Letter to the Editor

Effect of oxygen supplement during targeted temperature management on acute lung injury in the early stage of traumatic hemorrhagic shock

Tai-Wen Rao,1,2* Ye-Hua Shen,3,4* Xiao-Gang Zhao1,2 and Shou-Yin Jiang1,2*

Abstract
Ideal concentrations of inhaled oxygen with regard to lung protection during early traumatic hemorrhagic shock (THS) remain unknown especially in the era of targeted temperature management (TTM). We speculated that a significant increase in oxygen supply in early stage of THS would magnify the protecting role of hypothermia in acute lung injury. Forty male New Zealand rabbits were randomly divided into four groups (n = 10): sham group, control group, group 1, and group 2. Except for sham group, all other animals were submitted to 30 min of uncontrolled THS and received limited fluid resuscitation for 60 min. During resuscitation, in addition to 34°C of TTM, animals in group 1 inhaled 21% oxygen while animals in group 2 inhaled 50% oxygen. Animals in control group inhaled room air and were kept normothermia. Oxidative stress, inflammation, and apoptosis parameters in the lung tissues were determined. THS induced higher expression of malondialdehyde, surfactant protein A, nuclear factor kappa B, and caspase 3 as well as lower expression of Bcl-2 mRNA and superoxide dismutase activity. Compared with inhalation of 21% oxygen, inhalation of 50% oxygen during TTM significantly improves oxidative stress, inflammation, apoptosis, and acute lung injury. Oxygen supplement during TTM therapy alleviated acute lung injury in the early stage of THS. Further studies are required to explore the ideal combination forms of TTM and oxygen supplement with the purpose of maximizing therapeutic effect while minimizing adverse effects.

Keywords
acute lung injury, hemorrhagic shock, oxidative stress, oxygen supplement, targeted temperature management

Introduction
Oxygen therapy constitutes the critical components of intervention in the early management of traumatic hemorrhagic shock (THS). Guidelines recommend that high concentrations of oxygen should be administered immediately for patients with shock and major trauma. However, hyperoxia may induce enhanced reperfusion injury during shock and resuscitation. A recent meta-analysis based on cohort studies concluded that arterial hyperoxia in various subsets of critically ill patients is associated with poor outcome. Actually, the
ideal fraction of inspiration oxygen (FiO₂) during early THS remains unknown.

Our previous study showed that arterial oxygen partial pressure (PaO₂) in hemorrhaged rabbit was slightly increased after targeted temperature management (TTM), which was found to be associated with alleviated acute lung injury (ALI) and improved early survival. Further cluster analysis based on published data showed that animals that had been treated with 34°C of TTM and inhaled room air was associated with an average of 11.2 mm Hg’s increase in PaO₂ after adjusting for temperature factor. This implies that increased oxygen supply during hypothermia may have a role in lung protection. However, previous studies were not specially designed to explore the effect of oxygen intervention on ALI after THS, not to mention its actual effect under TTM. Consequently, the purpose of this study was to investigate the influence of oxygen supplement during TTM on ALI in the early stage of THS.

**Materials and methods**

*Animals*

Male New Zealand rabbits aged 6–8 weeks, weighing 2.0 ± 0.03 kg, were purchased from Xinchang rabbit market. Before the experiment, rabbits were fasted overnight but were allowed to drink freely. The study protocol was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. Animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Institutes of Health publication, 2011).

*Surgical preparation*

Animals were anesthetized with xylazine (2.5 mg/kg, i.m.) and pentobarbital sodium (30 mg/kg, i.v.) and secured to a backboard in a supine position. Normal rectal temperature (Tr) was maintained using a heating pad. A 20-gauge Angiocath (Becton-Dickinson, Sandy, UT, USA) was inserted into the left femoral artery which was connected to MedLab-U organism signal system (Nanjing Medease Science and Technology Co., Ltd, Nanjing, China) for monitoring blood pressure and bloodletting. Another 20-gauge Angiocath was inserted into the right femoral vein for fluid resuscitation. A midline laparotomy was performed and one branch of the ileocecal artery was isolated; this was to simulate abdominal trauma with uncontrolled bleeding. The abdomen was temporarily closed through tensioning the suture.

**Uncontrolled THS model**

Rabbit model of THS was established according to our previously work and was simplified not to include limb fracture. Animal experiment in this study comprised two phases: phase 1 (uncontrolled THS, 0–30 min) and phase 2 (limited fluid resuscitation, 30–90 min). Uncontrolled hemorrhage was initiated at 0 min via bloodletting from the left femoral artery at a rate of 2 mL/kg/min. Mean arterial pressure (MAP) was kept at 25 mm Hg until the end of phase 1. At 30 min, the prepared branch of the ileocecal artery was punctured with a 25-gauge syringe needle, and animals were infused intravenously with Ringer’s lactate (RL) solution to maintain a stable MAP of 40 mm Hg until the end of phase 2. After that, rabbits were sacrificed with an overdose of pentobarbital sodium.

**Experimental protocol**

Based on our previous data, we expected the total ALI score in higher FiO₂ group to be 4 to 6. Sample size of 10/group was adequate to detect a relative difference of 80% between treated groups with an α of 0.05 and power (1 − β) of 0.80. Thus, 40 rabbits were randomly divided into four equal groups: sham group, control group, group 1 (TTM with FiO₂ of 0.21), and group 2 (TTM with FiO₂ of 0.5). Animals in sham group did not received fluid therapy except for basic procedures. Animals in control group inhaled room air during shock and fluid resuscitation (detailed earlier), and Tr was maintained at normothermia throughout the experiment. Fluid resuscitation strategies for animals that were allocated into groups 1 and 2 were similar to control group. Tr was kept normothermia for all groups during phase 1.

In phase 2, TTM was induced in animals in groups 1 and 2 by spraying alcohol onto the abdomen, with electric fan accelerating cooling. After achieving target temperature of 34°C, Tr was maintained stable via intermittent use of heating pad and cooling method. Under well-fitted animal mask,
animals in group 1 inhaled 21% oxygen, while ani-
mals in group 2 inhaled 50% oxygen. At the end of
phase 2, all animals were humanely killed and the
lung tissues were harvested for histopathologic and
biochemical analyses.

**Malondialdehyde and superoxide dismutase**
**activity determination**

Malondialdehyde (MDA), a common marker of
oxygen free radical damage, is one of the final
products of polyunsaturated fatty acid peroxidation
in cells. In contrast, superoxide dismutase (SOD) is
an important antioxidant enzyme which protects
organ from damage induced by reactive oxygen
species (ROS). In this study, MDA level and SOD
activity were measured by spectrophotometric
method via commercial assay kits (Jiancheng
Bioengineering Institute, Nanjing, China). Briefly,
MDA concentration in tissue homogenates was
determined via the thiobarbituric acid method. The
assay was based on the conjugation ability of MDA
with thiobarbituric acid, to form a red product
which has maximum absorbance at 532 nm. SOD
activity was determined at 450 nm which was
based on the generation of oxygen by xanthine and
xanthine oxidase.

**Western blot analysis**

Lung tissues were crushed and washed with phos-
phate-buffered saline and homogenized in lysis
buffer for 30 min. The extracts were cleared by cen-
trifugation at 12,000g for 5 min. Bicinchoninic acid
assays were used for accurate determination of pro-
tein concentration. β-Actin was used as the loading
control. The resolved proteins were separated by 10%
sodium dodecyl sulfate–polyacrylamide gel
electrophoresis and transferred to polyvinylidene
difluoride membrane. After being blocked with 5%
nonfat milk in phosphate-buffered saline for 1.5 h at
room temperature, membranes were incubated with
primary antibodies such as anti-surfactant protein A
(SP-A) (1:2000 dilution; Bioss, China) and anti-
nuclear factor kappa B (NF-κB) (1:2000 dilution;
Bioss, China) at 4°C overnight. Blots were washed
twice in tris-buffered saline with Tween over 30 min.
Horseradish peroxidase-anti-rabbit immu-
noglobulins (1:5000; HuaBio, China) were incu-
bated for 1 h at room temperature. All membrane
images were acquired using the ChemiDoc™ MP
imaging system (Bio-Rad, Hercules, CA). Band
intensities were quantified using the Quantity One
software (Bio-Rad).

**Quantitative real-time polymerase chain**
**reaction analysis**

TRizol reagent (Kangwei Century Biological
Technology Co., Ltd, Beijing, China) was applied
to extract total lung RNA. RNA was assessed for
purity and concentration and cDNA was synthe-
sized using the Verso cDNA synthesis kit (Takara,
Japan). The sequences of primers used in the
study were as follows: caspase 3, forward
5’-GAGAAACAACGAAACCTCGTG-3’ and
reverse 5’-CCCAGAGTCATTGCTTT-3’;
NF-κB, forward 5’-GAGGCGAGATGACCTCA
ACA-3’ and reverse 5’-ATTCTTGAAAGCAGC
GCTT-3’; Bcl-2, forward 5’-TGGTACCTCAG
CTTCTTCCC-3’ and reverse 5’-CTTCACTC
GATCTCCCAGT-3’; β-actin, forward 5’-CATGG
ATGATGATATCGCCGC-3’ and reverse 5’-CTGG
TCGCCACATAGAAT-3’. The mRNA expres-
sions were determined with the SYBR Prime Script
RT-PCR Kit (Takara) in a Mastercyler® ep realplex
thermocycler (Eppendorf, Hamburg, Germany)
with the following thermal cycling conditions:
94°C, 1 min; 95°C, 10 s; 58°C, 10 s; 72°C, 10 s (40
cycles). Expression of each studied gene was nor-
malized to that of the β-actin gene. All mRNA lev-
els were calculated using the 2−ΔΔCT method.

**Histopathological examination**

The lung specimens were fixed in 10% formalin,
embedded in paraffin, and sectioned at 4.0 μm
thickness. Tissue slices were stained with hema-
toxylin and eosin (HE) and observed with light
microscopy (Nikon Eclipse Ti-SR, Japan). The
severity of lung injury was assessed by a patholo-
gist who was blinded to grouping base on a previ-
ous method.4 In short, four characteristics of
histological changes of the lung tissue were used to
assess ALI: alveolar congestion, hemorrhage, infil-
tration or aggregation of neutrophils in airspaces or
vessel walls, and thickness of alveolar wall/hyaline
membrane formation. Each characteristic was
quantitatively divided into four scales (0, normal;
1, light; 2, moderate; 3, strong; and 4, intense). The
total score of ALI was calculated as the sum of
scales of the four parameters.
Table 1. Effect of oxygen supplement on SOD activity and MDA content in rabbit pulmonary tissues after THS treated with TTM.

| Groups   | SOD activity (U/mL) | MDA content (nmol/mL) |
|----------|---------------------|-----------------------|
| Sham     | 67.39 ± 4.755       | 0.50 ± 0.038          |
| Control  | 47.41 ± 3.710       | 0.87 ± 0.049          |
| Group 1  | 53.60 ± 2.957*      | 0.69 ± 0.048*         |
| Group 2  | 60.34 ± 1.470***    | 0.60 ± 0.032***       |

SOD: superoxide dismutase; MDA: malondialdehyde; THS: traumatic hemorrhagic shock; TTM: targeted temperature management.

Data are presented as mean values ± SD.

*P < 0.01 versus control and sham group.

**P < 0.05 versus group 1.

Statistical analysis

Data are presented as mean values ± standard deviation. Variables were analyzed by one-way or two-way analysis of variance with repeated measures followed by post hoc Turkey’s tests for multiple comparisons (SPSS 19; SPSS Inc., Chicago, IL, USA). A value of P < 0.05 was considered statistically significant.

Results

No significant differences were observed in body weight and baseline Tr, MAP, and heart rate between groups. The volume of blood loss and volume of RL infused were not significantly different between group 1 and group 2. All rabbits survived over 90 min.

MDA contents and SOD activity

MDA content in the lung tissues was elevated significantly after THS (control group vs. sham-operated group, P < 0.01). MDA content was found to be decreased after TTM treatment as shown in data from group 1 and group 2 (compared to control group, P < 0.01). Raising oxygen supplement from 0.21 to 0.5 on the basis of TTM significantly decreased MDA content (group 2 vs. group 1, P < 0.05). In contrast, SOD activity in the lung tissues was significantly decreased (control group vs. sham-operated group, P < 0.01). SOD activity in group 1 and group 2 was higher than that in control group (both P < 0.01). Raising oxygen supplement from 0.21 to 0.5 increased SOD activity (group 2 vs. group 1, P < 0.05) (Table 1).

Expressions of SP-A and NF-κB protein

Expressions of SP-A and NF-κB protein in control group were significantly increased compared with sham group (both P < 0.01). TTM treatment significantly reduced SP-A and NF-κB expression (compared to control group, both P < 0.01). Raising oxygen supplement from 0.21 to 0.5 during TTM significantly decreased expression levels of SP-A and NF-κB protein (group 2 vs. group 1, both P < 0.05) (Figure 1).

Expressions of caspase 3, NF-κB, and Bcl-2 mRNA

The relative expressions of caspase 3 and NF-κB mRNA in control group were significantly elevated compared with sham group (both P < 0.01). TTM treatment significantly reduced caspase 3 and NF-κB mRNA levels (compared to control group, both P < 0.01). Raising oxygen supplement from 0.21 to 0.5 on the basis of TTM significantly decreased expression levels of caspase 3 and NF-κB mRNA (group 2 vs. group 1, both P < 0.05). In contrast, expression of Bcl-2 mRNA was significantly decreased after THS (control group vs. sham group, P < 0.01). TTM therapy increased Bcl-2 mRNA expression in group 1 and group 2 compared with control group (both P < 0.01). Raising oxygen supplement further increased Bcl-2 mRNA expression (group 2 vs. group 1, P < 0.05) (Figure 2).

Histopathologic changes

Histological examination showed that the structure of lung tissue was normal in sham group. After THS, the pulmonary tissues changed significantly as shown in control group, which included dilated vessels and hyperemia, thickened alveolar walls, dilated alveolar capillaries, and interstitial edema (Figure 3). Following therapy with TTM, inflammatory changes induced by THS were significantly improved, which were further ameliorated after elevating FiO2 from 0.21 to 0.5. Quantitative analysis revealed that the total ALI score was significantly lower in group 2 compared with control group (P < 0.01) and group 1 (P < 0.05).

Discussion

THS activates microvascular endothelium and promotes generation of ROS which can lead to
ALI in the early stage.\textsuperscript{5} TTM has been shown to decrease excitotoxicity, limit inflammation, prevent adenosine triphosphate (ATP) depletion, and reduce ROS.\textsuperscript{6} Based on TTM, our study indicated that oxygen supplement during TTM therapy after THS can reverse proinflammatory and apoptotic responses and alleviate ALI, with higher concentrations of oxygen therapy resulting in better ALI profiles.

The molecular mechanisms for beneficial effect of oxygen supplement remain unknown. Under physiological hypoxia and ischemia/reperfusion, ROS is derived primarily via mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. When cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, protein, and DNA may ensue.\textsuperscript{7} The nuclear transcription factor hypoxia-inducible factor \(1\alpha\) (HIF-1\(\alpha\)) is a key regulator of gene expression under hypoxic and inflammatory conditions.\textsuperscript{8} Research has proposed a role of mitochondrial ROS in the regulation of HIF-1\(\alpha\) stability.\textsuperscript{9} HIF-1\(\alpha\) triggers hypoxia-related downstream target genes involved in inflammation, such as NF-kB activation.\textsuperscript{10} ROS/HIF-1\(\alpha\) pathway maybe an important molecular mechanism leading to ALI after the THS. In a recent study, inhibition of HIF-1\(\alpha\) ameliorated lung injury which was induced by trauma in rats.\textsuperscript{11} Oxygen supplement improves hypoxemia and microcirculation metabolism, which may reduce production of ROS and stability of HIF-1\(\alpha\), thereby protects lung from injury after THS. Further studies are required to specially investigate this pathway. This study enriches our knowledge on the role of oxygen therapy during TTM in early management of THS, since severely injured patients may still die despite current numerous interventions.\textsuperscript{12}

This study has limitations. First, we did not measure arterial blood gas analysis, thus the exact arterial oxygen partial pressure (\(\text{PaO}_2\)) was unknown. Second, we did not design other groups in which animals would inhale higher concentrations of oxygen (\(\text{FiO}_2 > 0.5\)), as it has been known
Figure 2. Expressions of (a) NF-κB, (b) caspase 3, and (c) Bcl-2 mRNA in rabbit lung tissues after resuscitation from THS based on TTM therapy. Values were normalized to β-actin gene.

*P < 0.01 versus control and sham group.

#P < 0.05 versus group 1.

##P < 0.01 versus group 1.

Figure 3. Representative pathologic figures of pulmonary tissues in normal rabbits and those who were subjected to THS (stained with hematoxylin and eosin, magnification of 200 ×). Following TTM, acute inflammatory changes induced by THS were significantly improved as shown obviously in group 1, which were further ameliorated after elevating inhaled oxygen concentration from 0.21 to 0.5 (group 2).
that inhalation of 100% oxygen would be unfavorable. Nonetheless, the optimal concentrations of oxygen for ameliorating ALI may need to be studied in the future. Finally, we did not measure downstream expressions of inflammatory biomarkers such as interleukin-1 (IL-1), IL-6, or tumor necrosis factor-α (TNF-α) that can be expressed following activation of NF-κB; however, pathologic changes of the lung tissues have indicated inflammatory differences between groups.

Conclusion

Oxygen supplement during TTM therapy alleviated ALI in the early stage of THS. Rabbits achieved the best ALI profiles under combined treatment with TTM and oxygen supplement. Further studies are required to explore the ideal combination forms of TTM and oxygen supplement with the purpose of maximizing therapeutic effect while minimizing adverse effects.

Animal welfare

Animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Institutes of Health publication, 2011).

Authors’ note

This article was presented at the 12th National Trauma Conference of China (26–28 April 2019, Sichuan).

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (approval no. No11. 2017).

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Zhejiang Provincial Natural Science Foundation of China (LQ17H150001) and the Natural Science Foundation of China (81701933).

ORCID iDs

Tai-Wen Rao https://orcid.org/0000-0001-5354-4667
Shou-Yin Jiang https://orcid.org/0000-0001-6298-2431

References

1. O’Driscoll BR, Howard LS, Earis J et al. (2017) BTS guideline for oxygen use in adults in healthcare and emergency settings. Thorax 72(Suppl. 1): ii1–ii90.
2. Helmerhorst HJF, Roos-Blom MJ, van Westerloo DJ et al. (2015) Association between arterial hyperoxia and outcome in subsets of critical illness: A systematic review, meta-analysis, and meta-regression of cohort studies. Critical Care Medicine 43(7): 1508–1519.
3. Jiang S, He X, Wang J et al. (2013) Therapeutic mild hypothermia improves early outcomes in rabbits subjected to traumatic uncontrolled hemorrhagic shock. Journal of Surgical Research 183(2): 752–759.
4. Xu L, Bao HG, Si YN et al. (2013) Effects of adiponectin on acute lung injury in cecal ligation and puncture-induced sepsis rats. Journal of Surgical Research 183(2): 752–759.
5. Torres Filho I (2017) Hemorrhagic shock and the microvasculature. Comprehensive Physiology 8(1): 61–101.
6. Andresen M, Gazmuri JT, Marin A et al. (2015) Therapeutic hypothermia for acute brain injuries. Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine 23: 42.
7. Spirlandeli AL, Deminice R and Jordao AA (2014) Plasma malondialdehyde as biomarker of lipid peroxidation: Effects of acute exercise. International Journal of Sports Medicine 35(1): 14–18.
8. Yeh CH, Cho W, So EC et al. (2011) Propofol inhibits lipopolysaccharide-induced lung epithelial cell injury by reducing hypoxia-inducible factor-1alpha expression. British Journal of Anaesthesia 106(4): 590–599.
9. Li L, Tan J, Miao Y et al. (2015) ROS and autophagy: Interactions and molecular regulatory mechanisms. Cellular and Molecular Neurobiology 35(5): 615–621.
10. Palazon A, Goldrath AW, Nizet V et al. (2014) HIF transcription factors, inflammation, and immunity. Immunity 41(4): 518–528.
11. Jiang H, Huang Y, Xu H et al. (2012) Inhibition of hypoxia inducible factor-1alpha ameliorates lung injury induced by trauma and hemorrhagic shock in rats. Acta Pharmacologica Sinica 33(5): 635–643.
12. Blackbourne LH, Baer DG, Cestero RF et al. (2011) Exsanguination shock: The next frontier in prevention of battlefield mortality. Journal of Trauma 71(Suppl. 1): S1–S3.