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Ozone Improves Oxygenation and Offers Organ Protection after Autologous Blood Transfusion in a Simulated Carbon Dioxide Pneumoperitoneal Environment in a Rabbit Hemorrhagic Shock Model

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Keywords
Ozone · Trioxygen · Autologous blood transfusion · Hemorrhage shock

Abstract
Objectives: Autologous blood transfusion techniques are well applied in surgery, but the red blood cells (RBCs) collected during laparoscopic surgery may forfeit their ability to oxygenate. O_3 is a potent oxidation gas. This study investigates whether O_3 could improve the oxygen-carrying capacity of RBCs, reduce inflammatory reactions, and offer organ protection. ***Methods:*** We established a hemorrhagic shock model in rabbits, and simulated CO_2 pneumoperitoneum and O_3 were applied before autologous blood transfusion. Perioperative mean arterial pressure and arterial blood gas were recorded, blood gas and RBC morphology of collected blood were analyzed, plasma IL-6, ALT, AST, CRE, and lung histopathology POD0 and POD3 were tested, as well as postoperative survival quality. ***Results:*** Autologous blood that underwent simulated CO_2 pneumoperitoneum had a lower pH and SaO_2 and a higher $PaCO_2$ than the control group. After O_3 treatment, PaO_2 and SaO_2 increased significantly, with unchanged pH values and $PaCO_2$. RBCs in autologous blood were drastically deformed after CO_2 conditioning and then reversed to normal by O_3 treatment. Rabbits that received CO_2-conditioned autologous blood had a compromised survival quality after surgery, higher plasma IL-6 levels, higher lung injury scores on POD0, higher ALT and AST levels on POD3, and O_3 treatment alleviated these adverse outcomes. ***Conclusion:*** O_3 can restore RBC function, significantly improve blood oxygenation under simulated CO_2 pneumoperitoneum, offer organ protection, and improve the postoperative survival quality in the rabbit hemorrhage shock model.

Introduction

Patient blood protection, also known as patient blood management, was first proposed by the Society for the Advancement of Blood Management in the United States. Its role in reducing allogeneic blood transfusion and improving patient outcomes has been well-proven [1]. Intraoperative autologous blood recovery technology is a blood protection technology that has developed rapidly in recent decades and has been widely accepted by many disciplines. Its clinical application and research results in orthopedics [2–4], neurosurgery [5–7], cardiac surgery [8, 9], and other subjects show that it plays a positive role in reducing allogeneic blood transfusion, reducing postoperative

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infection, shortening the length of hospital stay and reducing hospitalization costs. Preliminary studies have also been conducted on its safety in obstetrics [10, 11] and oncology surgery [12, 13].

Autologous blood transfusion techniques are well applied in laparoscopic surgery, although specific issues should be addressed. For example, a previous study suggested that during pneumoperitoneum laparoscopic surgery, the pH value and PaO_2 of autologous blood collected intraoperative decreased significantly, along with an increase in $PaCO_2$. At the same time, the centrifugal washing process hardly improved this result [14]. However, the decrease in blood pH value and the increase in $PaCO_2$ will directly affect the morphology of red blood cells (RBCs) and the distribution of hemoglobin in RBCs [15, 16]. At the same time, the ability of hemoglobin to bind oxygen also decreases with decreasing pH and increasing $PaCO_2$ [17]. Therefore, whether the oxygen-carrying capacity of the blood recovered from laparoscopic surgery is severely weakened, and its effect on the internal environment and organ function after transfusion should be further studied.

Ischemia/reperfusion injury is prevalent in shock resuscitation and can cause extensive damage to vital tissues and organs. Trioxygen (O_3), also known as ozone, is a gas with strong oxidation. As a new treatment method, it has been applied in the adjuvant treatment of various diseases, especially ischemic and inflammatory diseases [18]. O_3 has a significant antioxidative stress effect [19–21] and can freely diffuse into RBCs. It can increase the content of ATP and 2,3-DPG through the activation of phosphofructokinase [22], enhance the oxygen supply of blood to hypoxic tissues, and play an anti-inflammatory role by reducing the production of inflammatory factors such as TNF-α, IL-1β, and IL-6 [22]. O_3 also contributes to the treatment of organ ischemia-reperfusion injury [22], septic shock [23], acute cerebral infarction [24], gout [25], and other diseases. Therefore, we assumed that O_3 treatment might improve the oxygen-carrying capacity of RBCs, reduce oxidative stress after an autologous blood transfusion during laparoscopic surgery, reduce inflammation, and offer organ protection.

Our study mimicked CO_2 pneumoperitoneum in a rabbit hemorrhagic shock model, observed recovery from shock after autologous blood transfusion, and further evaluated organ damage and postoperative survival quality. In particular, we used O_3 to treat recovered autologous blood before transfusing it. Meanwhile, we evaluated the inflammatory indicators and RBC morphology to discuss the underlying mechanism further. This study provides a theoretical reference for blood protection's safety and practical implementation in clinical practice. Furthermore, it provides new approaches for further exploration of quality improvement of perioperative autologous blood transfusion and better perioperative organ protection.

Materials and Methods

Animals

All procedures followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study protocol was reviewed and approved by the Ethics Committee of the Peking University People's Hospital, approval number 2016PHC035. Twenty-four male New Zealand rabbits weighing 3 kg~3.5 kg were housed individually in standard raised-flooring cages. Animals were maintained at approximately 25°C and 12 h light and 12 h dark for a minimum of 1 week in the animal colony before the experiment. They were fasted overnight but had free access to water before the experiment. According to the different treatments, 24 rabbits were randomly assigned to four groups with 6 rabbits each: sham group (Sham surgery), control group (blood without special treatment), CP group (blood treated with simulated CO_2 pneumoperitoneum), and CP + Oz group (blood treated with simulated CO_2 pneumoperitoneum and then treated with O_3).

Surgical Preparations

General anesthesia was induced using 4% isoflurane and maintained by isoflurane (2%~3%, balance 50% O_2); the depth of anesthetic was assessed every 5 min by the corneal reflex response. Airway support was provided with a veterinary supraglottic airway (Rabbit V-gel R4; Docsinnovent, London, United Kingdom). A 20-gauge Angiocath (Becton-Dickinson, Sandy, UT, USA) was inserted via the left femoral artery for bloodletting, blood sampling, and pressure monitoring. In addition, left femoral vein catheterization was performed for resuscitation. Animals were stabilized for 20 min after the procedure completed.

Hemorrhagic Shock Model

Animals were hemorrhaged from the femoral artery at 3 mL/(kg·min) until the mean artery pressure (MAP) declined to 40 mm Hg and then continued at 1 mL/(kg·min) to maintain MAP from 30 mm to 35 mm Hg for 120 min. Autologous blood was supplemented with heparin (15 U/mL). To keep the MAP from 30 mm Hg to 35 mm Hg during the shock period, autologous blood was reinfused several times at 0.5 mL~1 mL each time. For the Sham group, rabbits were maintained and anesthetized for 120 min without hemorrhage.

Simulated CO_2 Pneumoperitoneum Model in vitro

We established a model of simulated CO_2 pneumoperitoneum in vitro using a medically used blood storage bag (20153660283, Sichuan, China). Autologous blood was collected in a blood storage bag and supplemented with heparin (15 U/mL). After blood was injected into the blood storage bag, medically used carbon dioxide was led into the bag, and the air pressure in the bag was maintained at 12–15 mm Hg for 60 min. For the control group, collected blood stayed for 60 min without CO_2 inflation.

O_3 Treatment

For the CP + Oz group, collected blood was conditioned with simulated CO_2 pneumoperitoneum for 60 min. Then CO_2 was vented out and treated with 30 μg/mL O_3 for 10 min before reinfusion (equal volume as blood) [26]. The O_3 was produced by Medozon (Herrmann, Germany). The O_3 was insufflated into a syringe then injected into the blood storage bag immediately after produced. The storage bag was gently shaken, and O_3 was allowed to contact the blood for 10 min before reinfusion.

Resuscitation

For the sham group, rabbits were maintained and anesthetized for 60 min without resuscitation. For the control group, CP group, and CP + Oz group, animals were resuscitated with autologous blood under different treatments via the femoral vein at approximately 3 mL/min. After resuscitation for 60 min (POD0), 3 rabbits from each group were killed by intravenous 10% potassium chloride. The general state, weight change, and food intake of another 3 rabbits were observed 3 days after the surgery.

Blood Gas Analysis

Arterial blood was collected through the left femoral artery at the beginning of hemorrhagic shock, the end of 120 min hemorrhagic shock, and 60 min after resuscitation for blood gas analysis. Under different procedures, as described above, autologous blood was tested for blood gas before transfusion. The arterial blood was stored in the arterial blood gas needle (BD, America) and immediately tested by automatic blood gas analysis (pHox, Nova, America).

Scanning Electron Microscope for RBCs

Autologous blood in each group was sampled for an scanning electron microscope (SEM) exam before infusing. According to a previous study, scanning electron microscopic studies of RBC were performed using an electron microscope (model S-800, Hitachi) [27, 28]. Blood samples of the control group, CP group, and CP + Oz group were fixed in phosphate-buffered (pH 7.2–7.4) 2.5% glutaraldehyde for 1 h, washed twice in 0.1 M phosphate buffer (pH 7.2–7.4), and mounted on poly-L-lysine-coated glass slides. The glass slides were kept in a moist atmosphere for 1 h, washed in phosphate buffer, and postfixed in 1% osmium tetroxide for 1 h. After critical-point drying with liquid CO_2 in a vacuum apparatus and covering with a gold-palladium layer, the samples underwent scanning electron microscopic analysis. Reversible and irreversible shapes were determined according to the previous study [29]. The morphological index (MI) was adopted to express the degree of echinocytic transformation of RBCs [16]: MI = 0, 0.5, 1, and 2 correspond to diskocytes, echinocyte I (irregularly speculated), echinocyte II (equally spaced blunt projections), and echinocyte III (equally spaced pointed projections), respectively. Three rabbit blood specimens were collected in each group, and three randomly selected fields, including at least 10 RBCs for each rabbit, were scored. Two researchers who were blinded to the groups and worked independently examined all the samples.

Enzyme-Linked Immunosorbent Assay of IL-6

To estimate the intensity of the inflammatory reaction after the shock model, the concentrations of IL-6 in the plasma were measured using an enzyme-linked immunosorbent assay (ELISA) kit (CSB-E06903Rb, CUSABIO, Wuhan, PR China). This kit uses the Sandwich-ELISA method. The enzyme-substrate reaction was terminated by adding a sulfuric acid solution, and the color turned yellow. The optical density (OD) was measured spectrophotometrically at 450 nm. The OD value is proportional to the concentration of IL-6. The results are expressed as pg/mL.

Blood Biochemical Analysis

To evaluate liver and renal function after the shock model, glutamic-pyruvic transaminase (ALT), glutamic oxalacetic transaminase (AST), and creatinine (CRE) in plasma were measured using a biochemical analyzer (Hitachi LST008, Japan). The rate assay method was used for ALT and AST measurement, and the enzymatic method was used for CRE measurement.

Histopathological Analysis

Lung injury was evaluated according to a previous study [30, 31]. Lung tissue harvested from rabbits was immediately fixed in formalin. Then, 48 h after fixation, the tissue samples were dehydrated and embedded in paraffin blocks. The sections were cut to 4 μm thickness, stained with hematoxylin and eosin, and examined by two pathologists blinded to the groups who worked independently. For each slide, six microscopic fields were randomly selected for examination. A score quantified pulmonary histological damage according to previous studies [30, 31], which involved seven variables: alveolar inflammation, interstitial inflammation; alveolar hemorrhage; interstitial hemorrhage; edema; atelectasis, and necrosis. For each of those seven variables, the severity was graded as follows: 0 = no injury; 1 = injury in 25% of the field; 2 = injury in 50% of the field; 3 = injury in 75% of the field; and 4 = diffuse injury. Therefore, the maximum possible score was 28. Three rabbit lung tissues were collected from each group, and three inconsecutive slides for each tissue were scored.

Statistical Analysis

All values are presented as the mean ± standard deviation, and $p < 0.05$ was considered statistically significant. SPSS 25.0 software (SPSS, Inc., Software, Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance (ANOVA), repetitive measurement ANOVA, *t* test, and the Kruskal-Wallis test were employed to determine group differences.

Results

Hemodynamics

No animals died prior to sacrifice in the experiment. The MAP in the sham group was stable during the whole surgery. Except for the sham group, the MAP was not different at baseline or during the hemorrhagic shock period among the groups. In the shock period, the MAP decreased from the target of 30 mm Hg–35 mm Hg and was successfully maintained for 120 min. During resuscitation, the MAP of the Control group quickly recovered to 50 mm Hg and stayed between 50.0 mm Hg ± 3.2 mm Hg at the lowest and 51.0 mm Hg ± 3.4 mm Hg at the highest. The MAP of the CP group was between 53.3 mm Hg ± 1.6 mm Hg and 54.0 mm Hg ± 3.5 mm Hg, which was significantly higher than that of the control group ($p < 0.05$). The MAP of the CP + Oz group fluctuated between 50.3 mm Hg ± 1.4 mm Hg and 52.0 mm Hg ± 1.7 mm Hg, and there was no significant difference compared to the control and CP group (Fig. 1).

Pre-Hemorrhage and Post-Hemorrhage Characteristics

Group descriptive characteristics for pre-hemorrhage and post-hemorrhage volume and blood chemistry values are summarized in Table 1. The body weight, average shed blood volume, and average resuscitation volumes were not significantly different among the control group, CP group, and CP + Oz group. In addition, the pH values, lactate, and hemoglobin of the Control group, CP group, and CP + Oz group during the pre-hemorrhage period were not significantly different from those of the sham

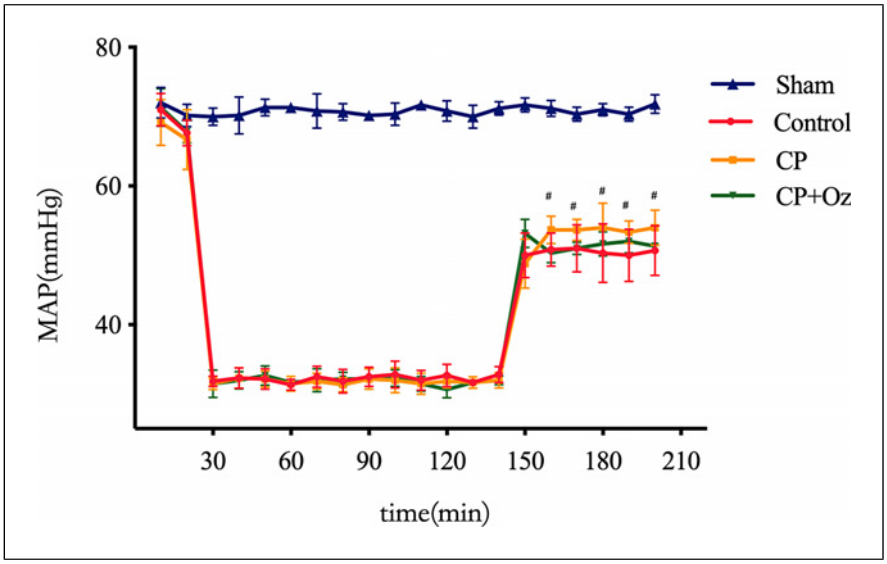

Fig. 1. MAP during surgery in the sham, control, CP, and CP + Oz group. $n = 6$. $^{\#}p < 0.05$, versus control group.

Table 1. Pre-hemorrhage and post-hemorrhage characteristics ($n = 6$)

	Sham	Control	CP	CP + Oz
Body weight, kg	3.42±0.05	3.40±0.03	3.33±0.13	3.34±0.10
Shed blood volume, mL	–	71±4	69±5	69±4
Resuscitation volumes, mL	–	81±4	78±4	79±4
Blood chemistry				
Pre-hemorrhage				
pH	7.39±0.04	7.40±0.01	7.42±0.04	7.45±0.04
$PaCO_2$, mm Hg	43±4	46±4	44±5	41±3
PaO_2, mm Hg	239±15	249±8	235±10	240±7
Glu, mmol/L	8.64±1.32	11.05±2.00	10.50±2.79	8.34±2.46
K^+, mmol/L	3.75±0.50	3.33±0.09	3.34±0.33	3.44±2.34
Lac, mmol/L	1.5±0.2	1.2±0.4	1.7±0.2	1.3±0.7
HCO_3^-, mmol/L	24.6±4.4	28.5±2.4	28.1±2.4	29.6±2.5
Hb, g/dL	12.4±1.3	12.3±0.6	12.5±0.3	12.9±0.6
Post-hemorrhage				
pH	7.39±0.05	7.22±0.04*	7.28±0.06*	7.28±0.05*
$PaCO_2$, mm Hg	41±6	59±7*	49±14	49±6
PaO_2, mm Hg	248±17	255±12	234±19	245±9
Glu, mmol/L	8.72±0.72	21.85±7.84*	14.96±3.32*	19.37±5.32*
K^+, mmol/L	3.60±0.32	3.50±0.32	3.46±0.41	3.40±0.11
Lac, mmol/L	1.4±0.2	5.1±1.8*	4.7±2.0*	4.6±0.9*
HCO_3^-, mmol/L	24.3±4.3	24.6±3.4	22.7±4.4	25.2±3.0
Hb, g/dL	12.3±1.3	8.3±1.0*	8.8±0.8*	9.4±0.5*

*$p < 0.05$ versus sham group.

group. However, they were significantly lower at the end of the shock period, representing the successful hemorrhage shock model.

Autologous Blood Characteristics

Blood Gas

After treatment with the simulated CO_2 pneumoperitoneum, the pH values and SaO_2 of blood were significantly lower than those of the control group ($p < 0.05$), while the $PaCO_2$ was significantly higher ($p < 0.05$), but CO_2 pneumoperitoneum did not affect PaO_2, suggesting damage to the RBC-binding O_2 ability. Then, when the CO_2-conditioned blood was treated with O_3 (CP + Oz group), the pH values and $PaCO_2$ of blood were not significantly different compared to those of the CP group, but the PaO_2 and SaO_2 increased significantly (Table 2). Indicating O_3 treatment improves blood oxygenation.

Table 2. Autologous blood characteristics (n = 6)

	Control	CP	CP + Oz
pH	7.33±0.03	6.66±0.05$^{\#\#}$	6.78±0.06
PaO_2, mm Hg	175±20	156±21	266±40$^{\Delta\Delta}$
$PaCO_2$, mm Hg	39±3	293±14$^{\#\#}$	274±27
SaO_2, %	99.9±0.8	77.9±3.6$^{\#\#}$	89.4±9.8$^{\Delta\Delta}$

$^{\#\#}p < 0.01$ versus control group. $^{\Delta\Delta}p < 0.01$ versus CP group.

RBC Morphology

SEM analysis of RBCs demonstrated the creation of erythrocytes after the shock model and an apparent echinocytic change in the CP group. This deformation was abolished by O_3 treatment (Fig. 2). Compared with the control group, the CP group had a significantly increased MI (1.61 ± 0.57 vs. 0.11 ± 0.21, $p < 0.01$). However, after O_3 treatment, the MI in the CP + Oz group was markedly reduced (0.28 ± 0.42 vs. 1.61 ± 0.57, $p < 0.01$).

Resuscitation Characteristics

Arterial blood gas parameters for each treatment group at 60 min of resuscitation and postoperative day three are given in Table 3. After 60 min of resuscitation, the pH values and lactate did not return to baseline, indicating that the shock state had prolonged effects. Different autologous blood treatments for resuscitation had no evident influence on arterial blood gas parameters on POD3.

Postoperative Survival Quality

Food intake and weight changes were recorded on POD3 and are shown in Figure 3a and 3b. Compared to the sham group, the control group, CP group, and CP + Oz group had less food intake and more significant weight loss ($p < 0.01$). The intake of the CP group was significantly lower than that of the control group and CP + Oz group (158 g ± 26 g vs. 386 g ± 4 g vs. 436 g ± 17 g; $p < 0.01$, $p < 0.01$). The weight loss in the CP group was greater than that in the control group without significant difference, while significantly more remarkable compared to the CP + Oz group (90 g ± 10 g vs. 67 g ± 15 g vs. 33 g ± 15 g; $p = 0.074$, $p < 0.01$). This result suggests that although the internal environment seemed to recover on POD3, the survival quality was still compromised by shock and probably aggregated by simulating CO_2 pneumoperitoneum, and O_3 may alleviate this damage.

Blood Biochemical Analysis

Blood biochemical analysis of ALT, AST, and CRE in each group is shown in Figure 3c–e. Compared to the sham group, the ALT and AST levels did not show significant differences in POD0. On POD3, ALT and AST levels in the CP group were significantly increased compared with those in the sham group ($p < 0.05$, respectively), and this adverse effect on the liver was abolished by O_3 treatment. CRE increased in all three surgery groups compared with sham ($p < 0.05$, respectively) and decreased to baseline on POD3. There was no significant difference in CRE between groups at POD3.

Plasma IL-6 Level

To further investigate the underlying mechanism of O_3 treatment on autologous blood collected from simulated CO_2 pneumoperitoneum, we examined the inflammatory indicator IL-6. At 60 min after resuscitation, the IL-6 levels of sham, control, CP, and CP + Oz groups were 64.70 ± 8.62, 86.16 ± 5.04, 79.17 ± 6.47, and 65.55 ± 5.98, respectively, and both control and CP groups had higher IL-6 levels than sham group ($p < 0.05$). Compared to the control and CP groups, IL-6 levels in the CP + Oz group were significantly lower ($p < 0.05$). On POD3, the IL-6 levels of sham, control, CP, and CP + Oz groups were 66.68 ± 10.43, 72.50 ± 7.91, 64.74 ± 12.46, and 66.92 ± 12.06, respectively. IL-6 levels in peripheral blood were not significantly different among the groups (Fig. 4), indicating that O_3 decreased the inflammatory reaction in the shock model.

Histopathologic of Lung

Typical lung injury pathologic aspects were observed in the CP group, such as severe edema, alveolar wall thickening, hyaline membrane formation, hemorrhage, and neutrophil infiltration in the lung parenchyma. These lung tissue injuries were notably reduced in the CP + Oz group (Fig. 5a–h). On POD0, lung injury scores in the sham, control, CP, and CP + Oz groups were 2.0 ± 0.4, 5.2 ± 0.9, 9.9 ± 1.7, and 4.3 ± 0.4, respectively. Lung injury scores were significantly higher in the control, CP, and CP + Oz groups than in the sham group. Compared to the control group, the CP group had a significantly higher lung injury score, while after O_3 treatment, the CP + Oz group's injury score was significantly reduced ($p < 0.01$). On POD3, the lung injury scores in the sham, control, CP, and CP + Oz groups were 1.9 ± 0.4, 4.7 ± 1.8, 9.4 ± 2.2, and 3.7 ± 0.4, respectively. There was no significant difference among the groups on POD3 (Fig. 5i).

Discussion

To investigate the oxygen-carrying capacity changes of the autologous blood collected during laparoscopic surgery and the consequent effect on the internal environment and organ function after transfusion, we mimicked the clinical environment of CO_2 pneumoperitoneum in laparoscopic surgery in a rabbit hemorrhage shock model. We choose the rabbit hemorrhage shock model

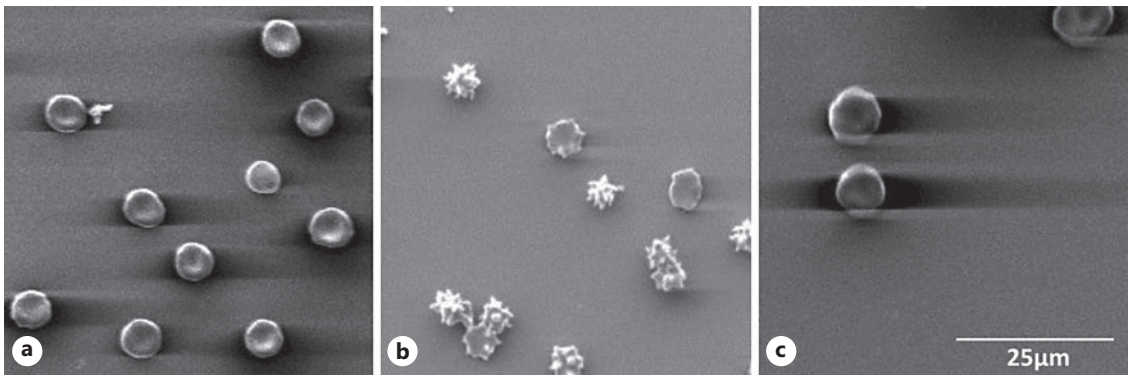

Fig. 2. RBCs morphology in autologous blood under SEM. **a** Control group. **b** CP group. **c** CP + Oz group.

Table 3. Resuscitation and postoperative characteristics

Blood chemistry	Sham	Control	CP	CP + Oz
Resuscitation (n = 6)				
pH	7.39±0.04	7.25±0.08*	7.26±0.07*	7.25±0.05*
$PaCO_2$, mm Hg	42±6	70±5*	57±14*	61±8*
PaO_2, mm Hg	244±17	242±15	248±21	257±25
Glu, mmol/L	8.80±0.91	15.87±6.38	10.5±2.46	13.33±5.28
K^+, mmol/L	3.58±0.29	4.04±0.36	3.96±0.34	3.79±0.14
Lac, mmol/L	1.5±0.2	3.6±1.5	3.5±1.0*	2.9±0.4*
HCO_3^- mmol/L	24.0±4.0	27.9±3.5	26.0±3.1	27.2±4.0
Hb, g/dL	12.3±1.3	10.6±0.8	10.7±0.3	10.6±0.8
POD3 (n = 3)				
pH	7.47±0.02	7.44±0.03	7.40±0.08	7.49±0.07
$PaCO_2$, mm Hg	42±1	38±6	43±2	34±4
PaO_2, mm Hg	417±16	413±21	328±62	368±39
Glu, mmol/L	8.34±0.61	9.33±0.17	10.72±3.98	7.16±0.30
K^+, mmol/L	4.57±0.30	4.31±0.31	2.97±1.27	4.48±0.57
Lac, mmol/L	1.6±0.2	1.8±0.8	1.7±0.2	1.9±0.6
HCO_3^-, mmol/L	29.2±1.7	23.9±1.6	28.1±3.12	25.8±1.04
Hb, g/dL	11.6±0.9	11.6±0.9	10.8±0.6	11.4±1.2

*p < 0.05 versus sham group.

for the experiment for the following reasons: (1) the rabbit hemorrhagic shock model is mature and reliable and can mimic clinical practice very well. (2) The in vitro simulated CO_2 pneumoperitoneum and resuscitation model we developed requires sufficient blood for processing. For these reasons, we chose the rabbit model over the rat model. In this model, in previous studies, we successfully represented the characteristics of a significantly lower pH value and high $PaCO_2$ caused by autologous blood transfusion during clinical laparoscopy [14]. Although no significant decrease in oxygen partial pressure was found after CO_2 inflation, the oxygen saturation in autologous blood in the CP group was significantly decreased. According to the Bohr effect [32], the reduced pH in RBCs will reduce the affinity of hemoglobin to oxygen. In addition, the blood pH will also affect the morphology of RBCs and the distribution of hemoglobin in the cell [15, 16].

After O_3 treatment, the oxygen partial pressure and oxygen saturation of autologous blood increased significantly, which we believe is O_3's effect on erythrocytes. O_3 molecules, as a strong oxidizer, once infused with the blood, do not fully follow Henry's law by immediately reacting with ions and biomolecules. O_3 is approximately tenfold more soluble than oxygen. As it dissolves in the plasma, it instantaneously reacts with hydrophilic antioxidants. At the same time, the remaining O_3 performs the peroxidation of available unsaturated fatty acids, which represent an elective substrate and are mostly albumin-bound, and continue with their reaction chains.

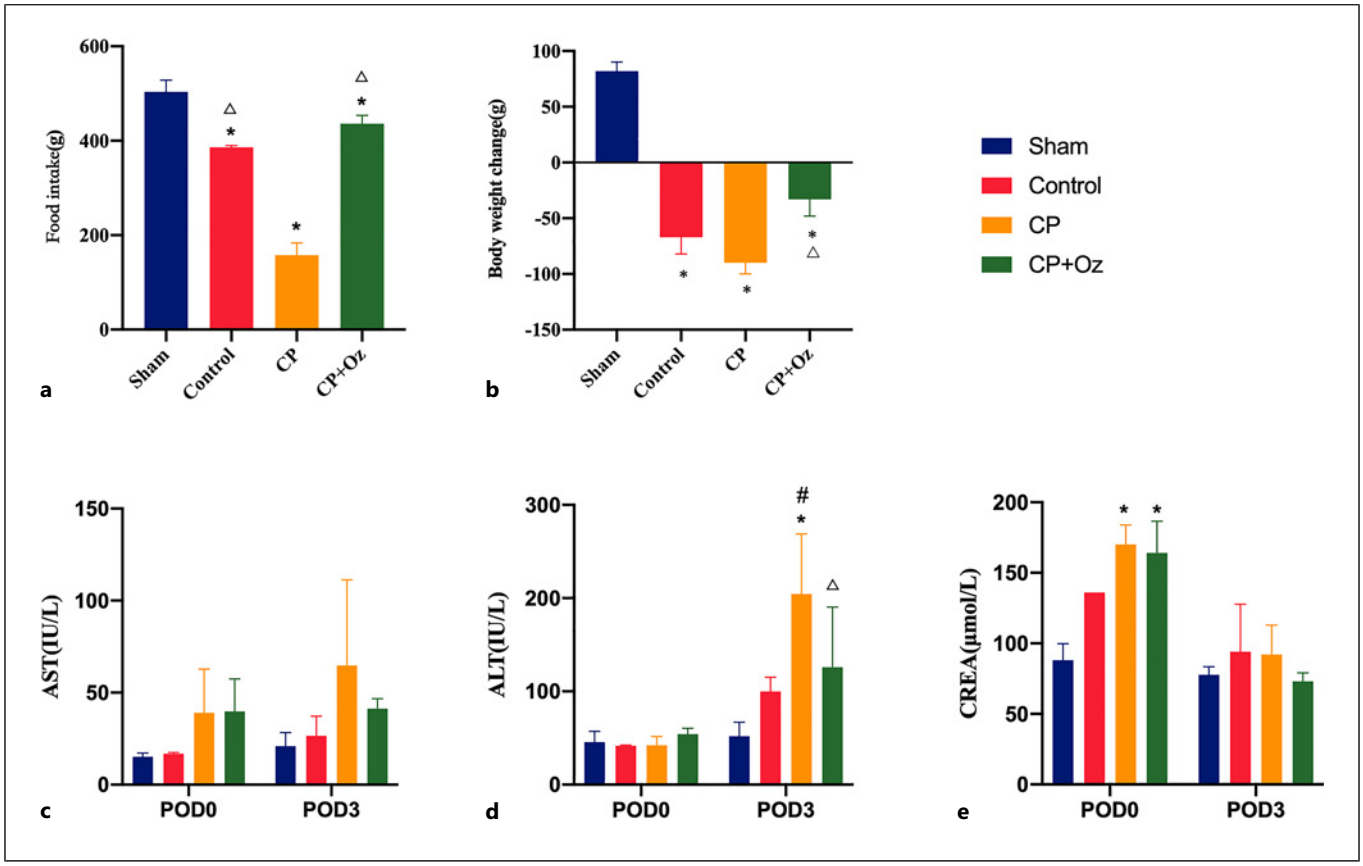

Fig. 3. Postoperative survival quality. $n = 3$. **a** Food intake. **b** Body weight change. **c** Plasma AST levels. **d** Plasma ALT levels. **e** Plasma CRE levels. $^{*}p < 0.05$ versus sham group; $^{\Delta}p < 0.05$ versus CP group.

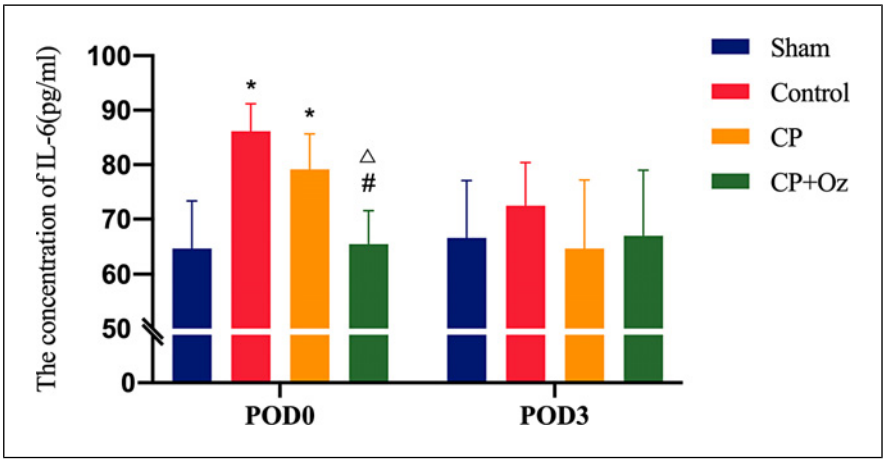

Fig. 4. Plasma IL-6 levels on POD0 and POD3. $^{*}p < 0.05$ versus sham group; $^{\#}p < 0.05$ versus control group, $^{\Delta}p < 0.05$ versus CP group.

These reactions occur briefly and continue as O_3 dissolves in the plasma. The quick and sequenced reaction releases ATP and 2,3-DPG as well as reactive oxygen substances, which may promptly improve oxygenation [22]. Therefore, in this study, we demonstrated that using O_3-treated blood can significantly improve oxygenation after an autologous blood transfusion during simulated CO_2 pneumoperitoneum. O_3 treatment is also related to leukocytes and platelets' performance [22]. O_3 acts as cysteine oxidation. It is also a very modest inducer of some cytokines. Moreover, platelets are exquisitely sensitive to progressive acute oxidative stress.

In this study, the changes in blood gas and electrolytes before and after hemorrhagic shock modeling indicated that the establishment of the hemorrhagic shock model was effective, and all animals in each group were in a state

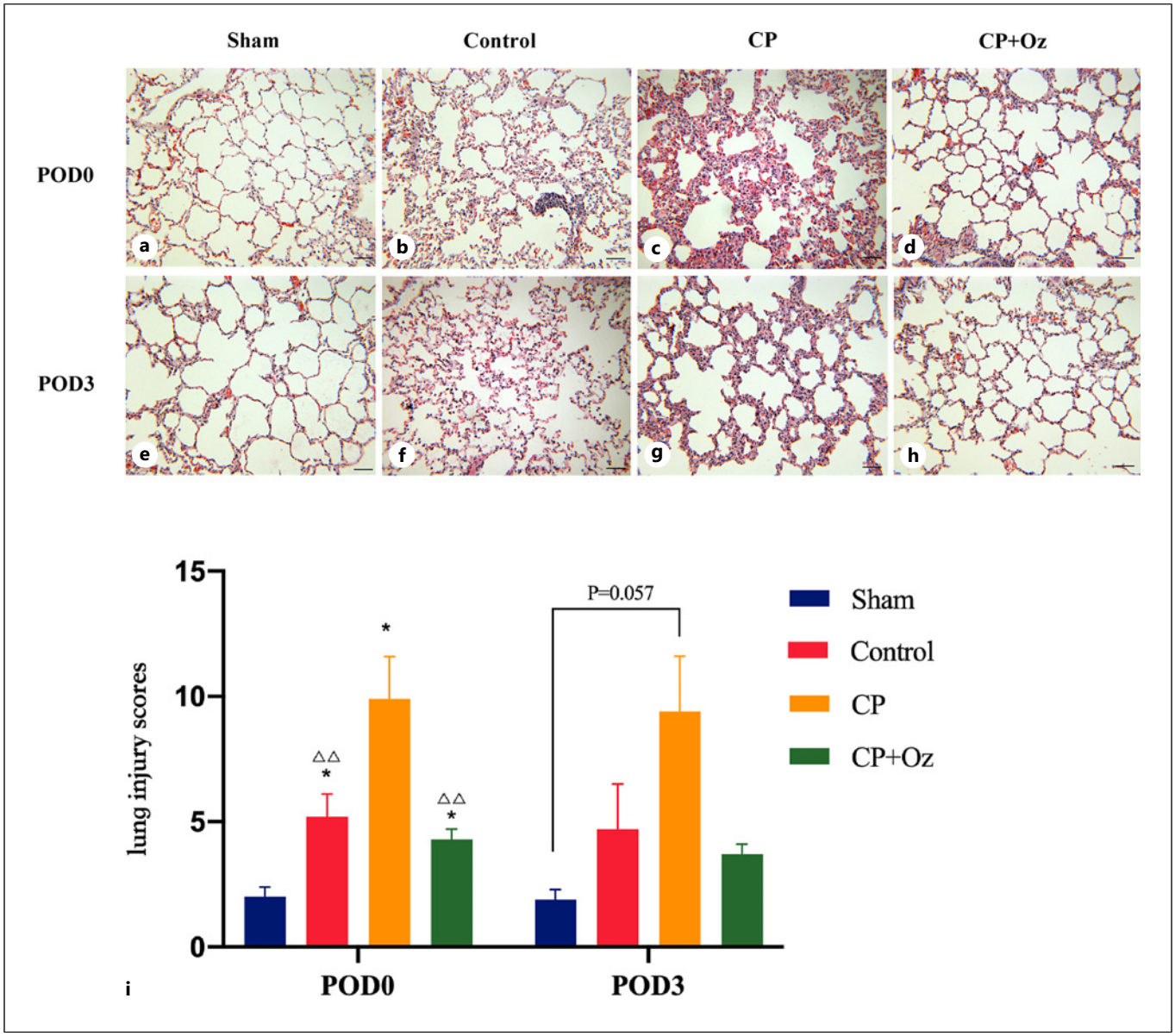

Fig. 5. Histopathological analysis of lung tissue (×200, $n = 3$). **a–d** POD0; (**e–h**) POD3. **a**, **e** sham group, (**b**, **f**) control group, (**c**, **g**) CP group, (**d**, **h**) CP + Oz group. **i** Lung injury scores of each group on POD0 and POD3. $^{*}p < 0.05$ versus sham group; $^{\Delta\Delta}p < 0.01$ versus CP group.

of shock hypoperfusion after modeling. No statistically significant differences were observed in blood gas and electrolyte among the groups on POD3, indicating that the influence of blood transfusion after different treatments on the internal environment could be self-adjusted at a given time. During the resuscitation period, the different treatment groups found statistically significant differences in MAP. However, the difference stayed within 3 mm Hg, which we considered not to reach clinical significance. In this case, we assume that different blood treatments do not significantly impact hemodynamics. Therefore, by evaluating the changes in MAP and the internal environment, the transfusion of blood collected from a simulated laparoscopic pneumoperitoneum environment may perform poorly in improving tissue hypoxia. In contrast, this situation can be significantly improved after O_3 treatment. However, due to the solid compensatory function of the body, the changes mentioned above did not lead to clinically significant changes in hemodynamics or the internal environment, suggesting that in this study, blood transfusion after various treatments in the hemorrhagic shock model did not have a significant impact on hemodynamics or the internal environment.

Postoperative quality of life is affected by various factors, including postoperative pain, infection, ischemia-reperfusion

injury, inflammatory reaction, postoperative stress reaction, and other matters. In this study, we observed that blood transfusion after simulated CO_2 pneumoperitoneum treatment in a rabbit hemorrhagic shock model significantly reduced rabbit food intake and increased weight loss, indicating the poor postoperative quality of life. Next, we demonstrated that O_3 treatment could reverse these impacts. The reason for the significant improvement in postoperative quality of life remains unclear. Previous studies suggest a few possible reasons: (1) O_3 alleviates ischemia-reperfusion injury of the gastrointestinal tract and therefore attenuates mucosal barrier function impairment caused by hemorrhagic shock [33–36] as well as reperfusion injury of the gastrointestinal mucosa [36–38]. In the hemorrhagic shock model, blood collected under a simulated CO_2 pneumoperitoneum environment is poor in supplying oxygen to hypoxic tissues due to its pH and hypoxic characteristics, which may aggravate ischemia/reperfusion injury of the gastrointestinal tract. O_3 can significantly improve blood oxygenation, enhance blood oxygen supply to hypoxic tissue, improve microcirculation, reduce ischemia/reperfusion injury of hemorrhagic shock, and thus improve postoperative quality of life, which is manifested in increased food intake and decreased weight loss (Fig. 3). (2) O_3 is an anti-inflammatory factor. We observed an immediate decrease in the plasma concentration of IL-6 in the CP + Oz group compared to the control and CP groups on POD0. We are indicating an anti-inflammatory effect of O_3. The plasma concentration of IL-6 on POD3 was not significantly different between the groups, which may be explained by self-recovery. It is also possible that in a prolonged state of ischemia or hypotension, continued O_3 could show long-term effects. (3) Reduction of the stress response. The effect of O_3 on enhancing the antioxidant stress capacity of the body has been thoroughly studied [23]. In hemorrhagic shock, septic shock, and other types of shock, O_3 has shown good antioxidant stress capacity. (4) Restore RBC structure. In our study, blood under a simulated CO_2 pneumoperitoneum environment presented a severe echinocytic transformation of RBCs, consistent with a previous study [16]. Extreme deformation compromises the oxygen transport capacity of RBCs. O_3 treatment abolished this effect in our study and recovered RBC morphology to the pre-CO_2 state. The underlying mechanism is unclear, but a normal shape of RBCs affiliates their oxygen binding.

Organ injury directly caused by hemorrhagic shock and ischemia/reperfusion injury caused by the blood transfusion process were the possible leading causes of organ injury in this study. We chose ALT and AST to reflect liver function and CRE for renal function after recovery from shock. Simulated CO_2 pneumoperitoneum is associated with worsened liver function on POD3, which was reversed by O_3 treatment. On lung pathology, we observed more severe inflammatory injury and significantly higher lung injury scores in the CP group than in the Control group. Previous studies have also suggested that in severe hypoxic ischemia, brain tissue, and lung tissue aggravate the local inflammatory response, increase inflammatory factors, and massive infiltration of inflammatory cells, resulting in inflammatory injury to target organs [39]. In our study, the pathological damage of lung tissue in the CP + Oz group was significantly reduced compared with that in the CP group, as was the lung injury score. In the blood transfusion process, locally enhanced oxidative stress induced by organ reperfusion is the critical factor leading to ischemia/reperfusion injury [38]. For the CP + Oz group, the reduction in organ damage was possibly related to the decrease in inflammation. We observed a decrease in plasma IL-6 concentration in the CP + Oz group at the end of surgery compared with the control and CP groups, indicating a decrease in the overall inflammatory reaction. Therefore, we assume that by transfusion of blood treated with O_3, the systemic inflammatory response can be attenuated, and ischemia/reperfusion injury may be alleviated by this anti-inflammatory effect on all tissues and organs across the body. O_3 has been reported to be applied for protecting ischemia/resuscitation injury. The underlying mechanism may include the regulation of HSP70 by activating the JAK2/STAT3 pathway [40].

This study has certain limitations. (1) The autologous blood collected from rabbits did not undergo centrifugal washing. Although, according to our previous study [14], the process may have little help with oxygenation, it may have a protective effect by wash-off contaminants introduced by the procedure and filtering out white blood cells to attenuate the inflammatory reaction. Nevertheless, the results and conclusion still stand since all four groups used whole blood for resection. (2) Studies on the effect of O_3 on RBC structure and function are lacking. Our study preliminarily proved that O_3 could reverse RBS deformation caused by CO_2 pneumoperitoneum, and the underlying mechanism remains to be elucidated in the future.

In conclusion, blood from a simulated CO_2 pneumoperitoneum environment could show significantly low pH, high $PaCO_2$, and low SaO_2. In a hemorrhagic shock model, the autotransfusion of hypercarbia blood does not cause dramatic changes in hemodynamics or the internal environment. However, it will affect the postoperative quality of life and cause severe inflammatory organ injury. Treatment with O_3 can restore RBC function, significantly improve blood oxygenation after simulated CO_2 pneumoperitoneum, and improve postoperative survival quality. O_3 also plays a protective role in preventing organ injury caused by an autologous blood transfusion after a simulated CO_2 pneumoperitoneum environment.

Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee of the Peking University People's Hospital, approval number 2016PHC035.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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None.

Author Contributions

Yu Gan and Xue Tian designed and carried out the experiments and prepared data and manuscripts. Han Yao carried out part of the ELISAs and prepared the SEM samples. Fei Huo helped prepare the revised manuscripts. Finally, Yi Feng designed and implemented this study.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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