Effects of Various Body Temperatures After Lipopolysaccharide-Induced Lung Injury in Rats*

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Study objectives: In this study, we determined the effects of various body temperatures (BTs) after initiation of lipopolysaccharide (LPS)-induced lung injury.

Design and setting: Forty-nine, adult, male, Sprague-Dawley rats each weighing 300 to 400 g were used.

Methods: The treated rats were challenged with intraperitoneal (IP) administration of 5 mg/kg LPS. Control animals received IP saline solution injections. After 16 h, treated and control animals were anesthetized. The animals received direct intratracheal (IT) injection of LPS (1 mg/0.2 mL) or saline solution (control animals). A cooling or heating blanket was then used to control BT. The rats were randomly assigned to three control groups of mild hypothermia (34°C) plus saline solution, normothermia (37°C) plus saline solution, and mild hyperthermia (39°C) plus saline solution, and three LPS groups of mild hypothermia plus LPS, normothermia plus LPS, and mild hyperthermia plus LPS, where each condition was maintained for 5 h. The mean arterial pressure (MAP) and blood gas concentrations were measured. BAL was done in the left lung 5 h after the IT injection of LPS with temperature control. Parts of the right lung were excised for myeloperoxidase (MPO) and malondialdehyde (MDA) measurements, whereas the rest was collected for wet/dry (W/D) ratio determination.

Results: Normothermia plus LPS caused significantly increased W/D ratio, LDH activities, protein concentrations, and tumor necrosis factor-α concentrations in BAL fluid, and MPO activities and MDA levels in lung tissues when compared to saline solution control group. MAP and PaO₂ were significantly decreased. The pathologic picture also showed increased neutrophil infiltration in lung tissues. In contrast, treatment with mild hypothermia but not hyperthermia significantly attenuated these parameters when compared with the normothermia-plus-LPS group.

Conclusions: These experimental data suggest that mild hypothermia applied after initiation of acute lung injury induced by LPS in rats had a protective effect by inhibiting the inflammatory reaction.

Key words: acute lung injury; ARDS; hyperthermia; hypothermia; lipopolysaccharide; tumor necrosis factor-α

Abbreviations: BALF = BAL fluid; BT = body temperature; IL = interleukin; IP = intraperitoneal; IT = intratracheal; LDH = lactate dehydrogenase; LPS = lipopolysaccharide; MAP = mean arterial pressure; MDA = malondialdehyde; MPO = myeloperoxidase; PMN = polymorphonuclear neutrophil; TNF = tumor necrosis factor; W/D = wet/dry

Gram-negative bacterial sepsis may lead to severe hypoxemia and noncardiogenic pulmonary edema due to lung vascular injury and increased permeability (ARDS).1 In the ICU, ARDS is still an important cause of morbidity and mortality. Many investigations revealed that lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, has an important role in the development of ARDS and induced a series of cell activations and production of inflammatory mediators.2 Investigations focused on the
potential roles of numerous cellular agents and their mediators that resulted in ARDS and acute edematous lung injury. Nonetheless, several interventions and pharmacologic treatments such as antioxidants, inhaled nitric oxide, and various anti-inflammatory agents in ARDS showed no success in human studies.\(^1\)\(^,\)\(^3\) Currently, the only lung protection strategy is the use of low tidal volume ventilation, which has proved to reduce mortality by 22\%.\(^1\)

The manipulation of temperature has been used clinically and experimentally as a therapeutic modality. Mild hypothermia is reported to be effective attenuating traumatic head injury and ischemic brain injury in a number of studies.\(^4\)\(^,\)\(^5\) In contrast, hyperthermia may exacerbate brain ischemic injury following global ischemia and traumatic brain injury.\(^6\)\(^,\)\(^7\) Previously, there have been few investigations\(^8\)\(^,\)\(^9\) concerning pretreatment with hypothermia for acute lung injury. Nonetheless, it is impractical for clinical use. The effect of hypothermia after initiation of acute lung injury was only restricted to one small human study and several case reports.\(^5\) The effect of hyperthermia in LPS-induced acute lung injury has not been reported. The purpose of this study was to examine the effects of various body temperatures (BTs) immediately after LPS-induced lung injury. The degree of lung injury was assessed by various parameters including wet/dry (W/D) weight ratio, myeloperoxidase (MPO) activity, malondialdehyde (MDA) level, pathologic change in lung tissue, and protein concentration, LDH level, and tumor necrosis factor (TNF)-\(\alpha\) level in BAL fluid (BALF). Blood oxygenation and mean arterial pressure (MAP) were also measured.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats weighing 300 to 400 g were used in this study. All the experimental procedures were in accordance with the National Institutes of Health guidelines,\(^10\) and approval of the project protocol was obtained from the National Science Council and Animal Review Committee at the National Defense Medical Center.

**LPS-Induced Lung Injury**

In preliminary experiments, to determine the appropriate method necessary to produce acute lung injury, the rats were challenged with intraperitoneal (IP) administration of 5 mg/kg LPS (Escherichia coli, serotype 055, B5; Sigma Chemical; St. Louis, MO). Control animals received IP normal saline solution injections at 40 mL/kg. After 16 h, treated and control animals were anesthetized. The trachea was surgically exposed, and the animals received direct intratracheal (IT) injection of LPS (1.0 mg/0.2 mL) or saline solution. Five hours later, the rats underwent tracheal intubation and midline thoracotomy. The right lung was clamped and excised for MPO measurement and W/D weight ratio determination. BAL was done to the left lung. The results showed the rats with IP and IT LPS injections had significant lung injury when compared to the saline solution control group, only IP injection, or only IT injection (data not shown).

**Temperature Control**

During 16 h after IP injection of LPS, the rats were housed in temperature-controlled units (25°C) with food and water freely accessible. Rectal temperature was measured every 4 h; there was no significant difference between LPS and saline solution groups at any of time points examined. After IT injection, the rats were placed on a heating or cooling blanket with their bellies down. In the normothermic groups, the rectal temperature was maintained at 37 ± 0.5°C (mean ± SEM). In the mild hypothermia group, the rectal temperature was reduced to 34 ± 0.5°C. In the mild hyperthermia group, the rectal temperature was elevated to 39 ± 0.5°C. The target temperature was reached in 10 to 20 min.

**Experimental Protocol**

Rats were classified into six experimental groups, with seven rats per group, as follows (Fig 1): (1) mild hypothermia plus saline solution, where the rats received IP and IT injections of normal saline solution; the rats were maintained at a rectal temperature of 34°C for 5 h; (2) normothermia plus saline solution, where the rats received IP and IT injections of normal saline solution; the rats were maintained at a rectal temperature of 37°C for 5 h; (3) mild hyperthermia plus saline solution, where the rats received IP and IT injections of normal saline solution; the rats were...
maintained at a rectal temperature of 39°C for 5 h; (4) normothermia plus LPS, where after IP and IT injections of LPS, the rats were maintained at a rectal temperature of 37°C for 5 h; (5) mild hypothermia plus LPS group, where after IP and IT injections of LPS, the rats were maintained at a rectal temperature of 34°C for 5 h; and (6) mild hyperthermia plus LPS, where after IP and IT injections of LPS, the rats were maintained at a rectal temperature of 39°C for 5 h.

BAL

BAL was performed on the left lung using 5 mL of phosphate-balanced saline solution in 2.5-mL aliquots after cannulation of the left trachea. The recovered BALF was centrifuged at 250g for 10 min. Lactate dehydrogenase (LDH) activity was measured using the method described by Vassault. In brief, part of the supernatant was incubated with 0.24 mM nicotinamide adenine dinucleotide in Tris/NaCl buffer (pH 7.2) at room temperature for 5 min. The reaction was then started by the addition of 0.8 mM pyruvate and followed by spectrophotometry at 340 nm for 2 min. The protein concentration of the supernatant was determined using bicinchoninic acid protein assay reagents (Pierce; Rockford, IL). The concentration of TNF-α in the BALF was measured using an enzyme-linked immunosorbent assay kit (Diaclone; Canton, MA).  

Determination of MPO Activity

A spectrophotometric method was used to determine MPO activity in the lung tissue. In brief, the specimen was freeze thawed and sonicated three times. Homogenates were centrifuged at 15,000g for 10 min at 4°C. A 100-μL aliquot of supernatant was mixed with 900 μL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL of o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. One unit of peroxidase activity equaled the amount of enzyme decomposing 1 μmol of hydrogen peroxide per minute at 25°C. Decomposition of hydrogen peroxide was calculated from the oxidation of o-dianisidine using an absorption coefficient of 11.3/mM/cm at 400 nm.

Determination of MDA Levels

MDA levels in lung tissue were determined as an indicator of lipid peroxidation. Lung tissue was homogenized in 1.15% KCL solution. An aliquot (100 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1% thiobarbituric acid and 700 μL of distilled water. Samples were then boiled for 30 min at 100°C and centrifuged at 3,000g for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm.

W/D Weight Ratio

Lungs excised at the end of the experiment were weighed for determination of the final wet lung weight. The right lung was placed in an oven at 60°C for 48 h to allow determination of the W/D weight ratio.

MAP and Oxygenation

MAP was monitored by cannulating the femoral artery with a catheter connected to a pressure transducer (Model 023XL; Spectramed; Stratham, CA). The arterial blood samples were measured for PaO₂ and carbon dioxide, pH, and base excess using a blood gas analyzer (BGM IL-112; Instrumentation Laboratory; Milan, Italy) during the baseline period, and 3 h and 5 h after temperature assignment.

Lung Histology

The right lower lung lobes were taken for histologic examination. The tissues were immersed in 10% formaldehyde fixative for 24 h. The lung lobes were then washed for 8 h with tap water to remove the formaldehyde. For light microscopy, the lung tissue was dehydrated with graded alcohols (70%, 80%, 90%, 95%, and absolute alcohol, each concentration for 45 min), put into xylene for 1 h, and then embedded in paraffin at 60°C. A series of 5.0-μm sections were cut and stained with hematoxylin-eosin. Slides were viewed using an Axiosplan Microscope (Zeiss; Oberkochen, Germany). The number of polymorphonuclear neutrophils (PMNs) in the lung interstitium was determined as the average number of PMNs per high-power field (400 ×). A minimum of 10 fields were randomly examined by a blinded observer.

Statistical Analysis

The data are expressed as mean ± SEM. Statistical differences among groups were determined using one-way or two-way, repeated-measures analysis of variance, followed by a post hoc comparison using the Newman-Keuls test. Comparisons within each group for a given parameter were performed using paired Student t test. We considered a value of p < 0.05 to be statistically significant.

Results

IP and IT injections of LPS significantly increased the mean protein concentrations, LDH activity, and TNF-α levels in BAL fluid when compared to the control group. Treatment with mild hypothermia significantly ameliorated the increase in protein concentrations, LDH activity, and TNF-α level in BALF in normothermia-plus-LPS groups (Fig 2, 3) [p < 0.05].

Figure 4 shows the changes in W/D lung weight ratios. IP and IT injections of LPS induced a significant increase in W/D ratio when compared to control group. Treatment with mild hypothermia but not mild hyperthermia lowered W/D ratios when compared with the normothermia-plus-LPS groups (p < 0.05).

LPS induced significant increases of MPO and MDA activities in lung tissues compared to that in saline solution control group. Treatment with mild hypothermia significantly attenuated the increase in MPO activity and MDA level in LPS-treated lung tissues when compared with the normothermia-plus-LPS group (p < 0.05). However, the MDA activity of lung tissue significantly increased in the mild hyperthermia-treated LPS group when compared with the normothermia-plus-LPS group.

Hyperthermia treatment significantly increased the PaO₂ by 3 h and 5 h after LPS injection compared to the normothermia-plus-LPS group (105.4 ± 7.5 mm Hg vs 73.0 ± 10.4 mm Hg, and 108.0 ± 7.8 mm Hg vs 64.0 ± 8.3 mm Hg, respectively) [p < 0.05]. The PaO₂ in the hyperthermia-treated LPS groups did...
not differ from that of the normothermia-plus-LPS group at any time point. Hypothermia treatment significantly increased the MAP by 3 h and 5 h after LPS administration compared to the normothermia-plus-LPS group (108.0 ± 4.5 mm Hg vs 80.0 ± 5.4 mm Hg, and 92.0 ± 4.1 mm Hg vs 73.0 ± 5.1 mm Hg, respectively) \( p < 0.05 \). The MAP in the hyperthermia-treated LPS groups did not differ from the normothermia-plus-LPS group at any time point.

In the lung pathology of the normothermia-plus-LPS group, marked inflammatory cell infiltration was observed, with neutrophils in the interstitium and alveoli of the lungs. Interstitial edema and vascular congestion were also observed. Mild hypothermia significantly attenuated neutrophil accumulation in the lungs of the rats receiving normothermia plus LPS. The numbers of neutrophils per high-power field (400-fold magnification)
were significantly higher (p < 0.05) in the normothermia-plus-LPS group (324.6 ± 15.6) than normothermia-saline solution group (162.6 ± 8.0). In contrast, hypothermic treatment of LPS-injected rats significantly attenuated neutrophil numbers (221.3 ± 14.0) when compared with the normothermia-plus-LPS group (p < 0.05). The number of neutrophils (336.3 ± 13.8) in the hyperthermic-treated LPS groups did not differ from the normothermia-plus-LPS group (Fig 7).

Figure 3. Effects of various BTs (mild hypothermia [34°C], normothermia [37°C], and mild hyperthermia [39°C]) on the TNF-α concentration in the BALF. *p < 0.05 as compared with the normothermia-plus-LPS group.

Figure 4. Effects of various BTs (mild hypothermia [34°C], normothermia [37°C], and mild hyperthermia [39°C]) on the W/D weight ratio of rats. *p < 0.05 as compared with normothermia-plus-LPS group.
**DISCUSSION**

LPS have been administered intraperitoneally or intratracheally to evoke an acute lung injury in rats.\(^{14,15}\) Previously, we found that acute lung injury induced by the IP route prominently resulted in cardiovascular collapse with mild acute lung injury rather than significant hypoxemia and pulmonary edema.\(^{16}\) Variability in the response of animal to a different approach of LPS can arise from the activity of commercially available LPS and dosage. Different
lots of the same LPS product can vary in its activity. This might be the reason for disparate observations reported in studies using similar doses of LPS. Because IP or IT injection of LPS can cause accumulation of PMNs in the lung tissue, it is reasonable to postulate that IT injection of LPS after IP injection of LPS may result in a more robust PMN influx into the alveolar space and increase in vascular permeability. Therefore, we tried to add IT instillation of LPS, producing more severe lung injury. Our pilot experiment showed the rats with IP and IT LPS injections had significant lung injury when compared to the saline solution control group, only IP injection, or only IT injection (data not shown). A disadvantage of the model is that it needs close observation of the reaction of rats between IP and IT injection.

The present study demonstrated that our LPS-induced lung injury model significantly increased W/D ratio, LDH level, protein concentration, and TNF-α level in BALF, and MPO activity and MDA level in lung tissue, and decreased PaO₂ and MAP. After initiation of LPS-induced lung injury, treatment with mild hypothermia but not mild hyperthermia attenuated the increase of W/D ratio, LDH level, protein concentration, and TNF-α level in BAL fluid, MPO activity and MDA level in lung tissue, and MAP, thus improving lung pathology. The increase in PaO₂ in the mild hypothermia-treated group revealed that mild hypothermia indeed attenuated lung injury and improved lung oxygenation. These promising results obtained for LPS-induced lung injury raise the possibility that mild hypothermia may also be of therapeutic use when applied after acute lung injury.

The lower W/D ratio, LDH level, and protein concentration in the BALF of the mild hypothermia treatment group, compared to the normothermia or mild hyperthermia treatment groups, suggests that hypothermia decreases lung permeability in LPS-induced lung injury. This result is comparable to that of data showing that hypothermia protects against the increase of capillary permeability after intestine ischemia-reperfusion injury. Although our results did not explore the mechanism responsible for hypothermia protection following LPS administration, several possible mechanisms are known. The metabolic rate of all enzyme reactions is known to decrease during hypothermia. Many mediators such as cytokines, histamine, bradykinin, or proteases are involved in capillary permeability injury; hypothermia may decrease their synthesis or their release from cells. Evidence has accumulated to implicate oxygen radicals in the mechanism of acute lung injury caused by LPS. We showed that hypothermia significantly reduced lipid peroxidation. Our result was comparable to studies that showed that mild intraoperative or in vitro hypothermia significantly decrease the production of reactive oxygen intermediates from PMNs, and the production of reactive oxygen intermediates was closely associated with the change of temperature. Hypothermia is also speculated to diminish the degradation of adenosine triphosphate to hypoxanthine due to its effect...
on metabolic rate. Furthermore, hypothermia has been shown to greatly slow the rate of conversion of xanthine dehydrogenase to xanthine oxidase. Therefore, hypothermia may prevent the production of oxygen radicals, or slow the release of mediators of increased capillary permeability, thereby limiting capillary endothelial injury and edema.

PMNs are an important factor in the development of acute lung injury in animals. PMN accumulation after LPS-induced lung injury precipitated the pulmonary microvascular injury and subsequent ARDS. Depletion of PMNs in experimental models significantly decreases lung injury. Many animal experiments have documented that hypothermia decreases PMN accumulation in various conditions such as meningitis, peritonitis, and ischemia-reperfusion brain injury, consequently preventing cytotoxic and oxidative cell injury to the endothelium and epithelium. Our results are consistent with above reports that mild hypothermia attenuates PMN infiltration and MPO activity in lung tissue. Although the precise cellular mechanism responsible for decreasing PMN accumulation is not clear, it has been suggested that expression of endothelial and leukocyte adhesion molecules plays an important role. Diminished neutrophil recruitment following hypothermia treatment could be a result of inhibition of neutrophil adhesion to endothelium. Indeed, hypothermia is known to suppress endothelial and leukocyte adhesion molecular expression. On human umbilical vein endothelial cells treated with LPS, interleukin (IL)-1, or TNF-α, both expressions of E-selectin and neutrophil adherence were inhibited at 25°C. In an animal model of meningitis, hypothermia significantly decreased β1-integrin expression and activation of extravasated PMNs, and then decreased PMN infiltration. The expression of intercellular adhesion molecule-1 was also inhibited with mild hypothermia in a focal model of transient cerebral ischemia, experimental stroke, and brain inflammation in rats. In IL-1 stimulated human cerebral endothelial cells, hypothermia (32°C) reduced leukocytes rolling and adhesion; at the same time, the gene expressions of IL-1 and IL-8 were decreased. The transcription factor nuclear factor-κB was also down-regulated.

Many reports document an increase in inflammatory cytokines on BALF and serum in LPS-induced acute lung injury. The TNF-α level in BALF has been reported to increase in both experimental and clinical acute lung injuries. Release of TNF from the lungs after LPS stimulation amplified lung inflammation initiating the cytokines cascade, through release of chemotactic factors and by up-regulating the expression of leukocyte and endothelial cell adhesion molecules, resulting in activation of neutrophils. In our model, mild hypothermia treatment decreased TNF-α level in BALF. Similarly, hypothermia also decreased the level of TNF-α in the cerebral spinal fluid of experimental meningitis. Furthermore, moderate hypothermia decreased TNF-α production of peripheral blood mononuclear cells stimulated by LPS. It is probable that hypothermia reduced the metabolic rate and energy requirement of infiltrating cells, thereby decreasing cytokine release. The decreased level of TNF-α in the hypothermia-treated group may be, at least in part, responsible for the reduction in intrapulmonary neutrophil infiltration and consequent microvascular injury.

The decrease in MAP induced by LPS administration was significantly attenuated in the mild hypothermia-treated group. This result is similar to that in the report by Taniguchi et al., in which mild hypothermia prevented the reduction of systolic arterial pressure and decreased concentrations of nitrate/nitrite and TNF-α in plasma in endotoxin-induced shock. In addition, another study showed that hypothermia can decrease inducible nitric oxide synthase in lungs of endotoxemic rats. Overproduction of nitric oxide by endotoxin-mediated expression of inducible nitric oxide synthase had been demonstrated to induce hypotension. Whether or not the same mechanism was at work in our study requires further investigation.

It is not uncommon for patients with ARDS to present with elevated BT attributable to infection or other reasons. Nonetheless, whether or not there are beneficial or detrimental effects of mild hyperthermia in humans is not clear. Increasing the brain temperature to 39°C appeared to accentuate the severity of ischemic brain injury in rats and increase vascular permeability, exacerbate intraacellular acidosis and high energy phosphate dysfunction, and activation and adherence of neutrophils. In our study, the mild hyperthermia-treated LPS group increased lipid peroxidation when compared to the normothermia-plus-LPS group. These results were the same as those reporting that mild intraschema hyperthermia potentiated the increase of reactive oxygen species production, and this may be due to increase cerebral metabolic rate for oxygen. Recently, it was reported that hyperthermia increased pulmonary neutrophil recruitment and accelerated pulmonary oxygen toxicity.

Although our study showed that hypothermia attenuated LPS-induced lung injury, many questions remain to be answered regarding potential mild hypothermia treatment in ARDS before it is implemented in humans. After rewarming, whether or not the lung injury progressed again, infiltrating neutrophils reactivated, and the protection was temporarly
effective remains a question. We also cannot exclude delayed hypothermia from being beneficial in our model of acute lung injury. No other studies have described a time course of progression of lung injury after hypothermia treatment. The appropriate depth and the duration of hypothermia, and the speed and the extent of rewarming should be considered in future studies. Furthermore, many investigators are concerned with adverse effects when using hypothermia treatment, because hypothermia was an indicator of poor outcome in patients with sepsis and major trauma.5

In summary, our study showed that mild hypothermia applied after LPS administration can be beneficial. Further investigations needs to be done to understand the mechanism of lung protection by hypothermia, and possible clinical applications in ARDS should be considered.

References
5 Bernard SA, Buist M. Induced hypothermia in critical care medicine: a review. Crit Care Med 2003; 31:2041–2051
29 Rowin ME, Xue V, Irazuzta J. Hypothemia attenuates β1 integrin expression on extravasated neutrophils in an animal model of meningitis. Inflammation 2001; 25:137–144