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Effect of Hypothermia on Splenic Leukocyte Modulation and Survival Duration in Severely Septic Rats

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Abstract

Background—Therapeutic hypothermia in severe septic shock is associated with prolonged survival. We hypothesized that moderate hypothermia would prolong survival and modulate the inflammatory response in rats with septic shock by exerting its therapeutic effect on splenic leukocytes.

Materials and Methods—Severe septic shock was created in rats by cecal ligation and incision (CLI). One hour after CLI or laparotomy, rats were randomized to sham, normothermia (NT), or 4 hours of hypothermia (HT) followed by 2 hours of rewarming. Hypothermia ($31\pm1^{\circ}\text{C}$) was induced using a cooling blanket, and monitored via a rectal temperature probe.

Results—Survival duration was 2.78 ± 1.0 hours in NT rats and 8.33 ± 0.32 hours in HT rats ($n=8/\text{group}$, $p<0.0001$). In separate groups, three hours after CLI, the spleen weight was significantly smaller in NT rats (769 ± 100 mg) than in HT rats (947 ± 157 mg, $p=0.04$). Fluorescent immunostaining of formyl peptide receptors (FPR) on leukocytes in spleen tissue showed considerably higher FPR expression in HT rats than in NT rats. Significantly elevated pro-

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DISCLOSURE

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inflammatory cytokines and myeloperoxidase (MPO) enzyme in plasma were found in NT rats compared with HT rats. Anti-inflammatory cytokine, IL-10, was significantly higher in HT rats. Both pro-inflammatory cytokines and plasma MPO were significantly reduced in splenectomized NT rats.

Conclusions—Moderate hypothermic therapy significantly prolongs the survival duration of rats with severe septic shock. Hypothermia dampens the inflammatory response during septic shock by modulating the spleen to an anti-inflammatory mode and preventing the spleen from releasing activated splenic leukocytes into the blood.

Keywords

Hypothermia; septic shock; spleen; cytokines; inflammatory response

INTRODUCTION

Sepsis has become a major public health concern and its incidence is increasing.¹ Infection-induced sepsis is a leading cause of mortality and critical illness worldwide.^{1,2} An initial sepsis-related inflammatory response may quickly develop into septic shock and multiple organ dysfunctions — a leading cause of morbidity and mortality in these critically ill patients.^{3,4} The current treatment of septic patients mainly consists of source control, broad-spectrum antibiotics, and overall supportive care. This treatment protocol has been essentially unchanged over the past decade.³ The Surviving Sepsis Campaign, published in 2012, advocates early and aggressive fluid resuscitation, empiric antibiotics, identifying the source of infection and targeted therapy when applicable.⁵

The concept of therapeutic hypothermia during septic shock was advocated over forty years ago when Blair, et al. first described its therapeutic use in 1961.⁶ Since then, there have been multiple studies supporting and/or condemning the use of therapeutic hypothermia.^{7–12} In experimental studies, therapeutic hypothermia is protective against inflammatory responses and improves survival in animals with severe septic shock.¹³ Recent studies have shown that splenectomized animals that undergo cecal ligation and incision (CLI) have improved survival compared with their intact counterparts.^{14,15} Spleens harbor inflammatory cells such as T and B cells, monocytes and neutrophils. When the body detects a bacterial load in the bloodstream, the spleen is stimulated and plays a key role in clearance of bacteremia.^{16,17}

In the current study, we sought to identify the role of moderate hypothermia in the treatment of severe septic shock induced by CLI in rats. The role of the spleen in mediating the inflammatory responses during severe septic shock and the effect of moderate hypothermia on the spleen were investigated.

MATERIALS AND METHODS

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Eighth Edition, revised 2011) and was conducted under

protocols approved by the University of Virginia's Institutional Animal Care and Use Committee.

Animals

Adult male Sprague-Dawley rats (13–15 weeks old, Charles River Laboratories International, Inc, Wilmington, MA, USA) were used in the study. The rats were housed in a controlled environment with free access to food and water. They had a minimum of 7 days after arriving to become adjusted to their surroundings in accordance with protocol. All animals underwent either CLI or sham surgery (laparotomy only).

Cecal ligation and incision procedure

We used a severe sepsis model based on a previous study designed by Rim, et al.¹³ Given previous experiences with rat survival time, we used a CLI model targeting 100% mortality within 12 hours in the control group. Animals were placed in an induction chamber and general anesthesia was initiated with 3% isoflurane. Once fully anesthetized, an intramuscular (IM) injection of ketamine-HCL (50 mg/kg) and xylazine-HCL (10 mg/kg) was administered, with a repeat dose administered 4 hours later.

Under sterile conditions, a 2 cm vertical midline incision was made through the abdominal wall and the cecum was carefully extruded. The blind-ending distal half of the cecum was ligated with 4-0 silk suture (Ethicon, Inc, Somerville, NJ, USA) and a 0.5 cm incision was created along the antimesenteric portion of the cecum. Figure 1 illustrates the CLI procedure. The cecum was gently manipulated so stool spilled into the abdomen and 5 mL of sterile saline was flushed into the abdominal cavity to ensure diffusion of the stool. Abdominal fascia and skin were closed in two layers with running 5-0 Polysorb sutures (Medtronic, Minneapolis, MN, USA). We then gently massaged the abdomen to stimulate a diffuse peritonitis. The rats were then resuscitated with a dorsal subcutaneous injection of normal saline (warmed to 37°C) between the shoulders at a dose of 5 mL/100 g. Additional fluid (same dose and temperature) was administered at 3 and 5 hours following CLI. Additionally, the rats were administered SQ buprenorphine (0.05 mg/kg) for pain control at the conclusion of the surgical procedure.

Thermal therapy and survival analysis

Three groups of rats (n=8/group) were used for this portion of the experiment. Sham animals underwent laparotomy only, followed by continuous normothermia. All animals that underwent CLI were observed for 1 hour in their cage under a heat lamp in order to maintain normothermia (36–37.5°C via rectal temperature probe), and were then randomly divided into two groups: continuation of normothermia (NT), or 4 hours of hypothermia followed by rewarming to normothermia (HT), similar to the model of therapeutic hypothermia used by Rim, et al.¹³

Hypothermia was achieved initially by laying the rats on a bag of crushed ice until their core temperature dropped to 31°C. Target temperature was reached within 5–10 minutes of cooling. They were then placed in a cooling blanket and wrapped. Rats were maintained at a temperature of 30–32°C via rectal thermometer. After 4 hours of hypothermia, rewarming

was achieved over a 2-hour period by increasing the cooling/heating blanket by 1°C every 30 minutes to a goal of 36–37.5°C. Once the rats were adequately rewarmed, they were placed back in their cages. Normothermia was maintained thereafter with a heat lamp and confirmed by obtaining periodic rectal temperatures. Figure 2 summarizes the experimental protocol.

Harvesting spleen and plasma

Separate groups of rats (n=6/group) were randomized to Sham, NT, or HT. Three hours after surgery (laparotomy or CLI), animals were re-dosed with ketamine and xylazine and a repeat midline laparotomy was performed. The splenic hilum was located, clamped, and ligated with 3-0 silk suture. The spleen was excised and weighed. Following splenectomy, a right or left groin cut-down was performed to expose the femoral vein. A total of 3 mL of blood was obtained using a syringe rinsed with heparin. All of the blood samples were then centrifuged 3000 RPM for 20 minutes at 4°C. The plasma was harvested, stored at –80°C, and subsequently used for cytokine analysis. Plasma levels of IL-1 β , IL-6, IL-10, IL-17, TNF- α , MCP-1, and IFN- γ were measured using a multiplex enzyme-linked immunosorbent assay (ELISA) rat cytokine panel (Bio-Rad Laboratories, Hercules, CA, USA).

Fluorescence imaging of the spleen

Separate groups of rats (Sham, NT and HT groups, n=3/group) were used for near infrared fluorescence imaging of the spleen, along with measuring myeloperoxidase (MPO) levels in plasma and spleen. Two and half hours after laparotomy or CLI, the femoral vein was exposed through a groin cut-down and 10% cFLFLF-PEG-Cy7, a peptide probe specific to leukocytes expressing formyl peptide receptor-1 (FPR-1),^{18,19} was injected as a bolus dose (1 μ L/g body weight). Thirty minutes after injection, rats were re-anaesthetized and a repeat laparotomy was performed. Spleens were harvested as previously described and then imaged together for comparison and semi-quantitative signal intensity analysis. The imaging data was acquired on the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA, USA) and processed with Living Image software (Caliper, Hopkinton, MA, USA). Plasma was obtained as previously described. Plasma and spleen levels of MPO enzyme were measured using a commercially available ELISA assay (TSZ ELISA, Minneapolis, MN, USA).

Splenectomy group

One additional group of animals underwent splenectomy just prior to CLI, followed by the normothermia protocol (NT+Spx). Three hours after CLI, plasma was obtained from these animals as previously described and used as an additional control group for measurement of MPO and cytokine levels.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation. Survival data were analyzed using the Kaplan-Meier method. All other data were compared using one-way ANOVA followed

by t-test for unpaired data with Bonferroni correction. $P < 0.05$ was used to determine statistical significance.

RESULTS

Thermal therapy and survival analysis

Severe septic shock by CLI in this study created 100% mortality within 4 hours in control rats (NT), with mean survival duration of 2.78 ± 1.0 hours. Moderate hypothermia nearly tripled the duration of survival in HT rats (8.33 ± 0.32 hours, $p < 0.0001$) (Figure 3). HT rats were visibly more active after rewarming and required 2–3 times more anesthetic drugs than NT rats at the time of blood and spleen harvest.

Spleen weights

Mean spleen weight was significantly less in NT rats than in Sham rats (769 ± 100 mg vs. 1085 ± 74 mg, $p = 0.0008$). In HT rats, spleen weight was significantly increased compared with NT rats (HT: 947 ± 157 mg, $p = 0.04$, Figure 4A). The mean body weight for all animals used in this part of the study ($n = 18$) was 482.6 ± 69.6 g, with no significant differences between groups ($p = 0.5$, Figure 4B).

Fluorescence imaging of the spleen

cFLFLF, a short peptide probe that selectively binds to leukocytes expressing FPR-1, was used to evaluate leukocyte activation and release from spleens. In rats that received cFLFLF-PEG-Cy7, near-infrared fluorescence imaging at 3 hours after CLI showed remarkably smaller spleens in NT rats with much less fluorescence compared with HT and Sham rats (Figure 5A). The semi-quantitative analysis of fluorescence density, as assessed by regions of interest, revealed a significant decrease in density in NT spleens (23% of Sham spleens) as compared to HT spleens (50% of Sham spleens, Figure 5B).

MPO and cytokine levels

Levels of MPO in plasma and splenic tissue were measured 3 hours after CLI (Figure 6). There were very low levels of MPO enzyme in the Sham animals (plasma: 26.1 ± 9.8 ng/100 μ L, spleen: 1.2 ± 1.3 ng/100 μ g protein). In plasma, both NT and HT rats had significantly elevated MPO enzyme compared to Sham (NT: 289.1 ± 20.8 , $p < 0.001$; HT: 235.1 ± 15.9 ng/100 μ L, $p < 0.001$). However, the elevation in HT rats was significantly less when compared with NT rats ($p = 0.04$). Splenectomy before CLI further reduced the MPO level in the plasma (NT+Spx: 112.5 ± 23.6 , $p < 0.001$ vs. NT and HT, Figure 6A). Spleen MPO levels were similar in groups NT and HT (NT: 23.5 ± 7.4 vs. HT: 23.3 ± 11.2 , $p = 1.0$), and both were significantly elevated compared to Sham ($p = 0.037$ vs. NT, $p = 0.039$ vs. HT, Figure 6B).

Cytokines/chemokines were measured from the peripheral blood at 3 hours after CLI using a multiplex ELISA rat cytokine panel. The pro-inflammatory cytokines, IL-1 β , IL-6, IL-17, TNF- α , and IFN- γ , were significantly elevated in NT rats compared with Sham and HT rats. IL-10 and MCP-1 were significantly more elevated in HT rats than in NT and Sham rats (Figure 7). Splenectomy in NT rats reduced all the pro-inflammatory cytokines measured (Figure 7).

DISCUSSION

The present study confirmed that moderate hypothermia is beneficial in the setting of severe septic shock, and in our rat model, lead to significantly longer survival compared with normothermia. Additionally, septic rats exposed to normothermia demonstrated significantly less splenic leukocytes as measured by Cy7 near-infrared fluorescence imaging compared with HT or sham rats. When plasma was subsequently evaluated, levels of MPO were higher in NT rats compared with HT rats, indicating greater neutrophil activation in NT rats. NT rats that received preoperative splenectomy (NT+Spx), demonstrated a significant reduction in plasma MPO levels. Levels of proinflammatory cytokines were decreased in HT rats, along with an increase in IL-10. Likewise, proinflammatory cytokine levels were decreased in NT+Spx rats. Taken collectively, these results indicate that the spleen plays a significant role in the proinflammatory response during severe sepsis, and that hypothermia may modulate the spleen to a more anti-inflammatory role, inhibiting it from releasing leukocytes and proinflammatory cytokines.

The high incidence of morbidity and mortality in septic shock is primary due to the body's initial, and often massive, cytokine release and the inflammatory response to the offending bacterial agent. In a related study, the Hypothermia After Cardiac Arrest Study Group demonstrated that mild hypothermia is recommended in patients with post-resuscitation syndrome to prevent ischemia-reperfusion injury, resulting in better neurological outcome and reduced mortality.²⁰ The dysregulated production of cytokines, the central feature of post-resuscitative syndrome, mimics the immunological disorders observed in sepsis.^{21,22} Significant proinflammatory cytokine release (IL-1, IL-6, TNF- α , IL-10) ultimately results in the damage to various organs in the septic patient.²³ Prior studies have shown that the inflammatory cytokine, IL-6, has been proven to be a predictive marker of disease severity in CLI models.¹³ In addition, a high level of IL-6 has been shown to be associated with a higher mortality in animal and human sepsis studies.^{23,24} The ratio of IL-6 to IL-10 (an anti-inflammatory cytokine) was also reported to be a predictor of sepsis outcome, as it represents equilibrium between pro-inflammatory and anti-inflammatory responses.¹³ An increase in the ratio of IL-6 to IL-10 correlated with poor outcome.²⁵

In the current study, we found a significant surge of pro-inflammatory responses in a CLI-induced severe sepsis rat model. Consistent with studies in the literature, we found that pro-inflammatory cytokines, IL-1 β , IL-6, IL-17, IFN- γ and TNF- α , were significantly elevated at 3 hours following CLI in normothermic control rats (NT), which all died within 4 hours following CLI. MPO levels were also significantly increased in the plasma and spleen after CLI. MPO activity correlates with increased neutrophil activity, the presence of inflammation, and the onset of sepsis.²⁶ Hypothermia significantly reduced the levels of these pro-inflammatory cytokines and decreased MPO in the plasma, and also increased the levels of IL-10 and MCP-1. Additionally, hypothermia significantly prolonged the duration of survival. It appears as though hypothermia moderated the inflammatory balance towards an anti-inflammatory state, thus prolonging survival. In addition to reduced cytokine and MPO release, hypothermia may have extended survival through additional mechanisms, such as reduced cellular metabolism, reduced oxygen consumption, and minimization of

cellular hypoxia, all of which have been shown to contribute to neuroprotection during cardiac resuscitation.^{27,28}

We also found that splenectomy abrogated the pro-inflammatory responses in NT rats, implicating the spleen as an important contributor to the inflammatory response during CLI-induced septic shock (Figures 6 and 7). Interestingly, IL-10 remained elevated in splenectomized NT rats. This indicates that IL-10 is also secreted by non-splenic sources during septic shock. The activation of the spleen during septic shock was characterized by an exodus of splenic leukocytes, likely neutrophils, as represented by splenic weight loss, elevated plasma MPO levels, and reduced expression of FPR positive cells in the spleen (Figures 4–6).

FPRs are members of the seven-transmembrane, G-protein coupled receptor superfamily and are highly expressed on neutrophils and mononuclear phagocytes. FPRs are involved in host defense and in sensing cellular dysfunction. Activated monocytes and neutrophils overexpress FPRs.^{29,30} In this study, FPRs were visualized using a ligand of FPR1, cFLFLF-PEG-Cy7.^{18,19} We found the density of fluorescence in the spleen of NT rats was remarkably less than Sham and HT rats (Figure 5). This is potentially due to the exodus of activated splenic neutrophils during sepsis, which correlates with the changes observed in spleen weights and MPO levels (Figures 4 and 6).

There is little published literature on the role of the spleen in sepsis and the risk/benefit of splenectomy in severe sepsis. It has previously been shown that splenectomy reduces the inflammatory responses and protects animals against septic lethality.^{15,31} The spleen is a reservoir for lymphocytes, neutrophils and monocytes and may release these cells in response to inflammatory signals.³² The cytokines measured in the current study could be released from all leukocyte subtypes. The effect of hypothermia on the inflammation derived from circulating leukocytes is minimal considering that splenectomy decreased cytokine levels significantly more (Figure 7). However, the splenectomy could not completely abrogate the rise in MPO in the plasma (Figure 6). This increase in plasma MPO in splenectomized rats is likely due to the upregulation of either circulating leukocytes or released bone marrow leukocytes. Possibly these leukocytes are either immature or aged compared to splenic leukocytes and thus are less important to the inflammatory responses. It is reported that immature circulating neutrophils have reduced phagocytic capacity compared with mature neutrophils, and likely lower levels of MPO secretion.³³ Overall, we found that the spleen contributes importantly to and mediates the inflammatory responses during septic shock. Hypothermia may modulate spleen-derived cytokines and block the release of inflammatory cells into the circulation, thus reducing the inflammatory response.

Limitations

Besides therapeutic hypothermia, we did not treat this post-CLI severe septic shock with broad-spectrum antibiotics or source control with abdominal washout. The duration of 4-hour hypothermia is arbitrary. It would be more clinically relevant to study the effect of brief hypothermic therapy versus prolonged hypothermia under the background of infectious source control and antibiotic treatment. In future studies, we plan to evaluate the role of IL-10 in the prolongation of survival. Given the spleen as a major source of inflammatory

responses, early application of hypothermic therapy would be superior to delayed treatment, but the timing and the duration of the hypothermia remain to be determined. Also, it would be wise to determine how to modulate the spleen into an anti-inflammatory mode without inducing hypothermia, as hypothermia certainly comes with its own set of risks and side effects.

Conclusions

In conclusion, we found that inducing moderate hypothermia in a rat model of severe septic shock lead to modulation of the spleen to a more anti-inflammatory state and prolonged the duration of survival. The spleen contributed importantly to the pro-inflammatory responses during septic shock and was a major source of inflammatory cytokines. Moderate hypothermia may inhibit the spleen from releasing leukocytes and pro-inflammatory cytokines into the bloodstream and lead to production of more anti-inflammatory cytokines.

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Figure 1.
Illustration of cecal ligation and incision procedure through vertical midline laparotomy
(created by Anita Impagliazzo).

Figure 2.

Experimental protocol. NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision; Sham, laparotomy only.

Figure 3.
Role of moderate hypothermia on duration of survival in rats with severe septic shock (n=8/group). NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision.

Figure 4.

(A) Spleen and (B) body weights at 3 hours after cecal-ligation and incision (n=6/group). # p<0.001 vs. Sham, p=0.04 vs. HT. NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision; Sham, laparotomy only.

Figure 5.

(A) Representative *ex vivo* images of splenic formyl peptide receptors (FPRs) in the spleen at 3 hours after cecal-ligation and incision in Sham, NT and HT rats. FPRs bound to Cy7-labeled cFLFLF-PEG and were imaged with fluorescent camera. (B) Fluorescence densities of imaged spleens (n=3/group). X p<0.05 vs. Sham, # p<0.05 vs. NT. NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision; Sham, laparotomy only; ROI, region of interest.

Figure 6.

(A) Plasma and (B) splenic tissue myeloperoxidase (MPO) levels at 3 hours after cecal-ligation and incision (n=3/group). X p<0.05 vs. Sham, # p<0.05 vs. NT, ^ p<0.05 vs. HT. NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision; Sham, laparotomy only.

Figure 7.

Plasma cytokines/chemokines at 3 hours after cecal-ligation and incision (n=4/group). X p<0.05 vs. Sham, # p<0.05 vs. NT, ^ p<0.05 vs. HT. NT, rats exposed to normothermia after cecal-ligation and incision; HT, rats exposed to 4-hour period of hypothermia after cecal-ligation and incision; Sham, laparotomy only.