Hypothermia Reduces Microvascular Permeability and Reactive Oxygen Species Expression after Hemorrhagic Shock

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**Background:** Hypothermia is a frequent manifestation after trauma-induced hemorrhagic shock. Clinical studies have suggested that hypothermia is an independent risk variable predisposing patients to an increase in morbidity. Thus, most of the current goal-directed resuscitation protocols are aimed at the establishment of euthermia. However, recent data suggest that hypothermia may provide protection by attenuating the inflammatory response after hemorrhagic shock. The purpose of this study was twofold: to examine the effects of mild to moderate hypothermia on barrier function after hemorrhagic shock, and to determine the role of reactive oxygen species (ROS) in this process.

**Methods:** After a control period, blood was withdrawn to reduce the mean arterial pressure to 40 mm Hg for 1 hour in urethane-anesthetized rats. Mesenteric postcapillary venules in a transilluminated segment of small intestine were examined to quantify changes in permeability and ROS expression. Sprague-Dawley rats received an intravenous injection of fluorescein isothiocyanate (FITC-albumin) during the control period. The fluorescent light intensity emitted from the FITC-albumin was recorded with digital microscopy within the lumen of the microvasculature and compared with the intensity of light in the extravascular space. The images were downloaded to a computerized image analysis program that quantitates changes in light intensity. This change in light intensity represents albumin-FITC extravasation.

**Results:** Our results demonstrated a marked increase in albumin leakage after hemorrhagic shock that was significantly attenuated with mild (34°C) and moderate (30°C) hypothermia. In addition, hypothermia attenuated ROS expression after hemorrhagic shock.

**Conclusion:** These data suggest that hypothermia may protect barrier integrity after hemorrhagic shock by inhibition of oxygen radical expression.

**Key Words:** Hypothermia, Hemorrhagic shock, Reactive oxygen species, Vascular permeability.

The objectives of this study were to examine the relationship of hypothermia to microvascular permeability in a hemorrhagic shock model and to determine whether hypothermia altered the expression of reactive oxygen species (ROS) after hemorrhagic shock, using a real-time in vivo hemorrhagic shock model to quantitate ROS expression. Hypothermia is defined as follows: mild, 33° to 36°C; moderate, 28° to 32°C; deep, 10° to 20°C; profound, 5° to 10°C; and ultraprofound, 0° to 5°C. Our results demonstrated that both mild and moderate hypothermia provided protection to the endothelium and preserved barrier integrity after hemorrhagic shock. In addition, hypothermia attenuated the expression of ROS, suggesting a possible mechanism for this protection.

**MATERIALS AND METHODS**

**Care of Animals**

The surgical procedures and experimental protocols were conducted at the Texas A&M University System Health Science Center College of Medicine, Scott & White Hospital after approval by the animal care and use committee. The facility is approved by the American Association for Accreditation of Laboratory Animal Care in accordance with National Institutes of Health guidelines.

**Chemicals and Solutions**

The test solute for the permeability measurements was fluorescein isothiocyanate-bovine albumin (FITC-albumin; Sigma). The test solution was prepared by dissolving the FITC in saline for a final concentration of 50 mg/kg. The
fluorescent probe used to detect ROS generation was dihydorhodamine 123 (DHR) (Molecular Probes, Eugene, OR) dissolved in DMSO for a final concentration of 0.6 g/kg.

**Animal Surgery and Intravital Microscopy**

Male Sprague-Dawley rats weighing 275 to 325 g were used. The rats were fasted for 18 hours and given water ad libitum before each experiment. The rats were anesthetized by intramuscular injections of 50% urethane (1.5 g/kg). Polyethylene (PE) cannulas (PE-50, 0.58-mm inside diameter) were placed in the right internal jugular vein to give fluids and in the right carotid artery for withdrawal of blood. Mean arterial pressure (MAP) was measured continuously using a PE-50 cannula in the left femoral artery connected to a blood pressure analyzer (Dig-Med, BPA 400A, Micromed, Louisville, KY). A midline laparotomy incision was performed to expose a section of small bowel mesentery. The rats were placed in the lateral decubitus position on a temperature-controlled Plexiglas plate mounted to an intravital upright microscope (Nikon Eclipse E 600, Tokyo, Japan). The temperature of the animal was measured continually with a rectal probe (Fisher, Springfield, NJ). Hypothermia was recorded for the whole animal. An exteriorized segment of mesentery from the proximal ileum was draped over a temperature-controlled Plexiglas stage and used for microscopic examination. Normal saline at 2 mL/min was applied topically (superfused) to the mesenteric vessels to provide moisture. In addition, the mesenteric vessels were covered with plastic wrap to reduce evaporation. Venules with diameters of 20 to 35 μm were selected for study with a Nikon 20× objective, 0.45- to 2.16-mm working distance (Nikon Instruments, Inc., Natick, MA). Images were obtained with a Photometrics Cascade Camera (Tucson, AZ). A video time and date generator (WJ-810, Panasonic, Secaucus, NJ) provided on-screen time, date, and stopwatch functions. The image was projected onto a video monitor (Trinitron 20-inch monitor, Sony, New York, NY) and captured digitally on computer and stored on CD (compact disk). The data were analyzed using MetaMorph 4.5/4.6 (Universal Imaging Corp., Downingtown, PA).

**Measurement of Vascular Permeability and ROS Expression**

The extravasation of FITC-albumin was measured by determining the changes in integrated optical intensity by image analysis. The labeled albumin represented relative changes in permeability. DHR is an oxygen-sensitive probe that is used to quantify ROS generation in vivo as described by Wood et al. DHR freely permeates cellular membranes and, when oxidized, forms rhodamine, a fluorescent compound easily detected by fluorescent microscopy. Areas in the small bowel mesentery, postcapillary venules, and the adjacent extravascular space were selected for study. Each experimental frame was digitized into 512 × 512 pixels. Each pixel was associated with a 16-bit gray-scale value. Gray-scale values were measured inside the postcapillary venules and in the extravascular space adjacent to that venule. Two measurements were recorded, using the MetaMorph image analysis systems, at these predetermined sites. The area inside and outside the vessel was constant. The images were standardized to images taken at the beginning of each experiment within the same animal and at set timed intervals between different animals. This method of standardization was selected to minimize the bias incurred with changes in room lighting and hematocrit concentration within each animal throughout the study period. This method of measuring vascular permeability was previously validated by Bekker et al.

**Experimental Protocols**

The experimental groups for permeability changes consisted of sham/control (n = 5), hemorrhagic shock (60 minutes) alone (n = 5), hemorrhagic shock (60 minutes) at 34°C (mild hypothermia) (n = 5), and hemorrhagic shock (60 minutes) at 30°C (moderate hypothermia) (n = 5). The experimental groups for ROS measurements consisted of sham/controls (n = 5), hemorrhagic shock alone, and hemorrhagic shock at 34°C and 30°C. The rats were allowed to recover from surgical manipulation for 30 minutes before the start of all experiments. This was followed by a 10-minute recording of baseline parameters: MAP, red cell centerline velocity, and vessel diameter. During this period, animals were dosed with either FITC-albumin at 50 mg/kg for permeability determination or DHR 0.6 g/kg intravenously for reactive oxygen expression, and baseline integrated optical intensities were obtained intra- and extravascularly. The animals then underwent 60 minutes of hemorrhagic shock. To produce hemorrhagic shock, the MAP was decreased to 40 mm Hg by withdrawing blood from the right carotid artery into a syringe containing 100 units of heparin. To maintain this level of hemorrhagic shock required approximately 50 to 60% of the animal’s blood volume (level IV shock). After the shock period, the shed blood plus two times the volume in lactated Ringer’s solution was reinfused. This resuscitation protocol maintained an MAP at or above 90 mm Hg. Parameters were recorded postshock at 5, 30, and 60 minutes into reperfusion. Minimal exposure for less than 15 seconds per recording was performed to prevent quenching of the fluorescent indicators.

**Statistical Analysis**

For each experimental condition, permeability was measured by light intensity taken at two different sites within the vessel and adjacent to the vessel with the same area. The data were initially analyzed to determine significance between groups by analysis of variance. When significance was found, post hoc t tests were performed to determine the site of significance within the data sets. Comparisons were made regarding permeability in the hemorrhagic shock versus sham operated, and the hemorrhagic shock and hypothermia groups. The differences were considered significant at a value of p < 0.05. All data are presented as means ± SE.
RESULTS

Effects of Hypothermia on Microvascular Permeability after Hemorrhagic Shock

Figure 1 is a composite photograph of a rat’s mesenteric venule before the shock period, labeled sham preshock, demonstrating minimal fluorescence in the extravascular space, and a photograph taken at 60 minutes into resuscitation of a sham-operated animal. In the third photograph taken at 60 minutes postshock, during resuscitation, there is a significant increase in fluorescent intensity in the extravascular space, suggesting a marked increase in albumin (FITC) extravasation. The cumulative results of hemorrhagic shock on the extravasation of FITC are shown in Figure 2. The hemorrhagic shock period was for 60 minutes’ duration (MAP of 40 mm Hg). There was essentially no change in fluorescent extravasation during hemorrhagic shock, possibly because of decreased flow. However, there was a significant increase in extravasation starting at 10 minutes into resuscitation and continuing throughout the experiment.

Figure 3 examined hypothermia at 30°C and 34°C versus hemorrhagic shock (temperature maintained at 37°C). Our results showed attenuation of microvascular extravasation of FITC during hypothermia at both 30°C and 34°C. The temperature was held constant during volume depletion in the hemorrhagic shock control group as discussed in the Materials and Methods section.

Effects of Hypothermia on ROS Expression after Hemorrhagic Shock

Figure 4 is a composite photograph of a rat’s mesenteric venule before the shock period (preshock) demonstrating minimal fluorescence of DHR (ROS expression). The second photograph shows hemorrhagic shock at 60 minutes into resuscitation. Note the marked increase in ROS expression in both the endothelial cells and leukocytes, labeled A and B, respectively. Figures 3 and 4 demonstrate the attenuation of ROS expression of 30°C and 34°C, respectively.

Figure 5 represents the cumulative data of hemorrhagic shock on the expression of ROS expression versus hypothermia at 30°C and 34°C. ROS expression increased significantly at 5 minutes into resuscitation, and the increase was sustained throughout the experiment. Figure 6 shows the temporal relationship of ROS expression versus permeability,
FITC extravasation. These data suggest ROS expression preceded the development of increased vascular permeability. Baseline represents a ratio of change versus values at the start of each experiment.

**DISCUSSION**

Previous studies from our laboratory implicate reactive oxygen species as a major modulator of microvascular injury after hemorrhagic shock. The oxygen stress caused by the ischemic insult resulted in an increase in ROS during reperfusion, which subsequently activated leukocytes and caused direct damage to microvascular endothelial cells. This damage to the endothelium caused by up-regulation of ROS can actually cause an increase in microvascular permeability after hemorrhagic shock. Hypothermic conditions have been reported to reduce the oxidative stress in various in vitro and in vivo settings.

**Fig. 4.** Composite photograph demonstrating DHR fluorescence of ROS in a pre- and posthemorrhagic shock animal (original magnification, ×20). Note the marked expression of ROS after shock. This expression was attenuated in the hypothermic animals at both 30°C and 34°C.

**Fig. 5.** Effect of hemorrhage shock on ROS expression at 37°C (—), 34°C (—), and 30°C (—). *p < 0.05, hemorrhagic shock versus hypothermia.

**Fig. 6.** Temporal relationship of ROS expression versus microvascular permeability changes after shock. Baseline represents an internal comparison of both ROS and permeability at the start of each experiment. Start resuscitation (time 0) is after the hemorrhagic shock phase.
vivo studies. Slikker and colleagues demonstrated that hypothermia lowers the risk of oxidative stress-induced cellular damage and programmed cell death by increasing the activity of glutathione peroxidase and by the induction in the expression of the antiapoptotic protein bcl-2. \(^\text{10}\) Zar and Lancaster concluded that mild hypothermia (34°C) protected against postischemic endothelial cell injury by decreasing ROS formation using lucigenin and luminol-enhanced chemiluminescence in isolated rat hearts. \(^\text{11}\) The data of Yoshioka et al. indicated that high-grade partial ischemia in skeletal muscle is accompanied by significant phospholipid peroxidation and that antecedent regional hypothermia markedly attenuated subsequent and further reperfusion-associated oxidative injury. \(^\text{12}\) Baiping et al. recorded similar results demonstrating hypothermia significantly inhibited the accumulation of lipid peroxidation products and the consumption of free radical scavengers in brain tissue after cardiac arrest and resuscitation. \(^\text{13}\) Hassoun et al. indirectly demonstrated the modulation of oxidative stress proteins during intraschematic hypothermia, showing a decrease in activation of nuclear factor-κB and inducible nitric oxide synthase but no effect on hemeoxygenase-1 during hypothermia. \(^\text{14}\)

Several investigators have demonstrated the deleterious effects of hypothermia after hemorrhagic shock, including coagulopathy \(^\text{15}\) and depressed cardiovascular, hepatocellular, and immune functions. \(^\text{16,17}\) These studies suggested that hemorrhagic shock and the hypothermic effect on hemodynamic and coagulation parameters were additive. The effects of hypothermia were thought to persist despite the arrest of hemorrhage and adequate volume replacement. Several clinical studies have concluded that hypothermia is an ominous predictor of survival. \(^\text{18,19}\) Jurkovich et al. demonstrated that hypothermia increased with higher Injury Severity Score, and at temperatures of 32°C or below, they had no survivors. There were 71 patients in that study with multiple confounding factors including length of time in the field, regulation of temperature of the resuscitative fluids, and an Injury Severity Score that ranged from 25 to greater than 50. As indicated by the authors, it was impossible to determine whether hypothermia contributed to the injury or whether the severe injuries produced the hypothermia. Gentilello et al. \(^\text{19}\) subsequently determined that hypothermia was an independent variable that predicted morbidity and/or mortality. Again, the study population was low at 57 patients randomized into two groups: rapid continuous arteriovenous rewarming or slower standard rewarming. They reported a 7% mortality (2 of 29) in the rapid recurring group versus 43% (12 of 28) in the standard group and concluded that rapid rewarming was beneficial and decreased mortality after major trauma.

Hansen hypothesized that hypothermia may result in an increase in permeability by cytoskeletal disruption and altered endothelial cell shape resulting in endothelial cell-cell barrier integrity loss. \(^\text{20}\) These studies were performed on cultured cells at 4°C for 3 to 8 hours and therefore not truly representative of mild or moderate hypothermia. Zhang and Wolf showed that an increase in the viscosity of blood and peripheral vasoconstriction resulted in extravasation of plasma proteins and water. \(^\text{21}\) They measured postcapillary resistance and isogravimetric capillary pressure in isolated cat hindlimbs and determined at a low temperature (5°C) increased permeability 3.4 times over baseline (37°C). These experiments, similar to those of Hansen, were performed at extremely low temperatures. Temperatures below 28°C require cardiopulmonary bypass for induction and reversal, and deep hypothermia below 10°C stops the heart from pumping. Our study demonstrated that both mild (34°C) and moderate (30°C) temperatures had beneficial effects by decreasing permeability and suggested that ROS may be one of the mechanisms responsible.

Recent data support our findings and have shown benefit to hypothermia in controlled and uncontrolled (spontaneous, accidental) hypothermia. The literature is replete with examples of therapeutic benefit in patients with controlled hypothermia (i.e., for cardiac procedures and neurologic protection) but limited in information regarding uncontrolled hypothermia (hemorrhagic shock). Kim et al. used an ongoing rat hemorrhagic model to demonstrate that moderate hypothermia (30°C) and limited resuscitation improved survival in these animals. \(^\text{22}\) Takasu et al. repeated these studies in rats using both mild and moderate hypothermia and showed a benefit in both groups. \(^\text{23}\) Takasu et al., in contradiction to the above-mentioned clinical studies, actually found that hypothermia increased blood pressure. \(^\text{24}\) These findings were pursued further to determine whether animal survival was improved secondary to an increase in blood pressure or from the “metabolic” effects of hypothermia. Using a pressure-controlled hemorrhagic shock model, Prueckner et al. documented that hypothermia, not the blood pressure effect, increased the survival time and rate in this hemorrhagic shock model, suggesting a possible metabolic mechanism for the blood pressure protective effect after hemorrhagic shock. \(^\text{25}\)

As alluded to, our laboratory demonstrated a role of ROS in the pathophysiology of hemorrhagic shock. \(^\text{26}\) Our study demonstrated that ROS generation preceded the activation of leukocytes in a hemorrhagic shock model. The attenuation of ROS production with superoxide dismutase and catalase significantly inhibited the activation of leukocytes after hemorrhagic shock. This study provides the basis for us to examine the hypothesis that hypothermia may exert its beneficial “metabolic” effect by attenuating ROS production and/or expression. Hypothermia has been shown to attenuate ROS generation, thereby inhibiting oxidative injury. Although the studies were performed in vitro or by measuring byproducts of reactive oxidants, it provided the background necessary to formulate our hypothesis.

This study is of particular importance because it provides a direct method of quantitating ROS expression in vivo. Our previous study describes the utility and specificity of DHR in detecting superoxide anion and hydrogen peroxide.
production. DHR has been used to detect changes in ROS production in both neutrophils and endothelial cells. DHR is cell membrane permeable, with minimal cellular toxicity, converts from the nonfluorescent to the fluorescent form with low rates of cross-reactivity and spontaneous conversion, and sequestrates in the fluorescent oxidized form. The fluorescent product of DHR oxidation, rhodamine-123, is a positively charged lipophilic compound that concentrates in the mitochondria, with minimal loss to the extracellular space. These properties of DHR make it an ideal fluorescent marker. We address the specificity of DHR by evaluating the effects of antioxidants on shock-induced DHR fluorescence, which showed that pre-treatment with superoxide dismutase and catalase almost completely attenuated DHR fluorescent signal after hemorrhagic shock. We acknowledge that the inhibition of superoxide anion and hydrogen peroxide may alter the eventual production of other reactive intermediates. Previous studies from this laboratory also demonstrated a relationship between ROS expression and leukocyte adherence. ROS generation became significant at 5 minutes into resuscitation followed by a significant increase in leukocyte adherence at 5 to 10 minutes of resuscitation. In studies not shown, ROS expression plateaued at 90 minutes after resuscitation and continued to be significantly elevated at 3 hours when compared with sham-generated rats. This study examines the relationship of ROS expression to vascular permeability. ROS expression appears to begin before we detect a significant change in vascular permeability. Permeability changes correlated with the onset of leukocyte adherence. Hypothermia blocked ROS generation and an increase in permeability, and (in data not shown) attenuated leukocyte adherence.

This observed effect of hypothermia on ROS and permeability may be temporary and afford early protection. Wu et al. have shown recently that long-term survival may not be altered in hypothermic animals. They demonstrated that a higher mean arterial pressure, lower heart rate, and less resuscitation was necessary in the hypothermic animals versus the normothermic animals. The survival of animals in the hypothermic group was improved at 24 and 48 hours after shock, but the survival was similar at 72 hours after moderate shock. However, during severe shock, the survival at 72 hours was greater in the hypothermic group. They concluded that hypothermia supports arterial pressure during resuscitation from hemorrhage during the early phases and may have benefit late in severe cases of hemorrhage. Condejo et al. demonstrated the beneficial effects of controlled hypothermia in preventing tissue injury when imposed during reperfusion as compared with ischemia. The results of Jurkovich et al. were similar; however, they showed an increase in injury and permeability during rewarming after reperfusion.

We conclude that hypothermia—30°C and 34°C—provides protection of the microvasculature by decreasing permeability. A possible mechanism for this protection could be its effect on reactive oxygen species expression. The potential for detrimental effects during the resuscitative phase from shock seem to be minimal, and early maintenance of hypothermia may be beneficial. Additional studies will need to be developed to understand the delicate balance of “early” beneficial versus “late” detrimental effects of hypothermia and to define its clinical role and effectiveness.

REFERENCES


