# Hepatic Transcription Factor Activation and Proinflammatory Mediator Production is Attenuated by Hypertonic Saline and Pentoxifylline Resuscitation After Hemorrhagic Shock

*Jessica Deree, MD, William H. Loomis, BS, Paul Wolf, MD, and Raul Coimbra, MD, PhD*

***Background:*** **Fluid resuscitation can contribute to postshock inflammation and the development of end organ injury. We have previously observed an attenuation in pulmonary and ileal inflammation when hypertonic saline and pentoxifylline (HSPTX) were concomitantly administered after hemorrhage. We hypothesized that the attenuation in hepatic injury observed with HSPTX is associated with the reduction of transcription factor activation and proinflammatory mediator production when compared with Ringer's lactate (RL).**

***Methods:*** **Male Sprague-Dawley rats were resuscitated with racemic RL (32 mL/kg) or HSPTX (4 mL/kg 7.5% NaCl + PTX 25 mg/kg) and killed at 4 hours and 24 hours after resuscitation. Liver injury was determined by histology and serum aminotransferases. Nitrite, tumor necrosis factor-$\alpha$, interleukin (IL)-1$\beta$, and IL-6 were measured with enzyme-linked immunosorbent assay. High mobility group box 1, inducible nitric oxide synthase, nuclear factor (NF)-$\kappa$B phosphorylation, and signal transducers and activators of transcription-3 phosphorylation were determined by Western blot. Transcription factor activation was verified with Electrophoretic Mobility Shift Assay.**

***Results:*** **RL resuscitation led to significant increases all measured parameters when compared with control. In contrast, HSPTX did not induce elevations in histologic liver injury or alanine aminotransferase levels. HSPTX attenuated inducible nitric oxide synthase by 23% ($p < 0.01$), nitrite by 25% ($p < 0.05$), tumor necrosis factor-$\alpha$ by 25% ($p < 0.05$), IL-1 by 63% ($p < 0.01$), IL-6 by 35% ($p < 0.05$), and high mobility group box 1 by 39% ($p < 0.05$) when compared with RL. HSPTX reduced I$\kappa$B-$\alpha$ phosphorylation by 34% ($p < 0.05$), NF-$\kappa$B p65 phosphorylation by 75% ($p < 0.01$), and signal transducers and activators of transcription-3 phosphorylation by 52% ($p < 0.01$).**

***Conclusions:*** **The reduction in liver injury observed with HSPTX resuscitation after hemorrhage is associated with attenuation transcription factor activation and proinflammatory mediators. HSPTX has the potential to be a superior resuscitation fluid with significant immunomodulatory properties.**

***Key Words:*** **Hypertonic saline, Pentoxifylline, Ischemia reperfusion, Hemorrhagic shock, Hepatic injury.**

*J Trauma.* 2008;64:1230–1239.

Death after traumatic injury can be divided into early and late phases. Although the majority of early death is a consequence of acute blood loss and hypovolemic shock, death late in the hospital course is often due to widespread inflammation and subsequent organ failure.[1,2] The liver, with its critical involvement in homeostasis, host defense, and metabolism, is one organ particularly affected by the systemic inflammatory response. Therefore, in the setting of an already injured patient, a considerable degree of morbidity and mortality can result from inflammation-induced hepatic dysfunction.[3,4]

Submitted for Publication September 22, 2007.

Accepted for publication January 23, 2008.



From the Division of Trauma and Critical Care, Department of Surgery, University of California-San Diego.

Presented at the 66th Annual Meeting of the American Association for the Surgery of Trauma, September 27–29, 2007, Las Vegas, Nevada.

Supported by Division of Trauma and Surgical Critical Care, Department of Surgery, University of California School of Medicine, San Diego, California.

Oral Presentation at the 2007 Annual Meeting of the American Association for the Surgery of Trauma, September 27–29, Las Vegas, NV.

Address for reprints: Raul Coimbra, MD, PhD, FACS, University of California-San Diego, 200 W. Arbor Drive, #8896, San Diego, CA, 92103-8896; email: rcoimbra@ucsd.edu.

**DOI: 10.1097/TA.0b013e31816a4391**

The fact that hepatic injury after trauma is the result of both ischemia and reperfusion has been described extensively.[5–7] The induction of inducible nitric oxide synthase (iNOS), the activation of iNOS-dependent transcription factors such as nuclear factor (NF)-$\kappa$B and signal transducers and activators of transcription (STAT)-3, and the resultant production of proinflammatory cytokines have all been identified as late events contributing to the propagation of the inflammatory cascade in the liver.[8–12] Although the early events of hepatic injury are less understood, studies suggest that the extracellular release of damage-associated molecular pattern signals such as high mobility group box 1 (HMGB1) may be the uniting event between injury and inflammation.[13,14]

In addition to the depth and duration of shock, there is evidence suggesting that the choice of resuscitative fluid can also modulate the severity of the inflammatory response after hemorrhage.[15] The administration of racemic Ringer's lactate (RL), the current standard of fluid resuscitation, has been shown to enhance neutrophil activation, inflammation, and organ injury in both animal and human studies of ischemia-reperfusion injury.[16–18] Therefore, alternative resuscitation strategies designed to restore perfusion and downregulate inflammation may ultimately reduce the incidence and severity of hepatic dysfunction after severe hemorrhage.

Two novel methods of resuscitation that may potentially aid in the attenuation of ischemia-reperfusion injury are the use of small volume hypertonic agents such as hypertonic saline (7.5% NaCl; HS) and use of anti-inflammatory pharmacologic adjuncts such as the phosphodiesterase inhibitor pentoxifylline (1-[5-oxohexyl]-3,7-dimethylxanthine; PTX). Because several similarities exist between the immunomodulatory effects of HS and PTX,[19,20] our laboratory has postulated that the combined administration of hypertonic saline and pentoxifylline (HSPTX) may serve as a potent low volume anti-inflammatory fluid that is capable of reducing resuscitation-induced organ dysfunction. In vitro, HSPTX has been shown to downregulate neutrophil oxidative burst and CD11b expression more efficiently than HS infusion alone.[21] Additionally, in a small animal model of hypovolemic shock, HSPTX has been shown to markedly reduce intestinal and pulmonary injury and inflammation when compared with RL.[22–24] The effects of HSPTX on resuscitation-induced hepatic inflammation remain unknown. In this series, we hypothesized that resuscitation with HSPTX would attenuate hepatic injury, transcription factor activation, and the production of local proinflammatory mediators when compared with RL after hemorrhagic shock.

## MATERIALS AND METHODS

The experiment was approved by the University of California Animal Subjects Committee and in accordance with guidelines established by the National Institutes of Health.

### Experimental Model

Male Sprague-Dawley rats (300–400 g) were purchased from Harlan Sprague-Dawley (San Diego, CA). A 12-hour light and dark cycle was instituted, and food and water were provided ad libitum. Animals were anesthetized with ketamine and xylazine by intraperitoneal injection. A right inguinal incision was performed, and the femoral artery and vein were cannulated with polyethylene catheters (PE50). The venous catheter was used for injection of resuscitative fluids, and the arterial catheter was used to withdraw blood and monitor the mean arterial pressure. Blood was withdrawn during a period of 10 minutes until a mean arterial pressure of 35 mm Hg was obtained. Controlled hypotension was then maintained at 35 mm Hg ± 5 mm Hg for 1 hour by withdrawal or reinfusion of blood as necessary.

The animals were randomly divided into three groups according to the treatment received. Sham animals (n = 5) underwent cannulation without shock or resuscitation and served as negative controls. RL-resuscitated animals (n = 7) received 32 mL/kg of racemic RL. HSPTX treated animals (n = 7) received 4 mL/kg of 7.5% NaCl + 25 mg/kg of PTX (Sigma, St. Louis, MO). PTX was dissolved in HS without the infusion of any additional fluid, and its dose was chosen based on studies from our laboratory demonstrating its safety and lack of hypotension at this concentration.[22] The volume of RL infusion was calculated to give equivalent sodium loads to both the RL- and HSPTX-treated animals. The body temperature of the animals was maintained at 37°C throughout the experiment with a heating blanket.

At the end of resuscitation, the catheters were removed, 1 mL of subcutaneous 0.125% bupivacaine was administered, and the incision was closed. Animals were killed 4 hours or 24 hours after the completion of shock and resuscitation via an intraperitoneal injection of Nembutal (120 mg/kg). Blood was immediately collected via a right ventricular puncture and centrifuged at 300*g* for 15 minutes. The resultant serum was stored at −70°C. The right hepatic lobe was snap frozen in liquid nitrogen and stored at −70°C. The left hepatic lobe was also harvested and stored in 10% phosphate buffered saline (Irvine Scientific, Santa Ana, CA) buffered formalin for histologic examination.

Since it was our objective to examine the effects of HSPTX on both the early and late events of hepatic inflammation, two time points of animal sacrifice were chosen. The specific time points of 4 hours and 24 hours were selected based on our previous work in an identical hemorrhagic shock model in which similar early and late mediators of inflammation were accurately detected.[22,23]

### Hepatic Injury

Hepatic tissue from animals killed 24 hours after resuscitation was embedded in paraffin using an automated processing unit (Autotechnicon, Tarrytown, NY). Sections 5-$\mu$m thick were transferred onto glass slides and stained with hematoxylin and eosin ($n \geq 5$ per group; Richard Allen Scientific, Kalamazoo, MI). A pathologist, blinded to the treatment groups, subjected the slides to microscopic examination and scored the sections for injury according to the system developed by Sneed et al.[25] from 0 (normal) to 4 (severe) in the following four categories: necrosis, hemorrhage, hepatic parenchymal inflammatory infiltrate, and sinusoidal inflammatory infiltrate. The score from each category was summed to produce the total hepatic injury score. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements were determined through previously described techniques.[26]

### Protein Extraction

Liver samples were homogenized in 1 mL of ice-cold T-PER Tissue Protein Extraction Reagent containing 1% protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Homogenates were centrifuged at 10,000*g* for 5 minutes, and the supernatant was aliquoted and stored at −70°C for later analysis. To determine the effects of resuscitation of NF-$\kappa$B and STAT3 activation, the extraction of cytoplasmic and nuclear proteins was performed using NE-PER nuclear and cytoplasmic extraction reagents containing 1% protease inhibitor cocktail per the manufacture's instructions (Pierce). The protein concentration of all extracts was determined using the bicinchoninic acid protein assay (Pierce). Absor-

bance was measured on a standard curve for albumin with a microplate reader (Molecular Devices, Sunnyvale, CA).

### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

In separate experiments, whole tissue lysates from animals killed 4 hours after shock and resuscitation (iNOS, HMGB1), cytoplasmic extracts (I$\kappa$B-$\alpha$), or nuclear extracts (p65 NF-$\kappa$B, STAT3) containing 10 $\mu$g of protein per sample were suspended in sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) and collected by boiling the samples at 100°C for 5 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8% to 16% Tris-glycine polyacrylamide gradient gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% milk in Tris-buffered saline/Tween 20 for 1 hour. Primary antibodies specific for iNOS (1:500; BD Biosciences, San Diego, CA), HMGB1 (1:1000; Abcam, Inc., Cambridge, MA), phosphorylated I$\kappa$B-$\alpha$ (1:250; Cell Signaling), phosphorylated NF-$\kappa$B p65 (1:500; Cell Signaling), and phosphorylated STAT3 (1:500; Cell Signaling) were incubated with the membranes overnight at 4°C in Tris-buffered saline/Tween 20 supplemented with 3% bovine serum albumin along with the appropriate loading controls. The membranes were washed and incubated for 1 hour at room temperature with horseradish peroxidase-linked anti-rabbit immunoglobulin G (1:2000) prepared in blocking solution. After thorough washing, the Pierce Supersignal West Pico Chemiluminescent Kit was applied for antibody detection with X-ray film. Data are expressed as the mean band pixel total after adjustment for the density of its respective control band ($n \geq 4$ per group).

### NF-$\kappa$B and STAT3 Electophoretic Mobility Shift Assay

The nonradioactive Light Shift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) Kit was used to detect DNA-transcription factor interactions. The 3′ biotin end-labeled oligonucleotide used as a probe for the NF-$\kappa$B EMSA was a 42-bp double-stranded construct (5′-TTGTTACAAG*GGGACTTTCC*GCTG*GGGACTTTCC*-AGGGAGGC-3′) containing two tandomly repeated NF-$\kappa$B binding sites (italics). The 3′ biotin end-labeled oligonucleotide used in the STAT3 EMSA was a 24-bp double stranded construct (5′-GATCC*TTCTGGGAATT*CCTAGATC-3′). Specificity for each experiment was determined by competition assay with the addition of 200 mol/L excess of unlabeled double-stranded NF-$\kappa$B or STAT3 oligonucleotide, respectively.

Nuclear extracts (10 $\mu$g) of animals killed at the 4-hour time point were incubated with 5 nM NF-$\kappa$B or STAT3 probe (1× binding buffer, 150 mmol/L potassium chloride, 0.1 mmol/L ethylenediaminetetraacetic acid, 2.5 mmol/L dithiothreitol, 0.05% nonyl phenoxylpolyethoxylethanol, 10% glycerol, and 50 ng/mL poly[dI-dC]) and subjected to 6% DNA retardation gel at 100 V for 90 minutes. Gels were electrophoretically transferred at 380 mA for 1 hour on ice to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN) and immediately ultraviolet cross-linked for 15 minutes with an ultraviolet transilluminator equipped with a 312-nm bulb. Streptavidin-horseradish peroxidase conjugate and the light shift chemiluminescent substrate were used to detect the biotin end-labeled DNA. The nylon membranes were then exposed to X-ray film for 1 to 3 minutes for detection. Data are expressed at the mean band pixel total ($n \geq 4$ per group).

### Nitrite Levels

In aqueous solution, nitric oxide rapidly degrades into its stable metabolites, nitrate and nitrite. Using a commercially available kit nitrate is first reduced to nitrite via nitrate reductase for total nitrite measurements (Oxis Research, Portland, OR). Nitrite quantification ($n \geq 4$ per group) using Greiss reagent was performed and expressed as the mean concentration in animals killed 4 hours after shock and resuscitation ($\mu$mol/L).

### Proinflammatory Cytokine Levels

The concentration of tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1$\beta$, and IL-6 was measured from hepatic lysates from animals killed 24 hours after shock and resuscitation ($n \geq 5$ per group) using commercially available sandwich enzyme immunoassay techniques (enzyme-linked immunosorbent assay kit, R&D Systems, Minneapolis, MN). Results are expressed in pg/mL.

### Statistical Analysis

All values are expressed as the mean + standard error (SE) of $n$ observations, where $n$ represents the number of animals in each group. Each assay was performed in duplicate or triplicate where appropriate. Statistical significance of differences among groups was determined by analysis of variance with Bonferroni correction. A $p$ value $<0.05$ was considered statistically significant.

## RESULTS

### Hepatic Injury

Animals treated with standard RL resuscitation developed significant histologic changes including neutrophil infiltration, necrosis, and subcapsular hemorrhage (Fig. 1). In contrast, the HSPTX infusion after shock reduced hemorrhage and cellular infiltration to a level observed in the Sham group. The total hepatic injury score was significantly greater in the RL group ($8 \pm 0.4$) than either the Sham or HSPTX groups ($2.8 \pm 0.5$ and $3.2 \pm 0.5$, respectively; $p < 0.05$). Hepatic injury was also estimated by determining the concentration of serum AST and ALT at 24 hours (Fig. 2). Resuscitation with HSPTX resulted in markedly lower levels of ALT when compared with RL ($110 \pm 20$ vs. $211 \pm 22$ IU/L; $p < 0.01$). AST levels were unchanged in all three treatment arms (data not shown).

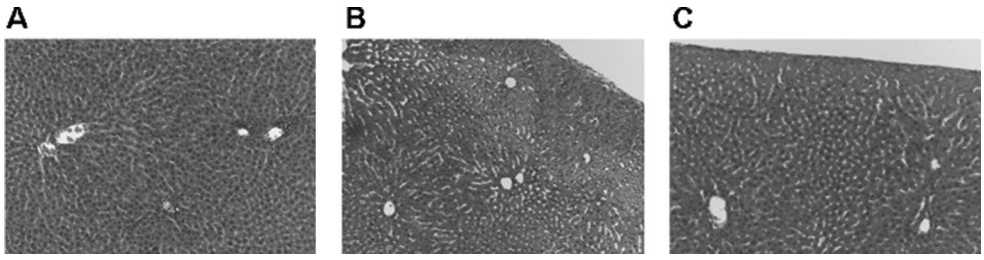


**Fig. 1.** *Resuscitation-induced hepatic injury. Livers harvested 24 hours after shock and resuscitation were evaluated by a pathologist blinded to the treatment each animal received* (n ≥ *5 per group). Panel A demonstrates the normal appearance of tissue specimens from Sham animals. Panel B shows the appearance of representative tissue from RL-resuscitated animals (32 mL/kg). Note the substantial areas of subcapsular hemorrhage, neutrophil infiltration, and necrosis. Panel C shows the appearance of tissue from HSPTX-treated animals (4 mL/kg 7.5% NaCl + 25 mg/kg PTX). Note the improvement in inflammation and injury when compared with the RL group (40× magnification).*

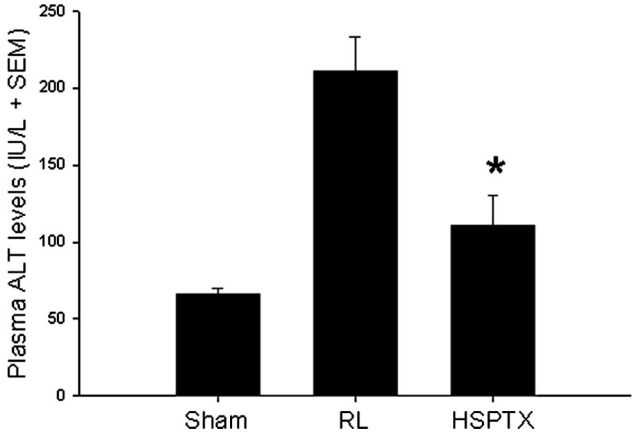


**Fig. 2.** *Serum ALT levels. Quantification of serum ALT levels was determined as described in the Methods section. Data are presented as in IU/L in animals killed 24 hours after hemorrhage and resuscitation with either RL (32 mL/kg) or HSPTX (4 mL/kg 7.5% NaCl + 25 mg/kg PTX). HSPTX animals had a 52% decrease in serum ALT levels when compared with those administered RL.* *$p < 0.01$ *versus RL.*

## iNOS Expression and Nitrite Production

Resuscitation with RL significantly upregulated hepatic iNOS content when compared with HSPTX (105,473 ± 3,033 vs. 82,411 ± 3,953; $p < 0.01$). No difference in iNOS induction was observed between the Sham group (77,889 ± 3,389) and those who underwent postshock resuscitation with HSPTX (Fig. 3A and B).

In vivo, nitric oxide (NO) is rapidly metabolized to nitrite ($NO_2^-$) and nitrate ($NO_3^-$) through an interaction with the heme group in hemoglobin.[27] To determine whether the modulation in iNOS expression correlated with NO production after ischemia and reperfusion, hepatic nitrite levels were determined. Nitrite levels declined by 26% when HSPTX was used for resuscitation versus RL (1,895 ± 82 vs. 2,561 ± 157 μmol/L, respectively; $p < 0.05$; Fig. 3C). No statistically significant difference was seen between animals treated with HSPTX and the negative control (1,708 ± 79 μmol/L).

## NF-κB Activation

In its inactive state, NF-κB remains quiescent in the cytoplasm associated with the inhibitory complex, IκB. Reactive oxygen species generated through reperfusion is one stimulus capable of activating the cascade responsible for IκB-α phosphorylation, which, in turn, allows for ubiquitination and proteolytic degradation of this inhibitory subunit and results in translocation of the transcriptionally active p50–p65 NF-κB heterodimer to the nucleus where it modulates transcription.[28–30] Resuscitation with RL led to an 28% increase in hepatic cytoplasmic IκB-α phosphorylation over the Sham group (92,716 ± 10,508 vs. 67,998 ± 4,153; $p < 0.05$). An increase in IκB-α phosphorylation was not observed after HSPTX resuscitation (61,207 ± 3,828) and remained comparable with that seen in the negative control (Fig. 4).

To assess whether the attenuation I-κBα phosphorylation observed with HSPTX after hemorrhagic shock affected nuclear translocation of NF-κB, we analyzed the relative amounts of NF-κB p65 subunit phosphorylation in hepatic nuclear extracts. As expected, RL-treated animals had a significantly higher degree of nuclear translocation over that of the Sham group (72,640 ± 7,051 vs. 17,545 ± 3,832; $p < 0.01$). There was 75% less p65 phosphorylation in the HSPTX-treated group (18,357 ± 2,605) when compared with their RL-treated counterparts ($p < 0.01$). NF-κB-DNA binding assayed by EMSA was also shown to correlate with changes in phosphorylation observed in the Western blots (Fig. 4C).

## STAT3 Activation

The effects of HSPTX on STAT3, the transcription factor responsible for the upregulation of IL-6 and granulocyte-colony stimulating factor after shock, were also explored (Fig. 5).[31] In the liver, HPTX resuscitation completely abrogated the observed increase in STAT3 phosphorylation seen after conventional RL infusion (53,897 ± 9,740 vs. 111,977 ± 9,459; $p < 0.01$). STAT3 activation and DNA binding was observed to parallel the changes in STAT3 phosphorylation (Fig. 5C).

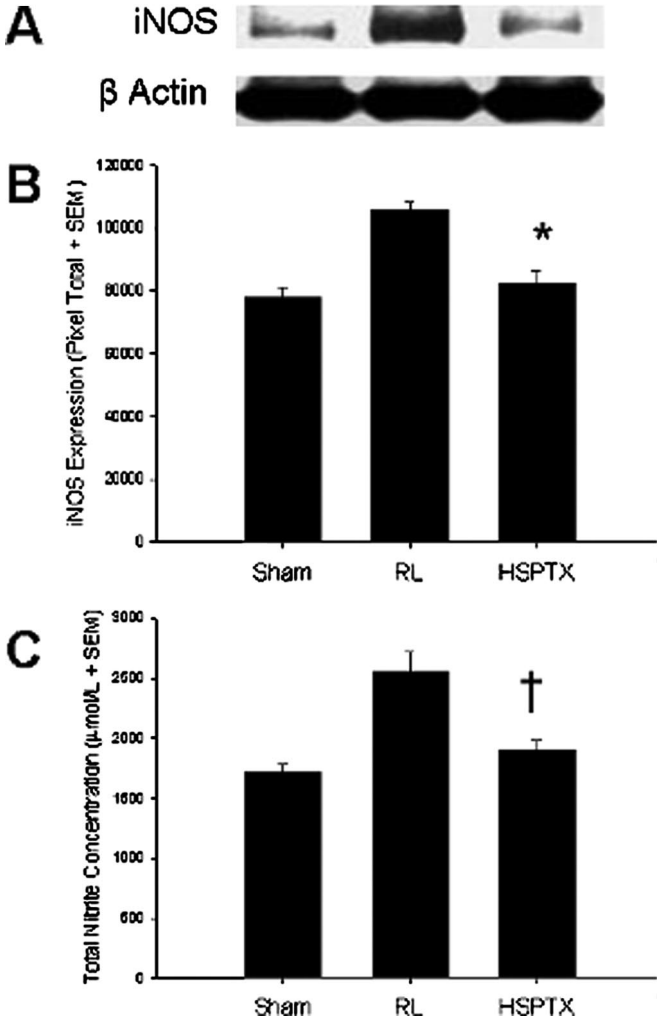


**Fig. 3.** *Hepatic iNOS and nitrite expression after hemorrhage and resuscitation with RL (32 mL/kg) or HSPTX (4 mL/kg 7.5% NaCl + 25 mg/kg PTX). (*A*) Western blots for iNOS expression were performed in animals killed 4 hours after resuscitation as described in Methods section, and results are expressed as the mean ± SE (*n ≥ *4 per group). (*B*) RL-treated animals had a greater than 20% rise in iNOS expression when compared with the Sham group or those given HSPTX. *p < 0.01 versus RL. (*C*) Total nitrite levels were assayed to determine the correlation between iNOS expression and NO production (*n ≥ *4 per group). HSPTX administration resulted in a 26% reduction of hepatic nitrite levels when compared with RL (32 mL/kg). †p < 0.05 versus RL.*

### Effect of HSPTX on Proinflammatory Cytokine Levels

To determine whether the reduction in transcription factor activation observed with HSPTX was associated with a decrease in proinflammatory cytokine levels, the hepatic concentration of TNF-$\alpha$, IL-1$\beta$, and IL-6 were measured at 24 hours (Fig. 6). Animals resuscitated with HSPTX had levels of TNF-$\alpha$ similar to that of the Sham group (71 ± 9 vs. 65 ± 6 pg/mL). RL resuscitation led to a 24% increase in TNF-$\alpha$ when compared with HSPTX (94 ± 9 vs. 71 ± 9 pg/mL; $p < 0.05$). RL treatment also resulted in a 63% increase in IL-1$\beta$ (1,294 ± 300 vs. 482 ± 52 pg/mL; $p < 0.01$) and a 36% increase in IL-6 (730 ± 15 vs. ± 34 pg/mL; $p < 0.05$) when compared with those who received HSPTX after shock.

### Hepatic HMGB1 Content

HMGB1 has been demonstrated to be an early mediator of inflammation and cell injury after hepatic ischemia-reperfusion injury.[32] Therefore, we sought to determine whether HSPTX resuscitation affected the relative levels of HMGB1 in the liver after hemorrhage (Fig. 7). The use of conventional RL resulted in a marked elevation in hepatic HMGB1 when compared with the negative control (100,043 ± 6,414 vs. 63,648 ± 2,314; $p < 0.05$). In contrast, HSPTX resuscitation did not result in an increase in HMGB1 content over that of the Sham animals (61,993 ± 1,291).

## DISCUSSION

The liver plays a major role in regulating biochemical, immunologic, and metabolic functions of the host. In the setting of trauma, it has been reported that approximately 20% of patients admitted in hypovolemic shock exhibit some evidence of liver dysfunction.[33,34] Although it is most often associated with hemorrhage, hepatic ischemia-reperfusion injury can also be a consequence of elective liver resection, transplantation, and other forms of shock. Therefore, strategies aimed at effectively limiting the severity of hepatic ischemia-reperfusion injury by either shortening the period of hypoperfusion or by ameliorating reperfusion-induced inflammation resulted are warranted.

During the past decade, conventional fluid resuscitation with RL has been increasingly challenged by numerous studies documenting its proinflammatory nature.[16–18] In addition to its well-documented effects on neutrophils and endothelial cells, an increase in the number of iNOS mRNA transcripts has been observed after hemorrhage and RL administration.[35] The proinflammatory properties of racemic RL have been attributed to the D-form of lactate, an isomer not normally encountered in humans. This theory is supported by the observation that neutrophil activation does not occur upon exposure to RL strictly composed of the L-isomer.[36] Resuscitation with RL strictly composed of the L-isomer, however, does not circumvent the other major disadvantages of isotonic fluid resuscitation that are the large infusion volumes necessary to achieve adequate perfusion and the time-consuming process of infusing these large volumes.

Both HS and the addition of PTX to conventional RL resuscitation have been independently proposed as unique reperfusion strategies capable of attenuating the postshock inflammatory response. Both regimens have been shown to independently ameliorate hepatic injury, neutrophil activation, endothelial dysfunction, and lung injury after hemorrhagic shock.[19,20,37–39] On the contrary, only PTX has the capacity to consistently downregulate TNF-$\alpha$ production,[40] implying that despite their numerous similarities, PTX and HS modulate different steps in the inflammatory cascade and

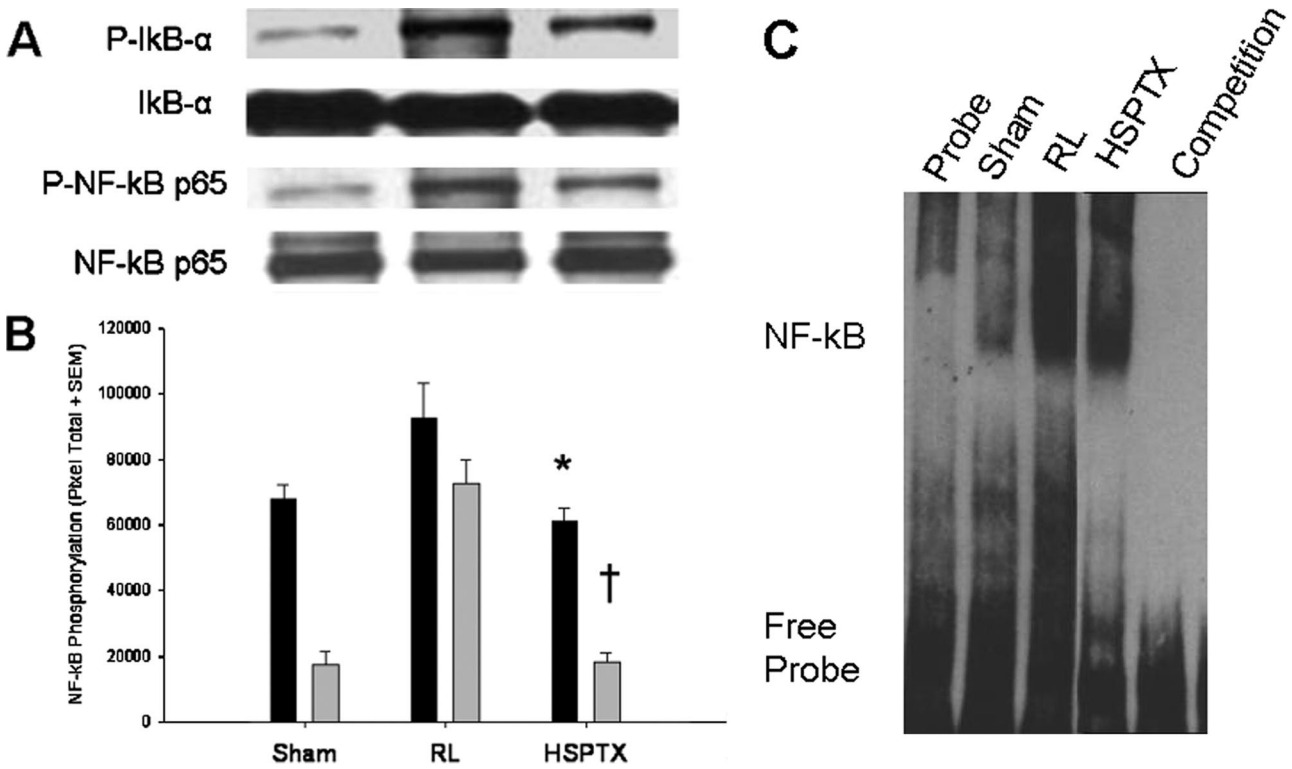


**Fig. 4.** *Effects of resuscitation on hepatic NF-κB activation after hemorrhagic shock. Cytoplasmic IκB-α and nuclear NF-κB p65 subunit phosphorylation were determined 4 hours after shock and resuscitation with RL (32 mL/kg) or HSPTX (4 mL/kg 7.5% NaCl + 25 mg/kg of PTX). Results are expressed as the mean ± SE (*n ≥ 4 per group). (A) Representative Western blots of phosphorylated and total protein. (B) The HSPTX-treated animals had 35% and 75% decline in IκB-α and NF-κB p65 phosphorylation, respectively, when compared with their RL-treated counterparts. *$p < 0.05$ versus RL and †$p < 0.01$ versus RL. (C) Representative EMSA correlating hepatic NF-κB activation with the observed modulation in phosphorylation.*

have the potential to act synergistically when administered concomitantly.

The role of iNOS as an early mediator of inflammation is supported by several animal studies in which inhibition of iNOS abrogates liver inflammation after ischemia-reperfusion injury.[41,42] In the present study, we determined that HSPTX alleviated hepatic injury, the induction of iNOS, and the excessive production of NO, in contrast to RL. Published work by Hierholzer et al.[10,42] has previously reported that later events in the inflammatory cascade including the activation of NF-κB and STAT3 and the production of proinflammatory cytokines are dependent on the induction of iNOS. Therefore, we sought to determine whether HSPTX also had an effect on these downstream events as well. Indeed, HSPTX effectively ameliorated RL-associated increases in NF-κB and STAT3 activation and the subsequent production of TNF-α, IL-1β, and IL-6. These findings are not surprising since TNF-α and IL-1β contain regulatory binding sites for NF-κB in their promoter regions and that STAT3 activation is a critical step in the production of IL-6.[43,44]

Linking the lesser known proximal with the well-documented distal events of ischemia-reperfusion injury has been the focus of a vast amount of recent research. Hierholzer et al.[42] have proposed that a critical step between the initial cellular damage and activation of the inflammatory cascade is the release of damage associated molecular pattern signals from ischemic cells. HMGB1 is a 30 kDa nuclear DNA-binding protein that stabilizes nucleosomes and allows the bending of DNA that facilitates gene transcription.[13] In the case of cellular ischemia and necrosis, large amounts of HMGB1 are released into the extracellular medium and are capable of inducing the early phases of the inflammatory response such as iNOS synthesis in pulmonary macrophages.[45,46] Therefore, therapeutic agents such as HSPTX that limit HMGB1 release may be extremely useful in the prevention of hepatic ischemia-reperfusion injury. The observation that HSPTX affects HMGB1 release also implies that HSPTX modulates events that occur very early in the inflammatory cascade.

The primary clinical endpoint of fluid resuscitation after traumatic injury is the restoration of intravascular volume and normalization of hemodynamic parameters. Interestingly, studies have shown that gastric acidosis persists once normotension is achieved, indicating continued splanchnic hypoperfusion.[47] Therefore, strategies aimed at limiting intestinal ischemia should also limit iNOS induction and injury both locally and remotely in organs such as the lung and the liver. Therefore, it is plausible

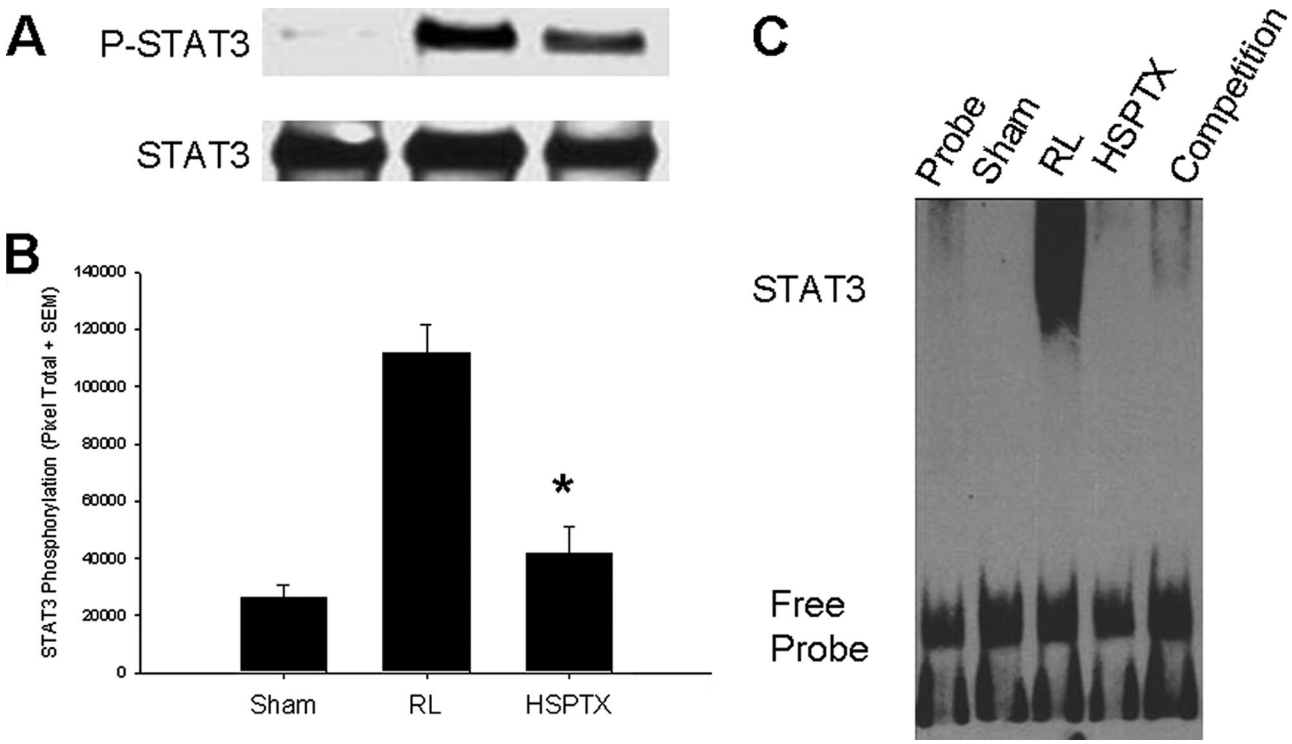


**Fig. 5.** *Effects of resuscitation strategy on hepatic STAT3 phosphorylation.* (A) *Western blots for phosphorylated STAT3 was performed on animals killed at 4 hours after resuscitation as described in Methods section, and results are expressed as the mean ± SE (n ≥ 4 per group).* (B) *The HSPTX-treated animals had a 52% decrease in hepatic STAT3 phosphorylation when compared with those animals that received RL.* *p < *0.01 versus RL.* (C) *Representative EMSA correlating STAT3 transcription factor activation with the observed modulation in phosphorylation.*

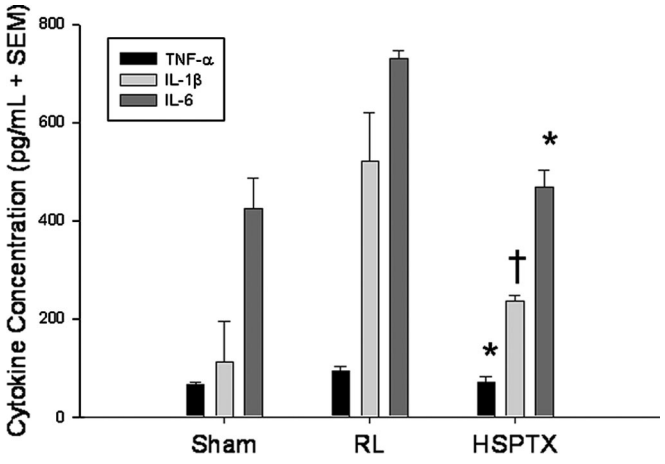


**Fig. 6.** *Effects of resuscitation strategy on hepatic cytokine levels. Quantification of hepatic proinflammatory cytokines in animals killed 24 hours after shock and resuscitation was performed by enzyme-linked immunosorbent assay as described in the Methods section. Data are presented as the mean ± SE in pg/mL* (n ≥ *5 per group). HSPTX resuscitation after hemorrhagic shock (4 mL/kg 7.5% NaCl + 25 mg/kg PTX) resulted in a 23% decrease in TNF-α, a 63% decrease in IL-1β, and a 36% decrease in IL-6 at 24 hours when compared with RL (32 mL/kg).* *p < *0.05 versus RL and* †p < *0.01 versus RL.*

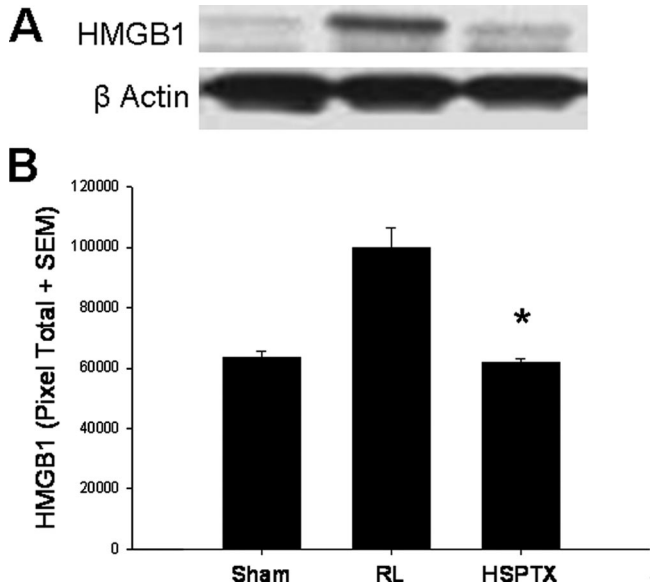


**Fig. 7.** *Hepatic HMGB1 content 4 hours after hemorrhage and resuscitation with RL (32 mL/kg) or HSPTX (4 mL/kg 7.5% NaCl + 25 mg/kg PTX).* (A) *Western blots were performed as described in the Methods section.* (B) *Results are expressed as the mean ± SE* (n ≥ *4 per group). The HSPTX-treated animals had a 39% decrease in HMGB1 levels when compared with those animals that received RL resuscitation.* *p < *0.05 versus RL.*

that resuscitation HSPTX previously shown by Roche e Silva et al. to synergistically improve gastric oxygenation when compared with standard RL treatment[48] may decrease hepatocyte death, the release of HMGB1, and the subsequent induction of iNOS and iNOS-mediated events through a perfusion dependent mechanism. Further investigation is necessary to determine whether these events are indeed related.

Both HSPTX are well-known modulators of late occurring events in inflammation such as neutrophil activation.[21] When compared with RL, HSPTX resuscitation has been shown to markedly reduce neutrophil degranulation and adhesion molecule expression, two parameters of neutrophil function. Therefore, because of its effects on both early and late steps in the inflammatory cascade, HSPTX may have an advantage over other proposed resuscitation strategies that target only one specific portion of the inflammatory cascade.

Although the advantages of postshock resuscitation with HSPTX seem promising at this time there are some limitations to this study, which deserve mention. In our model, we have fashioned our resuscitation in both the RL and HSPTX groups to have a calculated equivalent sodium load based on the amount of fluid administered and known concentration of sodium in each fluid. However, the serum sodium concentrations in each experimental group were not determined in this model. Because the serum sodium concentration is well known to have an impact on inflammation, it would be beneficial to examine this in future experiments. The second and perhaps largest limitation in this study is the omission of additional control group and treatment arms. The main objective of this study was to compare our combined resuscitative fluid against the clinically used crystalloid RL. We did not compare the effects of HSPTX with the effects of HS or PTX alone but plan to do so in the future. Although we have observed a synergistic effect with HSPTX when compared with HS or PTX in vitro, this has not been performed in vivo. Doing so would significantly support our theory that the best strategy for resuscitation after shock may be one that combines the beneficial properties of multiple agents. In addition to this, HSPTX was not compared with other crystalloids, specifically L-isomer RL, which is now commercially available. It would indeed be interesting to see how HSPTX compares with the "less inflammatory" RL and if the large infusion volumes needed with RL would still be a disadvantage despite the removal of its inflammatory component.

In summary, the attenuation hepatic injury observed with HSPTX is associated with the downregulation of HMGB1 release, iNOS activity, transcription factor activation, and proinflammatory cytokine production after hemorrhage. This novel resuscitation strategy may have future therapeutic potential in the fight against ischemia-reperfusion injury and hepatic failure.

## REFERENCES


1. Vincent JL. Prevention and therapy of multiple organ failure. *World J Surg.* 1996;20:465–470.
2. Deitch EA. Multiple organ failure: pathophysiology and potential future therapy. *Ann Surg.* 1992;216:117–134.
3. Donelly SC, Robertson C. Mediators, mechanism, and mortality in major trauma. *Resuscitation.* 1994;28:87–92.
4. Roumen RM, Redl H, Schlag G, et al. Inflammatory mediators in relation to the development of multiple organ failure in patients after severe blunt trauma. *Crit Care Med.* 1995;23:474–480.
5. Hassoun HT, Kone BC, Mercer DW, et al. Post-injury multiple organ failure: the role of the gut. *Shock.* 2001;15:1–10.
6. Pastores SM, Katz DP, Kvetan V. Splanchnic ischemia and gut mucosal injury in sepsis and multiple organ dysfunction syndrome. *Am J Gastroenterol.* 1996;91:1697–1710.
7. Swank GM, Deitch EA. Role of the gut in multiple organ failure: bacterial translocation and permeability changes. *World J Surg.* 1996;20:411–417.
8. Taylor BS, Alarcon LH, Billiar TR. Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry.* 1998;63:766–781.
9. Menezes JM, Hierholzer C, Watkins SC, et al. The modulation of hepatic injury and heat shock expression by inhibition of nitric oxide synthase after hemorrhagic shock. *Shock.* 2002;17:13–18.
10. Hierholzer C, Harbrecht B, Menezes JM, et al. Essential role of inducible nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J Exp Med.* 1998;187:917–928.
11. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol.* 2003;284:G15–G26.
12. Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology.* 2003; 125:1246–1257.
13. Wang H, Yang H, Tracey KJ. Extracellular role of HMGB1 in inflammation and sepsis. *J Int Med.* 2004;255:320–331.
14. Erlandsson H, Andersson U. The nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol.* 2004;34:1503–1512.
15. Masuno T, Moore EE, Cheng AM, et al. Bioactivity of post-shock mesenteric lymph depends on the depth and duration of hemorrhagic shock. *Shock.* 2006;26:285–289.
16. Alam HB, Stanton K, Koustova E, et al. Effect of different resuscitation strategies on neutrophil activation in a swine model of hemorrhagic shock. *Resuscitation.* 2004;60:91–99.
17. Alam HB, Sun L, Ruff P, et al. E- and P-selectin expression depends on the resuscitation fluid used in hemorrhaged rats. *J Surg Res.* 2000;94:145–152.
18. Savage SA, Fitzpatrick CM, Kashyap V, et al. Endothelial dysfunction after lactated ringer's solution resuscitation for hemorrhagic shock. *J Trauma.* 2005;59:284–290.
19. Angle N, Hoyt DB, Liu F, et al. Hypertonic saline resuscitation diminishes lung injury by suppressing neutrophil activation after hemorrhagic shock. *Shock.* 1998;9:164–170.
20. Barroso-Aranda J, Schmid-Schonbein GW. Pentoxifylline pretreatment decreases the pool of circulating activated neutrophils, in-vivo adhesion to endothelium, and improves survival from hemorrhagic shock. *Biorheology.* 1990;27:401–418.
21. Coimbra R, Loomis WH, Melbostad H, et al. Role of hypertonic saline and pentoxifylline on neutrophil activation and tumor necrosis factor-$\alpha$ synthesis: a novel resuscitation strategy. *J Trauma.* 2005; 59:257–265.
22. Coimbra R, Porcides R, Loomis WH, et al. HSPTX protects against hemorrhagic shock resuscitation-induced tissue injury: an attractive alternative to ringer's lactate. *J Trauma.* 2006;60:41–51.
23. Deree J, Martins JO, Leedom A, et al. Hypertonic saline and pentoxifylline educes hemorrhagic shock resuscitation-induced pulmonary inflammation through attenuation of neutrophil degranulation and pro-inflammatory mediator synthesis. *J Trauma.* 2007;62:104–111.

24. Deree J, de Campos T, Shenvi E, et al. Hypertonic saline and pentoxifylline attenuates gut injury after hemorrhagic shock: the kinder, gentler resuscitation. *J Trauma.* 2007;62:818–827.
25. Sneed RA, Grimes SD, Schultze AE, et al. Bacterial endotoxin enhances the hepatotoxicity of allyl alcohol. *Toxicol Appl Pharmacol.* 1997;144:77–87.
26. Coimbra R, Porcides RD, Melbostad H, et al. Nonspecific phosphodiesterase inhibition attenuates liver injury in acute endotoxemia. *Surg Infect.* 2005;6:73–85.
27. Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis–an overview. *Acta Anaesthesiol Scand.* 1999;43:275–288.
28. McDonald PP, Bald A, Cassatella MA. Activation of the NF-kB pathway by inflammatory stimuli in human neutrophils. *Blood.* 1997; 89:3421–3433.
29. Renard P, Percherancier Y, Kroll M, et al. Inducible NF-kB activation is permitted by simultaneous degradation of nuclear IkB-α. *J Biol Chem.* 2000;275:15193–15199.
30. Schmitz ML, Baeuerle PA. The p65 subunit is responsible for the strong transcription activating potential of NF-kB. *EMBO J.* 1991; 10:3805–3817.
31. Brundage SI, Schreiber MA, Holcomb JB, et al. Amplification of the pro-inflammatory transcription factor cascade increases with severity of uncontrolled hemorrhage in swine. *J Surg Res.* 2003; 113:74–80.
32. Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med.* 2005;210:1135–1143.
33. Heckbert SR, Vedder NB, Hoffmann W, et al. Outcome after hemorrhagic shock in trauma patients. *Am J Physiol.* 1998;45:545–549.
34. Helling TS. The liver and hemorrhagic shock. *J Am Coll Surg.* 2005; 201:774–783.
35. Alam HB, Stegalkina S, Rhee P, et al. cDNA array analysis of gene expression following hemorrhagic shock and resuscitation in rats. *Resuscitation.* 2002;54:195–206.
36. Koustova E, Stanton K, Gushchin V, et al. Effects of lactated ringer's solutions on human leukocytes. *J Trauma.* 2002;52:872–878.
37. Pascual JL, Ferri LE, Seely AJ, et al. Hypertonic saline resuscitation of hemorrhagic shock diminishes neutrophil rolling and adherence to endothelium and reduced in vivo vascular leakage. *Ann Surg.* 2002; 236:634–642.
38. Coe DA, Freischlag JA, Johnson D, et al. Pentoxifylline prevents endothelial damage due to ischemia and reperfusion injury. *J Surg Res.* 1997;67:21–25.
39. Deree J, Loomis WH, Putnam JG, et al. Pentoxifylline-supplemented resuscitation attenuates both early and late mediators of hepatic inflammation after hemorrhagic shock. *J Organ Dysfunct.* In press.
40. Coimbra R, Melbostad H, Hoyt DB. Effects of phosphodiesterase inhibition on the inflammatory response after shock: role of pentoxifylline. *J Trauma.* 2004;56:442–449.
41. Takamatsu Y, Shimada K, Yamaguchi K, et al. Inhibition of inducible nitric oxide synthase prevents hepatic, but not pulmonary, injury following ischemia-reperfusion injury of rat liver. *Dig Dis Sci.* 2006;51:571–579.
42. Hierholzer C, Billiar TR, Tweardy DJ, et al. Reduced hepatic transcription factor activation and expression of IL-6 and ICAM-1 after hemorrhage by NO scavenging. *Arch Orthop Trauma Surg.* 2003;123:55–59.
43. Ndengele MM, Muscoli C, Wang ZQ, et al. Superoxide potentiates NF-kB activation and modulates endotoxin-induced cytokine production in alveolar macrophages. *Shock.* 2005;23:186–193.
44. Song M, Kellum JA. Interleukin-6. *Crit Care Med.* 2005;33:S463–S465.
45. Chen G, Ward MF, Sama AE, et al. Extracellular HMGB1 as a proinflammatory cytokine. *J Interferon Cytokine Res.* 2004;24:329–333.
46. Ren D, Sun R, Wang S. Role of inducible nitric oxide synthase expressed by alveolar macrophages in high mobility group box 1-induced acute lung injury. *Inflamm Res.* 2006;55:207–215.
47. Tamion F, Richard V, Sauger F, et al. Gastric mucosal acidosis and cytokine release in patients with septic shock. *Crit Care Med.* 2003; 31:2137–2143.
48. Cruz RJ, Yada-Langui MM, Poli de Figueiredo LF, et al. The synergistic effects of pentoxifylline on systemic and regional perfusion after hemorrhage and hypertonic resuscitation. *Anesth Analg.* 2006;102:1518–1524.


## DISCUSSION

**Dr. Eileen M. Bulger** (Seattle, Washington): What is most impressive in this study is the fact that for all parameters assessed the hypertonic saline pentoxifylline group was comparable to the sham group that did not undergo hemorrhagic shock. But I do have some questions for the authors.

My first question regards the control groups. Your study compared three groups, a sham, an hemorrhagic shock with racemic LR, and hemorrhagic shock with hypertonic saline and pentoxifylline.

Why did you choose to include only a racemic LR group? As acknowledged by the authors, racemic LR has been shown to be associated with increased inflammation. Why not include an L lactate LR group which would be clinically relevant?

Secondly, why not have a hypertonic saline alone group? That would assure us that the changes seen are additive and not merely due to the use of hypertonic saline which has been shown to have these effects?

The third question, you observed a much greater reduction in transcription factor expression than was seen in cytokine production.

For example, there was a 75 percent reduction in NFkB expression but only a 25 percent change in TNF levels. Can you speculate on the reasons for this discrepancy? Could this be related to the timing of these studies?

Finally, there are many human studies that demonstrate the safety of hypertonic resuscitation. Is there any human data with the use of pentoxifylline?

What are the potential side effects of pentoxifylline in humans? And is this fluid combination ready for pilot studies in humans?

**Dr. Peter Rhee** (Tucson, Arizona): Yes, this is an excellent study with a lot of useful information and I think some of it is an interpretation, just like the "glass half full/half empty."

The word attenuation is the only one I had a problem with. I think otherwise the study was very clean.

And I think from what I saw the data shows that the lactated ringer caused a lot of issues but there wasn't necessarily an attenuation, per se. It just didn't, your hypertonic saline resuscitation just didn't cause any effect.

**Dr. Robert N. Cooney** (Hershey, Pennsylvania): I'm curious, what were the sodium levels when you harvested the animals and did you look at how the resuscitation affected the sodium levels?

Could you clarify how many of the data elements you presented were from the 5-hour versus the 24-hour time point. That wasn't clear as you were going through.

**Dr. Jessica Deree** (San Diego, California): To address Dr. Bulger's first question, we did not include an L-lactate group or a hypertonic saline alone control group because we wanted to test our fluid specifically against the conventional fluid that is used across the country.

We do know that L-lactate fluid induces a significantly smaller inflammatory response than racemic; however, L-lactate resuscitation still involves the infusion of a large volume of fluid. I do believe that a hypertonic saline alone control group may be needed, although we did not compare the two here. We plan on looking at hypertonic saline alone versus HSPTX in the future.

We have tested an RL-PTX group previously, and HSPTX has actually been shown to be a better fluid than RL with or without PTX. However, we don't have that treatment data here. Additionally, in in-vitro studies, we have shown that the combination of HS and PTX down regulates MAP-kinase phosphorylation and oxidative burst synergistically when compared to either component alone.

To address Dr. Cooney's question, the 4-hour group was basically used for all of the signaling cascades such as the transcription factor and cytokine data while the 24-hour group was basically used for histology and ALT levels, the later markers of hepatic injury.

When looking at the cytokine and the transcription factor data there is a discrepancy but I do think that this is probably a product of the time period we chose to check our measurements. It would be nice if all things in medicine were a linear pathway but as we all know this is not the case the majority of time. His is another explanation as to why our changes in transcription factors were not the same as observed in cytokine levels. Although I do stress that the changes in cytokine levels were still statistically significant but less so.

The PTX dose we used in this study is 25mg/kg. This basically is equivalent to the dose that is given to vascular patients that receive PTX for intermittent claudication, the original and current use of this drug. The only side effect seen patients that take PTX is nausea, not something that we've actually tested in our animal population.

In human studies, PTX has been used as an attenuator of inflammation and has been tested in neonatal sepsis, nonalcoholic hepatitis, and in patients undergoing cardiac bypass with good results. In those patients we haven't seen any increased reports of hemorrhage or hypotension as has been exhibited with some of the other phosphodiesterase inhibitors.

To address Dr. Rhee, I do think attenuation is probably not the best word to use since we don't see a change with HSPTX. But in comparison to their RL group I do often use the word attenuation so that may just be a misnomer on my part.

And the last question by Dr. Cooney, we did not specifically test in the actual sodium concentration in each animal group, but the amounts given were equivalent in each group. Although we know from in vitro studies not only in our lab but in many others the sodium increase is approximately 40 meq over normal but I do not have that data as I related to this specific experiment.