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ORIGINAL ARTICLE

Hyperbaric Oxygen Ameliorated Acute Pancreatitis in Rats via the Mitochondrial Pathway

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Abstract

Background Acute pancreatitis (AP) is a common disease of the digestive system. The mechanism of hyperbaric oxygen (HBO) therapy for AP is not completely clear.

Aims This study investigated the effects of HBO in AP and whether it acts through the mitochondria-mediated apoptosis pathway.

Methods Eighty male Sprague–Dawley rats were randomly assigned to four groups: control (8 rats), sham (24 rats), AP (24 rats), or AP + HBO (24 rats). AP was induced by ligating the pancreatic duct. The AP + HBO group was given HBO therapy starting at 6 h postinduction. Eight rats in each group were killed on days 1, 2, and 3 postinduction to assess pancreatic injury, mitochondrial membrane potential, ATP level, and expression levels of BAX, Bcl-2, caspase-3, caspase-9, and PARP in pancreatic tissue and blood levels of amylase, lipase, and pro-inflammatory cytokines.

Results HBO therapy alleviated the severity of AP and decreased histopathological scores and levels of serum amylase, lipase, and pro-inflammatory cytokines. Compared to AP induction alone, HBO therapy increased expression of the apoptotic protein BAX, caspase-3, caspase-9, and PARP and ATP levels in tissues and decreased antiapoptotic protein Bcl-2 expression levels and the mitochondrial membrane potential on the first day; the results on the second day were partly consistent with those on the first day, while there was no obvious difference on the third day.

Conclusions HBO therapy could induce caspase-dependent apoptosis in AP rats to alleviate pancreatitis, which was possibly triggered by mitochondrial apoptosis pathway regulation of Bcl-2 family members.

Keywords Acute pancreatitis · Hyperbaric oxygen · Apoptosis · Mitochondrial pathway

Introduction

Acute pancreatitis (AP) is the most common pancreatic disease in the world, and the rough incidence of AP is 33.74 per 100,000 people every year [1]. Most patients present with self-limiting mild pancreatitis. However, 10–20% of AP may develop into severe acute pancreatitis (SAP). SAP is a dangerous disease that is characterized by rapid onset, rapid change, numerous complications, poor prognosis, and tremendous costs, and the mortality rate is up to 20–30% [2, 3].

Apoptosis is thought to induce either mild or no inflammatory response. Apoptosis is an energy-consuming process, so it is rare in hypoxic environments such as SAP [4]. However, necrosis destroys the integrity of cell membranes, leading to the release of digestive enzymes and inflammatory mediators and ultimately causing local and systemic damage. Necrosis is mainly associated with SAP [4, 5]. The severity of SAP can be reduced by inducing apoptosis of

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damaged acinar cells or converting the death type of acinar cells from necrosis to apoptosis [6–8]. Therefore, we speculate that "apoptosis disorder" may be a significant mechanism for the pathogenesis of SAP.

Hyperbaric oxygen (HBO) is a therapy in which pure oxygen or a high concentration of oxygen is inhaled into the body under the pressure of more than 1 absolute atmosphere (1.0 ATA). It provides numerous functions, including increasing oxygen supply, improving blood flow, affecting energy metabolism, resisting inflammation, affecting cell apoptosis, and promoting wound healing [9–11]. Many studies have confirmed that HBO has a positive effect in the treatment of AP in rats [12–15]. However, the role of HBO in AP is still an observational study, and the specific mechanism of HBO mitigates AP is not fully understood. Therefore, our experiment is to further explore whether HBO can increase mitochondrial energy reserve and improve the apoptotic disorder of AP in rats through the mitochondrial apoptosis pathway.

Methods

Animal

Eighty male Sprague–Dawley rats (250–300 g) were housed in comfortable and safe boxes and were given standard chow and water ad libitum under a 12-h light/dark cycle. The cages were filled with autoclaved sawdust, which was replaced three times a week. All experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University.

Design

The rats were randomly assigned to four groups. The control group with eight rats was untreated and served as a baseline for normal apoptosis. Then, the remaining seventy-two rats were equally divided into three groups: sham, AP and AP + HBO, with twenty-four rats in each group. Animals in the sham group underwent laparotomy only to eliminate the effect of surgery on apoptosis. AP was induced in the rats in the intervention groups, and some animals were randomly treated with HBO. They were placed in hyperbaric oxygen chambers for animals and then were given routine oxygen inhalation with 99% oxygen at 2.5 atm for 90 min, including compression and decompression times of 15 min. The first treatment started 6 h postinduction of AP, and subsequent treatments were performed twice daily. These three groups were each divided into three subgroups, with each subgroup containing eight rats. The rats in each group were killed on days 1, 2, and 3 after induction of AP. Animals were monitored throughout by health assessment guidelines.

Induction of Acute Pancreatitis

Based on the study of Meyerholz and Samuel [16], the model of AP induced by ligation of the rat pancreatic duct was established. All animals were fasted overnight before surgery and anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). A 2–3 cm median laparotomy was made under the xiphoid. Then, the pancreas was found under the stomach, and the bile–pancreatic duct was ligated close to the duodenum. All surgical procedures were performed by one investigator.

Tissue Collection and Histopathological Analysis

Animals were anesthetized on days 1, 2, and 3 after induction of AP. The pancreas tissue was collected and divided into three parts, which were separately fixed, frozen at − 80 °C or placed in precooled phosphate-buffered saline (PBS) for the follow-up experiments. The fixed part was sent to the Department of Pathology of Liaoning Provincial People's Hospital for paraffin embedding. Tissues were cut at 4 μm using a microtome and stained with hematoxylin and eosin. The severity of each rat's pancreatic pathology was determined by a professional histologist who used a double-blind method. The pancreatic tissue was graded based on edema, acinar necrosis, hemorrhage and fat necrosis, inflammation, and perivascular infiltrate using histopathological scores ranging from 0 to 4 according to Schmidt et al. [17], and the maximum possible score was 16.

Mitochondrial Transmembrane Potential Analysis

The mitochondrial membrane potential (MMP) was detected using the MMP assay kit with JC-1. Fresh pancreatic tissue was removed from anesthetized rats and then washed in precooled PBS, rapidly cut into pieces, and digested with trypsin in incubators at 37 °C for 10 min. Then, the digestion was terminated with medium containing 10% serum of equal volume, and the digested tissue was filtered through a 40 μm single-cell filter. After collection and washing with PBS, the single-cell suspension was counted under the microscope to adjust the number of cells to 10^6. The cells were stained in accordance with the protocol of the MMP assay kit with JC-1 (Beyotime, Shanghai, China) using flow cytometry and fluorescence microscopy for analysis.

Detection of ATP Levels

ATP levels in tissues were measured with an ATP assay kit (Beyotime, Shanghai, China). Briefly, tissues were washed with PBS and homogenized in ATP lysate. The supernatant

was collected after centrifugation at 12,000 × g at 4 °C for 5 min. Then, 100 µl of detection solution was added to each well in a black 96-well plate. Next, 20 µl of sample or standard was added to the corresponding wells. A SpectraMax i3x apparatus was used to measure the samples. Thereafter, the protein concentration was detected to eliminate the errors caused by the difference in protein content in each sample, and the final ATP concentration was expressed in nmol/mg units.

Protein Isolation and Western Blot

Total protein from pancreas tissues was extracted with lysis buffer (Beyotime, Shanghai, China), which included a protease-inhibitor cocktail. Then, the tissues were homogenized and centrifuged at 12,000 rpm at 4 °C for 20 min, and the supernatant was obtained and quantified using the Bradford method. Proteins were separated using 10% SDS–PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Germany). Thereafter, the membranes were sealed with 5% skim milk (Becton Dickenson, Franklin Lakes, NJ, USA) in TBST (Tris–HCl buffer solution with 0.05% Tween-20) for 1 h at room temperature before incubation overnight at 4 °C with primary antibodies against cleaved caspase-3 (CST, Boston, USA), cleaved caspase-9, PARP, Bcl-2 (Wanleibio, Shenyang, China), and BAX (Proteintech, Wuhan, China). Membranes were washed with TBST 3 times for 10 min and incubated with horseradish peroxidase-conjugated antirabbit IgG (1:5000; Proteintech, Wuhan, China) at room temperature for 2 h. β-actin (Absin, Shanghai, China) was used as a loading control to measure relative protein expression.

RNA Isolation and Quantitative Real-Time PCR (qPCR)

Rat pancreatic tissues (100 mg) were homogenized for RNA isolation using RNAisol reagent (Takara, Japan) according to the manufacturer's protocol. The RNA was quantified using spectrophotometry, and 1 µg RNA per sample was reverse-transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (RR047A Takara) into cDNA. The cDNA was subjected to quantitative real-time PCR in a 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A Takara) under the following cycling conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. β-actin was used as a reference. Primer sequences are listed in Table 1. The change in relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical Staining

Pancreatic tissues were cut to a 4 µm thickness. Slices were dewaxed in xylene and rehydrated in a graded series of ethanol. Then, slices were placed into a box containing citrate buffer solution for antigen repair via a high-temperature and high-pressure method. After 2.5 min, the box was removed and cooled to room temperature. The slices were washed 3 times with PBS for 5 min every time. The slices were moved to a wet box and incubated according to the instructions of the UltraSensitive™ SP IHC kit (Maxim, Fuzhou, China). And the sections were incubated with primary antibodies of anti-BAX (1:300; Wanleibio, Shenyang, China) or anti-Bcl-2 (1:200; Wanleibio, Shenyang, China) at 4 °C overnight. All sections were developed with a DAB plus kit (Maxim, Fuzhou, China) for 1–2 min. Slices were visualized under a light microscope to monitor the degree of staining. The sections were counterstained in hematoxylin for 8 min and then quickly rinsed with water 3 times. After differentiation with 1% hydrochloric–alcohol solution, the sections were washed with running water for 30 min, dehydrated in a graded series of ethanol, and made transparent by xylene. Finally, all slices were covered with coverslips and measured under a microscope. The intensity scores were assigned as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Percentage scores of positive cells were defined as follows: 0 (0–5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The two scores of each pancreas sample were multiplied to produce a final score of 0 to 12.

Biochemical Assays and Cytokine Levels in Blood

Blood samples were harvested from the heart before rats were killed, centrifuged at 3,000 rpm to obtain serum, and stored at − 80 °C. After a fivefold dilution with saline, amylase and lipase levels were assessed by an automated biochemical analyzer and were expressed as U/L. The serum levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using enzyme-linked

Table 1 List of primers

mRNA	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	CTAGAAGCATTTGCGGTGCACGAT	ATGTACCCAGGCATTGCTGACAG
Caspase-3	CACTGGAATGTCAGCTCGCA	TCAGGGCCATGAATGTCTCTC
Caspase-9	CTAGGGGTGTAACAGCTTCTGGAT	ATGATCGAGGATATTCAGCGGGC

immunosorbent assay (ELISA) kits according to the manufacturers' instructions.

Statistical Analysis

All data were analyzed using SPSS 22.0 software. Results were presented as the mean ± SD. Normally distributed data were analyzed using Student's *t* test or one-way analysis of variance (ANOVA). Non-normally distributed data were analyzed using the Mann–Whitney *U* test or Kruskal–Wallis *H* test. For all data, two-sided *p* values < 0.05 were considered statistically significant.

Results

Effects of HBO on Histopathological Changes

The pancreatic duct ligation model (Fig. 1) is easy to perform, and this method is similar to the pathogenesis of biliary AP. Moreover, assessments of histological changes such as edema, acinar necrosis, hemorrhage and fat necrosis, inflammation and perivascular infiltrate were conducted (Fig. 2a). As shown in Fig. 2b and Table 2, changes in pathology were significantly increased in the groups that underwent AP induction compared to the sham groups. The macroscopic scoring was lower in the AP + HBO groups than in the AP groups, but only scores on the first day were significantly different. The difference between the normal group and sham groups was not statistically significant.

Changes in Serum Amylase, Lipase and Cytokines

The changes in serum amylase and lipase are shown in Fig. 2c, d. At three different time points, the serum amylase and lipase levels of the AP groups were significantly elevated compared with those of the sham groups, but there was no significant difference between levels in the control and sham groups. The levels of amylase and lipase continued to decrease in the AP and AP + HBO groups from day 1 to day 3 after modeling. And the levels of serum amylase between the AP + HBO group and sham group on day 3 had not significant difference. HBO therapy significantly intensified the decline in lipase levels in the AP + HBO groups compared with the AP groups on days 1 and 2 postinduction of AP, while the differences between the AP + HBO and AP groups were not significant at day 3. The therapy of HBO significantly reduced the levels of serum amylase in the AP + HBO group compared with the AP groups at day 1 after AP induction. But there were no significant differences in the AP + HBO group compared with the AP group on days 2 and 3.

As shown in Fig. 2e, f, compared with the rats in the sham groups, serum pro-inflammatory cytokines (IL-6 and TNF-α) were significantly increased in the AP groups at each time point. However, this increase in TNF-α levels was inhibited by HBO treatment in the AP + HBO groups compared with AP groups on days 1 and 2, and the increase in IL-6 levels was also inhibited by HBO treatment in the AP + HBO groups at day 1 compared with the AP group, which indicates that HBO treatment may inhibit pro-inflammatory cytokine expression in blood. Though there were no significant differences between AP groups and AP + HBO groups in the serum levels of TNF-α on day 3 and the serum levels of IL-6 on days 2 and 3, the difference between the control group and sham groups was not statistically significant.

Analysis of the Mitochondrial Transmembrane Potential and ATP Level

It is generally believed that apoptosis is an active energy-consuming process and that the ATP level is a significant factor in apoptosis or necrosis. The cells will necrose if they do not have a sufficient energy supply after being damaged. In contrast, the cells will undergo apoptosis in a positive way [18]. The decrease in the MMP is an early manifestation of apoptosis. A fluorescent JC-1 probe was used to investigate the MMP by flow cytometry and fluorescence microscopy. As shown in Fig. 3a, b, the JC-1 fluorescence intensity red/green ratio of the acinar cells in the AP + HBO groups was

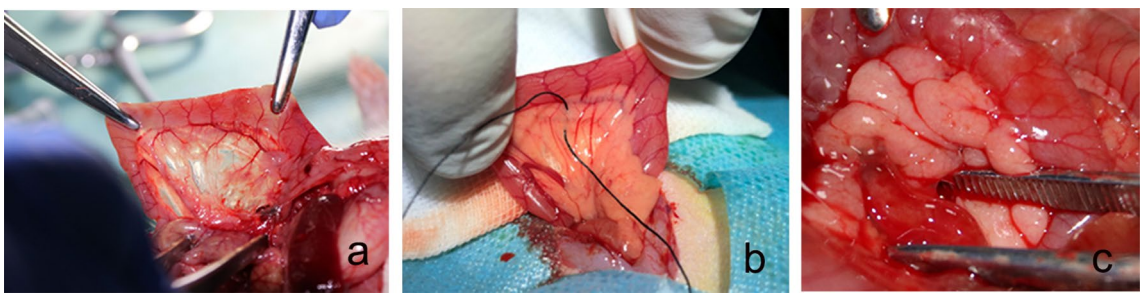


Fig. 1 Acute pancreatitis induction. **a** Exposed bile–pancreatic duct. **b** Bile–pancreatic duct ligation. **c** Observation on the third day after operation

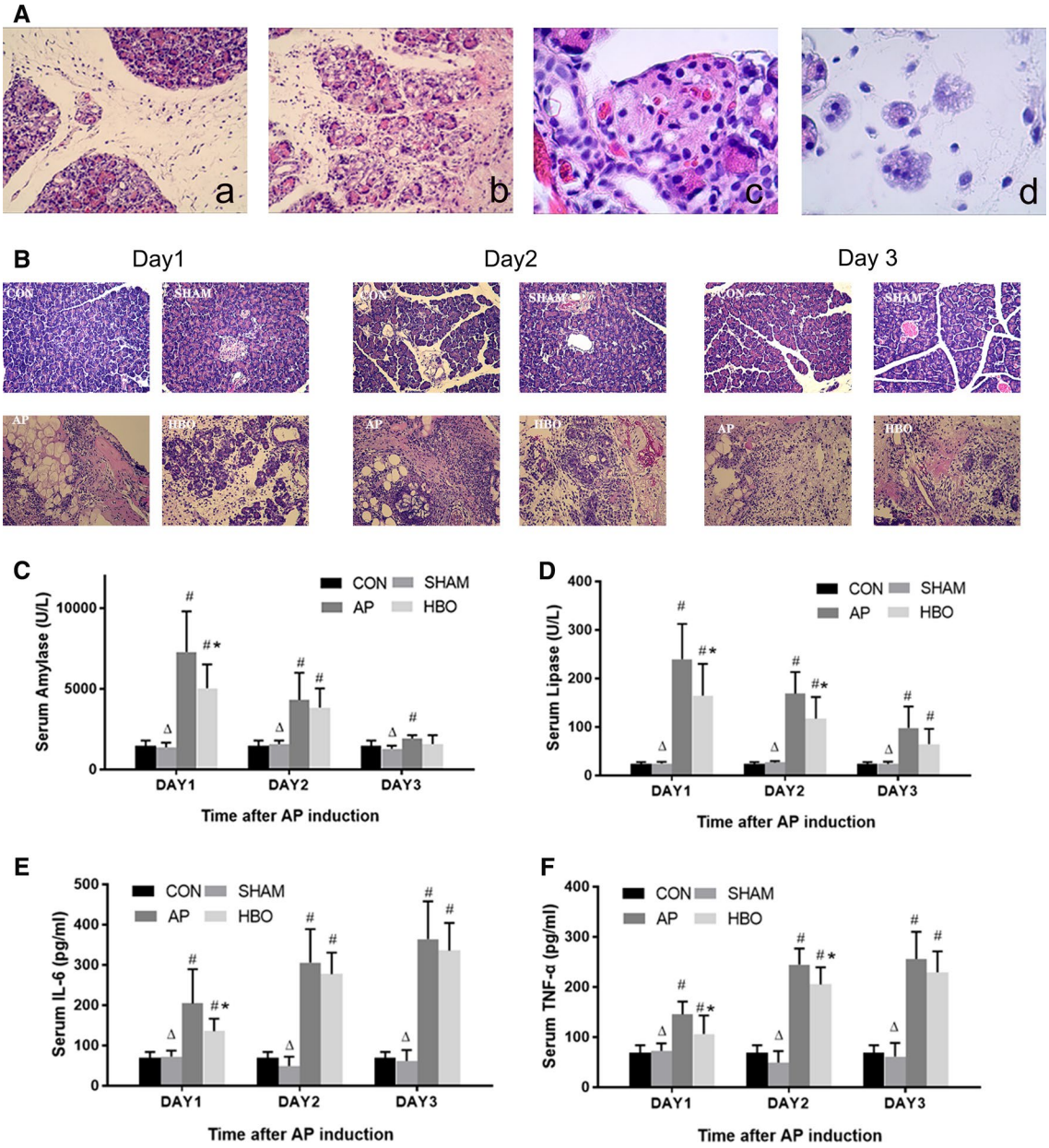


Fig. 2 Pathological changes in the pancreas and expressions of serum amylase, lipase, IL-6, and TNF-α. **a** Histological alteration in pancreatic edema (**a**), acinar cell necrosis (**b**), hemorrhage (**c**), substantial inflammatory infiltration (**d**), (HE × 100). **b** Pathological scoring of pancreatic tissues (HEX100). **c** Expression of serum amylase. **d** Expression of serum lipase. **e** Expression of serum IL-6. **f** Expression of serum TNF-α. $^{\#}p < 0.05$ in comparison with the sham group; $^{*}p < 0.05$ in comparison with the AP group; $^{\triangle}p > 0.05$ in comparison with the control group

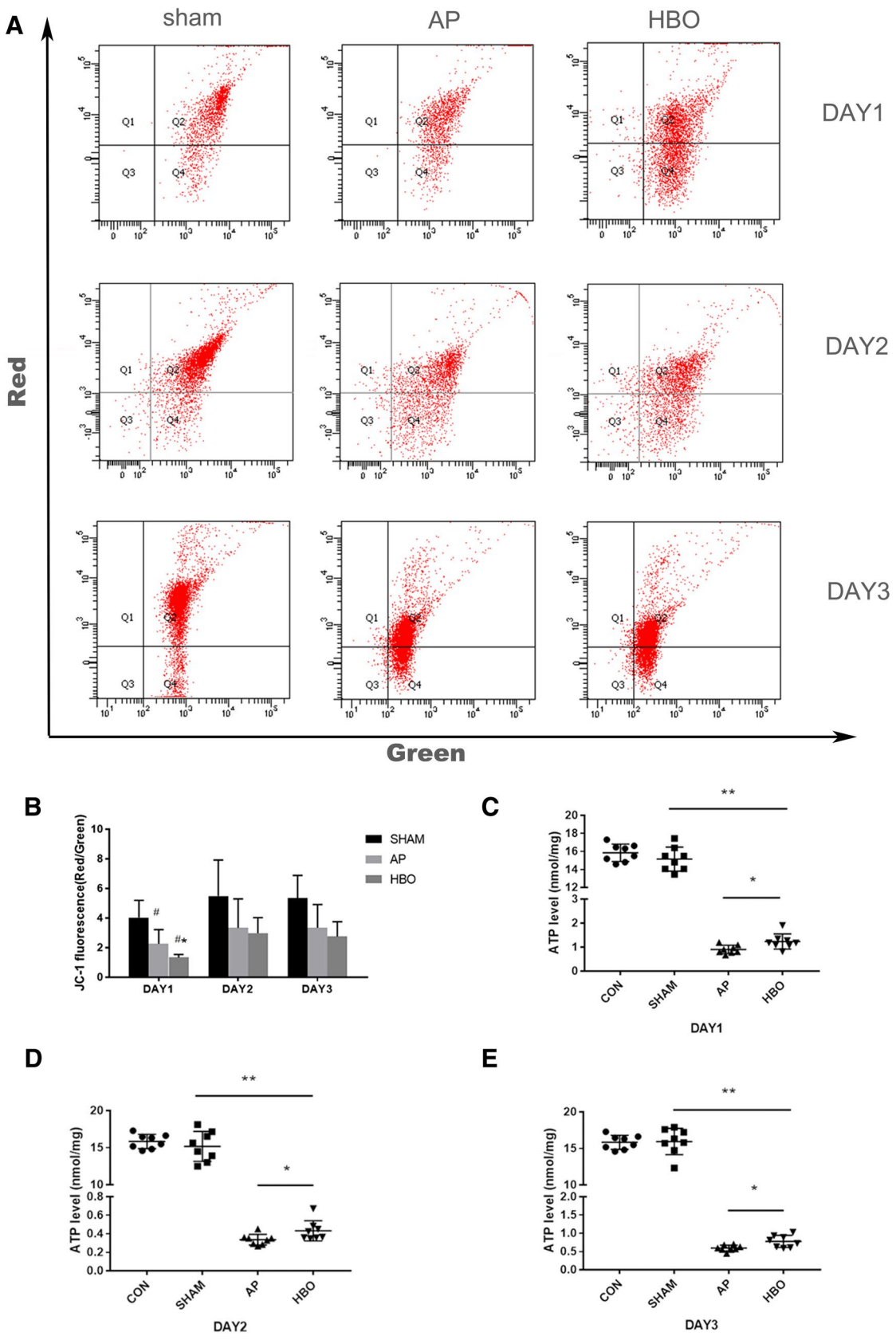
Table 2 Severity scores of HE staining at endpoints

Groups	Day 1	Day 2	Day 3
Con	0.6875 ± 0.5303	0.6875 ± 0.5303	0.6875 ± 0.5303
Sham	0.6875 ± 0.5939	0.875 ± 0.5175	0.9375 ± 0.6781
AP	11.13 ± 2.167*	9.75 ± 1.512*	7.813 ± 2.419*
AP + HBO	7.813 ± 1.668*#	9.25 ± 0.8018*	9.063 ± 3.659*

Histopathological score about pancreas. Compared with control group, sham group $^{\wedge}p < 0.05$. Compared with sham group, $^{*}p < 0.05$. Compared with AP group, HBO treatment group $^{\#}p < 0.05$

lower than that in the other groups, which was consistent with the fluorescence photograph (Fig. 4). The results suggested that the MMP of acinar cells in rats decreased after treatment with HBO.

As shown in Fig. 3c, e, compared to that in the sham groups, the ATP expression level in the AP and AP + HBO groups significantly decreased at three different time points ($p < 0.05$), while the ATP expression level in the control group was not significantly different from that in the sham groups ($p > 0.05$). The HBO-treated groups exhibited


A
sham
AP
HBO
DAY1
DAY2
DAY3
Red
Green
Q1
Q2
Q3
Q4
B
JC-1 fluorescence(Red/Green)
SHAM
AP
HBO
DAY1
DAY2
DAY3
C
ATP level (nmol/mg)
CON
SHAM
AP
HBO
DAY1
D
ATP level (nmol/mg)
CON
SHAM
AP
HBO
DAY2
E
ATP level (nmol/mg)
CON
SHAM
AP
HBO
DAY3

◂**Fig. 3** Levels of mitochondrial transmembrane potential and ATP. **a**, **b** Flow cytometry analysis of MMP. $^{*}p<0.05$ in comparison with the AP group. $^{\#}p<0.01$ in comparison with the sham group. **c**–**e** ATP levels of pancreas tissues. $^{*}p<0.05$ in comparison with the AP group, and $^{**}p<0.01$ in comparison with the sham group. The data are expressed as the mean ± SD

significant increases in the ATP level at three different time points compared to the AP groups ($p<0.05$).

Effect of HBO on the Expression of Apoptosis Pathway Proteins and mRNAs in Pancreatitis

To explore whether the mitochondrial apoptotic pathway actually plays a role in pancreatitis, tissues were collected and analyzed by Western blotting to measure the expression quantity of cleaved caspase-9, cleaved caspase-3 and PARP, which is the key protein in the mitochondrial apoptotic pathway [19, 20]. As shown in Fig. 5a, under stimulation of HBO, the levels of cleaved caspase-3, cleaved caspase-9, and PARP significantly decreased in the AP groups compared with the HBO groups ($p<0.05$) on the first day, and the corresponding bands on the second day had the same tendency, while only the cleaved caspase-9 was statistically significant ($p<0.05$). In addition, no significant differences were observed on the third day ($p>0.05$). There were no significant differences between the four groups (Fig. 7, control, sham1, sham2, sham3, $p>0.05$).

To further quantify the effect of HBO therapy on the apoptosis pathway, qPCR was used to measure the mRNA expression levels. As shown in Fig. 5b, the tendency of genetic expression was in accord with that of the protein. However, only the caspase-9 level on the first day showed a significant difference between the AP and AP + HBO groups ($p<0.05$).

Influence of HBO on Upstream Proteins

Compared with the AP groups, the AP + HBO groups showed higher expression of BAX/Bcl-2 on the first and second days in the Western blotting analysis (Fig. 6a, $p<0.05$), while there were no differences between the control and sham groups (Fig. 7, $p>0.05$). According to the principles of scores in immunohistochemistry, the significant changes in the expression levels of apoptotic proteins were computed and presented. As shown in Fig. 6b–d, the antiapoptotic gene Bcl-2 was more highly expressed in the AP groups than in the AP + HBO groups at all time points ($p<0.05$). In contrast, the apoptotic gene BAX was expressed at lower levels in the AP groups than in the AP + HBO groups at three time points ($p<0.05$). However, the expression levels of BAX and Bcl-2 were negative in the control group and sham groups.

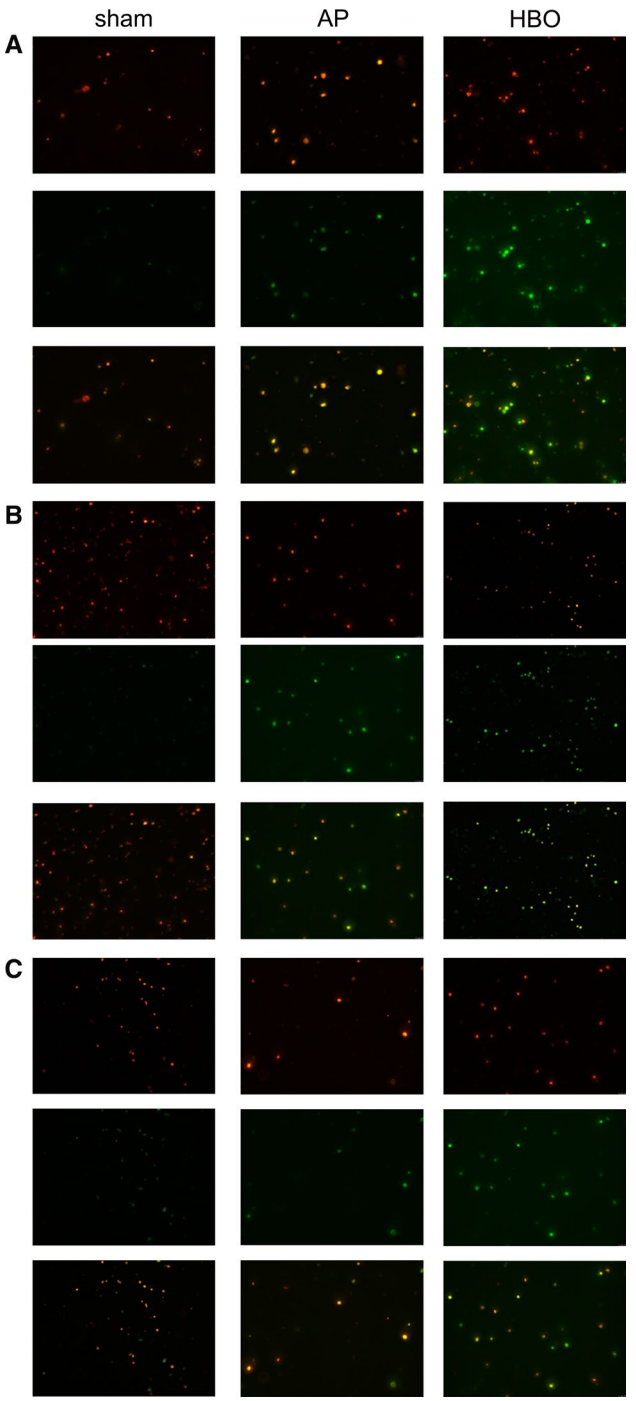


Fig. 4 Fluorescence photograph of the MMP of acinar cells. **a**: (day 1), **b**: (day 2), **c**: (day 3). More red fluorescence can be seen in the sham group, and more green fluorescence can be seen in the HBO group compared with the AP group

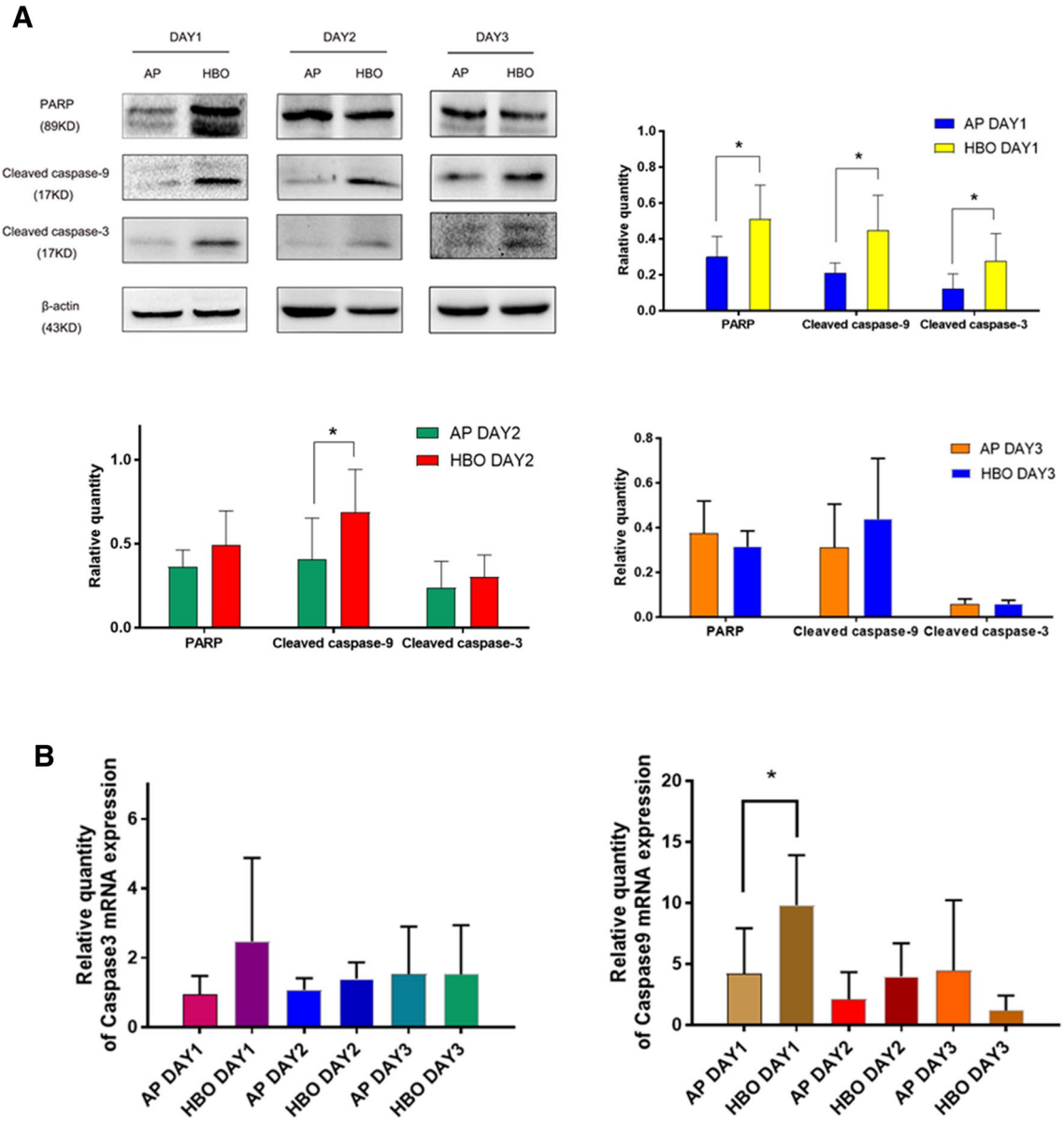


Fig. 5 Apoptosis of Western blotting and RT-PCR analysis. **a** Expression levels of cleaved caspase-9, cleaved caspase-3, and PARP were examined by Western blotting analysis of pancreatic tissues. The levels of cleaved caspase-9, cleaved caspase-3, and PARP protein at three time points, which were normalized with respect to the β-actin band density. The data are expressed as the mean ± SD. *$p < 0.05$ in comparison with the AP group. **b** RT-PCR results for caspase-3 and caspase-9 mRNA expression. The expression of caspase-9 mRNA increased significantly in the HBO group compared with the AP group on the first day. The data are expressed as the mean ± SD. *$p < 0.05$ compared with the AP group

Discussion

The purpose of this study was to further investigate the effect of HBO on the mitochondrial apoptosis pathway of AP. AP progresses rapidly and tends to develop into SAP, leading to systemic inflammatory reaction syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). In our study, we induced AP with pancreatic duct ligation, and a high histological score reflected the severity of pancreatic injury. Compared with the sham group, obvious changes in pancreatic pathology, the activity levels of serum amylase, lipase and pro-inflammatory factor in the AP group were significantly increased, suggesting successful modeling. IL-6 and TNF-α are crucial pro-inflammatory factors in AP, which are mainly involved in the regulation and amplification of the AP inflammation response [21, 22]. Studies have shown that the severity of AP was reduced by blocking the cascade of

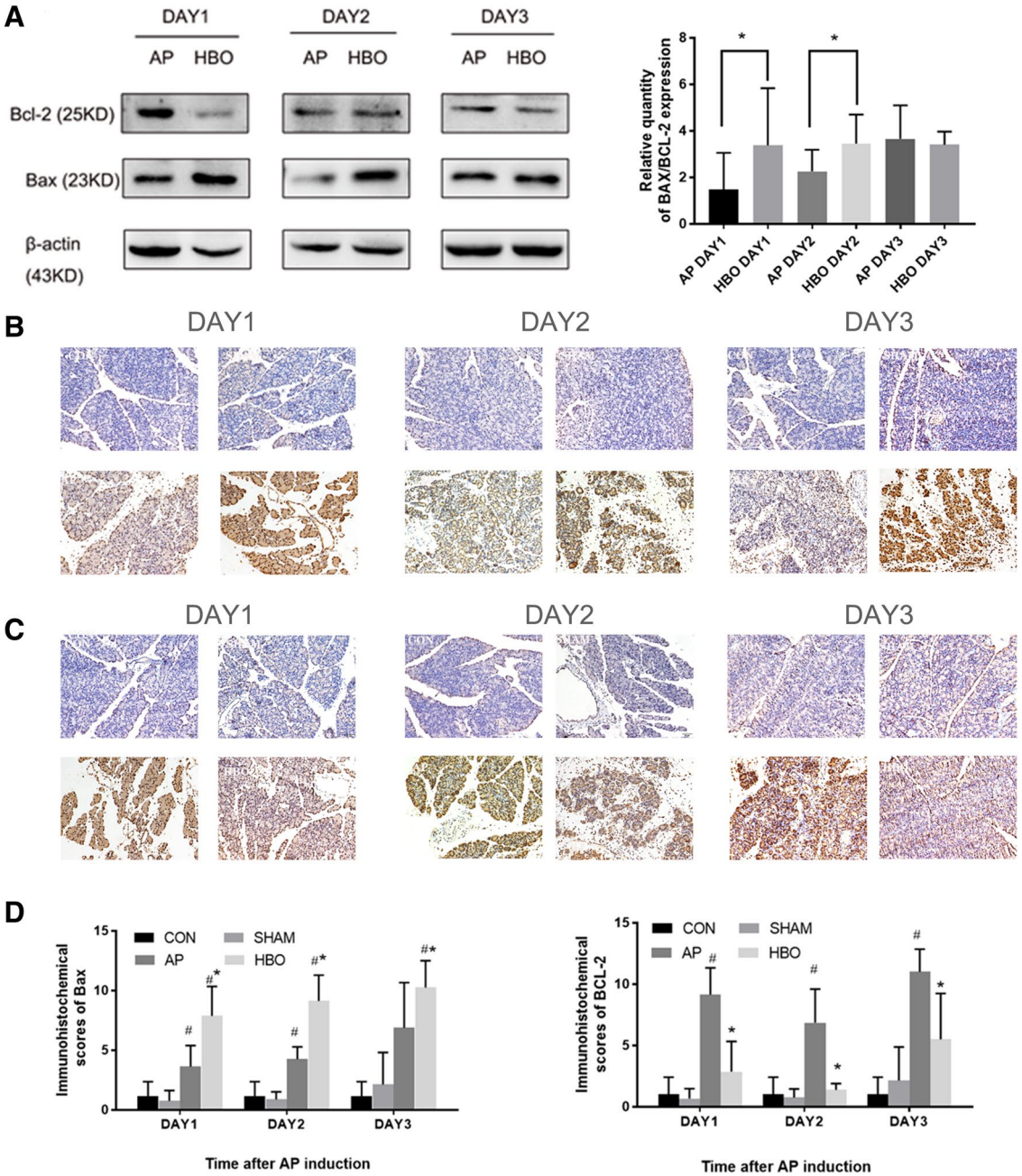


Fig. 6 The expression levels of BAX and Bcl-2 were examined by Western blotting analysis and immunohistochemical staining of pancreatic tissues. **a** The levels of BAX and Bcl-2 protein at three time points, which were normalized with respect to the β-actin band density. Data are expressed as the mean ± SD. $^{*}p < 0.05$ in comparison with the AP group. **b** Immunohistochemical staining of BAX. **c** Immunohistochemical staining of Bcl-2. **d** Immunohistochemical score of the levels of BAX and Bcl-2 in pancreatic tissues. $^{*}p < 0.05$ in comparison with the AP group; $^{\#}p < 0.01$ in comparison with the sham group

pro-inflammatory cytokine [23, 24]. Clinical studies have shown that HBO therapy was beneficial for SAP patients, and the disease severity of patients treated with HBO was improved according to clinical, biochemical, and radiological criteria [25]. Other experimental studies have also shown that HBO treatment improved survival rate, reduced cell necrosis, increased cell apoptosis, promoted cell proliferation, and thus reduced the severity of AP [26–28]. Our study also found that HBO significantly reduced the severity of pancreatic histopathology and levels of serum amylase, lipase, IL-6, and TNF-α in the blood of AP rats. However, the specific mechanism by which HBO promotes

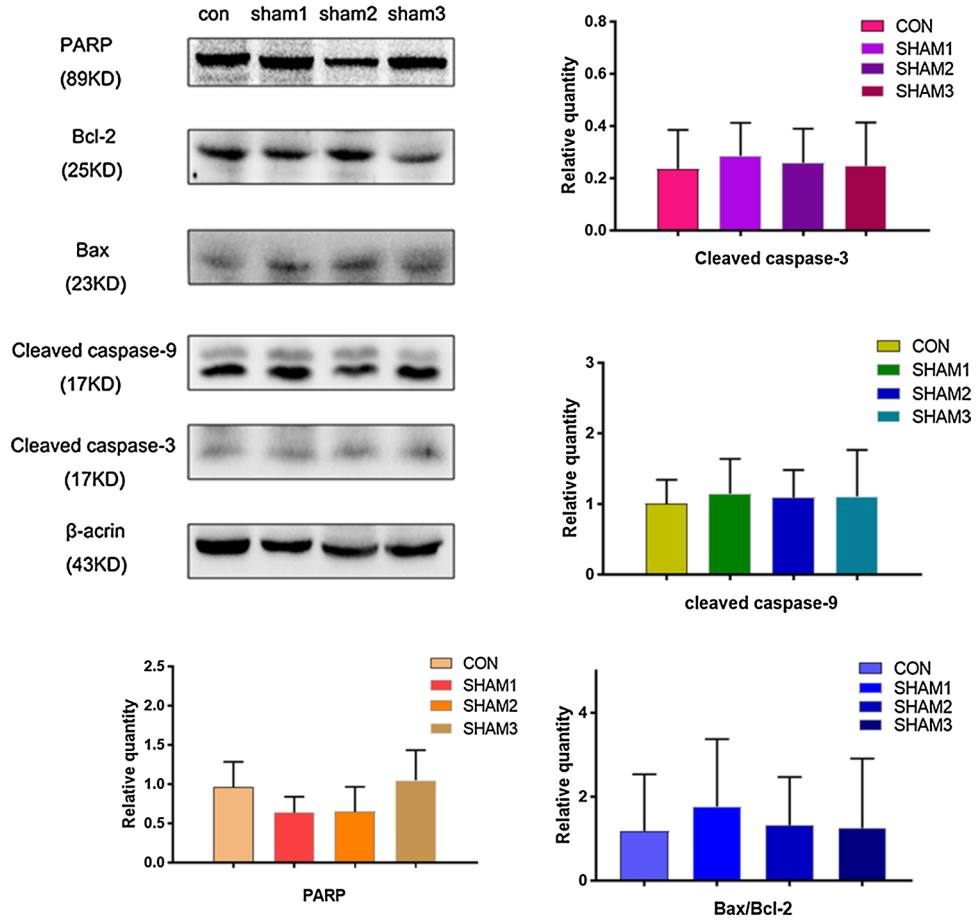


Fig. 7 Control group and sham group analysis by Western blotting. The control group was not different from the sham groups at three different time points ($p > 0.05$)

apoptosis of acinar cells remains unclear. In this study, we investigated the effect of HBO on the mitochondrial apoptosis pathway of AP in rats.

In AP, a large number of acinar cells are damaged, and the degree of damage reflects the severity of the disease. There are two death pathways for acinar cells in AP: apoptosis and necrosis. Some researchers believe that SAP acinar cells are largely necrotic and rarely apoptotic, while AP cells are the opposite [29]. Apoptosis is a process of programmed cell death, which is a process of energy consumption, and mitochondria play a key role [30]. If ATP is insufficient, apoptosis cannot proceed, and cells turn from apoptosis to necrosis [31], thus aggravating the severity of disease. Therefore, we recommend the use of HBO to maintain cell integrity, preserve and update cell energy stores, and promote apoptosis pathways rather than necrosis, which triggers inflammatory processes.

Mitochondria-mediated pathways are initiated in response to substantial cell damage and external stress signals. When the cell is damaged, the mitochondrial structure is damaged, and the environment provides appropriate ATP to promote the development of apoptosis [32]. The decrease in the MMP is an early response to apoptosis, and the caspase cascade system plays a key role in the induction, transduction, and amplification of apoptosis signaling [33, 34]. Caspase-3, activated by caspase-9, is a critical factor in systemic apoptosis [35]. Once caspase-3 is activated, PARP can be increased by caspase-3, which is an indicator of apoptosis [20]. HBO is a treatment in which pure oxygen is inhaled at more than one atmosphere of pressure. HBO upregulated ATP in unconscious rats and improved mitochondrial function [11, 36]. Furthermore, HBO significantly increased the levels of Apaf-1, BAX, cleaved caspase-3, and cleaved caspase-9 and decreased the expression of Bcl-2, promoting PDT-induced cell apoptosis [37]. Our results confirmed that HBO could induce apoptosis of pancreatic cells in rats, which was supported by several methods, including a JC-1 probe, immunohistochemical staining, and Western blotting. On the first day postinduction of AP, the AP + HBO groups exhibited a significant increase in the level of ATP and a decrease in the MMP compared with the AP groups. In addition, Western blotting analysis showed that HBO treatment upregulated the expression of cleaved caspase-3, cleaved caspase-9, and PARP. It is well known that the Bcl-2 family is the upstream regulatory member of the mitochondrial apoptosis pathway. As an important proapoptotic gene, BAX is present in the cytoplasm and is

activated when it receives apoptotic information. Activated BAX is present on the mitochondrial membrane, which changes the permeability of mitochondria and activates the caspase system. In addition, BAX can also form a complex with Bcl-2 through a specific domain, which causes Bcl-2 to lose its function of altering MMP and induces apoptosis [38–40]. Studies have shown that dexamethasone downregulated Bcl-2 and upregulated BAX to improve pancreatic acinar cell apoptosis [41]. Our study showed consistent results. Immunohistochemistry analysis showed that at three time points, the HBO groups had higher expression levels of BAX than the AP groups, sham groups, and control group, while the opposite situation occurred for Bcl-2.

Interestingly, hyperbaric oxygen can promote apoptosis of acinar cells [42], but there were no statistically significant differences on the third day. In other words, the high expression levels of the upstream protein did not lead to accordant expression of the downstream protein on the third day, which was consistent with the finding that hyperbaric oxygen therapy advanced the peak of apoptosis [24]. At the same time, with prolonged obstruction of obstructive pancreatitis, the pressure on the duct increased. Then, more severe necrosis and less apoptosis appeared, so it was difficult to see significant apoptosis after the first day of HBO treatment. We consider this observation to be closely related to the duration of pancreaticobiliary obstruction. Therefore, the optimal treatment time and frequency of HBO therapy in this study has not been determined. However, it is undeniable that early active treatment is effective.

In conclusion, the present study demonstrates that treatment with HBO could promote apoptosis in rats with AP, which was triggered through the mitochondria-mediated pathway by regulating Bcl-2 family members. In addition, the maintenance of ATP levels in tissues by HBO was an important precondition.

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Compliance with Ethical Standards

Conflict of interest All authors declare no conflicts of interest.

Ethical approval 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.

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