Original Article

Effects of hyperbaric oxygen on intestinal mucosa apoptosis caused by ischemia-reperfusion injury in rats

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BACKGROUND: Hyperbaric oxygen (HBO) is an effective adjuvant therapy for ischemia-reperfusion (I/R) injury of the brain, small intestine and testis in addition to crushing injury. Studies have shown that HBO increases the activity of villi of the ileum 30 minutes after I/R injury. The present study aimed to observe the effect of HBO on apoptosis of epithelial cells in the small intestine during different periods of I/R and to elucidate the potential mechanisms.

METHODS: Rats were subjected to 60-minute ischemia by clamping the superior mesenteric artery and 60-minute reperfusion by removal of clamping. The rats were randomly divided into four groups: I/R group, HBO precondition or HBO treatment before ischemia (HBO-P), HBO treatment during ischemia period (HBO-I), and HBO treatment during reperfusion (HBO-R). After 60-minute reperfusion, samples of the small intestine were prepared to measure the level of ATP by using the colorimetric method and immunochemical expression of caspase-3. The levels of TNF- α in intestinal tissue were measured using the enzyme-linked immunosorbent assay method (Elisa).

RESULTS: TNF- α levels were significantly lower in the HBO-I group than in the HBO-P ($P<0.05$), HBO-R and I/R groups; there was no significant difference between the HBO-R and I/R groups ($P>0.05$). The expression of caspase-3 was significantly lower in the HBO-I group than in the HBO-P group ($P<0.05$); it was also significantly lower in the HBO-P group than in the I/R and HBO-R groups ($P<0.05$). ATP level was significantly lower in the HBO-I group than in the HBO-P group ($P<0.05$), and also it was significantly lower in the HBO-P group than in the I/R and HBO-R groups ($P<0.05$).

CONCLUSIONS: There is an association between HBO, small intestinal I/R injury, and mucosa apoptosis. HBO maintains ATP and aerobic metabolism, inhibits TNF- α production, and thus prevents intestinal mucosa from apoptosis. Best results can be obtained when HBO is administered to patients in the period of ischemia, and no side effects are produced when HBO is given during the period of reperfusion.

KEY WORDS: Hyperbaric oxygen; Ischemia-reperfusion injury; Apoptosis

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INTRODUCTION

Ischemia-reperfusion (I/R) injury is common in tissues or/and organs.^[1,2] Bowel villi are extremely sensitive to I/R injury.^[3] The intestine is very active in metabolism and has a unique immune function, and it is also the largest body of bacterial libraries. Intestinal

I/R injury causes local intestinal injury and damage of the liver, lung, kidney and other organs.^[2,3] Intestinal I/R injury may induce multiple organ dysfunction syndrome (MODS), which is associated with damage of oxygen radicals, energy metabolism, overload of intracellular calcium, leukocyte adhesion, and endothelial cell injury.^[4]

During reperfusion, oxygen reaches the tissue and produces a large amount of reactive oxygen species (ROS), resulting in peroxidation of cell membrane and ultimately apoptosis due to exacerbation of necrosis and triggering of a series of biochemical reactions.^[4-6] Thus many methods have been used to prevent or reduce oxidative stress damage, including the use of low temperature, anti-oxidants, drugs such as vitamin C, allopurinol, free radical scavengers for reducing the active oxygen production, and ischemic pretreatment.^[5,7-9]

Hyperbaric oxygen (HBO) shows promising results in some models, which can reduce ischemic injury.^[3] HBO is used as an effective adjuvant therapy for the I/R injury of the brain, small intestine, and testis in addition to crushing injury. HBO increases the activity of villi of the ileum in 30-minute I/R injury and is effective in the treatment of I/R injury to other organs.^[10,11] This study was undertaken to observe the effect of HBO on apoptosis of epithelial cells in the small intestine in different periods of I/R and to elucidate its potential mechanisms.

METHODS

Animals and grouping

Forty healthy male SD rats, weighing 250-300 g, from the Experimental Animal Center of China Medical University, were randomly divided into four groups: I/R group, 1 hour intestinal ischemia by clamping the SMA followed by 1 hour reperfusion; HBO-P (HBO preconditioning) group, 1 hour HBO pre-treatment before ischemia; HBO-I (HBO during ischemia) group, 1 hour HBO treatment during ischemia; and HBO-R (HBO during reperfusion) group, 1 hour HBO treatment during reperfusion.

Intestinal ischemia/reperfusion injury model

An intestinal I/R injury model was established by the reported method.^[12] Male SD rats were anesthetized with chloral hydrate (0.2 mL/kg, i.p.) and fixed in a supine position. Subsequently, they were subjected to laparotomy, and the main branches of the superior mesenteric artery were clamped. Their abdominal cavities were closed. After 60 minutes, the clamp was removed and the abdominal cavity closed. After 60 minutes of reperfusion, the abdominal cavity of the rat was opened and the small bowel was removed. The I/R group underwent 1-hour ischemia and 1-hour reperfusion; the HBO-P group was given 1-hour HBO treatment and

then underwent intestinal I/R; the HBO-I group was given 1-hour HBO treatment during ischemia; and the HBO-R group was given 1-hour HBO treatment during reperfusion.

HBO treatment

In this study, the animal chamber (DWC400A/0.2-1, Huaxin Chamber Company, Weifang, Shandong) was washed with pure oxygen 5 minutes at first so that the oxygen concentration reached 90% in the chamber. Then the pressure inside the chamber increased to 2.4 ATA with a constant speed over 15 minutes and the oxygen concentration was higher than 95%. The pressure and oxygen concentration were maintained for 60 minutes. Finally, the pressure decreased with a constant speed over 15 minutes. When the pressure in the chamber was 1 ATA, the door was opened and the rats were taken out.

Measurement of intestinal TNF- α

Intestinal tissue of 1000 mg was taken from each rat and washed by saline at 37 °C at different time points, then kept at -70 °C for the preparation of frozen tissue. Intestinal tissue of 300 mg was rinsed in cold saline to remove blood and dried by filter paper. Tissue homogenate was prepared with normal saline, and cells were ruptured by an ultrasonic generator [14 μ m amplitude ultrasonic treatment (Soniprep150, Torbeo, USA)] for 30 seconds, and centrifuged by 10% prepared homogenates at low temperature and a low speed of 3000 r/min for 15 minutes. The supernatant was used for detection of intestinal tissue TNF- α by enzyme-linked immunosorbent assay.

Caspase-3 expression and quantitative analysis in intestinal mucosa

Expression of aspartate-specific cysteine protease (caspase-3) in rat small intestinal mucosa was detected by immunohistochemistry. Samples from the distal small bowel (5 cm to the ileocecum) were fixed with 10% formalin, paraffin-embedded after dehydration and cut into sections of 4 μ m thickness.

SABC method

The sections were dewaxed conventionally, incubated in 30 mL/L H₂O₂ methanol solution at room temperature for 15 minutes, and washed with PBS for 10 minutes. Then antigens were repaired for 15 minutes in 0.01 mol/L citrate buffer (pH 6.0), heated to 92-96 °C, closed by dropping of normal rabbit

serum 10 minutes after cooling, and then incubated with first anti-caspase-3 body overnight at 4 °C. The sections were washed with PBS solution next day, and then incubated with biotinylated secondary antibody, avidin-chain enzyme, and at last stained with DAB, restained with hematoxylin. The sections were dehydrated conventionally with ethanol, cleared with xylene, and mounted with neutral gum. ERK1 expression was observed and analyzed under a light microscope (1:200 dilution of caspase-3 antibody, PBS as negative control instead of primary antibody).

Image analysis

Image analysis was made with an automatic medical image HPIAS21000 color image analysis system quantitatively; five horizons in each section were randomly selected. The average value of absorbance A of caspase-3 of intestinal tissue was determined before and after treatment. Caspase-3 protein expression was detected by an immunohistochemical method and morphological changes of apoptotic cells in rat intestinal epithelial tissue were observed under a light microscope.

Measurement of intestinal ATP enzyme

Similar to the measurement of intestinal TNF- α , 1000 mg intestinal tissue was washed with saline at 37 °C at different time points. The tissue was frozen at -70 °C and 300 mg of them was rinsed in cold saline to remove blood, and dried by filter paper. Tissue homogenate was prepared with normal saline. Cells were treated with 14 μ m amplitude ultrasound in an ultrasonic generator (Soniprep150, Torbeo, US), 10% of prepared homogenates were centrifuged at low temperature and a low speed of 3000 r/min for 15 minutes. The supernatant was obtained for target detection including ATP.

Statistical analysis

Each section was placed under a microscope and computerized with a DIPAS2200 type cells image analysis system. Ten was selected on the screen for a positive rate of statistics. The data were processed using SPSS 13.0 for Windows software. They were presented

as mean $\pm$ standard deviation. After single factor analysis of variance, independent samples were used to compare the results of the SNK-q test ($P < 0.005$).

RESULTS

Changes of TNF- α level in I/R intestinal tissue

The TNF- α level in the HBO-I group was significantly lower than in the HBO-P group ($P < 0.05$). It was lower in the HBO-P group ($P < 0.05$) than in the HBO-R and I/R groups ($P > 0.05$) (one-way ANOVA and SNK) (Table 1).

Changes of ATP enzyme in I/R intestinal tissue

There was an increasing tendency in ATP enzyme content in different groups. ATP enzyme content was significantly higher in the HBO-I group than in the HBO-P group ($P < 0.05$); whereas it was significantly higher in the HBO-P group than in the HBO-R and I/R groups ($P < 0.05$). No statistically significant difference was observed in the HBO-R and I/R groups ($P > 0.05$) (Table 1).

Bcl-2 protein was mainly expressed in the cytoplasm or membrane of cardiomyocyte. The positive expression of Bcl-2 protein in the myocardium increased significantly in groups B, C and D compared with group A ($P < 0.01$). The positive expression decreased significantly in groups C and D compared with group B ($P < 0.01$). The expression of Bcl-2 protein decreased significantly in group D compared with group C ($P < 0.05$) (Table 1).

Bcl-2/Bax ratio in the myocardium decreased significantly in groups B, C and D compared with group A ($P < 0.01$). Bcl-2/Bax ratio in the myocardium increased significantly in groups C and D compared with group B ($P < 0.01$). Bcl-2/Bax ratio in the myocardium increased significantly in group D compared with group C ($P < 0.05$) (Table 1).

Caspase-3 expression in I/R intestinal tissue

Specimens of the small bowel from the I/R group showed loss of intestinal villi, interstitial edema,

Table 1. Effects of HBO on TNF- α content, ATP activity, and optical density of caspase-3 in each group ($n=10$)

Parameters	I/R group	HBO-R group	HBO-P group	HBO-I group	F value
TNF- α (pg/g)	1606.9 $\pm$ 103.25*	1536.2 $\pm$ 136.17*	1252.8 $\pm$ 109.02 Δ *	1021.9 $\pm$ 91.29 Δ	58.709
ATP enzyme (U/mg)	10.86 $\pm$ 1.04*	11.42 $\pm$ 0.84*	13.88 $\pm$ 1.05 Δ	16.97 $\pm$ 1.45 Δ	62.224
Optical density	22.059 $\pm$ 1.05*	21.320 $\pm$ 0.89*	15.581 $\pm$ 0.72 Δ	14.186 $\pm$ 0.61 Δ	226.954

Compared with the HBO-I group and the HBO-P group, * $P < 0.05$; compared with the other three groups, $\Delta P < 0.05$.

Figure 1. Immunohistochemistry results (magnification×400); A: I / R group; B: HBO-R group; C: HBO-P group; D: HBO-I group.

infiltration of inflammatory cells, and brown areas of expression of caspase-3 in mucosa (Figure 1). Histological examination of the small bowel showed loss of intestinal villi and brown areas of expression of caspase-3 in mucosa (Figure 1). In the HBO-R group, the above changes also appeared, but they were slighter than those in the I/R group. In the HBO-P group, specimens of the small bowel showed normal structure of villi and mucosa, mild interstitial edema, infiltration of a small amount of inflammatory cells, and brown areas of expression of caspase-3 in the mucosa at the top (Figure 1). In the HBO-I group, the structure of intestinal villi was normal, light brown areas of caspase-3 expression was seen in the mucosa at the top, but there were no interstitial edema and inflammatory cell infiltration (Figure 1).

There was a decreasing tendency for caspase-3 expression in different groups. The expression of caspase-3 was significantly lower in the HBO-I group than in the HBO-P group ($P<0.05$); it was significantly lower in the HBO-P group than in the HBO-R and I/R groups ($P<0.05$). No statistically significant difference was observed in the HBO-R and I/R groups ($P>0.05$) (Table 1).

DISCUSSION

The intestine is a continuously updated organ, which can be promoted by apoptosis, programmed cell death. Regulated by the physiological mechanism, apoptosis can eliminate the dead, old or damaged cells, and play an important role in embryogenesis, environmental balance, lymphocyte development and tumor regression.^[13] In the pathogenesis, apoptosis is associated with loss of intestinal villi at the top, intestinal adenocarcinoma, inflammatory bowel disease, radiation injury, and I/R injury.^[13,14] Thus, apoptosis plays an important role in normal and stable gastrointestinal tract environment, but it can cause gastrointestinal disease. The intestinal cell apoptosis can also regulate the balance between cell proliferation and death.^[15] When cells are subjected to death-inducing stimulation, apoptosis occurs.^[15] This change starts in the initial phase (Fas-R, cyt c, Bcl-2), and is followed by the signal phase (caspase-2, 8, 9 and 10) and the implementation phase (caspase-3, 6 and 7), eventually leading to cleavage of DNA and activation of chromatin endonuclease.^[14,15] Animal studies have confirmed that I/R can trigger apoptosis, suggesting that I/R of intestinal epithelial cells trigger apoptosis.^[16,17] Cell death results in damage to the intestinal

barrier.

The occurrence of apoptosis is related to mitochondrial function. The direct interference with mitochondrial function can induce apoptosis. Meanwhile, the potential ($\Delta \psi_m$) of mitochondrial transmembrane is decreased in cell apoptosis. Once $\Delta \psi_m$ is declined, apoptosis of cells will inevitably take place. Thus the inhibition of $\Delta \psi_m$ decline can prevent apoptosis. This finding indicates that $\Delta \psi_m$ decline is an early feature of apoptosis. In addition, cytochrome C, a composition of mitochondria could induce the characteristic apoptotic nuclear changes. As an essential component of the respiratory chain, mitochondrial cytochrome C can activate protease cPP32, making DNA fragmented. In the apoptosis induced by staurosporine in a variety of cells, cytosolic cytochrome C is increased significantly.^[18,19]

Pathological hypoxia in cells is an acquired intrinsic defect of cellular respiration. Despite oxygen perfusion is increased or overload oxygen is released into the organization, anaerobic metabolism is escalated.^[20] The mechanism of cell respiration disorder is related to various cytokines (IL-1 β , IL-6, and TNF- α), NO, ADP-ribose-polymerase, and mitochondrial damage induced by oxidative stress.^[21-23] During ischemia, ATP consumption and hypoxanthine accumulation occur. Reperfusion can lead to production of a large number of oxygen free radicals.^[24] Furthermore, intestinal I/R triggers accumulation of cytokines, platelet activating factor, IL-1 β , IL-6 and NO.^[25,26] Previous studies^[27,28] found mitochondrial damage in the intestinal I/R model, confirming that impaired intestinal mitochondria result in morphological changes and changes of respiratory function. The administration of oxygen saturated solution to the intestine may partially restore mitochondrial function.

Yamada et al^[29] reported that secretion of TNF- α was inhibited after HBO treatment. In another study, the level of TNF- α increased significantly after recovery from trauma/hemorrhagic shock in rats, whereas HBO significantly reduced TNF- α secretion and inhibited excessive inflammatory response.^[30] HBO can block the damage of liver cells by inhibiting the secretion of TNF- α to inhibit apoptosis.^[31] Kaelin et al^[32] demonstrated that HBO significantly increased SOD activity, thus reducing the oxygen free radical-mediated apoptosis in the mouse.

In our experiment, we applied HBO for I/R intestine in different periods, and found that HBO could inhibit TNF- α secretion significantly during and before ischemia, and maintain ATP enzyme in the intestinal mucosa. In the reperfusion period, however, application

of HBO showed no significant difference in the inhibition of TNF- α and can't effectively maintain ATP in the small intestine. Similarly, application of HBO during and before ischemia could inhibit expression of caspase-3 in the intestinal mucosa, but in the reperfusion period it was not effective at all. The possible mechanisms are as follows: 1) The administration of HBO during ischemia can replenish oxygen, resist hypoxia metabolism, maintain ATP level, and suppress excessive production of TNF- α ; 2) HBO or preconditioning can increase oxygen reserve in tissue during ischemia, and mitochondria can use oxygen reserves to maintain the level of ATP. But when prolonged ischemia or oxygen reserves is depleted, ATP level will decline.

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