Protective effects of hydrogen on oxidative stress and liver function during CO₂ pneumoperitoneum in dogs¹⁾

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Summary

The purpose of this study was to investigate the protective effects of hydrogen reducing ischemia-reperfusion injury during CO₂ pneumoperitoneum on oxidative stress and liver function. Eighteen healthy Beagle dogs were divided into three groups. Dogs in the control group were subjected only to anesthesia for 90 min. The pneumoperitoneum group was subjected to the pressure of CO₂ pneumoperitoneum with 12 mmHg intra-abdominal pressure for 90 min. The hydrogen group was subjected to the pressure of CO₂ pneumoperitoneum with 12 mmHg intra-abdominal pressure for 90 min after a subcutaneous injection of hydrogen gas (0.2 mL/kg) for 10 min. Blood samples were collected before the induction of pneumoperitoneum, as well as 2 h and 6 h after deflation, to evaluate oxidative stress and liver function in serum. Liver tissue samples were taken 6 h after deflation for histopathological examination. In comparison with group P, a milder histopathological change was found in group H2, and the levels of hepatic function and anti-oxidation in group H2 were higher. Hydrogen gas reduced liver ischemia-reperfusion injury due to CO₂ pneumoperitoneum by reducing oxidative stress and improving liver function. Hydrogen gas therapy can be considered as a way to reduce liver ischemia-reperfusion injury in laparoscopic surgery.

Keywords: dog, laparoscopy, pneumoperitoneum, hydrogen, oxidative stress, liver function

With improvements in equipment and instruments, laparoscopic surgery has become preferred in many diagnostic and therapeutic processes because of its advantages over traditional techniques for many surgical procedures (11), such as ovariectomy (4, 7), ureteronephrectomy, and cystostomy (18, 26).

In the process of laparoscopic surgery, CO₂ is usually used to offer exposure in the abdominal cavity (15). However, CO₂ pneumoperitoneum affects blood gas (9) and liver functions, and causes histological changes in the liver (25). It has also been shown that CO₂ pneumoperitoneum-induced liver injury is related to pressure (23). Pneumoperitoneum may result in decreased splanchnic perfusion, affecting hepatic and renal functions (16). It also causes acidosis, organ ischaemic injury (21), oxidative stress, and histological tissue injury (22). To reduce the adverse impact of

pneumoperitoneum, researchers have taken a number of measures. For some patients, a low-pressure technique could be used to reduce injury (16), and ischaemic preconditioning could be more useful than the low-pressure technique (3).

Hydrogen could be used as an effective antioxidant therapy to protect against oxidative damage (20). Rheumatoid arthritis and related atherosclerosis could be prevented by hydrogen (12). Hydrogen gas reduced oxidative stress in a rat model of traumatic brain injury (14), and experiments showed that 2% H₂ inhalation could reduce brain injury in sepsis (17), as well as spinal cord ischemia-reperfusion injury in rabbits (10).

To date, however, no controlled studies evaluating the protective effects of hydrogen reducing ischaemic injury on oxidative stress and liver function during pneumoperitoneum in dogs have been reported. In the present clinical study, we aimed to investigate oxidative stress markers and liver function upon insufflation with CO₂ under the protective effects of hydrogen.

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Material and methods

Animals. Eighteen healthy Beagle dogs (8 males and 10 females), aged about 2-3 years, weighing 5-10 kg were used. The dogs were obtained from the Experimental Animal Center of Harbin Medical University. They were acclimated for 2 weeks before the experiment.

Model establishment. All dogs were healthy upon examination. Food was given 2 times a day, and water was free to drink. The dogs were housed under an alternating 12-h light/12-h dark cycle, and the environmental conditions were constant. Food and water were withheld, respectively, about 8 h and 2 h before anesthesia. Before anesthesia, the dogs were subcutaneously injected with atropine sulfate (0.02 mg/kg) according to the manufacturer's instructions. Anesthesia was induced with propofol (5 mg/kg) according to the manufacturer's instructions. Then isoflurane in a 2% mixture of oxygen was delivered by an animal anesthetic respiratory system to maintain anesthesia. Heart rate, respiratory rate, oxygen saturation and rectal temperature were measured during the experiment. After anesthesia was stabilized, the animals were placed in a supine position, and the abdominal skin was shaved and disinfected. Except in the control group, pneumoperitoneum was created after anesthesia. A Veress needle was inserted into the dogs' abdomen at the middle line at 1 cm to the umbilicus. CO₂ gas was insufflated with an automatic device until the pressure reached 12 mmHg. Pneumoperitoneum was maintained for 90 min. All dogs were given saline (5 mL/kg × h) during anesthesia.

The eighteen dogs were divided into three groups. Dogs in the control group (group C) were subjected only to anesthesia for 90 min. The pneumoperitoneum group (group P) was subjected to the pressure of CO₂ with 12 mmHg intra-abdominal pressure for 90 min. The hydrogen group (group H₂) was subjected to the pressure of CO₂ with 12 mmHg intra-abdominal pressure for 90 min after a hypodermic injection with hydrogen gas (0.2 mL/kg) for 10 min.

Sampling and tissue preparation

Blood samples. Hepatic function and oxidative stress markers were determined in the serum of venous blood from the medial subcutaneous vein of the forelimb. Blood samples from groups P and H₂ were collected before induction of pneumoperitoneum (samples from group C were collected after anesthesia) (T1), 2 h after deflation (the end of anesthesia) (T2) and 6 h after deflation (the end of anesthesia) (T3). After clotting, blood samples were centrifuged at 3500 rpm for 10 min, and serum was stored at -80°C until analysis.

Tissue samples. All dogs were re-anesthetized at T3. After anesthesia was stabilized, liver tissue samples were taken immediately after laparotomy. The samples were placed in 10% buffered formalin solution, and paraffin-embedded sections were stained with hematoxylin and eosin. All specimens were examined under a light microscope.

Oxidative stress and liver injury index in serum. The concentration of malonyldialdehyde (MDA) in serum was measured by the thio-barbituric acid technique and expressed as nmol/mL. The activity of superoxide dismutase (SOD) was determined by colorimetry and expressed as U/mL. Glutathione (GSH) was determined by the colorimetric method and expressed as μmol/mL. Hydroxyl free radical inhibitor activity was determined by Fenton reaction and expressed

as U/mL. Hepatic function tests for determination of serum concentrations of alkaline phosphatase (AKP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were carried out at designated times by the Reitman-Frankel modified method and expressed as U/L. MDA, SOD, GSH, Hydroxyl free radical inhibitor activity, AST, ALT and AKP in serum homogenates were measured using appropriate detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Postoperative care. Water was provided ad libitum 6 h postoperatively, and food was provided 12 h postoperatively. The dogs were injected with cefazoline (20 mg/kg, IV) and an analgesic agent (butorphanol, 0.2 mg/kg, IM) after surgery. All dogs were adopted until they were healthy upon examination.

Statistical analysis. Values were expressed as means and standard deviation. We used one-way ANOVA between multiple groups. The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed with the SPSS version 22.0.

Results and discussion

All dogs were hemodynamically stable throughout the procedure and completed the study. There were no significant changes in heart rate and peripheral oxygen saturation. At the end of the experiment, respiratory rates (breath/min) in group P (22.0 ± 4.20) were higher than in group C (15.8 ± 3.60) and group H₂ (17.7 ± 3.67). The rectal temperature (°C) in group P (36.8 ± 0.43) was lower than in group C (37.7 ± 0.45) and group H₂ (37.4 ± 0.44).

Histopathological findings. Histopathological changes were examined under a light microscope by a pathologist. The results of examination are shown in Figure 1. There were no significant changes in group C. Hepatic sinusoid straitness, partial hepatocyte swelling, vacuolar degeneration, and mild granular degeneration could be found in group P. A few swollen hepatocytes and degenerated vacuoles could be found in group H₂.

Oxidative stress markers. The results of oxidative stress marker tests are shown in Figure 2. MDA values in groups P and H₂ increased significantly ($p < 0.05$) in comparison with those in group C at T2 and T3, and the values in group H₂ decreased significantly ($p < 0.05$) in comparison with those in group P at T2 and T3.

SOD, GSH and hydroxyl free radical inhibitor activity values in group P decreased significantly ($p < 0.05$) in comparison with those in group C at T2 and T3. All values in group H₂ decreased significantly ($p < 0.05$) in comparison with those in group C at T2 and T3 except GSH values at T2 and hydroxyl free radical inhibitor activity values at T2 and T3. All values in group H₂ increased significantly ($p < 0.05$) in comparison with those in group P at T2 and T3 except GSH values at T2 and SOD values at T3.

Hepatic function. The results of hepatic function tests are shown in Figure 3.

AST, ALT and AKP values in group P increased significantly ($P < 0.05$) in comparison with those in group

Fig. 1. The protective effects of hydrogen on liver tissue during CO₂ pneumoperitoneum in dogs

Explanations: Group C (A), anesthesia for 90 min; no significant changes. Group P (B), pneumoperitoneum 12 mmHg for 90 min; partial hepatocyte swelling, hepatic sinusoid straitness, vacuolar degeneration and mild granular degeneration. Group H₂ (C), pneumoperitoneum 12 mmHg for 90 min after hypodermic injection with hydrogen gas for 10 min; a few swollen hepatocytes and degenerated vacuoles

C at T2 and T3. All values in group H₂ also increased significantly ($P < 0.05$) in comparison with those in group C at T2 and T3 except ALT and AKP values

at T2. All values in group H₂ decreased significantly ($P < 0.05$) in comparison with those in group P at T2 and T3 except AKP values at T2 and AST values at T3.

Fig. 2. The protective effects of hydrogen on oxidative stress during CO₂ pneumoperitoneum in dogs

Explanations: MDA, malondialdehyde (A); SOD superoxide dismutase (B); GSH, glutathione (C); hydroxyl free radical inhibitor activity (D). Group C, anesthesia for 90 min; Group P, pneumoperitoneum 12 mmHg for 90 min; Group H₂, pneumoperitoneum 12 mmHg for 90 min after hypodermic injection with hydrogen gas for 10 min. T1, before induction of pneumoperitoneum (group C was after anesthesia); T2, 2 h after deflation (the end of anesthesia); T3, 6 h after deflation (the end of anesthesia). Values are mean $\pm$ standard deviation. *Significantly different ($p < 0.05$) from group C. #Significantly different ($p < 0.05$) from group P

Fig. 3. The protective effects of hydrogen on liver function during CO₂ pneumoperitoneum in dogs

Explanations: AST, aspartate aminotransferase (A); ALT, alanine aminotransferase (B); AKP, alkaline phosphatase (C). Group C, anesthesia for 90 min; Group P, pneumoperitoneum 12 mmHg for 90 min; Group H₂, pneumoperitoneum 12 mmHg for 90 min after hypodermic injection with hydrogen gas for 10 min. T1, before induction of pneumoperitoneum (group C was after anesthesia); T2, 2 h after deflation (the end of anesthesia); T3, 6 h after deflation (the end of anesthesia). Values are mean $\pm$ standard deviation. *Significantly different ($p < 0.05$) from group C. #Significantly different ($p < 0.05$) from group P

This study established a hepatic ischemia-reperfusion model adopting the method of canine laparoscopy (5, 16). The liver had a rich blood flow, and accepted dual blood supply from the hepatic artery and portal vein systems. The liver was quite sensitive to low perfusion, and it was one of the viscera that were most affected by ischemia in the body. Compared with normal pressure values in the portal system (7-10 mmHg), the intra-abdominal pressure of 12-14 mmHg of CO₂ traditionally used in laparoscopic surgery was higher. It could reduce portal flow and affect liver function (19, 25). Portal vein blood flows were reduced by more than 30% during laparoscopic pneumo-

peritoneum (8). We used 12 mmHg intra-abdominal pressure for 90 min, because the higher pressure of pneumoperitoneum and longer time were conducive to establishing a liver ischemia-reperfusion model. The results show that the levels of MDA, AST, ALT and AKP in group P, compared with those in group C, were increased, whereas the levels of SOD, GSH, and hydroxyl free radical inhibitor activity were decreased, suggesting that a canine hepatic ischemia-reperfusion model was established successfully.

Pneumoperitoneum can result in liver ischemia-reperfusion injury. It was reported that Kupffer cells were activated by ischemia to produce reactive oxygen species (ROS) after reperfusion for 2 h, causing acute hepatic cell injury. After reperfusion for 6 h, neutrophils which are the source of ROS can accumulate in the liver, which can produce more serious hepatic damage (1, 2, 13, 24). Hydrogen has the smallest relative molecular mass in the gas and spreads rapidly into mitochondria, nucleus and other subcellular organelles, which are main places where ROS are generated. Hydrogen can react with cytotoxic ROS and protect against oxidative damage (20). Inhalation of hydrogen gas can reduce the levels of serum AST, hepatic MDA, and hepatic cell death (6). In our study, after hypodermic injection with hydrogen gas prior to pneumoperitoneum, the levels of MDA, AST, and ALT in group H₂ were significantly decreased as compared with those in group P at 2 h after deflation, the levels of SOD and hydroxyl free radical inhibitor activity were significantly increased, and the levels of GSH and AKP were changed, but not significantly. Compared with group P at 6 h after deflation, group H₂ showed significantly decreased levels of MDA, ALT, and AKP, significantly increased levels of GSH and hydroxyl free radical inhibitor activity, as well as changes in the levels of SOD and AST, which were not significant. The degree of liver tissue injury in group H₂ was smaller than that in group P. The results show that hydrogen can improve hepatic function because the levels of AST, ALT and AKP were decreased. Hydrogen can also reduce the levels of oxidative stress markers because MDA was decreased, whereas SOD, GSH and hydroxyl free radical inhibitor activity were increased. This suggests that hypodermic injection with hydrogen gas can reduce hepatic ischemia-reperfusion injury caused by CO₂ pneumoperitoneum.

In summary, hypodermic injection with hydrogen gas can reduce hepatic ischemia-reperfusion injury due to CO₂ pneumoperitoneum by reducing oxidative stress and improving hepatic function in dogs. Hydrogen gas treatment can be considered as an option during laparoscopic surgery.

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