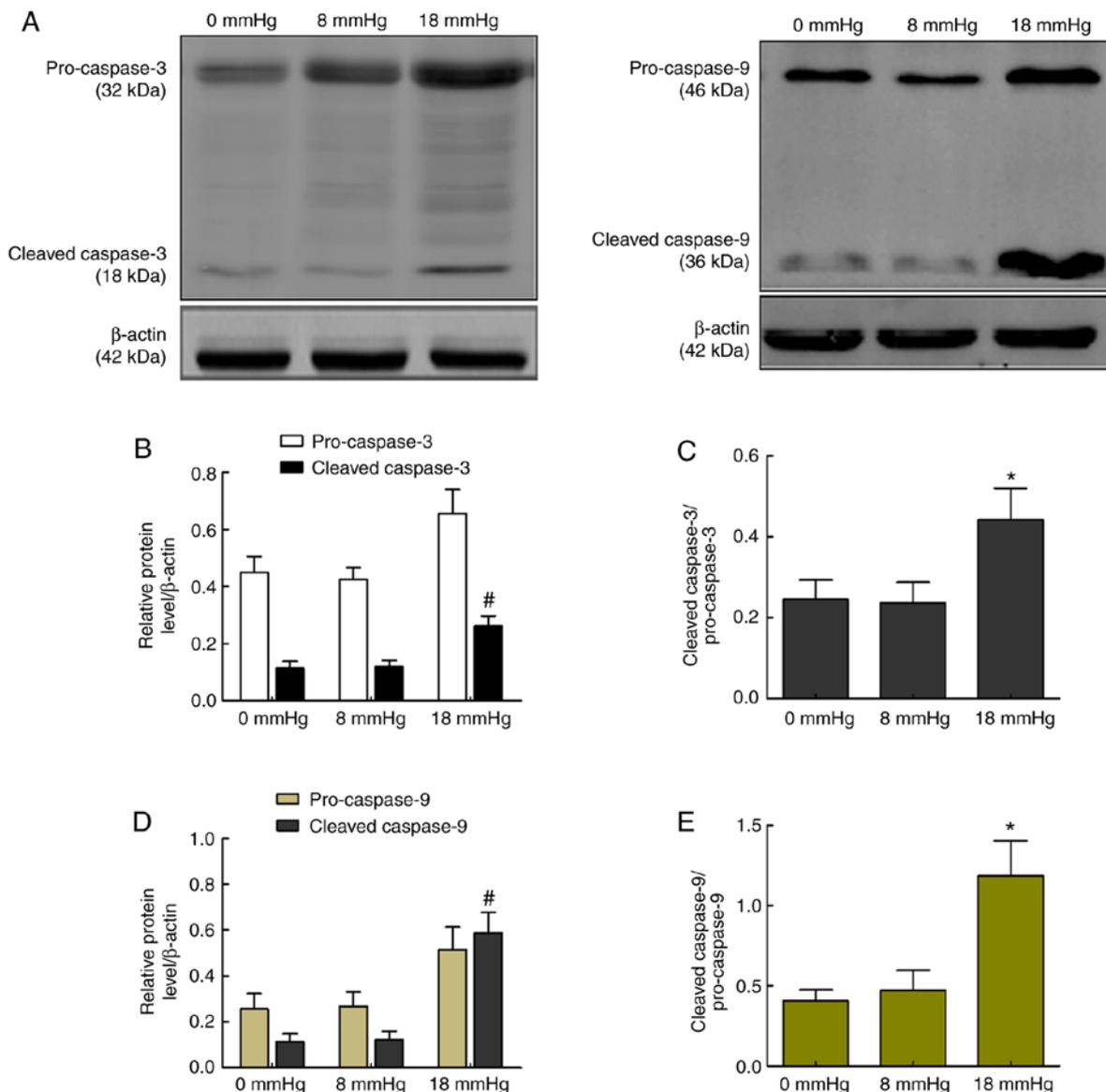


Figure 8. Expression levels of caspase-3 and caspase-9 in rabbit kidneys with severe hydronephrosis subjected to different pneumoperitoneum pressures. (A) Representative western blots demonstrating the protein expression levels of caspase-3, caspase-9 and  $\beta$ -actin in rabbit kidneys from different groups. (B) Relative expression of pro-caspase-3 and cleaved-caspase-3 in each group compared with  $\beta$ -actin. (C) Ratio of cleaved-caspase-3/pro-caspase-3 expression in each group. (D) Relative expression of pro-caspase-9 and cleaved-caspase-9 in each group compared with  $\beta$ -actin. (E) Ratio of cleaved-caspase-9/pro-caspase-9 expression in each group. All data are expressed as the mean  $\pm$  standard deviation, and each experiment was conducted in triplicate. <sup>#</sup> $P < 0.05$  vs. cleaved-caspase in 0 and 8 mmHg groups; <sup>\*</sup> $P < 0.05$  vs. cleaved-caspase/pro-caspase levels in 0 and 8 mmHg groups.

the nucleus, where it binds to DNA and stimulates chromatin condensation and DNA fragmentation (45). Additionally, the Bcl-2 family proteins, including anti-apoptotic (such as Bcl-2) and pro-apoptotic (such as Bax) members, serve an important role in the mitochondria-mediated pathway (46). The anti-apoptotic proteins inhibit the release of apoptosis-promoting factors, whereas the pro-apoptotic proteins promote the apoptotic process by regulating MMP and the permeability of membranes (47,48). Accordingly, the present study investigated a number of important proteins associated with the mitochondria-mediated pathway. The western blot analysis results revealed significant downregulation of Bcl-2 and upregulation of Bax, as well as marked release of Cyt c from mitochondria into the cytosol, in the 18 mmHg group. A significant increase in the expression of cleaved

caspase-3 and cleaved caspase-9 was also observed in the 18 mmHg group. Taken together, the current results provide evidence that apoptosis in rabbit kidneys with hydronephrosis induced by high CO<sub>2</sub> pneumoperitoneum is associated with the mitochondria-dependent pathway. An illustration of how high-pressure CO<sub>2</sub> pneumoperitoneum induces oxidative stress and mitochondria-dependent apoptosis in rabbit kidneys with severe hydronephrosis is shown in Fig. 9.

However, the present study has several important limitations. While the protocol following the method described by Wen *et al* (19) is an acceptable research model for kidney with hydronephrosis, it differs from hydronephrosis kidneys in humans. Therefore, how hydronephrosis affects the renal blood flow may differ between the species. Another issue to be stressed is the difference in the effects of pneumoperitoneum in

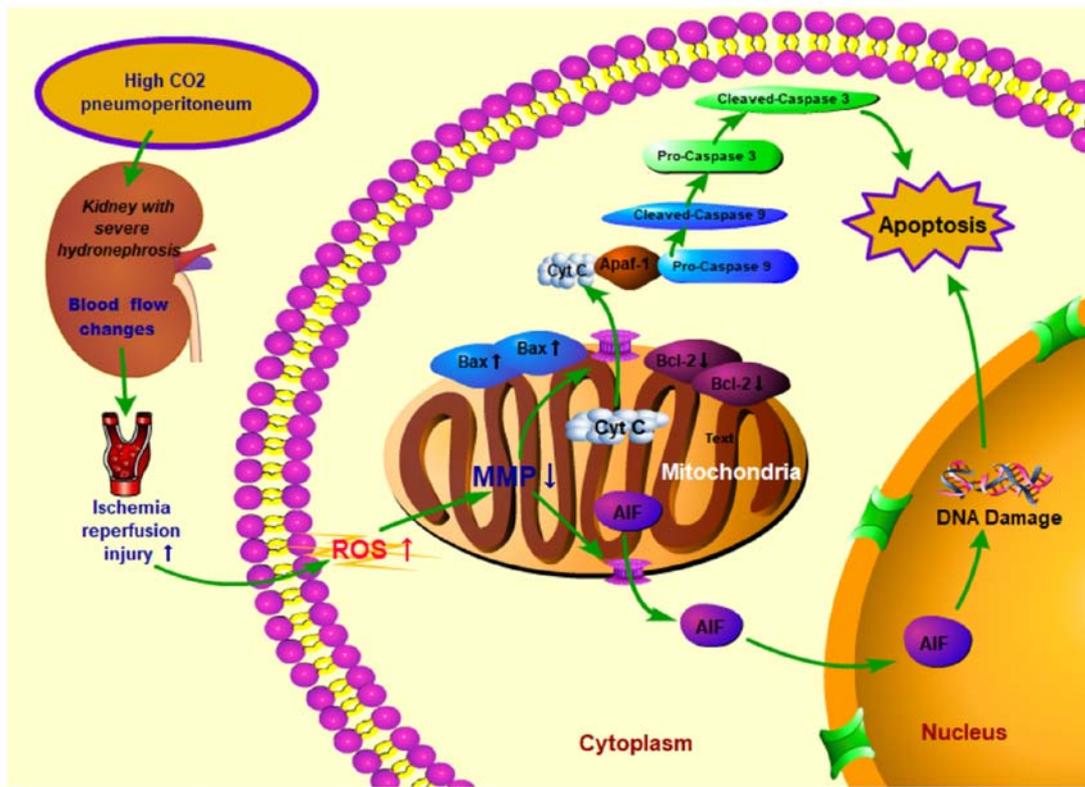


Figure 9. Proposed mechanisms of cell apoptosis induced by high CO<sub>2</sub> pneumoperitoneum pressure in rabbit kidneys with severe hydronephrosis. High CO<sub>2</sub> pneumoperitoneum pressure induced renal blood flow changes and increased the accumulation of ROS, resulting in upregulated Bax and downregulated Bcl-2 levels in kidneys. Consequently, the MMP was reduced, which then accelerated the release of Cyt c into the cytoplasm, leading to apoptosis via the caspase-3 and caspase-9-dependent pathway. CO<sub>2</sub>, carbon dioxide; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated x protein; Cyt c, cytochrome C; AIF, apoptosis inducing factor.

the rabbit abdomen compared with that in the human abdomen for a given pressure, since the level of cell tolerance to intra-abdominal pressure may differ between humans and rabbits (49). To the best of our knowledge, there has been no comparison between the kidney surface area in the two species; therefore, calculations of force per unit of surface area are not available. In humans, pneumoperitoneum pressure is recommended to be controlled to <8 mmHg (8). Considering the aforementioned important limitations, the experimental grouping was simplified, and three groups were set up with the pressures of 0 (no pressure), 8 (low pressure) and 18 mmHg (high pressure). We believed that these three groups were sufficient for to investigate how high-pressure CO<sub>2</sub> pneumoperitoneum affects kidneys with severe hydronephrosis. Furthermore, the insufflation with CO<sub>2</sub> in the present study was performed at room temperature (20-25°C) and dry conditions (0-5% relative humidity), and the intraabdominal pressure lasted for 90 min. According to a previous meta-analysis, warming and humidifying the gas used for insufflation has been proposed to reduce the iatrogenic effects of laparoscopic surgery, including pain, hypothermia and peritoneal alterations (50). However, a prospective randomized trial confirmed that heating and humidification of CO<sub>2</sub> during pneumoperitoneum is not indicated (51). Another experimental study also confirmed that the use of warming and humidification of insufflation gas had no effect on oxidative stress compared with the controls treated with unheated and non-humidified gas (52). Furthermore, the findings of Akbulut *et al* (53) indicated that the operating time should be limited to <120 min

during laparoscopic surgery, since the prolonged duration of pneumoperitoneal pressure may cause increased oxidative problems (54). The present study only explored how high-pressure CO<sub>2</sub> pneumoperitoneum affects kidneys with severe hydronephrosis. However, the properties of the gas and the duration of intraabdominal pressure should also be studied. Notably, a number of other important molecules or apoptotic factors also exist that should be detected in order to clarify the mechanism of the mitochondria-dependent pathway of apoptosis.

Bcl-2 homology domain 3 (BH3)-only proteins, such as Bad, Bim, Bid, Puma and Noxa, are known to be important proteins that initiate and regulate cell apoptosis (55). These proteins are involved in apoptosis by translocating from the cytoplasm to mitochondria, and then inhibiting the activity of anti-apoptotic members (such as Bcl-2) or activating the activity of pro-apoptotic members (such as Bax/Bak) (56,57). Other apoptosis pathways or certain other important molecules may also be associated with the activation of BH3-only proteins. For instance, the phosphorylation of Bad is associated with the PI3K-Akt pathway, while the cleavage of Bid is closely associated with the Fas death signaling pathway (58). P53, another important apoptotic member in DNA damage apoptosis, regulates the expression of Noxa and Puma (59). By contrast, a number of apoptotic molecules, such as Smac, AIF and Endo G, are also important apoptotic members that are released from mitochondria subsequent to a decrease of MMP. These molecules play apoptotic roles by activating the caspase-3 pathway or by causing DNA damage (60,61). In the present study, the

expression levels of Bcl-2, Bax, Cyt c, caspase-3 and caspase-9 were detected, and the results indicated that the apoptosis in severe hydronephrosis kidneys induced by high-pressure CO<sub>2</sub> pneumoperitoneum may be associated with the mitochondria-dependent apoptotic pathway. However, further studies regarding other aforementioned proteins or potential targets should be conducted to fully understand the exact mechanism underlying this type of apoptosis. The application of certain drugs to inhibit or promote mitochondria-dependent apoptosis in animals can also be investigated.

In conclusion, the results of the present study indicate that high-pressure CO<sub>2</sub> pneumoperitoneum induces oxidative stress and causes apoptosis in rabbit kidneys with severe hydronephrosis through the mitochondrial apoptotic pathway. These conclusions were supported by the increased generation of ROS, loss of MMP, increased percentage of swollen or vacuolar mitochondria, Cyt c release, altered expression of Bcl-2 family proteins and activation of the caspase-9/3 cascade in the high pressure (18 mmHg) group. Thus, these findings offer theoretical guidance for the application of pneumoperitoneum in laparoscopic surgery. These results also provide an insight into the molecular mechanism by which hydronephrosis protects the kidneys during laparoscopic surgery.

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#### 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

SZ and WL performed the experiment and were major contributors in writing the manuscript. FC conceived and designed the experiments. JN supplied the materials and analyzed the data. TR, WY and YR collected and analyzed the data. XY and RY supplied the materials and analyzed the data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Ethical and Research Committee of Wuhan University Medical School (Wuhan, China).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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