

# Anti-Inflammatory Treatment with Standardized Human Serum Protein Solution Reduces Local and Systemic Inflammatory Response after Hemorrhagic Shock

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## Key Words

Hemorrhage · Shock · Reperfusion · Inflammatory response · Microcirculation

## Abstract

**Objective:** Reperfusion after hemorrhagic shock leads to local and systemic inflammatory response. This study evaluates the effect of a short-term treatment with standardized human serum protein solution (SPS) on the local and systemic inflammatory response in the mesenteric microcirculation in the rat. **Methods:** Spontaneously breathing animals underwent median laparotomy and exteriorization of an ileal loop for intravital microscopy of the mesenteric microcirculation. Volume-controlled hemorrhagic shock was set by arterial blood withdrawal (2.5 ml/100 g body weight for 60 min), followed by reperfusion for 4 h. SPS (n = 10) or saline 0.9% (controls, n = 10) was given intravenously as a continuous infusion for 30 min at the beginning of reperfusion ('pre-hospital'). This was followed in both groups by substitution of blood and normal saline to support blood pressure ('in-hospital'). Systemic hemodynamics, mesenteric microcirculation and arterial blood gases were monitored before, during and after shock, and for 4 h after initiation of reperfusion. **Results:** SPS treatment markedly reduced leukocyte/endothelial interaction, and reduced the need for intravenous fluids compared to controls. For the entire observation period,

blood pH was unchanged from baseline only in SPS-treated animals. The improvement of base excess and abdominal blood flow persisted for 2 h after SPS infusion. **Conclusion:** Short-term SPS treatment of hemorrhagic shock improved mesenteric microcirculation, arterial blood gases and global hemodynamics, and attenuated the inflammatory response to reperfusion. It may provide clinical benefit when applied at an early phase of reperfusion after hemorrhagic shock.

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## Introduction

Reperfusion injury after hemorrhagic shock is orchestrated by a variety of mechanisms such as generation of oxygen radicals, complement activation, recruitment of neutrophils, and enhanced coagulation states [1]. Importantly, the inflammatory-type reactions of reperfusion injury significantly enhance tissue injury after hemorrhagic shock [1]. A number of different treatment strategies for hemorrhagic shock have been explored, and mainly solutions lacking anti-inflammatory properties were used [2]. Among them was the use of hydroxyethyl starch solution, which has provided significant benefit in some experimental and clinical situations [3, 4]. However, despite these favorable effects there is controversy as to whether the use of colloids has any advantages over

crystalloids [5], and there is evidence that repeated infusion of hydroxyethyl starch solutions might lead to an unfavorable hepatic dysfunction [6]. Our own previous work has highlighted the anti-inflammatory properties of albumin solution, and C1-esterase inhibitor after hemorrhagic shock. C1-esterase inhibitor bolus treatment significantly reduced the local inflammatory response without having a marked effect on systemic hemodynamics [7]. Compared to this, albumin infusion reduced leukocyte/endothelial interaction, and improved mesenteric and systemic hemodynamics after hemorrhagic shock substantially [8]. However, treatment of hemorrhagic shock with protein-containing solutions remains controversial. The resulting protein load might worsen pulmonary function in clinical conditions such as acute pancreatitis and abdominal sepsis [9, 10]. Yet, this effect was not seen with the use of albumin in early reperfusion after hemorrhagic shock [8]. Further studies have highlighted the anti-inflammatory effect of albumin when substituted for plasma protein during abdominal surgery [11], and in lung injury following shock [12].

Since reperfusion injury is a complex situation, the use of a 'reperfusion cocktail' counteracting major factors, which contribute to reperfusion injury, might be useful. A preparation, which naturally contains a variety of factors that might possibly exert beneficial effects in the setting of reperfusion injury, is fresh-frozen plasma [13]. A major disadvantage of fresh-frozen plasma is the limited availability in a 'pre-hospital' setting [14–16]. Alternatively, a standardized solution of human serum protein (SPS) [17–19] is commercially available for the 'pre-hospital' phase of treatment, which does not require typing, or delicate temperature management.

In the present study, we explored the effect of a short-term SPS treatment on leukocyte/endothelial interaction, and local and systemic hemodynamics after volume-controlled hemorrhagic shock. Further, we addressed the hypothesis whether SPS has beneficial effects on reperfusion injury after hemorrhagic shock in addition to some of its major components, such as albumin and C1-esterase inhibitor.

## Materials and Methods

### *Experimental Conditions*

Twenty-five male Sprague-Dawley rats were maintained on standard rat chow and water ad libitum until the night before the experiment. Rat chow was removed 10–12 h before the beginning of the experiment to reduce intestinal peristalsis during intravital microscopy. After anesthesia with urethane (1.25 g/kg BW i.m.,

single dose), the femoral artery and jugular vein were cannulated with small polyethylene tubes for arterial blood gas analysis, and arterial and central venous blood pressure recordings. From each rat, 250 µl of blood was drawn into heparinized syringes. Arterial blood gases (PaO<sub>2</sub>, SO<sub>2</sub>, PCO<sub>2</sub>, pH, and base excess), hemoglobin, hematocrit, lactate, potassium, and sodium, were analyzed with an arterial blood gas analyzer (ABL615, Radiometer, Copenhagen, Denmark). After median laparotomy, a Doppler flow transducer was placed around the abdominal aorta as previously described [20]. Hemodynamic data (abdominal blood flow in the descending aorta), heart rate, systolic, mean and diastolic arterial blood pressure, and central venous pressure, were recorded on a beat-to-beat basis using System 6 (Triton Technology, San Diego, Calif., USA). Analogue data were digitized and recorded online with a computer-based system (DASYLab, National Instruments Corporation, Austin, Tex., USA). An electrocardiogram in lead II was recorded during the experiment to detect electrocardiographic changes. Rectal temperature was kept constant at 37.5 ± 0.5°C by means of a feedback-controlled homeothermic blanket control unit (Harvard Apparatus, South Natick, Mass., USA). Animals were placed on a heating pad in a left lateral recumbent position on an adjustable Plexiglas microscope stage. A segment of the ileum was exteriorized through the abdominal incision and prepared for in vivo microscopic observation as published previously [7]. Intravital microscopy was performed with epi- and trans-illumination observing three to five unbranched mesenteric venules (diameter: 25–40 µm; length: 100–150 µm) (Axiotech fluorescence microscope with computer-controlled scanning table [Carl Zeiss, Jena, Germany], AttoArc 100 W light source [Atto Bioscience, Rockville, Md., USA]). Rhodamine-6G-labeled leukocytes were counted using epi-illumination. Cell counts were normalized to the endothelial surface assuming cylindrical geometry. Off-line analysis of coded video recordings was performed in a blinded manner.

Rats received a basal infusion with albumin in physiologic saline for compensation of intra-operative loss and evaporative water loss (0.3 ml/h/100 g body weight) after the central venous catheter was placed as previously described [11]. In this study, standardized human serum protein solution (SPS) (Biseko®, Biotest AG, Dreieich, Germany) was used during reperfusion (see 'Experimental Protocol'). Biseko is a coagulation factor-depleted, cold-sterilized serum preparation containing albumin, α<sub>1</sub>-antitrypsin, α<sub>2</sub>-macroglobulin, antithrombin III, and IgA, IgM and IgG.

Five animals died during the phase of systemic hypotension before reperfusion, and were excluded from the study. Mean body weights of SPS group and controls (saline 0.9%) were 290 ± 10 g and 286 ± 11 g, respectively.

All investigative procedures and the animal facilities conformed to the Guide for the Care and Use of Laboratory Animals. The regional animal care and use committee approved the protocol.

### *Experimental Protocol*

After exteriorization and fixation of the mesentery, rats were allowed to stabilize for 30 min and mesentery microcirculation was observed for 5 h of experimental time. Volume-controlled hemorrhagic shock was provoked during the first 10 min by withdrawing 2.5 ml/kg body weight of arterial blood from the femoral artery. The shed blood volume was transferred to citrate plasma

tubes (citrate to blood ratio 1:10). After 60 min of systemic hypotension, reperfusion was initiated. The reperfusion phase was divided into a 'pre-hospital' phase of 30 min and an 'in-hospital' phase. Before reperfusion, animals were randomized to two groups. The first group received SPS during the 'pre-hospital' phase (SPS group, n = 10), a second group (n = 10) receiving normal saline during this phase served as controls. During this 'pre-hospital' phase, either SPS or normal saline was administered intravenously until a mean arterial blood pressure of 70 mm Hg was reached. The 'in-hospital' phase started with administration of red blood cells to compensate for the initial blood loss. Afterward, all animals received fluid substitution (normal saline intravenously) at the beginning of the 'in-hospital' phase. The minimum infusion was normal saline at two times the shed blood volume infused for the first 30 min after initial treatment with SPS or normal saline. Thereafter, normal saline at the equivalent of at least the shed blood volume was infused over 2 h, followed by maintenance infusion with normal saline throughout the experiment, so that mean arterial blood pressure did not decrease below 70 mm Hg.

#### Statistical Analysis

Data are presented as means  $\pm$  SEM. Statistical analysis was performed with Sigma Stat<sup>®</sup> (SPSS Science Inc., Chicago, Ill., USA). Statistical significance of changes from baseline values within each group was tested with analysis of variance (ANOVA) for repeated measures. For nonparametric values, an ANOVA on ranks was applied. Differences between groups were statistically analyzed by one-way ANOVA. Likewise, ANOVA for nonparametric values was used (Kruskal-Wallis test) with multiple comparison method (Student-Newman-Keuls test) where applicable. Statistical significance was accepted at  $p < 0.05$ .

## Results

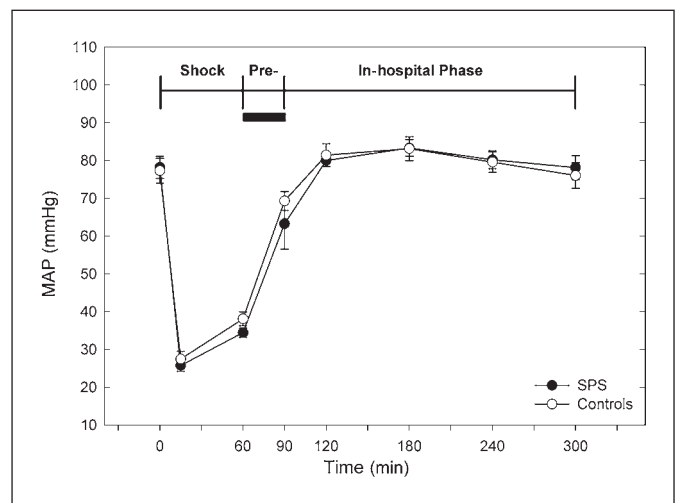
### Hemodynamics

**Mean Arterial Blood Pressure, Heart Rate, Central Venous Pressure, and Volume Substitution.** Immediately after induction of hemorrhagic shock, mean arterial blood pressure decreased to below 40 mm Hg until 60 min of the experimental time. After reperfusion, mean arterial pressure increased during the first 30 min, and no differences were observed between the two experimental

groups (fig. 1). Heart rate was similar between the groups at the beginning (SPS  $368 \pm 15$  bpm; controls:  $358 \pm 16$  bpm), increased significantly during systemic hypotension after 60 min (SPS:  $399 \pm 13$  bpm; controls:  $396 \pm 13$  bpm,  $p < 0.05$ ), and then plateaued until the end (SPS:  $437 \pm 14$  bpm; controls:  $414 \pm 15$  bpm).

Central venous pressure (CVP) increased in both groups at the beginning of reperfusion and was significantly higher at 60 min of reperfusion in the SPS group (data not shown). CVP equalized in both groups after an additional 60 min until the end of the observation period.

Total volume substitution was significantly lower in SPS-treated animals compared to controls (table 1). After reperfusion, the volume needed for an equal increase in mean arterial blood pressure was significantly smaller in the SPS-treated group compared to control animals. Be-



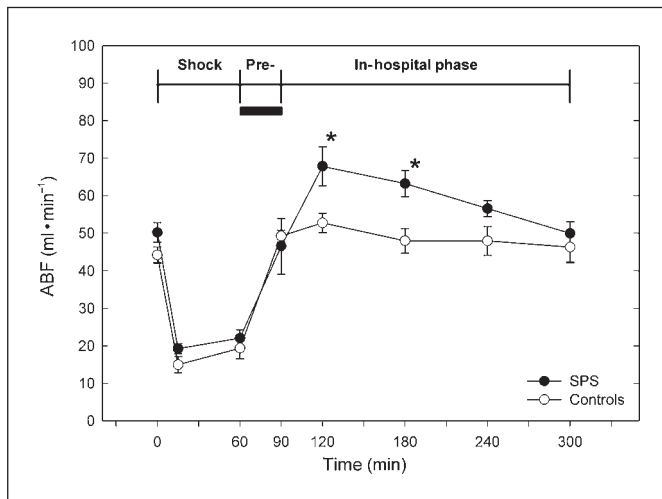
**Fig. 1.** Mean arterial blood pressure (MAP). Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls: saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%.

**Table 1.** Volume substitution during reperfusion at different time intervals

	Time interval					Total volume
	1-1.5 h	1.5-2 h	2-3 h	3-4 h	4-5 h	
SPS, ml	11 $\pm$ 0.5	11 $\pm$ 1	6 $\pm$ 0.2	6 $\pm$ 0.1	4 $\pm$ 2	38 $\pm$ 2
Controls, ml	18 $\pm$ 1*	12 $\pm$ 1	10 $\pm$ 2	11 $\pm$ 2	9 $\pm$ 2	60 $\pm$ 6*

SPS = Standardized human serum protein solution; controls = saline 0.9%.

\*  $p < 0.05$ .



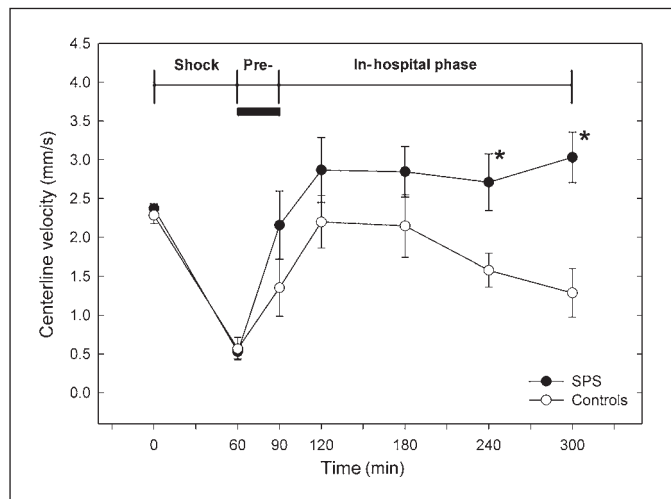
**Fig. 2.** Abdominal blood flow (ABF). Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%. \* Significant difference between the two groups,  $p < 0.05$ .

tween 3–4 and 4–5 h of reperfusion, the volume needed to support mean arterial blood pressure was comparable in both groups.

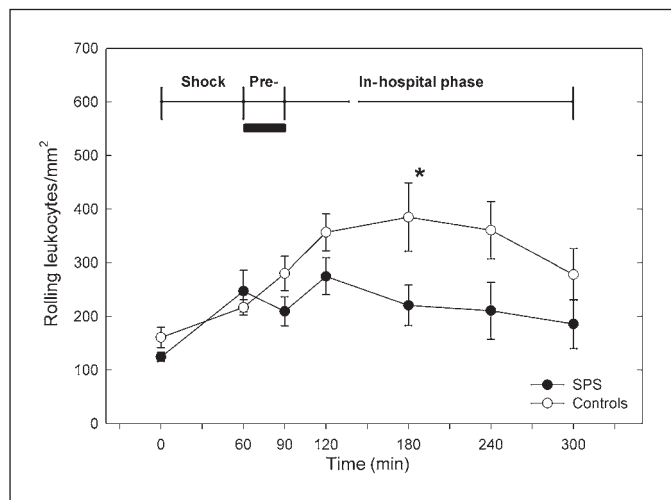
**Abdominal Blood Flow.** Abdominal blood flow in the descending aorta is depicted in figure 2. There were no differences between the two groups at the beginning and after 1 h of systemic hypotension. Abdominal blood flow increased after reperfusion at 90 min in both groups, and was significantly higher in the SPS group at 60 and 120 min after the initiation of reperfusion.

#### Microcirculation

**Centerline Velocity and Shear Rate.** Venular diameters did not differ significantly during the experiment. Centerline velocity and shear rate at baseline were similar in both groups and decreased in parallel during systemic hypotension (fig. 3, and data not shown). Sixty minutes after reperfusion, centerline velocity exceeded baseline values in the SPS-treated group, whereas it remained at or below baseline values in control animals. Elevation of centerline velocity in the SPS group continued until the end of the experiment, and was significantly higher than in controls 180 and 240 min after reperfusion (fig. 3). As venular diameters did not significantly differ during the observation period, shear rate paralleled centerline velocity values (baseline: SPS:  $452 \pm 12 \text{ s}^{-1}$ , controls:  $506 \pm 69 \text{ s}^{-1}$ ; 300 min: SPS:  $414 \pm 30 \text{ s}^{-1}$ , controls:  $217 \pm 61 \text{ s}^{-1}$ ,  $p < 0.05$ ).

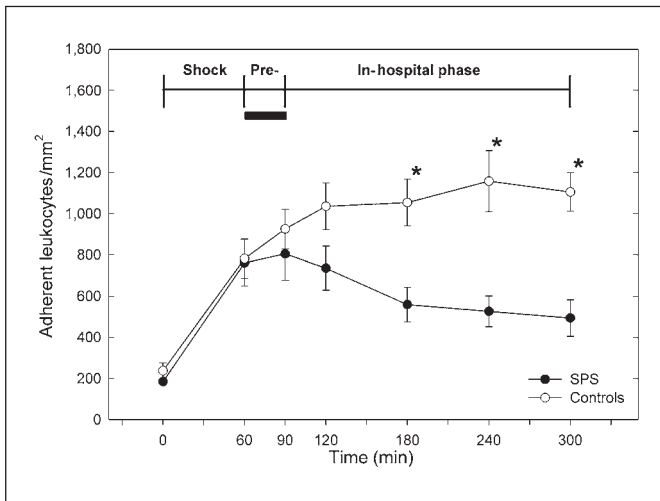


**Fig. 3.** Centerline velocity. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%. \* Significant difference between the two groups,  $p < 0.05$ .

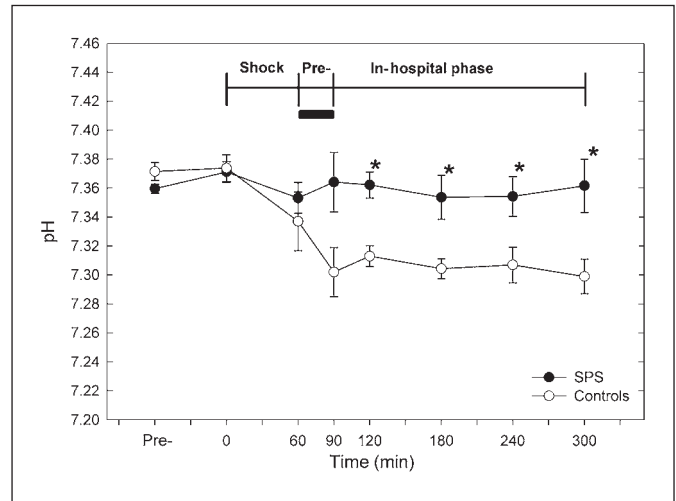


**Fig. 4.** Rolling leukocytes. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%. \* Significant difference between the two groups,  $p < 0.05$ .

**Rolling and Adherent Leukocytes.** Number of rolling and adherent leukocytes showed no significant differences between groups at the beginning of the experiment. A marked increase of rolling leukocytes was registered during systemic hypotension, with a further increase in the control group over the following 4 h of reperfusion. The



**Fig. 5.** Adherent leukocytes. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%. \* Significant difference between the two groups,  $p < 0.05$ .



**Fig. 6.** Blood pH. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; control = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%; Pre- = before abdominal surgery. \* Significant difference between the two groups,  $p < 0.05$ .

number of rolling leukocytes approximately doubled from baseline values in this group (fig. 4), and the increase of adherent leukocytes was even more pronounced in controls (five to six times baseline values) in contrast to the SPS group (fig. 5).

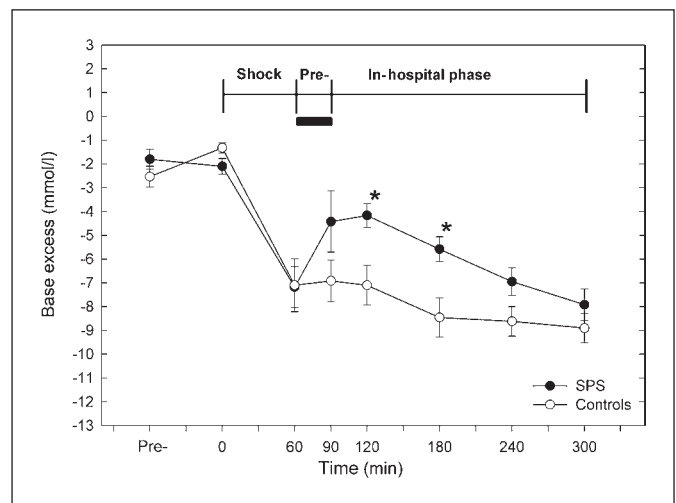
#### Arterial Blood Gas Values

PaO<sub>2</sub> values for both groups did not differ at the beginning (SPS: 85  $\pm$  4 mm Hg, controls: 86  $\pm$  2 mm Hg) or the end of the experiments (SPS: 92  $\pm$  10 mm Hg, controls: 87  $\pm$  2 mm Hg). Similar values were obtained for SaO<sub>2</sub> (data not shown).

Arterial PaCO<sub>2</sub> of all groups were comparable at baseline (SPS: 41  $\pm$  1 mm Hg, controls: 41  $\pm$  1 mm Hg), and at the end of the experiments (SPS: 30  $\pm$  2 mm Hg, controls: 34  $\pm$  2 mm Hg). PaCO<sub>2</sub> decreased significantly during the experiments in both groups ( $p < 0.05$ ).

Blood pH did not differ between the groups at the beginning of the experiments (fig. 6). In control animals, blood pH decreased after reperfusion, whereas it remained at baseline values in the SPS-treated group until the end of the experiments.

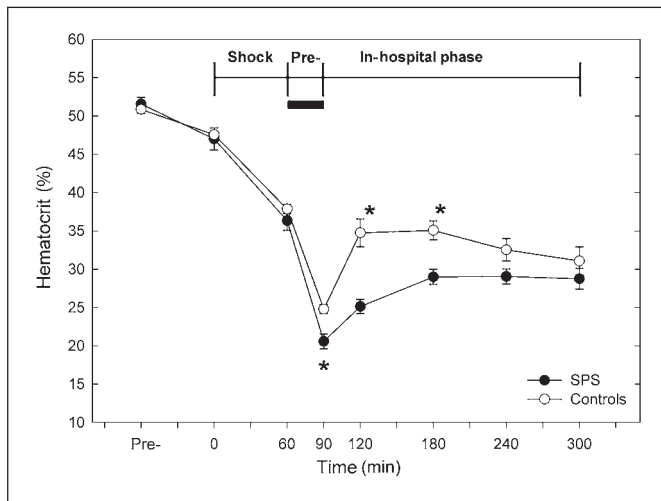
Base excess values are depicted in figure 7. In both groups, base excess decreased 4-fold during systemic hypotension compared to baseline values. Base excess remained at this level in controls until the end of the experiments, whereas it significantly increased until 60 min



**Fig. 7.** Base excess. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%; Pre- = before abdominal surgery. \* Significant difference between the two groups,  $p < 0.05$ .

after reperfusion in SPS-treated animals, and remained significantly higher until 120 min after reperfusion compared to controls ( $p < 0.05$ ).





**Fig. 8.** Hematocrit. Data are presented as means  $\pm$  SEM. SPS = Standardized human serum protein solution; controls = saline 0.9%. Horizontal bold black bar = Infusion of SPS or saline 0.9%; Pre- = before abdominal surgery. \* Significant difference between the two groups,  $p < 0.05$ .

Hematocrit values were comparable at baseline, and decreased 60 min after induction of hemorrhagic shock (fig. 8). Hematocrit values were significantly different between both groups after the first 30 min of reperfusion (SPS:  $20.6 \pm 1.0\%$ , controls:  $24.8 \pm 0.6\%$ ,  $p < 0.05$ ), which persisted for 2 h during reperfusion. At the end of the 'in-hospital' phase, hematocrit returned to approximately 30% in both groups.

## Discussion

Early infusion of standardized human serum protein solution (SPS) significantly reduced reperfusion injury after hemorrhagic shock. The infusion of SPS improved mesenteric microcirculation, systemic hemodynamics and prevented acidosis. Further, SPS treatment significantly reduced leukocyte recruitment to the mesenteric microcirculation for the entire observation period.

Leukocyte-endothelium cell-cell interaction is known to be one important step at the beginning of an inflammatory response syndrome, in parallel to contact activation and cytokine release [21–24]. In the acute phase of reperfusion injury, neutrophils are recruited to the damaged tissue, and since neutrophils promote acute inflammation, leukocyte/endothelial interaction and neutrophil recruitment are usually used as a surrogate for

inflammatory tissue damage [25]. Reperfusion injury may be an important source of organ failure and is applicable to a wide variety of problems (aortic cross-clamping, myocardial reperfusion injury, late resuscitation) [26]. Reductions of adherent and rolling leukocytes in the mesenteric microcirculation after SPS administration suggest an anti-inflammatory effect in the treatment of reperfusion injury after hemorrhagic shock. SPS is a serum preparation containing mainly albumin plus  $\alpha_1$ -antitrypsin,  $\alpha_2$ -macroglobulin and antithrombin III [19]. The observed anti-inflammatory properties of SPS could therefore be the sum of several effects, such as free radical scavenger activity and suppression of the release of reactive oxygen species, effects that are well described for albumin treatment [27–29]. Antithrombin is known to reduce the inflammatory response [30–32], and might therefore exert a synergistic effect with albumin and other components of SPS.

The intermittent increase in central venous pressure and abdominal blood flow observed during reperfusion with SPS can be explained with a hyperoncotic effect of SPS and an increase of intravascular volume. There is good evidence that the use of hyperoncotic solutions without direct anti-inflammatory properties during resuscitation can improve microcirculatory perfusion and reduce reperfusion injury [33]. The question to be answered is whether this could apply also for the beneficial effects seen with SPS during resuscitation. In our study, SPS was used to reverse systemic hypotension within a short period, and the anti-inflammatory effect with a reduced number of rolling and adherent leukocytes persisted until the end of the experiments. Further, centerline velocity and shear rate in the microcirculation did not differ significantly between the groups until 180 min after initiation of reperfusion. Then again, abdominal blood flow in the SPS-treated group reaches control group levels 120 min after initiation of reperfusion. Hence, the beneficial effect for the microcirculatory perfusion did not show while abdominal blood flow was increased, and, therefore, the observed effect cannot be sufficiently explained with enhanced global perfusion after SPS treatment. Further studies are needed to show a long-term impact of hyperoncotic solutions on the microcirculatory perfusion.

Animals treated with SPS needed significantly smaller volumes of intravenous fluids to reverse systemic hypotension, and significantly smaller total volumes to support blood pressure until the end of the experiments. Clinical studies have shown that a requirement of large volumes of crystalloid solutions in shock is associated

with increased mortality [34], although no differences in mortality were observed in the present study. Reperfusion treatment in our study of volume-controlled hemorrhagic shock was blood pressure guided (approximately 70 mm Hg after 30 min of reperfusion) as this is common in clinical practice. With the applied resuscitation policy, mean arterial blood pressure was comparable in both groups during the entire observation period. The beneficial effects of aggressive fluid resuscitation to normalize mean arterial blood pressure rapidly after hemorrhagic shock in our study may not be copied to uncontrolled shock situations, as this may promote mortality with uncontrolled bleeding [35–38]. Most likely because of the volume expansion with SPS, hematocrit was substantially lower in SPS-treated animals during the first 2 h of reperfusion with an increase to comparable levels as in control animals thereafter. However, our results did show any unfavorable effects on arterial oxygen pressure and oxygenation during the first hours of hemorrhagic shock therapy. Animals in our study were not intubated or mechanically ventilated. As unconscious patients with profound hypotension usually receive airway management, the model does not mimic clinical practice in this point. However, refraining from the use of ventilatory support allows studying the effect of compensatory hyperventilation on acid-base metabolism. Oxygen uptake was unimpaired throughout the whole observation period even without ventilatory support. The early reperfusion strategy with infusion of protein-containing solutions (SPS, albumin [8]) for treatment of hemorrhagic shock is different from those encountered in abdominal sepsis, where systemic inflammatory response syndrome is already completely developed and infusion of protein-containing solutions can worsen pulmonary function [9, 10].

As we showed in previous studies, either the use of a C1-esterase inhibitor or albumin markedly reduced reperfusion injury in the mesenteric microcirculation after hemorrhagic shock [7, 8]. However, there was no effect on blood pH after hemorrhagic shock as seen with SPS treatment. SPS-treated animals and control animals hyperventilated to comparable PaCO<sub>2</sub> levels. However, blood pH was unchanged from baseline levels only in SPS-treated animals until the end of the observation period, which might be explained with an additional buffer effect of SPS [16]. From the data, it remains unclear why base excess is only temporarily increased in SPS-treated animals. The most likely explanation for this phenomenon is an initial buffer effect of SPS that does not persist until the end of the experiments with an increasing bicarbonate use to balance pH after the effect of SPS faded.

There is ongoing debate on the optimal reperfusion strategy in various shock situation and whether colloids are superior to crystalloids, and most strategies aim at an improved microcirculatory perfusion [3, 5]. However, there is evidence that these reperfusion strategies do not reduce the inflammatory component of reperfusion injury [2]. As reperfusion injury is a complex situation with activation of complement and other systems, a promising strategy might be the use of an enriched 'reperfusion cocktail' counteracting major routes that contribute to reperfusion injury. Although the exact mechanisms by which SPS improved acid/base balance and microcirculation need to be further explored, the effects of SPS shown in our functional study might guide towards an optimized reperfusion solution.

During and after cardiopulmonary resuscitation, attention is usually only paid to cardiac function, and sufficient oxygen supply. After successful resuscitation, patients are then explored for brain damage as this usually determines the ultimate fate of a patient. However, significant complications after successful resuscitation include pulmonary infection and sepsis with multiple organ failure [39]. The role of mesenteric hypoperfusion and reperfusion injury in the setting of circulatory shock are probably underestimated. Further studies are needed to determine the impact of gut reperfusion injury on complications and outcome, and to find a treatment to prevent excessive gut reperfusion injury after circulatory shock.

In conclusion, SPS infusion during early reperfusion after hemorrhagic shock improved microcirculatory perfusion, systemic hemodynamics, reduced leukocyte/endothelium interaction, and prevented metabolic acidosis. Our results point to the potential clinical relevance of early SPS administration in patients with hemorrhagic shock.

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