**Manuscript Number:** ALN-D-15-01038R4  

**Full Title:** Therapeutic Whole-body Hypothermia Protects remote lung, liver and kidney injury following Blast Limb Trauma in Rats  

**Article Type:** Critical Care Medicine  

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**Abstract:** Background: Severe blast limb trauma (BLT) induces distant multiple organ injuries. In the current study, we determined whether whole-body hypothermia (WH) and its optimal duration (if any) afford the protection to the local limb damage and distant lung, liver and kidney injuries following blast limb trauma in rats.  
Methods: Rats with BLT, created by using chartaceous electricity detonators, randomly treated with WH for 30, 60 min, 3, 6hrs (n = 12/group). Rectal temperature and arterial blood pressure were monitored throughout. Blood and lung, liver and kidney tissue samples were harvested for measuring TNFα, IL-6 and 10, MPO activity, hydrogen sulfide and bio-markers of oxidative stress at 6hrs after BLT. The pathologic lung injury and the water content of the lungs, liver and kidneys and blast limb tissue were assessed.  
Results: Unlike 30min, WH for 60min reduced lung water content, lung and kidney MPO activity by 10%, 39 % and 28% (all p < 0.05), respectively. WH for 3 hrs attenuated distant vital organs and local traumatic limb's damage, reduced MPO activity, H2O2 and MDA concentration, TNFα and IL-6 level by up to 49% (p all < 0.01). Likewise, WH for 6hrs also provided protects to such injured organs but increased blood loss from traumatic limb.  
Conclusions: Our data indicated that whole-body hypothermia may provide protections for distant organs and local traumatic limb following blast trauma but warrants further study.
Dear Dr. Jerrold H. Levy,

Re: ALN-D-15-01038R3, entitled "Therapeutic Whole-body Hypothermia Protects remote lung, liver and kidney injury following Blast Limb Trauma in Rats"

Thank you so much for informing us to publish our manuscript in Anesthesiology. We have amended our work accordingly. All new changes made for this revision are distinguished by using red font.

We replaced "prior to" with "immediately after" in the section of "What This Article Tells Us That Is New" as following.

What We Already Know about This Topic
Blast limb trauma induces organ injury due to multiple mechanisms that produce a systemic inflammatory response.

What This Article Tells Us That Is New
Whole-body hypothermia for 3hrs immediately after injury in an experimental animal model provides multiorgan protection for traumatic injury following blast trauma.

Best wishes!

Yours sincerely,

Jiaolin Ning and Daqing Ma
On behalf of all authorship
Therapeutic Whole-body Hypothermia Protects Remote Lung, Liver and Kidney Injury Following Blast Limb Trauma in Rats

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Financial Disclosure

The authors have not disclosed any potential conflicts of interest. This study was supported by the grant of National Natural Science Foundation of China (Grant No. 81470267).

Acknowledgements

We thank the team at the Institute of Surgery Research, Department of Traumatic Surgery, Third Military Medical University, China for their help.

Running title

Hypothermia, remote organ injury and trauma

Words count

Abstract: 226; Introduction: 400; Discussion: 1450

What We Already Know about This Topic

Blast limb trauma induces organ injury due to multiple mechanisms that produce a
systemic inflammatory response.

**What This Article Tells Us That Is New**

Whole-body hypothermia for 3hrs immediately after injury in an experimental animal model provides multiorgan protection for traumatic injury following blast trauma.
ABSTRACT

Background: Severe blast limb trauma (BLT) induces distant multiple organ injuries. In the current study, we determined whether whole-body hypothermia (WH) and its optimal duration (if any) afford the protection to the local limb damage and distant lung, liver and kidney injuries following blast limb trauma in rats.

Methods: Rats with BLT, created by using chartaceous electricity detonators, randomly treated with WH for 30, 60 min, 3, 6 hrs (n = 12/group). Rectal temperature and arterial blood pressure were monitored throughout. Blood and lung, liver and kidney tissue samples were harvested for measuring TNFα, IL-6 and IL-10, MPO activity, hydrogen sulfide and bio-markers of oxidative stress at 6 hrs after BLT. The pathologic lung injury and the water content of the lungs, liver and kidneys and blast limb tissue were assessed.

Results: Unlike 30 min, WH for 60 min reduced lung water content, lung and kidney MPO activity by 10%, 39% and 28% (all p < 0.05), respectively. WH for 3 hrs attenuated distant vital organs and local traumatic limb’s damage, reduced MPO activity, H₂O₂ and MDA concentration, TNFα and IL-6 level by up to 49% (p all < 0.01). Likewise, WH for 6 hrs also provided protects to such injured organs but increased blood loss from traumatic limb.

Conclusions: Our data indicated that whole-body hypothermia may provide protections for distant organs and local traumatic limb following blast trauma but warrants further study.

Key words: Blast limb trauma; Distant organ injury; Whole-body hypothermia; Inflammatory response; Cytokine; Hydrogen sulfide.
Background

With the development of armour, survivors with blast limb trauma increased in modern military conflicts and terrorist attacks\(^1,2\). When multiple blast trauma is very severe and complicated, it often induced remote multi-organs injuries including the lungs, liver and kidneys via systemic inflammatory responses and oxidative stress and suppression of cystathionine gamma-lyase (CSE)/hydrogen sulfide (H\(_2\)S) pathway as reported previously\(^3,4\). The distant remote multiple organs injuries not only perplex the condition and treatment, but also prolong recovery. These injuries could develop towards multiple organs dysfunction (MODS) or even failure if no proper interventions are promptly being in-placed.

Whole-body hypothermia (WH) is a therapeutic intervention of the controlled reduce of core temperature to provide therapy, e.g. protecting organs at risk of injury\(^5\). To date, WH has principally been used as a protective therapy for various brain injuries\(^6\); moreover, there is emerging evidence that it may also be useful to protect other organs when at risk of injury. For example, WH can improve the prognosis of severe sepsis and severe hemorrhage shock\(^7\). It was also reported that WH attenuates the heart\(^8\), the lungs and kidneys\(^9,10\) and liver injury\(^11,12\). It has been recognized that the therapy window and the duration of WH are very important for achieving promising protective outcome. In general, the early application of therapeutic hypothermia can be more effective than later application\(^13,14\). Mild hypothermia is preferable because it can provide considerable protective effects and has minimal adverse effects\(^15,16\). It was reported that prolonged
hypothermia did not improve the protection effects but increased adverse effects\textsuperscript{17,18}.

Mild hypothermia for 24 hrs is recommended for brain protection following cardiac arrest\textsuperscript{19}. It has also been demonstrated that mild hypothermia for 1 hr improved the final outcome for uncontrolled hemorrhagic shock rats\textsuperscript{20} and 2.5hrs of hypothermia afforded hepatic protection in multiple trauma swine model\textsuperscript{21,22}. Interestingly, regional cooling for 30 minutes to traumatic limb was enough to afford maximum protection for the remote lung injury\textsuperscript{4}. Clearly, the optimal duration of hypothermic therapy varies with different diseases or pathological conditions.

To extend our previous findings\textsuperscript{4}, we hypothesized that WH could also attenuate organs injuries following BLT but may be even more effective than regional hypothermia.

Therefore the aim of the current study is to determine whether WH affords the protection to the local limb damage and distant lung, liver and kidney injuries and the optimal duration of WH for such injuries following blast limb trauma in rats.
**Materials and Methods**

After approval of the protocol by the Third Military Medical University Animal Care Committee, the experiments were performed according to the Chinese Institutes of Health Guidelines on the Use of Laboratory Animals.

**Animal model**

Anesthesia and methods of creation of blast limb trauma in rats have been previously described in detail\(^4\)\(^23\). Briefly, anesthesia was induced with intra-peritoneal (I.P.) injection of pentobarbital (50mg/kg of weight) and adequate anesthesia was achieved via injection of pentobarbital I.P. according to the lash flash throughout the experiment. Two catheters (OD, 0.965mm; ID, 0.58) were inserted into right femoral artery to monitor blood pressure and collect the blood sample and right jugular vein for fluid infusion, respectively. Subsequently, rats were then randomized into the Sham, which received identical treatment (anesthesia) and manipulations (surgery) but without blast injury, and the Blast group.

Blast limb trauma was induced by using chartaceous electricity detonators (845 Factory, Chongqing, China) containing 80mg diazodinitrophenol. The injuries were manifested as hemorrhage, open comminuted fracture, Tscheme-Gotzen 3-4 degree soft tissue injury and first degree burn injury. The blasted limbs were packed with sterile sponges after injury. Normal saline at 4ml/kg/hr was infused and spontaneous breathing kept throughout the experiment.
Experiment Protocol

After injury, the BLT rats were randomized by a random number generated by a computer to the normothermia group (BLT) and the groups of hypothermia (32± 0.5°C) for 30min (BLT-WH30), 60 min (BLT-WH60), 3 hrs (BLT-WH3h) and 6 hrs (BLT-WH6h)(n = 12/group) (Supplemental Figure 1). WH was initiated for the hypothermic group immediately after blast by spraying alcohol onto abdominal skin while an electric fan circulated cool air until rectal temperature reached to 33.5°C and maintained at 32 ± 0.5°C for 30, 60min, 3 or 6hrs. Then the rats in the group BLT-WH30, BLT-WH60 and BLT-WH3h were dried with electric hair drier and then placed onto the thermo mattress (40°C) for rewarming to over 35°C, and maintained this rectal temperature until termination of experiment, the speed of rewarming was kept at 1.5-2°C/hr (Supplemental Table 1). Adequate anesthesia was kept to avoid shivering during the period of cooling and rewarming.

Measurements

Arterial blood pressure (ABP) and rectal temperature with PowerLab (AD Instruments, Colorado Springs, CO, USA) were continuously monitored throughout the experiments.

Blood loss induced by blast was calculated by the following formula (blood loss = wet gauze weight - dry gauze weight).After the animals were sacrificed by injection I.P. of overdose pentobarbital at 6hrs post-injury, blood and tissue samples of the damaged limb, the right lower of lung, the right kidney and left hepatic lobe were harvested for following measurements.
**Histology:** The right lowing robe of lung and damaged limb muscle tissue were fixed in 10% formalin for 24hrs and then embedded in paraffin. The blocks were sectioned at 5μm thickness and then stained with hematoxylin-eosin. The pathologic changes of the lungs were scored in a blinded manner through analyzing with the following variables: lung edema, hemorrhage, infiltration of the inflammatory cells, thickness of alveolar wall and pulmonary architecture, as described previously\textsuperscript{4,23}. Each variable was graded as a scale of 0-4 (0. absent, 1.mild, 2.moderate, 3.severe, 4. very severe injury). The total histopathology score was expressed as the sum of the scores for statistical analysis.

**Biological measurements:** TNFα, IL-6, 10 levels were measured by enzyme-linked immunosorben assay according to instructions from the manufacturer (R&D systems, Minneapolis, USA).H\textsubscript{2}S level was determined using a modified methylene blue assay described previously\textsuperscript{4}. Other parameters, including serum Creatinine (Crea), AST and ALT were measured by using commercial kits (Jiancheng Biotechnology CO, Nanjing, China), respectively. All the biological measurements were determined in a blinded manner.

**Statistical Analysis**

The group size(n = 12) was determined at set of 1-Beta 0.9 and Alpha 0.05 by one way Analysis of Variance (PASS 10.0 software) based on the data of lung histopathology scores from the pilot experiments. No data were missing for final analysis and data were expressed as mean ± SD unless indicated otherwise. Histological injury scoring data was
expressed as a Box-and-Whisker plot and analyzed by Kruskal-Wallis nonparametric test followed by Dunn's test for comparison. The rest data was analyzed by two tailed Analysis of Variance followed by Tukey comparison (GraphPadPrism 5, San Diego, CA). A p <0.05 was considered to be statistically significant.
Results

Physiological Variables

The blood loss from the traumatic limb in the BLT, BLT-WH30, BLT-WH60 and BLT-WH3h groups do not differ significantly (11.4%, 16.3%, 16.1%, and 17.1% of the body weight, respectively), but WH for 6 hrs significantly increased the blood loss when compared with that of group-BLT (11.4% vs 17.8%, p=0.037).

BLT resulted in reduced mean ABP (MBP), and this MBP level was kept throughout experiments and hypothermia for any duration did not affect the MBP further (Supplemental Table 1).

WH treatment attenuated muscle and remote lung injury following blast limb trauma

In contrast to the normal muscle in the naïve rats (Fig 1A), muscle myofilament was ruptured in the BLT rats, together with hemorrhage and muscle swell (Fig 1B). The rats treated with WH for 3hrs (Fig 1E) and 6hrs (Fig 1F) attenuated muscle swelling compare to the BLT rats. These observations were corroborated with the traumatic muscle water content (Fig 1G).

In contrast to the normal lung in the Sham rats (Fig 2A), lung histological changes including congestions, hemorrhage, alveolar wall thickening and cell infiltration were found in BLT rats (Fig 2B). WH treatment for 30min (Fig 2C), 60min (Fig 2D), 3hrs (Fig 2E) and 6hrs (Fig 2F) alleviated these lung morphological changes induced by BLT varying
degree, but only WH treatment for 3hrs and 6hrs significantly decreased the pathology scoring (Fig 2G). Lung water content was decreased in the rats treated with WH for 30min (p=0.001), 60min (p=0.002), 3hrs (p<0.0001) and 6hrs (p<0.0001) compared with that in the BLT rats (Fig 2H).

The effect of WH treatment on liver and kidney function

Liver water content was decreased in the BLT-WH 3 or 6h rats compared with that of the BLT rats (Fig 3A). WH for 3hrs and 6hrs attenuated increased serum AST (Fig 3B) and ALT (Fig 3C) induced by BLT. WH for 3hrs and 6hrs alleviated the increased kidney water content induced by blast limb trauma (Fig 3D), but serum Urea was found no change in the all groups (Fig 3E).

The effect of WH treatment on cytokines

As shown in Fig 4A, plasma TNF α was increased 20folds than that of the Sham rats (73.6±15.4pg/ml, p<0.0001). WH for 3hrs and 6hrs reduced plasma TNFα to 58% (p=0.002) and 66% (p=0.008) of that in the BLT rats, respectively. Plasma IL-6 was increased 8.7folds than that of the Sham rats (268.7 ± 31.7pg/ml, p<0.0001) which was reduced to 63% and 59% by WH for 3hrs (p<0.0001) and 6hrs (p<0.0001), respectively (Fig 4B). Plasma IL-10 was increased 2.6 folds than that of the Sham rats (139.8 ± 19.3pg/ml, p<0.0001) but WH for 6hrs decreased plasma IL-10 significantly (p=0.018) (Fig 4C).

The effect of WH on plasma H₂S
BLT resulted in a decreased plasma H$_2$S level (36.8 ±9.5μM vs 68.4±11.7 of controls, 
p<0.001). WH for 3hrs (59.2±4.2) almost revered this decrease (p=0.450), and plasma H$_2$S 
level was lower in the BLT-WH30 (p<0.001), BLT-WH60 (p=0.003) and BLT-6h (p<0.001) 
rats compared with that in the Sham rats (Fig 4D).

The effect of WH on MPO activity

MPO activity was determined as a surrogate of neutrophil activity. MPO activity in plasma 
(Fig 5A), muscle (Fig 5B), lung (Fig 5C), liver (Fig 5D) and kidney (Fig 5E) was increased in 
the BLT rats compared with that in the Sham rats. WH for 3 and 6hrs reduced plasma (Fig 
5A) and muscle MPO activity (Fig 5B) induced by BLT. WH for any durations reduced lung 
MPO when compared with that of BLT rats but WH for 6hrs further reduced lung MPO 
compared with WH for 3hrs (p=0.047) (Fig 5C). Unlike hypothermia for 30 min, 
hypothermia for 3hrs (p=0.014) and 6hrs (p<0.0001) reduced liver MPO (Fig 5D). WH for 
3hrs (p=0.002) and 6hrs (p=0.001) alleviated the increased kidney MPO activity induced 
by BLT, respectively (Fig 5E).

WH attenuated oxidative stress in the BLT rats

As shown in the Figure 6, BLT resulted in a decreased T-AOC in the muscle (Fig 6A), lung 
(Fig 6B), liver (Fig 6C) and kidney (Fig 6D).Both WH for 3and 6hrs elevated a decrease of 
T-AOC in the muscle (Fig 6A), lung (Fig 6B) and kidney (Fig 6D), and WH for 6hrs also 
increased T-AOC in the liver (Fig 6C).
BLT caused a decrease of SOD activity in plasma (Fig 7A), muscle (Fig 7B), lung (Fig 7C), liver (Fig 7D) and kidney (Fig 7E). All durations of WH had no effects on the SOD activity in plasma (Fig 7A) and kidney (Fig 7E), but the prolonged hypothermia for 3hrs and 6hrs elevated SOD activity in muscle (Fig 7B), lung (Fig 7C) and liver (Fig 7D).

BLT resulted in a decrease of the GSH level in the plasma and all the organs being measured (p<0.05), but WH treatment did not alter such changes (Fig 8).

BLT resulted in an increase of the MDA level in the plasma (Fig 9A), muscle (Fig 9B), lung (Fig 9C), liver (Fig 9D) and kidney (Fig 9E). WH for 30 (p=0.033) or 60min (p=0.04) reduced lung MDA level in BLT rats (Fig 9B, C) but for 3hrs or 6hrs decreased all MDA level in the plasma and all organs being measured (Fig 9).

BLT increased hydrogen peroxide (H$_2$O$_2$) in the plasma and all studies organs (Fig 10). WH for 60min reduced lung (p=0.016) and liver (p=0.011) H$_2$O$_2$ level in BLT rats (Fig 10C, D), WH for 3hrs and 6hrs decreased H$_2$O$_2$ in the plasma and concerned organs (Fig 10).
Discussion

BLT results in not only local injury but also distant organs, including the lung injury, as reported previously\textsuperscript{4,23}. The current data demonstrate that WH treatment for 3hrs and 6hrs protected multiple organ injuries such as the traumatic limb, the lungs, liver and kidneys following BLT. The underlying mechanism may be associated with its suppression of neutrophil infiltration, restoring the balance of pro-inflammatory and anti-inflammatory response, inhibition of lipid peroxidation and production of ROS, and elevating H\textsubscript{2}S production.

In the current study, the protective effects of different duration of mild hypothermia was assessed in the BLT rats; WH for 3hrs was shown to be effective and the optimal duration to protect the all organs tested without the remarkable adverse effects when compared with WH for 30,60min or 6hrs. Although WH for 30 or 60 min alleviated the distant lung injury and water content, but it seems not long enough to attenuate local traumatic limb, liver and kidney injury. WH for 3hrs corrected the changes of the most of biochemical variables while WH for 6hrs treatment could further correct T-AOC in the lungs and liver, but increased blood loss. Therefore, WH for 3hrs following spontaneous rewarming is sufficient to provide both local tissue and distant organ protection in the BLT rats and limits the adverse effects of hypothermia. Our data reported here and others reported previously strongly suggested that different conditions need different duration of mild hypothermia for optimal outcome to be achieved\textsuperscript{3,4,20,21}. 
H$_2$S is a powerful biological signal participating in many pathological and pathological conditions\textsuperscript{24} and was shown to be involved in the process of distant organs injury following the BLT\textsuperscript{4,23}. Endogenous H$_2$S is produced from L-cysteine by enzymes such as cystathionine $\beta$-synthase (CBS), CSE, 3-mercaptopyruvate sulfurtransferase (3MST) and cysteine aminotransferase (CAT)\textsuperscript{25}. Disturbance of H$_2$S metabolism were found in several disease conditions, such as ischemia-reperfusion injury\textsuperscript{26}, acute lung injury\textsuperscript{27}, hypertension\textsuperscript{28}, atherosclerosis\textsuperscript{29}, cirrhosis\textsuperscript{30} and kidney fibrosis\textsuperscript{31}. Unlike other periods of hypothermia, WH for 3hrs treatment reversed the decreased plasma H$_2$S level. The causes for this discrepancy remain unknown but rescue of CSE activity could be one of many mechanisms. CSE is mainly expressed in the liver, kidney and the lungs\textsuperscript{32} and its activity was found to be decreased in these organs in the BLT rats, indicating that the decreased CSE activity is likely the main cause of decreased plasma H$_2$S level following BLT\textsuperscript{23}. It is reasonable to assume that WH for 6hrs treatment was too long and resulted in decreased metabolism rate because H$_2$S is the metabolism product of L-cysteine, all of substantcemetabolism is closely associated with reaction temperature and thus reduced the H$_2$S production in the BLT rats but warrants further study. Therapeutic hypothermia for 3 hrs \textit{per se} may restore H$_2$S production via increasing release of dopamine and serotonin and other biogenic amines in the BLT rats\textsuperscript{33,34}. H$_2$S has been found to confer multiply organ protection and it is true that exogenous H$_2$S donor has been shown to alleviate ischemia-reperfusion injury of the lungs\textsuperscript{35}, liver\textsuperscript{36} and kidney\textsuperscript{37}. In addition, exogenous H$_2$S donor, NaHS, was found to attenuate distant lung injury following BLT in our previous study\textsuperscript{38}, which is very likely to be due to inhibiting NF$\kappa$B activity, suppression
of oxidative stress, preserving function and structure of mitochondrial, up-regulation of pro-survival signal pathway\textsuperscript{39}. Thus, restoring \( \text{H}_2\text{S} \) may be considered to be one of mechanisms to the multiple organ protection of WH for 3hrs in the BLT rats. However, WH for 6hrs also provided protection for the distant organs although it did not restore plasma \( \text{H}_2\text{S} \) level when compared with 3hrs, which may suggest that multi-mechanisms are responsible for organ protection of WH.

Inflammatory response is considered to play a key role in distant organ injury. An early increase of pro-inflammatory cytokines after tissue trauma and hemorrhage has been well described in animal and clinical studies. TNF\( \alpha \) and IL-6 were found to be increased earlier than IL-10 following BLT in rats\textsuperscript{23}. It is well established that excessive TNF\( \alpha \) plays a critical role in systemic inflammation and physiopathologic role in the development of multiple organ dysfunction. IL-6 is an important cytokine in the progress of inflammatory response via delaying apoptosis of neutrophil [32] and mediating the hepatic acute phase response [30], and it adopts a central regulatory role in primary cellular and humoral immune activation [33,32]. Anti-inflammatory cytokines that are up-regulated following hyper-inflammatory response triggered by insults have been considered to keep the balance between pro-inflammatory and anti-inflammatory reaction. IL-10 is the pleiotropic anti-inflammatory cytokine and its main biological function is to limit inflammatory response \textit{via} regulating the differentiation and proliferation of several immune cells such as T, B, natural killer and antigen-presenting cells\textsuperscript{40}. Both pro-inflammatory and anti-inflammatory cytokines have been reported to be involved into
the protective effects of hypothermia. Indeed, hypothermia has been reported to block the inflammatory "cascade" reaction and thus attenuate the organs damage. Plasma TNFα, IL-6 and IL-10 were found to be reduced by WH treatment in the current study; however, pro-inflammatory cytokines were prone to be suppressed by WH treatment when compared with anti-inflammatory cytokines, which is confirmed by the elevated TNFα/IL-10 ratios by WH treatment for 3hrs (BLT: 2.92 vs BLT-WH3h: 1.99), which could be at least partly contributed to the organs protection of whole-body hypothermia as well.

Oxidative stress is also an important component of systemic inflammatory response and multiple organ dysfunction. Oxidative stress damages the parenchyma cells and micro-vessel endothelium and results in increased capillary permeability and edema. It is also well known that suppression of oxidative stress is also a mechanism of WH by mean of reduction of metabolism rate and preservation of energy substance. WH treatment for 3 or 6hrs reduced production of H₂O₂ and MDA, and preserve the T-AOC in concerned organs, WH for 3hrs or 6hrs were shown to elevate SOD activity in the muscle, the lungs and liver, however, had no pronounced effects on the GSH level in all concerned organs. All these data suggested that WH treatment for 3 or 6hrs may be effective in suppression of production of oxidation production than promotion expression of endogenous anti-oxidative proteins, which is different from the regional hypothermia, and this viewpoint was supported by the data that WH for 3 or 6hrs were not found to increase of Nrf2 (a key transcription factor for anti-xenobiotic genes) activity in the lungs (data not shown), but regional hypothermia for 30min could up-regulated Nrf2 activity. Activated
macrophage is the main source of reactive oxygen species (ROS)\textsuperscript{44}, and reduced macrophage infiltration contributed the suppression of oxidative stress by WH in the BLT rats, which is confirmed that WH for 3 or 6hrs reduced MPO activity in concerned organs and blood circulation.

WH has been reported to improve the hemodynamics states in different disease conditions, such as cardiogenic shock syndrome\textsuperscript{45}, hemorrhagic shock\textsuperscript{46} and sepsis\textsuperscript{47}. WH could affect cardiac output, systemic vascular resistance, left ventricular contractility and heart rate\textsuperscript{45}. However, MAP was not profoundly affected by WH treatment in this study, which suggested that stabilization of hemodynamics did not contribute to the WH associated organs protection in the BLT rats. In the current study, although significant benefit could be achieved, WH treatment for 6hrs was found to increased blood loss because prolonged WH treatment may impair coagulation function\textsuperscript{48,49}. In addition, prolonged WH treatment also causes other well-known adverse effects including acid-base imbalance and immune suppression\textsuperscript{50,51}. Yet, it should be appreciated that “keeping warm” for traumatic patients for better tissue or cellular oxygenation is important clinically. Our data reported here may suggest cooling patients at earlier stage after trauma may have therapeutic value but, undoubtedly, data obtained from rodents in the current study is far away from clinical situation. Therefore, better designed preclinical studies with large animals to be study subjects and clinical trials for comparison of the therapeutic effectiveness of “warm”, global (current data) or regional hypothermia\textsuperscript{4} in treating traumatic patients are urgently needed.
Our study had several limitations. First, this blast model was not including penetrating wound and severe contamination, and debridement was not performed in this severe blast limb model, which are different from clinical situation. Second, the time course of this study was only 6 hours and the long term outcomes of the treatment are not known. Third, vital organ injuries observed in this study may not be necessarily due to subsequent blast limb trauma only but due to explosion wave produced by blast, or the combination of both. Fourth, our data are reflecting blast trauma specifically but interestingly, surgical “trauma” cause remote lung injury has also been reported recently. Lastly, pre-clinical rodent model is far away from clinical settings and also the “favorable” effects of hypothermia on the biomarkers being measured in this study are only “observable” changes; the causal relationship is unknown and, therefore, the translation of our findings to clinical condition, therefore, requires more studies in big animal models and preclinical studies.

In conclusion, the current data show that whole body hypothermia for 3hrs conferred multiply organ protection in the BLT rats via suppressing oxidative stress, restoring balance between pro- and anti-inflammatory reaction and elevated endogenous H₂S production without adverse effects. Our current study could facilitate more studies in this area of research; until then, we do not know any therapeutic values of systemic hypothermia for traumatic patients.
Authors’ contributions

JN and LM carried out the study. JN and DM wrote the manuscript. LW supervised the histological procedures. XL and KL designed, coordinated and supervised the study. BY, JG and HZ analyzed the data and had input into writing the manuscript. All authors read and approved the final manuscript.
Reference


Treatment Protects against Remote Lung Injury after Kidney Transplantation in Rats. Anesthesiology 2015; 122: 1312-1326

Legends

**Figure 1:** The effects of whole body hypothermia treatment on the muscle histopathology changes and traumatic tissue edema induced by blast limb trauma. Representative microphotographs were taken from Sham (A), Blast limb trauma (BLT) (B), Blast limb trauma + whole body hypothermia treatment for 30 min (BLT + WH 30) (C), 60 min (BLT + WH 60) (D), for 3 hours (BLT + WH 3h) (E), 6 hours (BLT + WH 6h) (F). Traumatic tissue water content was represented by wet weight/dry weight (G). Data are mean ± SD (n=12). # # p < 0.01 vs BLT.

**Figure 2:** The effects of whole body hypothermia treatment on the lung histopathology changes and pulmonary edema induced by blast limb trauma. Representative microphotographs were taken from Sham (A), Blast limb trauma (BLT) (B), Blast limb trauma + whole body hypothermia treatment for 30 min (BLT + WH 30) (C), for 60 min (BLT + WH 60) (D), 3 hours (BLT + WH 3h) (E) and for 6 hours (BLT + WH 6h) (F); Histopathological scoring data of lung injury were presented in a box-whisker plot (the boxes are constructed with 25% and 75% confident intervals, median and maximum or minimum individual values) (G); Lung water content represented by wet weight/dry weight (H). Data are mean ± SD (n=12). * p < 0.05, ** p < 0.01 vs BLT.

**Figure 3:** The effects of whole body hypothermia treatment on liver and kidney water content, biochemistry marker of liver and kidney function in blast limb trauma rats. Liver water content (A); Serum aspartate aminotransferase (AST) (U/L) (B); Serum alanine aminotransferase (ALT) (U/L) (C); Kidney water content (D); Serum Creatinine (Crea) (µmol/L) (E). Data are presented as mean ± SD (n=12). * p < 0.05, ** p < 0.01 vs BLT. Data are presented as mean ± SD (n=12). * p < 0.05, ** p < 0.01 vs BLT.
Figure 4: The effects of whole body hypothermia treatment on plasma cytokines and hydrogen sulfide level in blast limb trauma rats. Tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), IL-10 (C) and hydrogen sulfide (H₂S) (D) in plasma. Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 5: The effects of whole body hypothermia treatment on myeloperoxidase (MPO) activity in blast limb trauma rats. MPO activity in plasma (A), Muscle(B), Lung (C),Liver(D) and Kidney(E). Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 6: The effects of whole body hypothermia treatment on total anti-oxidation of capacity (T-AOC) activity in blast limb trauma rats. T-AOC in muscle(A), Lung (B),Liver(C) and Kidney (D).Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 7: The effects of whole body hypothermia treatment on superoxide dismutase (SOD) activity in blast limb trauma rats. SOD activity in plasma(A), Muscle(B), Lung (C),Liver(D) and Kidney(E). Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 8: The effects of whole body hypothermia treatment on glutathione (GSH) level in blast limb trauma rats. GSH activity in plasma(A), Muscle(B), Lung (C), Liver(D) and Kidney(E). Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 9: The effects of whole body hypothermia treatment on malondialdehyde (MDA) level in blast limb trauma rats. MDA activity in plasma (A), Muscle (B), Lung (C),Liver(D)and Kidney(E). Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.

Figure 10: The effects of whole body hypothermia treatment on hydrogen peroxide (H₂O₂) level in blast limb trauma rats. H₂O₂ activity in plasma (A), Muscle(B), Lung (C),Liver(D) and Kidney(E).Data are mean ± SD (n=12). *p< 0.05, ***p < 0.01 vs BLT.
Figure 1
Figure 3

A) Liver water content

B) Serum AST (U/L)

C) Serum ALT (U/L)

D) Kidney water content

E) Serum Crea (μmol/L)
Figure 4

(A) Plasma TNFα (pg/ml)

(B) Plasma IL-6 (pg/ml)

(C) Plasma IL-10 (pg/ml)

(D) Plasma H₂S (µmol/L)

*p < 0.0001

*p = 0.045

*p = 0.01

*p = 0.018

*p = 0.023

*p = 0.042

*p = 0.002

Legend:
- Sham
- BLT
- BLT-WH30
- BLT-WH60
- BLT-WH3h
- BLT-WH6h

Download Figure(s) FIG 4.jpg
Figure 5

A. Plasma MPO (U/L)
B. Muscle MPO (U/g protein)
C. Lung MPO (U/g protein)
D. Liver MPO (U/g protein)
E. Kidney MPO (U/g protein)
Figure 6

A. Muscle T-AOC (U/mg) for Sham, BLT, BLT-WH30, BLT-WH60, BLT-WH3h, and BLT-WH6h. The p-values are p<0.0001, p=0.001, and p=0.038.

B. Lung T-AOC (U/mg) for Sham, BLT, BLT-WH30, BLT-WH60, BLT-WH3h, and BLT-WH6h. The p-values are p<0.0001.

C. Liver T-AOC (U/mg) for Sham, BLT, BLT-WH30, BLT-WH60, BLT-WH3h, and BLT-WH6h. The p-values are p<0.0001 and p<0.0001.

D. Kidney T-AOC (U/mg) for Sham, BLT, BLT-WH30, BLT-WH60, BLT-WH3h, and BLT-WH6h. The p-values are p<0.0001, p=0.001, p=0.008, and p=0.014.
Figure 7
Figure 8
Figure 10
Figure 9
Supplemental Table 1: Rectal temperature and mean artery blood pressure (mean± SD; n = 12).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Sham</th>
<th>BLT</th>
<th>BLT-WH30</th>
<th>BLT-WH60</th>
<th>BLT-WH3h</th>
<th>BLT-WH6h</th>
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<tbody>
<tr>
<td>RT (°C) Baseline</td>
<td>36.7±0.7</td>
<td>36.7±1.3</td>
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<td>31.8±0.5</td>
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<td>32.6±0.1</td>
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<tr>
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</table>

BLT= blast limb trauma; WH = whole-body hypothermia; 30 = 30 minute; 60 = 60 minutes; 3h = 3 hours; 6h = 6 hours. # p<0.05 vs BLT.
Supplemental Figure 1. Experimental protocols. Blast limb trauma (BLT) was created by being blasted of chartaceous electricity detonators which were fixed on the junction between foot and limb of rats. BLT rats were randomly treated with whole-body hypothermic (WH) treatment for 30 min, 60 min, 3 hours and 6 hours, or normothermia. They were rewarmed spontaneously at about 1.5°C per hour at the end of each hypothermia period. The experiments were terminated at 6 hours after blast.