Induction of profound hypothermia modulates the immune/inflammatory response in a swine model of lethal hemorrhage

Zhang Chen a, Huazhen Chen a, Peter Rhee a,b, Elena Koustova a, Eduardo C. Ayuste a, Kaneatsu Honma a, Amal Nadel a, Hasan B. Alam a,c,d,∗

a Trauma Research and Readiness Institute for Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
b Department of Surgery, Los Angeles County-University of Southern California Medical Center, Los Angeles, CA, USA
c Department of Surgery, Georgetown University Medical School, Washington, DC, USA
d Department of Surgery, Washington Hospital Center, Washington, DC, USA

Received 24 August 2004; received in revised form 31 January 2005; accepted 31 January 2005

Abstract

Profound hypothermic arrest (“suspended animation”) is a new strategy to improve outcome following uncontrolled lethal hemorrhage (ULH). However, the impact of this approach on the immune/inflammatory response is unknown. This experiment was conducted to test the influence of profound hypothermia on markers of immune/inflammatory system.

Methods: ULH was induced in 32 female swine (80–120 lb) by creating an iliac artery and vein injury, followed 30 min later by laceration of the descending thoracic aorta. Through a left thoracotomy approach, total body hypothermic hyperkalemic metabolic arrest was induced by infusing organ preservation fluids into the aorta using a cardiopulmonary bypass machine (CPB). Experimental groups were (1) normothermic controls (no cooling, NC), or hypothermia induced at the following rates: (2) 0.5 °C/min (slow, SC), (3) 1 °C/min (medium, MC) and (4) 2 °C/min (fast, FC). Vascular injuries were repaired during 60 min of profound (10 °C) hypothermic arrest. Hyperkalemia was reversed by hypokalemic fluid exchange, and blood was infused for resuscitation during re-warming (0.5 °C/min). The surviving animals were monitored for 6 weeks. Levels of IL-1, TNFα, IL-6, IL-10, TGF-β and heat shock protein (HSP-70) were measured by ELISA in serum samples collected serially during the experiment and post-operatively.

Results: Some of the immune markers were influenced by the use of CPB, independent of hypothermia (decrease in TGF-β and increase in IL-1). Hypothermia caused a significant decrease in IL-6, and an increase in HSP-70 expression compared to normothermic controls, independent of the cooling rate. An increase in IL-10 levels was noted which was influenced by the rate of cooling (p < 0.05, MC versus NC).

Conclusions: Profound hypothermia modulates the post-shock immune/inflammatory system by attenuating the pro-inflammatory IL-6, increasing anti-inflammatory IL-10 and augmenting the protective heat shock responses.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Hypothermia; Cytokine; Heat shock; Bypass; Thoracotomy; Vascular injury; Shock; Trauma

1. Introduction

Severe hypotension following trauma is an ominous sign and is associated with almost certain death unless the source of hemorrhage can be controlled promptly [1,2]. Strategies to maintain organ (especially brain) viability long enough to allow for surgical repair of treatable injuries may change the dismal outcome following traumatic exsanguination. Theoretically, induction of systemic hypothermia is a very promising approach to achieve this goal. Controlled induction of hypothermia for cellular protection is well established in the fields of cardiac, transplant and neurologic surgery [3–6], but not in trauma. However, a number of pre-clinical studies have shown that induction of hypothermia following severe hemorrhage can improve the outcome [7]. Using com-
plex animal models of trauma/shock it has been demonstrated that total body preservation (with profound hypothermia) can improve the outcome following lethal hemorrhage [8,9]. Although the protective properties of profound hypothermia have been clearly demonstrated, the mechanisms by which hypothermia protect cells remains to be studied. A decrease in cellular metabolism (decreased oxygen requirement) remains the most well described property of hypothermia. However, this is only one of the many possible mechanisms. Hemorrhagic shock with resuscitation, in addition to ischemic injury, have clearly been associated with activation of immune cells, generation of free radicals and an increase in immune mediated cellular damage [10]. Whether these pathways can be modulated through induction of therapeutic hypothermia remains to be studied. A decrease in cellular metabolism (decreased oxygen requirement) has been shown to decrease organ damage and improve survival after hemorrhagic shock, with [11] or without [12] alterations in post-resuscitation immune activation/inflammatory responses. The effect of profound hypothermia (<15 °C) on the immune system is largely unknown. In a recent experiment, we evaluated the impact of cooling rates on long-term outcome in a swine model of lethal hemorrhage. The data obtained from this study were divided into complimentary manuscripts for ease of presentation. The first manuscript described the physiologic changes during hypothermic arrest, performance of different organs and post-operative complications [13]. The current manuscript provides information about the impact of profound hypothermia on post-traumatic inflammatory response in these animals. This information was obtained by measuring circulating pro and anti inflammatory cytokines and heat shock protein (HSP) serially. Our hypothesis was that profound hypothermic resuscitation would attenuate the post-hemorrhagic shock immune activation and inflammatory response.

2. Methods

The institutional Animal Care and Use Committee approved this study. All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations regarding experiments involving animals. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996 edition. Strict aseptic technique was used for all surgical procedures.

2.1. Animal preparation

Thirty-two (n=8 per group) female Yorkshire swine (weight, 85–110 lb; Tom Morris Farms, Reistertown, MD) were anesthetized with an intra-muscular injection of ketamine (10 mg/kg) and inhaled isoflurane. After placement of a tracheal tube, the animals were allowed to breathe spontaneously while light anesthesia was maintained by administering isoflurane (0.5–1%) using a Narkomed M anesthesia machine (North American Dräger, Telford, PA). The right carotid artery and external jugular vein were cannulated with a 22G angio-catheter and 9F introducer sheath, respectively, using a cut down technique. A 7.5F oximetric thermodilution pulmonary artery catheter (Baxter Health Care Corp., Irvine, CA) was positioned in the pulmonary artery. The catheters were attached to a hemodynamic monitoring platform (Hewlett Packard, Paolo Alto, CA) and Baxter system (Explorer™, Baxter, Edwards Critical Care, Irvine, CA) was used for continuous monitoring of blood pressure, mixed venous oxygen saturation, and the standard pulmonary artery catheter variables (measured and derived). A left anterior lateral thoracotomy was performed through the fourth intercostal space. The animals were paralyzed (pancuronium) and switched to full ventilator support, and minute ventilation was adjusted to keep PaCO2 between 35 and 40 mmHg. The fraction of inspired oxygen (FiO2) was kept at the lowest possible level to maintain pulse oximetry readings above 95%.

2.2. Hemorrhage protocol

Through a lower abdominal incision, iliac vessels were exposed and two standardized longitudinal (medial and lateral) lacerations were made in the common iliac artery by passing a number 15 scalpel blade through the arterial walls [14]. A venous injury was simultaneously created by performing a 50% transection of a large branch of the inter nal iliac vein (uncontrolled arterial and venous hemorrhage). Animals were kept in shock for 30 min (simulating transport time to the hospital) before creating a 1.5 cm descending aortic laceration that caused lethal uncontrolled hemorrhage, which in previous experiments had resulted in 100% mortality unless hemorrhage was controlled promptly or hypothermic arrest induced rapidly. Animals were heparinized at the time of aortic injury (100 units/kg) and given a dose of dexamethasone (0.25 mg/kg). After 5 min of aortic hemorrhage, a catheter was placed into the aorta for the induction of hypothermic metabolic arrest as described below. During the 60 min of profound hypothermia (10 °C) the injured branch of the iliac vein was ligated and the iliac artery lacerations were repaired using running 6-0 monofilament sutures. Because of the asanguineous low-flow state, vascular repairs were technically easy to perform. The total blood loss before induction of hypothermia ranged between 1000 and 1500 mL (approximately 50% of estimated blood volume). The shed blood was saved in citrate phosphate dextrose solution (Abbott laboratories, Chicago, IL) for auto-transfusion.

2.3. Induction of asanguineous hypothermic metabolic arrest

We used techniques that have been developed in our laboratory and published previously [8,9]. Asanguineous hyper-
kalemic hypothermic arrest was induced by placing a prototype double lumen catheter into the aorta (through the injury site) and a standard venous cannula into the right atrium to initiate cardiopulmonary bypass (CPB). Standard CPB equipment (Gish Biomedical Inc., Santa Ana, CA) and roller pumps (Sarns Inc., Ann Arbor, Michigan) were used for this experiment. The reservoir was primed with 3 L of cold (2°C) high potassium (70 meq/L) organ preservation solution (Unisol-I® “intracellular type” UHK, Organ Recovery Systems Inc., Charleston, SC). During cooling, flow rate was kept at 3–4 L/min and the temperature of the heat exchanger was adjusted to achieve desired cooling rates. Experimental groups included:

1. Normothermic controls (NC): Core temperature maintained at 37°C
2. Slow cooling (SC): Induction of profound hypothermia at a rate of 0.5°C/min.
3. Medium cooling (MC): Induction of profound hypothermia at 1.0°C/min.
4. Fast cooling (FC): Induction of profound hypothermia at a rate of 2.0°C/min.

When the core temperature reached 20°C, 2 L of reservoir fluid were exchanged for low potassium fluid (Unisol-I® “intracellular type”, 25 meq/L, ULK, Organ Recovery Systems Inc., Charleston, SC). Once the core temperature reached 10°C, flow rates on the bypass machine were reduced to 10–20 mL/kg/min and heat exchanger adjusted only to maintain this temperature. The reservoir fluid was reduced to 10–20 mL/(kg min) and heat exchanger adjusted to achieve desired cooling rates. Experimental controls included:

1. Normothermic controls (NC): Core temperature maintained at 37°C.

Venous blood samples were obtained at the following time points: before operation (baseline), 30 min of hemorrhagic shock, start and end of profound hypothermic period, end of warming period, end of experiment and weeks 1, 3 and 6 after the experiment (surviving animals). The serum was separated by centrifuging blood samples at 3000 rpm for 15 min and saved at −80°C. The experiments for IL-6, IL-10, IL-1β, TGF-1β and HSP70 were done according to manufacturer’s protocol. Briefly, 100 µL of standards and serum samples (diluted 1:1) for IL-10, IL-1β (BioSource, Camarillo, CA) and HSP70 (Stressgen, Victoria, Canada), IL-6 (diluted 1:2) (R&D System, Minneapolis, MN) and 200 µL of standards and serum samples for TGF-1β (diluted 1:2) (BioSource, Camarillo, CA) were incubated in 96-well microtiter plates coated with those antibodies at 4°C overnight for IL-10, IL-1β, IL-6 and TGF-1β, at room temperature 3 h for HSP70 and 2 h for IL-6. The optical density of each well was determined with a microplate reader set at 450 nm (Dynatech Laboratories, Billingshurst, UK). The concentrations of these cytokines were determined by interpolation from a standard curve. Results were expressed as nanograms of antigen per ml for HSP70, picograms of antigen per ml for IL-6, IL-1β and TGF-1β. These markers were chosen because (1) alterations in their levels have been well described in shock and (2) reliability of these ELISA kits in swine has been established in our laboratory. We realize that many more markers/mediators could have been measured, but we had to limit our selection for logistical reasons. These animals were not instrumented chronically and required repeated anesthesia for blood sampling. To avoid excessive/repeated administration of anesthesia the sampling time points were also limited.

2.5. Statistical analysis

All data are presented as group means ± S.E.M. The SPSS statistical software program (SPSS/Windows, SPSS Inc., Chicago, IL) was used. One-way analysis of variance with Dunnett’s test for multiple comparisons was performed for all continuous variables and χ²-test was used to compare the survival rates. Significance was defined as p < 0.05.

3. Results

3.1. Measurement of circulating cytokines and immune markers

3.1.1. Heat shock protein-70 (HSP-70) response

HSP-70 levels increased gradually during the experiment in all animals (normothermic and hypothermic). For the hypothermic animals, the increase in HSP-70 achieved statistical significance at the end of warming period, and became even more pronounced by the end of experiment (p < 0.01). The HSP-70 expression was not influenced by the rate of cool-
4. Discussion

The findings from this experiment support other studies that have reported marked improvement in survival when profound hypothermia is induced following severe hemorrhage. Furthermore, by utilizing a realistic model (uncontrolled hemorrhage, open body cavities, major vascular injuries, operative trauma and repair of lethal injuries), and by monitoring for delayed complications, our study has added the much needed clinical relevance. The intra-operative hemodynamic changes, post-operative organ functions, and survival data has been reported in detail separately [13]. Briefly, the oxygen delivery was minimal (+50 mL/min), and total body oxygen consumption was non-measurable during the 60 min of asanguineous profound hypothemic arrest. There was no spontaneous cardiac activity, and the low flow CPB generated minimal (+20 mmHg) blood pressure during this period. Induction of profound hypothermia significantly attenuated the degree of lactic acidosis and base deficit compared to the normothermic controls. The biochemical abnormalities during the post-operative period were transient and improved rapidly. These included an increase in the levels of liver aminotransferases, serum creatine kinase and lactate dehydrogenase, and a non-significant increase in serum creatinine. All of these returned to baseline within the first week and there was no significant long-term organ dysfunction. The 6-week survival rates were 0% (NC), 37.5% (SC), 62.5% (MC) and 87.5% (FC) respectively (p < 0.05, MC and FC versus NC). All of the normothermic control animals were found to be clinically brain dead on reversal of anesthesia. Most of the deaths in hypothemic groups were due to early cardiac failure. This manifested either as failure to come off CPB or as cardiac arrest soon after discontinuation of CPB. All of the surviving animals were neurologically intact. There were two late deaths due to sepsis (intra-tho-
Fig. 2. Levels of circulating pro-inflammatory cytokines in different experimental groups: (a) interleukin-1β and (b) interleukin-6. Data presented as group means ± S.E.M. (*) *p < 0.05 compared to its own baseline. (♦) ♦p < 0.05 compared to no cooling group at the same time point. Sampling time points are: B, baseline; AH, after 30 min of uncontrolled hemorrhage; SH, start of 60 min period of profound hypothermia (10°C); EH, end of 60 min period of profound hypothermia (10°C); EW, end of warming period; EE, end of experiment (death, or successful detachment from cardiopulmonary bypass and ventilator); W1–6, post-operative weeks 1–6.

Fig. 3. Levels of circulating anti-inflammatory cytokines in different experimental groups. (a) Transforming growth factor-β and (b) interleukin-10. Data presented as group means ± S.E.M. (*) *p < 0.05 compared to its own baseline. (♦) ♦p < 0.05 compared to no cooling group at the same time point. Sampling time points are: B, baseline; AH, after 30 min of uncontrolled hemorrhage; SH, start of 60 min period of profound hypothermia (10°C); EH, end of 60 min period of profound hypothermia (10°C); EW, end of warming period; EE, end of experiment (death, or successful detachment from cardiopulmonary bypass and ventilator); W1–6, post-operative weeks 1–6.

Influence of hypothermia on outcome depends upon multiple variables. These include: the clinical scenario, depth and duration of hypothermia, strategies used for induction of hypothermia, rate of cooling and warming and time lag between injury and initiation of hypothermia, just to name a few. Even slow induction of mild hypothermia (33–34°C) has been shown to improve the outcome in patients with out of hospital cardiac arrest in prospective randomized trials [15,16]. When induced in the setting of hemorrhagic shock, mild to moderate hypothermia can delay significantly the onset of cardiac arrest (2–4 folds) [17,18] and may even prevent it completely [19,20]. However, this approach fails to improve survival when massive blood loss has led to a state of arrest (or near arrest). In this scenario, rapid induction of deep (profound) hypothermia (<15°C) remains the only therapeutic approach with the potential to alter the dismal outcome [21,22]. Patients in profound hemorrhagic shock (pulless or systolic BP less than 50 mmHg) after penetrating truncal trauma are candidates for emergency department thoracotomy as a life saving measure. The overall survival following this procedure is approximately 7.0% [23]. However, thoracotomy can also provide an excellent access for induction of
profound hypothermia (infusion of cold fluids into the aorta, without or without CPB). Hypothermia exerts its beneficial effects through several pathways. A very predictable effect of temperature alteration is a change in cellular metabolism. The $Q_{10}$ (temperature coefficient) is a useful way of explaining this effect. For the whole body $Q_{10}$ is about 2.0, suggesting a 50% reduction in metabolism for each 1°C decrease in body temperature [24]. Furthermore, hypothermia has been shown to decrease the production of free radicals [25], cytokine release [26] and expression of adhesion molecules [27]. It also modulates the expression of various oxidative stress proteins after regional ischemia-reperfusion [28]. However, no studies have been published describing the immunological consequences of total body profound hypothermia, induced after uncontrolled hemorrhage and major injuries.

The findings of this study need explanation, as many variables known to have an impact on cytokine balance were part of the experimental model. Among the markers measured in the current study, some were not detected in the circulation (TNF-α), while other showed only a brief change (IL-10) from baseline. The anti-inflammatory cytokine, TGF-1β, decreased significantly during shock and this became even more pronounced after the use of CPB (independent of hypothermia). This was an unexpected finding. Hemorrhage without soft tissue injury increases the production of TGF-1β, primarily from Kupffer cells and macrophages, which reaches a peak at 24 h and this elevation persists until 72 h post-hemorrhage [29]. In the current experiment, we noted a very rapid decrease in TGF-1β levels during shock and while on CPB. Furthermore, this depression persisted post-operatively for many weeks. Although the decrease in TGF during CPB may be explained by hemodilution, the most likely explanation for the prolonged suppression is the severity and multiplicity of the injuries in our animal model.

Among the pro-inflammatory cytokine, IL-1β response was largely independent of temperature changes. It started to increase following hemorrhage and reached statistical significance after the initiation of CPB. There was no difference between the hypothermic and normothermic groups. An increase in circulating levels of IL-1 after CPB, although reported in the literature, is not a consistent finding [30]. The IL-6 levels in this experiment were influenced markedly by hypothermia. This cytokine is well recognized as a marker of injury severity [31] and among all cytokine, IL-6 is considered to be the most reliable prognosticator of outcome [32]. Furthermore, the degree of IL-6 increase can reflect the development of septic complications following trauma and blood loss [33]. In the current study, normothermic animals displayed progressively increasing levels of IL-6 in the circulation that reflected the sequential insults (hemorrhage, surgical trauma, ischemia, CPB, reperfusion and blood transfusion). The IL-6 levels in the hypothermic animals also increased compared to the baseline but were markedly attenuated (up to 12-fold) compared to the normothermic controls. This finding highlights the anti-inflammatory properties of profound hypothermia.

Heat shock proteins are highly conserved cytoprotective molecules present in all prokaryotes and eukaryotes. One of the major HSP with a molecular mass of 70kDa (HSP-70) is induced by a number of stressful stimuli. It protects the cells by acting as an intracellular chaperone for damaged or mutated proteins. In addition, it decreases the production of downstream inflammatory cytokines (e.g. TNF, IL-1 and IL-6), regulates NF-κB expression, attenuates nitric oxide production, decreases cellular apoptosis, and markedly improves survival from inflammatory shock [34,35]. Originally, induction of HSP was associated with hyperthermic insult. However, it has now been shown that the myocardial protective effect of hypothermia during cardiopulmonary bypass involves a rapid (within 30 min) up-regulation of HSP [36]. In our study, HSP-70 increased somewhat with the use of normothermic cardiopulmonary bypass, but a very significant increase was noted in the hypothermic animals. This induced HSP in turn may have been responsible for the attenuation of IL-6 response in these animals. Whether HSP was induced in this experiment by hypothermia, re-warming, or a combination of the two is difficult to ascertain. Induction of HSP 70 in the heart has been seen during periods of hypothermia, as well as during re-warming, and it has been postulated to offer cellular protection during recovery from hypothermia [37]. Clearly, in our study the hypothermic animals had an attenuated inflammatory response, better cardiac performance and dramatically superior survival compared to normothermic controls. However, a cause-effect association between the circulating levels of HSP and outcome cannot be made. The rate of cooling markedly influenced survival without causing any major differences in the cytokine and HSP profile (between different hypothermic groups). This suggests that modulation of immune/inflammatory system is only one of many possible mechanisms by which hypothermia provides cellular protection.

A number of factors must be considered while analyzing these data. Most cytokines have fairly broad spectrum of biological activities, and their labeling as either pro- or anti-inflammatory in this manuscript was based upon the most well published/predominant action. The complexity of the experimental model, while providing clinical realism, adds many variables that could have influenced the results. The animals were subjected to a number of insults, such as surgical trauma, vascular injuries, uncontrolled hemorrhage, CPB, hypothermia, re-warming, blood transfusion and they were given various drugs. In such situations, it is often difficult to attribute the findings to one specific variable. Also some of the drugs used during the experiment (heparin, steroids, organ preservation fluids and epinephrine) have the potential to alter the cytokine profile. However, as the normothermic control animals were subjected to identical injuries, CPB management, and were given the same doses of fluids, blood and drugs, we believe that the observed differences can clearly
be attributed to hypothermia. It must be noted that whole blood was transfused in these animals, which is rarely done in clinical practice, and the use of packed red blood cells may be associated with a very different profile \[38\]. The use of organ preservation fluids (total of 9 L/animal) during the experiment may have diluted the circulating cytokine levels and influenced the measurements. As the cytokines differ widely in their time of induction, duration of response and half-lives, our sampling time points may have failed to capture the peak levels. Finally, circulating levels only broadly reflect the production of cytokines and stress proteins in the tissues. Direct measurement of these molecules in tissue beds (biologically more relevant) is the obvious next step.

Induced hypothermia can be a double-edged sword and its therapeutic role in trauma is still controversial. Hemorrhagic shock is characterized by decreased delivery of oxygen and metabolites to the cells. Clearly hypothermia can protect the ischemic tissues by decreasing their metabolism to match the limited oxygen delivery. In our study, profound hypothermia dramatically decreased total body oxygen consumption, which protected key organs during prolonged periods of shock, and provided precious time for repair of lethal injuries. We now know that cellular injury sustained during shock can actually worsen following resuscitation. This post-injury period is characterized by two inter-related but opposing cascades \[39\]. There is an initial period of exaggerated immune activation (systemic inflammatory response syndrome or SIRS), which can lead to immune mediated organ damage. In those that survive long enough, the compensatory anti-inflammatory response syndrome (CARS) may occur. The period of CARS is characterized by immune suppression and an increased risk for the development of infections. Ideally, strategies for immune modulation should be able to attenuate the early immune activation without worsening the delayed immune suppression. Induction of hypothermia seems to fulfill these criteria. The immunologic changes (augmented HSP, IL-10 and attenuated IL-6) in our experiment did not extend beyond the early post-injury period, thus allowing the animals to mount an effective immune response against infections. Despite the severity of injuries in this model, only two animals developed infections complications.

In summary, we have demonstrated that induction of profound hypothermia protects key organs and improves survival in a porcine model of multiple injuries and uncontrolled hemorrhage. Furthermore, profound hypothermia modulates the post-shock immune system by attenuating the pro-inflammatory IL-6, and augmenting the protective heat shock responses.

Acknowledgments

Data presented in part at the 27th Annual Conference on Shock, Nova Scotia, June 5-8, 2004. This work was supported by a research grant (RO1 HL71698-01, to HBA) from the National Institutes of Health.

Conflict of interest

None of the authors have any conflicts of interest to declare.

References


