Resuscitation Affects Microcirculatory Polymorphonuclear Leukocyte Behavior After Hemorrhagic Shock: Role of Hypertonic Saline and Pentoxifylline

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We have previously shown that lung injury following fluid resuscitation either with hypertonic saline (HS) or lactated Ringer's (LR) plus pentoxifylline (PTX) attenuated acute lung injury when compared with LR resuscitation. The objective of the present study is to determine whether our previous observations are accompanied by changes in polymorphonuclear leukocyte (PMN) behavior. To study this, PMN–endothelial cell interactions, microcirculatory blood flow, lung histology, lung PMN infiltration (MPO, Myeloperoxidase), and lung intracellular adhesion molecule-1 (ICAM-1) expression were assessed in a controlled hemorrhagic shock model followed by LR, HS, and LR+PTX resuscitation in rodents. Rats (240–300 g) were bled to a mean arterial pressure (MAP) of 35 mm Hg for 1 hr and then randomized into three groups: HS (7.5% NaCl, 4 ml/kg); LR (3× shed blood); and LR+PTX (25 mg/kg). Additionally, total shed blood was reinfused. A sham group underwent no shock and no treatment. The internal spermatic fascia was exteriorized and the microcirculation was observed by closed-circuit TV coupled to a microscope, 2 and 6 hrs after treatment. The number of leukocytes sticking to the venular endothelium was determined 2 hrs after fluid resuscitation. Microcirculatory blood flow was measured by an optical Doppler velocimeter. Lung histology and lung MPO immunostaining were assessed at 6 hrs, and lung ICAM-1 expression was determined by immunostaining at 2 hrs following fluid resuscitation. Two hours after treatment, HS (1.4 ± 0.4), LR+PTX (1.7 ± 0.3), and sham (0.4 ± 0.2) groups presented significant reductions in leukocyte adherence (cells/100 μm venule length), compared with the LR group (4.0 ± 0.9, $P < 0.05$). No differences were observed 6 hrs after treatment on leukocyte adherence and microcirculatory blood flow. ICAM-1 expression was significantly higher in LR-treated animals compared with the HS, LR+PTX, and sham groups ($P < 0.01$). PMN infiltration and overall lung injury were significantly attenuated by HS and LR+PTX. These results support earlier studies that indicated the potential application of HS and PTX in shock therapy and the increase in PMN–endothelial cell interaction and lung injury after LR resuscitation. Exp Biol Med 229:684–693, 2004

Key words: hemorrhagic shock; microcirculation; hypertonic saline; Pentoxifylline; PMN

Hemorrhage is a major cause of death after trauma. In the immediate phase after injury, the amount of blood loss is one of the main determinants of outcome. In later phases, sepsis and multiple organ dysfunction are associated with mortality (1, 2).

This later phase is usually characterized by a generalized and overwhelming inflammatory response, upregulation of proinflammatory cytokines, and infiltration of polymorphonuclear leukocytes (PMN) into tissues, ultimately leading to the development of multiple organ dysfunction. The mechanisms by which hemorrhage triggers the inflammatory response have been extensively studied, but they have not been completely elucidated. PMN are essential components of the innate inflammatory response. During an excessive inflammatory response, the activity of PMN results in tissue damage and severe lung injury (3) that can lead to Acute Respiratory Distress Syndrome (ARDS), a common cause of mortality in patients with sepsis and trauma (4). A rapid release of tumor necrosis factor (TNF)-α plays a central role in the synthesis of adhesion molecules on the PMN (CD11b/CD18) and endothelial cells (ICAM-1; Refs. 5, 6). Furthermore, the impact of different resuscita-

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tion strategies in the modulation of inflammation has not been fully evaluated.

In recent years, small-volume resuscitation by means of hypertonic saline (7.5% NaCl, 4 ml/kg) infusion has gained attention, not only because of its beneficial effects on the restoration of hemodynamic parameters (7–9) and microcirculatory improvements (10), but also because of its effects on different cell populations involved in the complex inflammatory and immune cascade (11–13).

Pentoxifylline [PTX; 1-(5-oxohexyl)-3,7-dimethylxanthine], a methylxanthine derivative and nonspecific phosphodiesterase inhibitor, has been used for the treatment of intermittent claudication in patients with peripheral and cerebrovascular atherosclerotic disease (14). Through its hemorheologic properties, PTX changes the deformability of red blood cells (RBC) and improves microcirculatory blood flow in chronic arterial insufficiency. Clinically, PTX has also been used in the attenuation of the inflammatory response after cardiopulmonary bypass in open-heart surgery, sepsis, and ARDS in neonates. Recent studies have focused on PTX effects on the inflammatory response, more specifically on the PMN. In hemorrhagic shock models, PTX administered during and after hemorrhage improves tissue oxygenation (15), intestinal blood flow (16), and animal survival (17).

In a previous study we demonstrated that hypertonic saline (HS) and PTX significantly attenuated lung injury compared with lactated Ringer's (LR) after hemorrhagic shock in rats (18). With that background in mind, we postulated, as a possible explanation for our previous observations, that HS and PTX may downregulate PMN–endothelial cell interactions. To test this hypothesis we evaluated PMN–endothelial cell interaction as well as microcirculatory flow following fluid resuscitation using a controlled hemorrhagic shock model in rats. Because upregulation of ICAM-1 is an essential step for PMN adhesion, we investigated whether the expression of ICAM-1 in the lung tissue was affected by the type of fluid resuscitation by means of immunostaining. In addition, lung injury by means of histology and lung PMN infiltration by means of myeloperoxidase (MPO) staining were performed.

Materials and Methods

The experiments described herein were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. Approval of the Animal Subject Committee of the Heart Institute (InCor) of the University of São Paulo was obtained prior to initiating the experiments.

Hemorrhage Model. Male Wistar rats weighing 250–350 g were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally. A right inguinal incision was performed, and the femoral vessels (artery and vein) were cannulated with polyethylene catheters. The venous catheter was used for injection of solutions and test drugs, and the arterial catheter was used for monitoring the mean arterial pressure (MAP) and for blood withdrawal. Animals received 100 IU/kg heparin by the intravenous route.

Blood was withdrawn over a period of 10 mins until a MAP of 35 ± 5 mm Hg was reached. This level of hypotension was maintained for 50 mins by blood withdrawal or by reinfusion of shed blood.

At the end of the shock period, fluid resuscitation was given after randomization of the animals into one of four groups: (i) LR solution in a volume equivalent to three times the shed blood; (ii) HS (4 ml/kg NaCl 7.5%); (iii) LR+PTX (LR 3× shed blood + 25 mg/kg pentoxifylline; Pentox, FARMASA, São Paulo, Brazil); and (iv) sham (no shock, no treatment). Immediately after treatment, shed blood was re-infused in treated animals. At the end of the resuscitation, MAP was measured, catheters were removed, the incision was closed, and the animals were returned to their cages.

Direct Vital Microscopy of the Microcirculation. Microcirculatory blood flow as well as PMN–endothelial cell interactions were evaluated by means of intravital microscopy at 2 and 6 hrs after hemorrhagic shock and resuscitation. The animals were anesthetized with 50 mg/kg sodium pentobarbital ip, and the internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination *in situ* (19, 20). Briefly, a longitudinal incision was made on the skin and dartos muscle in the midline over the ventral aspect of the scrotum. The fibers of the cremaster muscle were separated and the internal spermatic fascia was exposed. The animals were maintained on a special board that included a transparent platform on which the tissue to be transilluminated was placed.

The body temperature of the animal was maintained constant at 37°C throughout the experiment. The tissue preparation allowed the internal spermatic fascia to be kept moist and warm by irrigating it with warmed (37°C) Ringer-Locke's solution (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$ñ2 H_2O, 6 mM $NaHCO_3$, and 5 mM glucose), pH 7.20–7.40, containing 1% gelatin.

A television camera was incorporated to a microscope to facilitate observation of the enlarged image (3400×) on the video screen. Images were recorded on a video recorder with a ×40 long distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope (21). The image splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image splitter in the phototube, the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. Vessels selected for the study were third-order venules defined according to their branch-order location within the microvascular network (22). These vessels corresponded to postcapillary venules, with diameters ranging from 12 to 18 μm. This method of evaluating the microcirculation does not require extensive surgical

manipulation for the observation of the vascular network and provides a valuable means of transilluminating a tissue for quantitative studies of the microcirculation. In addition, the preparation is not affected by the respiratory movements of the animal, and the microcirculatory characteristics remain unchanged throughout the course of the experiment.

Analysis of Leukocyte Adherence. The number of sticking leukocytes was evaluated by means of the recorded images. Cells were counted by two independent observers unaware of the resuscitation group the animals belonged to for a 10-min period of observation of recorded images. A leukocyte was considered to be adherent to the venular endothelium if it remained stationary for more than 30 secs (23). The number of cells adhering to the endothelium was evaluated in a 100-μm length of post-capillary venules. Three sections of the vascular bed were tested and averaged for each animal. Cells were counted on the recorded image. Three different fields were evaluated for each animal to avoid sampling variability. Data were averaged for each animal.

Measurement of Blood Flow Velocity. A separate set of animals were anesthetized with sodium pentobarbital (50 mg/kg) 2 hrs after fluid resuscitation and venular blood flow velocity was measured. Centerline RBC velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Venular blood flow was calculated from the product of mean RBC velocity (V_{mean} = centerline velocity/1.6) and microvascular cross-sectional area, with cylindrical geometry assumed. Venular wall shear rate (γ) was calculated from the Newtonian definition: $\gamma = 8(V_{mean}/Dv$, where Dv = vessel diameter; Refs. 24, 25).

Lung Histology. Rat lung tissue harvested 6 hrs after fluid resuscitation was stored in 10% PBS buffered formalin and embedded with paraffin. Five-micron sections were cut from paraffin blocks and transferred onto glass slides. Lung injury was assessed by histology of sections stained with hematoxylin and eosin.

Lung MPO Immunostaining. PMN accumulation in the lung at 6 hrs after fluid resuscitation was assessed by staining tissue sections for MPO. After deparaffinization, the slides were incubated in Target Retrieval Solution (DAKO, Carpinteria, CA) for 20 mins at 95°C and cooled at room temperature. All subsequent steps were conducted at room temperature in a humid chamber. Endogenous peroxide activity was quenched with 1.5% H_2O_2 for 5 mins. Sections were blocked for 20 mins (1.5% goat serum in PBS) and incubated for 2 hrs with rabbit polyclonal MPO antibody (Lab Vision Corporation, Fremont, CA) diluted 1:100 in blocking solution. Sections were washed with PBS and incubated with a biotinylated rabbit secondary antibody diluted 1:400 for 30 mins. Specific labeling was detected with an Elite ABC peroxidase kit and DAB substrate (Vector Laboratories, Burlingame, CA).

Lung ICAM-1 Immunostaining. Two hours after fluid resuscitation, animals were anesthetized with sodium pentobarbital as previously described. A midline laparotomy was performed, and the animals were exsanguinated by transecting the abdominal aorta and the inferior vena cava. After tracheostomy, 10 ml of OCT cryopreservative lung tissue freezing medium (Leica Instruments, Nussloch, Germany) were instilled into the airways. The lungs were harvested through a median sternotomy, snap frozen into liquid nitrogen, and sectioned at 5 μm for immunodetection of ICAM-1.

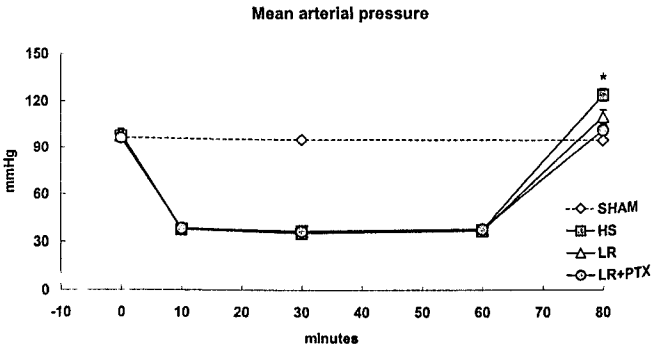

Figure 1. Mean arterial pressure. Hypertonic saline–treated animals (n = 14) had significantly higher mean arterial pressure (MAP) than sham (n = 10); lactated Ringer's (LR, n = 12); or LR plus pentoxifylline–treated animals (n = 13) 20 mins after resuscitation ($P < 0.05$).

Samples were fixed in acetone, hydrated in PBS solution, and exposed to 0.3% hydrogen peroxide (Merck, Sao Paulo, Brazil). Super Block blocking buffer in TBS (Pierce, Rockford, IL) was used to block nonspecific sites. The sections were incubated overnight at 4°C with the primary mouse IgG1 anti-rat ICAM-1 (CD54) monoclonal antibody (Seikagaku Corp., Tokyo, Japan). After washing in PBS, samples were incubated with a biotinylated anti-mouse IgG antibody; treated with avidin-biotin peroxidase complex (Vectastain ABC Elite Kit, Vector); developed in diaminobenzidine containing 0.01% hydrogen peroxide (Merck) solution; and counterstained with hematoxylin solution for light microscopic examination. Negative control samples were incubated with 0.1% bovine serum albumin (BSA) in PBS instead of the primary antibody. Analyses were performed using the Image Software (National Institutes of Health, Washington, DC) version 1.59.

Statistical Analysis. Data are presented as means ± SEM. Comparisons between groups was performed using the nonparametric Kruskal-Wallis test. Multiple comparisons were performed using analysis of variance (ANOVA). The significance level was set at $P < 0.05$.

Results

Hemodynamic Parameters. The initial MAP was higher than 90 mm Hg in all groups. There were no differences regarding MAP among groups before or during shock. However, MAP was significantly higher in HS-treated animals (n = 14) 20 mins after resuscitation

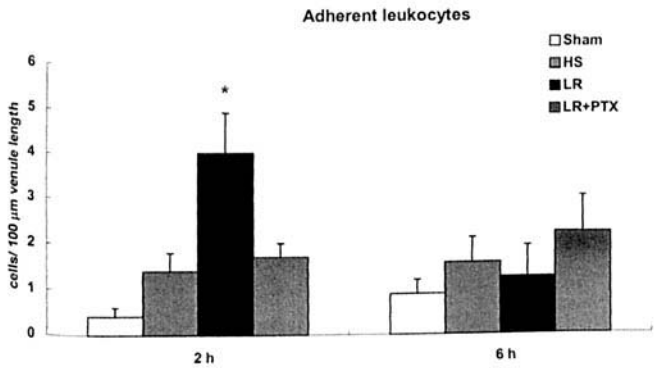

Figure 2. Adherent leukocytes. The number of cells adhering to the endothelium was significantly higher in lactated Ringer's (LR)-treated animals (n = 12) as compared with sham (n = 10)- hypertonic saline (HS, n = 14); and LR plus pentoxifylline (LR+PTX) groups (n = 13) 2 hrs after treatments ($P < 0.05$). No differences between groups were found at 6 hrs comparing LR-treated animals (n = 10) with sham (n = 10), HS- (n = 10), and LR+PTX-treated animals (n = 11).

compared with LR (n = 12), LR+PTX (n = 13), and sham (n = 10) groups ($P < 0.05$; Fig. 1).

There were no differences in the total volume of blood withdrawn after 60 mins of hemorrhage among treated groups. Values were 7.4 ± 0.2 ml for the HS group, 7.5 ± 0.1 ml for the LR group, and 7.6 ± 0.2 ml for the LR+PTX group.

Adherent Leukocytes. The number of leukocytes adhering to the endothelium 2 hrs after fluid resuscitation was significantly higher in LR-treated animals as compared with HS, LR+PTX, and sham animals ($P < 0.05$). At 6 hrs, no significant differences were observed between groups (Figs. 2 and 3).

Blood Flow Velocity and Venular Shear Rate. Vessels selected for study were post-capillary venules, and their diameters are indicated in Table 1. Results presented showed that there were no differences in blood flow velocity and venular shear rate among groups 2 hrs after fluid resuscitation.

Lung Histology. Lung specimens from LR-treated animals presented significant histological changes, including cellular inflammatory infiltrate, alveolar-capillary membrane thickening, hyaline membrane formation, hemorrhage, and edema. In contrast, the histological appearance of lung specimens from LR+PTX and HS-treated animals were similar to sham animals (Fig. 4).

Lung MPO Immunostaining. Polymorphonuclear leukocyte infiltration into the lung tissue was increased in LR-treated animals. LR+PTX- and HS-treated animals showed a decrease in MPO-stained cells in the lung compared with LR (Fig. 5).

Expression of ICAM-1 on Lung Vascular Endothelium. Intracellular adhesion molecule-1 expression in lung vessels evaluated by immune staining was significantly higher in LR-treated animals compared with other treatment

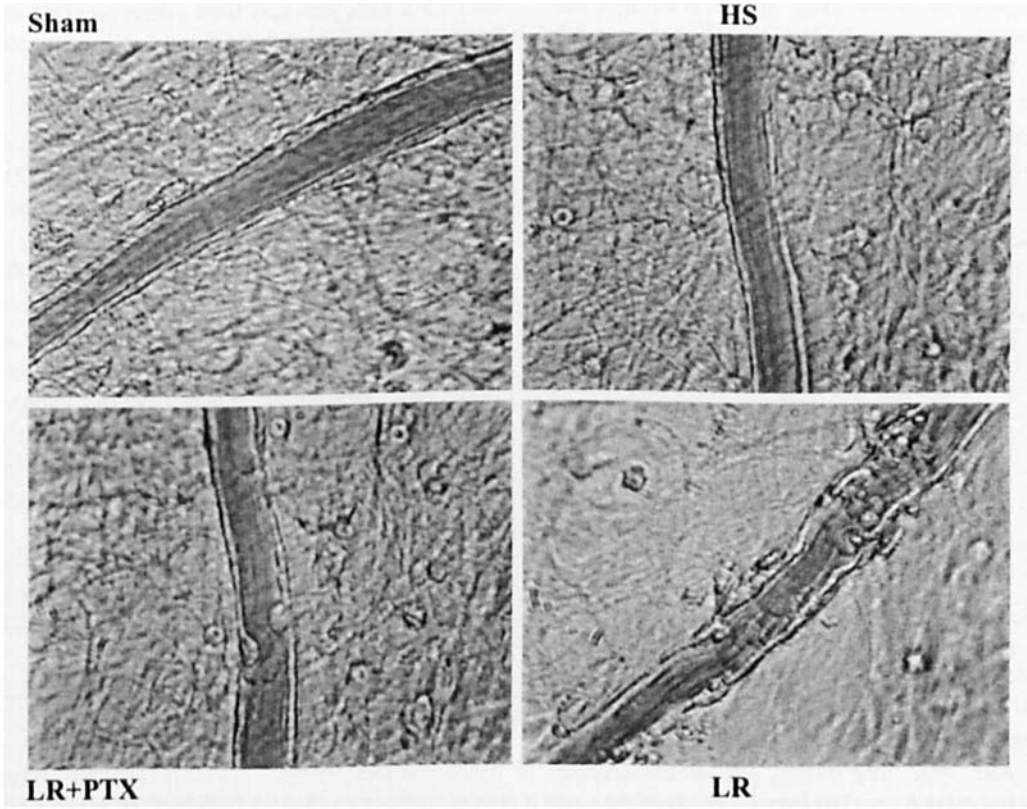

Figure 3. Representative images of intravital microscopy of the spermatic fascia vasculature 2 hrs after shock and fluid resuscitation. No adherent cells were seen in the sham group. Lactated Ringer's (LR) plus pentoxifylline (LR+PTX) and hypertonic saline (HS) treated animals had a very similar number of adherent polymorphonuclear leukocytes (PMN), which were markedly fewer than in LR-treated animals.

Table 1. Hemodynamic Parameters of the Microvascular Bed Measured at Two Hours After Fluid Resuscitation

Group	Diameter (μm)	Flow velocity (mm/s)	Shear rate (s^{-1})
Sham (n = 6)	14.6 ± 0.6	1.9 ± 0.1	1077 ± 60
Hypertonic saline (n = 7)	15.6 ± 0.5	1.9 ± 0.1	978 ± 69
Lactated Ringer's (n = 7)	15.9 ± 0.4	2.0 ± 0.1	1000 ± 60
Lactated Ringer's plus pentoxifylline (n = 7)	15.1 ± 0.6	2.0 ± 0.1	1050 ± 50

groups ($P < 0.01$). Values attained for HS- and LR+PTX-treated animals did not differ from sham animals. Results are summarized in Table 2. Representative sections of these preparations are shown in Figure 6.

Discussion

It has been shown that LR treatment leads to PMN activation, even in the absence of previous hemorrhage (26). Resuscitation with LR is also associated with increased lung injury when compared with HS and PTX resuscitation (11, 13, 18, 27). In a previous study we demonstrated that HS and LR+PTX attenuated lung injury characterized by a significant decrease in PMN infiltration in the alveolar-capillary membrane 24 hrs after hemorrhage and resuscitation compared with LR treatment (18).

The aim of the present study was to determine whether HS and LR+PTX alter PMN adherence and migration to tissues using real time analysis of cellular behavior in the microcirculation by means of intravital microscopy, assessing ICAM-1 expression in the lung, and evaluating PMN infiltration in the lung by means of MPO immunostaining. In addition, lung injury was also evaluated by histology.

Physical forces (shear rate, shear stress) generated by the blood flow within the microcirculation play an important role in the modulation of leukocyte-endothelium adhesion (28). To evaluate the possible interference of hemodynamic changes on leukocyte behavior, mean arterial pressure, microcirculatory blood flow velocity, and wall shear rate were analyzed. No differences were observed among groups with regard to arterial blood pressure levels, venular flow velocity, and shear rate 2 hrs after shock and fluid resuscitation. We therefore concluded that hemodynamic changes did not play a role on the enhanced leukocyte adherence and migration observed in LR-treated animals. This would lead to the upregulation of adhesion molecules as a likely putative mechanism.

The magnitude of leukocyte–endothelial cell interactions that takes place within postcapillary venules is controlled by complex interactions between surface receptors on leukocytes and their corresponding endothelial cell

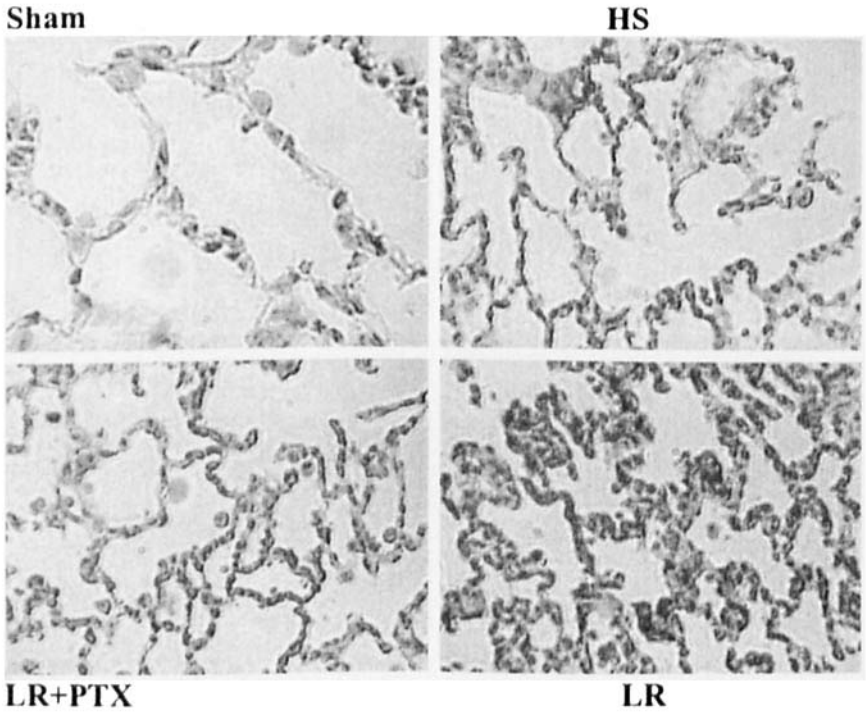

Figure 4. Lung histology at 6 hrs. The lungs of sham animals had a normal appearance at 6 hrs. Histology section of lungs from lactated Ringer's (LR)-treated animals revealed an intense inflammatory process with marked cellular infiltration, edema, and thickening of the alveolar-capillary membrane. Pentoxifylline plus LR and hypertonic saline–treated animals have decreased cellular infiltration, edema, and thickening of the alveolar-capillary membrane compared with LPS. (Magnification ×200, hematoxylin and eosin staining.)

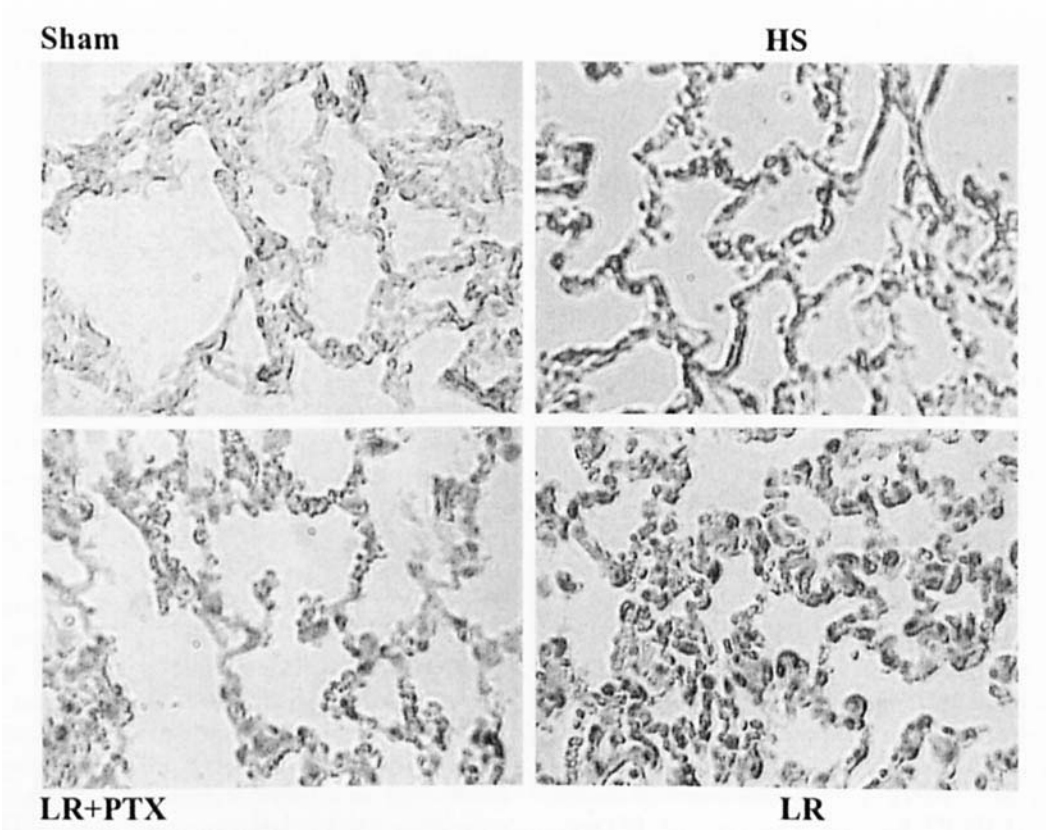

Figure 5. Lung myeloperoxidase at 6 hrs. Polymorphonuclear leukocyte infiltration into the lung tissue was markedly increased in lactated Ringer's (LR)-treated animals. The neutrophilic infiltrate observed in LR plus pentoxifylline and hypertonic saline–treated animals was minimal and similar to the sham group. (Magnification ×200.)

ligands. Leukocyte migration to sites of inflammation involves different sequentially expressed adhesion receptors at the cell surface level. First, selectins are involved in leukocyte rolling. Then, adherence takes place, mediated by β2 integrins (CD11b/CD18) expressed on the leukocytes that bind to the molecules of the Ig superfamily (ICAM-1 and ICAM-2) expressed on the vascular endothelium (28, 29). Firm adhesion associated with potent chemotactic stimuli will eventually lead to migration of inflammatory cells to the interstitial space where degranulation occurs.

The relationship between the resuscitation fluid, tissue injury, and expression of adhesion molecules and other cytokines remains unclear. Song *et al.* (30) demonstrated that lung PMN accumulation following hemorrhagic shock was not associated with ICAM-1 upregulation. Also, deletion of the ICAM-1 gene enhanced hemorrhagic shock–induced lung PMN accumulation (30). Sun *et al.* (31) reported ICAM-1 and VCAM-1 upregulation in animals receiving crystalloid solution, but not in those treated with fresh blood after hemorrhagic shock. These authors also did not observe differences on lung ICAM-1 expression comparing LR to HS resuscitation (31). In contrast, Alan *et al.* (32) showed that LR resuscitation caused a significant increase in pulmonary apoptosis and ICAM-1 expression after hemorrhagic shock. Oreopuolus *et al.* (33) demonstrated that HS pretreatment protects against hepatic ischemia/reperfusion injury in association with reduced hepatic ICAM-1 expression. Furthermore, hypertonicity was associated with inhibition of ICAM-1 expression in activated endothelial cell cultures (33). It has also been shown in a two-hit model (hemorrhagic shock followed by intratracheal endotoxin injection) that adhesion molecule expression was downregulated by HS compared with LR treatment (34).

Table 2. Intracellular Adhesion Molecule-1 (ICAM-1) Immunoreactivity in Lung Vessels

Group	ICAM-1 levels (arbitrary units)
Sham (n = 2)	3.46 ± 2.70
Hypertonic saline (n = 3)	7.27 ± 2.48
Lactated Ringer's (n = 3)	39.04 ± 2.42*
Lactated Ringer's plus pentoxifylline (n = 3)	5.80 ± 1.54

*P < 0.01, lactated Ringer's versus sham, hypertonic saline, and lactated Ringer's plus pentoxifylline.

In the present study, we observed that hemorrhagic shock resuscitation with either HS or PTX decreased ICAM-1 expression in lung vessels. This is a possible explanation of the mechanism by which HS- and PTX-treated animals presented less PMN infiltration into the lungs and decreased lung injury after hemorrhagic shock when compared with what their LR counterparts demon-

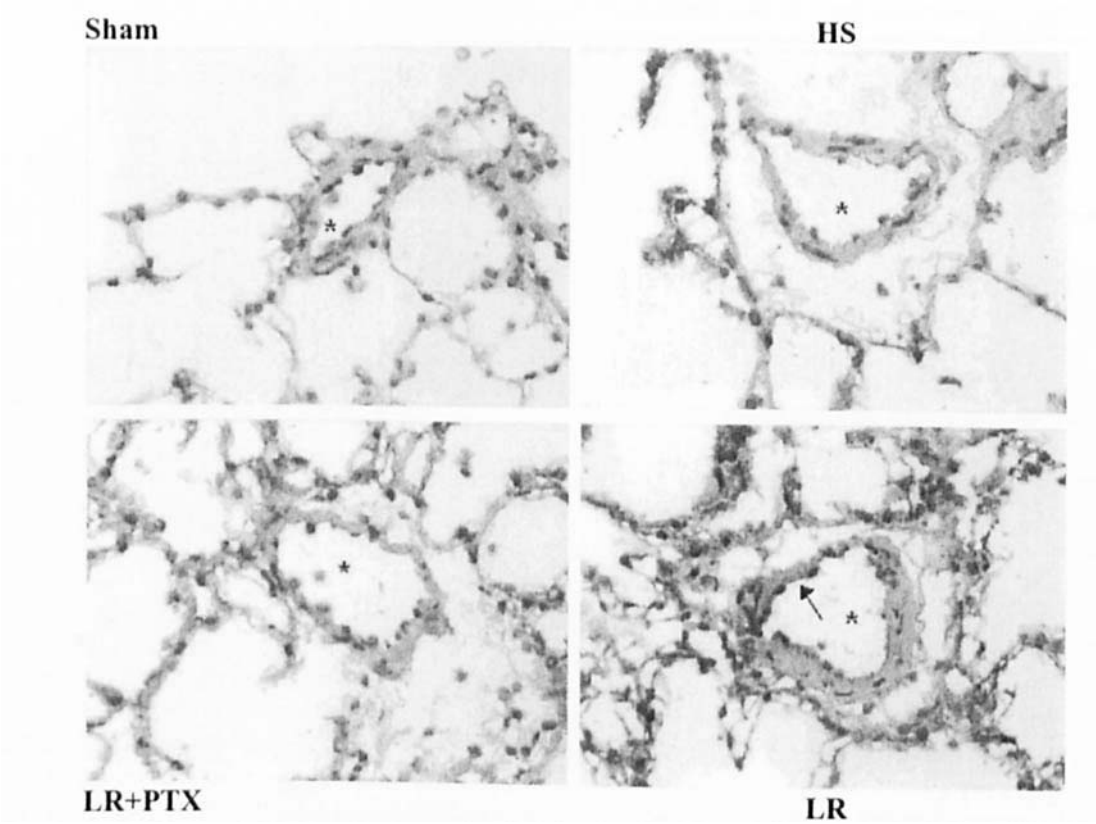

Figure 6. Lung intracellular adhesion molecule-1 (ICAM-1) expression. Relative to hypertonic saline, lactated Ringer's (LR) plus pentoxifylline, sham groups, ICAM-1 expression was increased in lung vascular endothelium of LR-treated animals. The asterisk indicates a vessel within the lung parenchyma. The arrow indicates ICAM-1 staining in the adventia of a small vein.

strated in a previous study (18). Furthermore, increased PMN adherence to the endothelium in LR-treated animals was observed at 2 hrs but not at 6 hrs postresuscitation, paralleling the peak of ICAM-1 expression in the lung.

Studies evaluating the effects of PTX on adhesion molecule expression have shown decreased P-selectin expression after hemorrhagic shock (35) and decreased proinflammatory cytokine-induced ICAM-1 expression in human pulmonary epithelial cells (36) and polymorphonuclear leukocytes (37). Tumor necrosis factor-α is involved in the regulation of ICAM-1 expression (37, 38). As a phosphodiesterase inhibitor, PTX increases intracellular cAMP, thus decreasing TNF-α synthesis, possibly leading to downregulation of ICAM-1 expression.

Hypertonic saline resuscitation has been shown to decrease leukocyte adhesion to the endothelium after hemorrhage. Pascual *et al.* (39) showed that HS resuscitation following hemorrhagic shock decreases PMN rolling and adherence to the endothelium and reduces vascular leakage compared with LR-treated animals. However, no differences were observed on microcirculatory blood flow and expression of ICAM-1 in the cremaster muscle between groups. Angle *et al.* (27, 40) demonstrated that peripheral PMN count was significantly higher in HS compared with LR resuscitation 2 hrs after hemorrhagic shock, suggesting that PMN margination or tissue sequestration is decreased following HS resuscitation. Those authors also observed that HS reduces PMN L-selectin expression. In a two-hit model of hemorrhagic shock and infection, HS resuscitation attenuated PMN lung sequestration and transmigration by decreasing leukocyte-endothelium interactions (41).

Pentoxifylline has been widely studied as an adjunct to fluid resuscitation in hemorrhagic shock, and many beneficial effects have been described. Pentoxifylline seems to restore cardiac output (42); downregulate the synthesis of important proinflammatory mediators such as TNF-α, IL-1, and IL-6 (43, 44); reduce shock-induced leukocyte adhesion to the sinusoidal endothelium of the liver; improve microvascular hepatic and intestinal blood flow (16, 43, 45, 46); restore hepatocellular function (43); and increase survival after hemorrhagic shock (47).

It is possible that, by a variety of mechanisms, PTX and HS are protective against organ injury development following hemorrhagic shock. We demonstrated that microcirculatory leukocyte adherence was significantly increased in LR-treated animals 2 hrs after shock as compared with HS, LR+PTX, and sham animals, paralleling ICAM-1 expression. In addition, PMN infiltration in the lung was markedly increased 6 hrs after fluid resuscitation in LR-treated animals. PTX- and HS-induced attenuation of PMN adherence and migration as well as decreased lung ICAM-1 expression are possible mechanisms responsible for the

protective effects of these resuscitation regimens in the development of lung injury. Other cellular and subcellular mechanisms related to the modulation of the inflammatory response by HS and PTX may take place and require further investigation.

This study has some limitations. Intracellular adhesion molecule-1 expression was assessed in the lung vasculature, but the intravital microscopy data was obtained in the spermatic fascia. This has been a limitation of several studies, because the methodology used for the evaluation of the lung microcirculation *in vivo* by means of intravital microscopy in rodents is underdeveloped and technically difficult. Most studies use PMN behavior data obtained from direct observation of the mesenteric circulation, cremaster muscle, or—as in our study—of the spermatic fascia to justify changes in adhesion molecule expression, PMN infiltration, and tissue injury in distant organs. In fact, Pascual *et al.* (39) evaluated the effects of HS on PMN-endothelial cell interaction, assessing PMN rolling and adhesion in the cremaster muscle, a preparation slightly different than ours. These authors found decreased PMN rolling and adherence in HS-treated animals, although ICAM-1 expression in the cremaster muscle was similar in both groups. They postulated that ICAM-1 may not be the principal target of HS-mediated effects or their model did not provide enough stimulation for ICAM-1 expression in the cremaster. Based on the available data on ICAM-1 expression following HS and PTX infusion, we strongly believe that ICAM-1 expression is decreased by those resuscitation regimens, which leads to less PMN adhesion and migration, as demonstrated by our data. Another limitation of the present study refers to the fact that the expression of other adhesion molecules on the PMN surface was not studied. However, studies from our laboratory and others have shown that HS and PTX downregulate L-selectin as well as CD11b expression under experimental conditions (27, 40, 48–50). Finally, a more in-depth, detailed, microcirculatory analysis, including functional capillary density and capillary wall swelling/deswelling, could have been done. Unfortunately, the software available at the time the experiments were done did not allow us to obtain the above-mentioned measurements.

Additionally, one may question whether differences in salt load comparing LR and HS resuscitation could have influenced the results of the present study. To equalize the salt load, the volume of LR must be increased, or the HS volume or concentration must be decreased. The former is a dangerous option because it may aggravate fluid overload, cellular swelling, pulmonary edema, and increase even further the inflammatory activation. The latter has not been shown to be an effective strategy to treat hemorrhagic shock. Both resuscitation regimens were used because they have been demonstrated to adequately resuscitate different animal species and humans following hemorrhagic shock (7, 9, 11, 13). More importantly, this study also compared conventional LR resuscitation with LR+PTX, solutions with the same sodium load but lead to completely opposed results in terms of PMN–endothelial cell interactions, ICAM-1 expression, and lung injury.

Based on our data, it is possible to establish a correlation between an early decrease in leukocyte adherence and attenuated lung inflammatory cell infiltration following HS and LR+PTX resuscitations. Intravascular volume expansion was probably no different between LR and LR+PTX, and it was probably similar comparing HS and LR at an early time point based on adequacy of resuscitation (hemodynamic and metabolic), which was shown in previous studies. In addition, blood flow velocities were no different between groups; this is the reason why early differences in intravascular volume expansion in and of itself cannot explain the differences in PMN adherence between groups.

Recent studies have shown that LR activates PMN and is associated with increased organ injury, particularly in the lungs (26, 51, 52). Recent studies have shown that LR solution adequately improves hemodynamic parameters after hemorrhagic shock, but it may cause detrimental effects on the immune response by altering leukocyte function (51, 53). These effects are most likely related to the composition of the racemic solution, which includes D- and L-lactate. Polymorphonuclear leukocyte margination; adhesion (39, 54); and the expression of adhesion molecules (51) was increased in animals treated with LR and decreased after HS treatment following hemorrhagic shock.

It is possible that LR upregulates lung ICAM-1 expression following hemorrhagic shock and PTX blunts that response, to the same extent that eliminating D-lactate from the racemic LR solution minimizes some of the "pro-inflammatory" properties of that solution. However, it is possible that other events, such as ischemia and reperfusion, are more relevant than the resuscitation solution in initiating the activation of the inflammatory cascade and the upregulation of adhesion molecules, which are blunted or downregulated by LR+PTX and HS. Previous studies from our laboratory and others have shown that HS and PTX have immunomodulatory properties when compared with LR (11–13, 34, 40, 48–50).

Other solutions have recently been used in experimental models of shock such as ethyl pyruvate, sodium pyruvate, and ketone bodies, all of which replace D-lactate and therefore attenuate the inflammatory response following shock (55–57). However, racemic LR is the standard of care used for fluid resuscitation following hemorrhagic shock and should be used for comparison to other solutions or drugs already in clinical use if development of clinically relevant resuscitation strategies based on translation research is a goal.

The use of isotonic saline (0.9% NaCl) instead of LR seems to be, at a glance, an attractive strategy to eliminate the effects of D-lactate on the inflammatory response. However, multiple *in vitro* experiments have shown that inflammatory cells suspended in salt-balanced media (with-

out D-lactate) are activated by multiple stimuli leading to an exaggerated proinflammatory response. The addition of PTX and HS significantly attenuates that response.

In conclusion, HS and PTX resuscitation following hemorrhagic shock downregulates PMN–endothelial cell interactions and lung ICAM-1 expression. Additional studies are necessary to determine if data obtained by evaluating the microcirculation distant to target organs such as the intestinal wall, the liver, and the lungs can be extrapolated to events occurring in the microcirculation of those organs. Further investigation on the mechanisms of PMN modulation following PTX and HS is necessary to elucidate whether these substances share the same mechanisms of action or if by different pathways they achieve similar results. These resuscitation strategies are not new but have not been implemented in clinical practice. Modulating the inflammatory response that follows major hemorrhage with different resuscitation regimens may prove beneficial in clinical practice.

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