Perfluorobron Emulsion in Prolonged Hemorrhagic Shock

Influence on Hepatocellular Energy Metabolism and Oxygen-dependent Gene Expression

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Background: Liver dysfunction as a result of impaired oxygen availability frequently occurs following hemorrhage and contributes to delayed mortality. Artificial oxygen carriers may improve oxygen supply to vital organs while avoiding the need for allogeneic transfusion.

Methods: Rats were subjected to hemorrhagic hypotension (mean arterial pressure = 35–40 mmHg for 120 min) and were subsequently resuscitated with (1) stored whole rat blood, (2) pentastarch, or (3) pentastarch combined with perfluorobron emulsion (PFE; 2.7 or 5.4 g/kg body weight), a second-generation artificial oxygen carrier. Recovery of liver adenosine triphosphate, hepatocellular injury, and expression of glutamine synthetase 1, a gene that is induced by exposure of hepatocytes to low partial pressure of oxygen, were studied at 4 h of resuscitation.

Results: Stored whole blood or pentastarch failed to restore liver adenosine triphosphate concentrations after prolonged shock as compared to sham controls and resulted in increased gene expression of glutamine synthetase 1. Addition of 2.7 g PFE/kg restored liver adenosine triphosphate to control, whereas 5.4 g PFE/kg resulted in adenosine triphosphate concentrations significantly above control. Improved hepatocellular oxygen supply was also confirmed by restoration of the physiologic expression pattern of glutamine synthetase 1. Serum enzyme concentrations were highest after resuscitation with stored blood, whereas addition of PFE failed to further decrease enzyme concentrations as compared to pentastarch alone.

Conclusions: Resuscitation with PFE is superior to stored blood or asanguineous resuscitation with respect to restoration of hepatocellular energy metabolism. The improved hepatocellular oxygen availability is reflected in normalization of oxygen-dependent gene expression. However, improved oxygen availability failed to affect early hepatocellular injury.

HEMORRHAGIC shock associated with trauma is the leading cause of death up to the age of 40 in most Western countries.1 Microvascular failure and depression of energy metabolism may persist despite seemingly adequate resuscitation, and these patients are at risk to develop multiple organ failure.2 Among failing organ systems, the liver reflects the second most frequently affected organ after severe and prolonged hemorrhagic shock.1 Alterations in cellular high-energy phosphate concentrations as a result of decreased availability of oxygen are likely to contribute to early and late hepatocellular injury and dysfunction. Tissue concentrations of adenosine triphosphate (ATP) are depleted during hemorrhagic shock3 and may recover in ischemic tissue when blood flow is restored. However, recovery is often incomplete, e.g., because of no reflow in the microcirculation.4–6 Intravenous volume replacement with asanguineous fluids may restore cardiac output but fails to restore microvascular perfusion,7,8 and severe hemodilution due to massive infusions of asanguineous fluids further impairs tissue oxygenation because of a decreased oxygen content. Thus, administration of stored allogeneic blood along with crystalloid- and colloid-containing fluids reflects the standard of care.2 However, there is evidence to suggest that storage of erythrocytes impairs their ability to improve tissue oxygenation at least for several hours after transfusion.9 The p50 value of stored erythrocytes is low because of the loss in 2,3-diphosphoglycerate,10,11 and deformability of erythrocytes decreases with storage, thereby impending access to the capillary bed.12 During the past decades, experimental and clinical studies have demonstrated that artificial oxygen carriers may improve tissue oxygenation and thus may be used as a temporary alternative to allogeneic blood transfusions.13–16 Perfluorochemicals are chemically and biologically inert compounds that dissolve large amounts of oxygen and carbon dioxide. Their oxygen-carrying capacity is directly proportional to partial oxygen pressure, and since perfluorochemicals exchange gases by simple diffusion, they load and unload oxygen two times faster than hemoglobin. Moreover, although only 25% of oxygen carried by erythrocytes is released under physiologic conditions, extraction of dissolved oxygen can be more than 90%. The half-life is dose-dependent. After administration of 2.7 g/kg of the 60% weight/volume perfluorochemical emulsion (PFE; Oxygent; Alliance Pharmaceuticals, San Diego, CA) in rats, a plasma concentration of approximately 80 mg perfluorocarbon/ml blood has been reported with a half-life of 4–8 h. Although PFE has classically been studied as a temporary blood substitute,17 e.g., until allogeneic blood is available, its small particle size (0.16–0.18 μm) may result in superior restoration of oxygen availability under conditions of severe microvascular failure. Thus, in the current study, we
compared PFE and stored blood with respect to recovery of hepatocellular ATP content, hepatocellular injury, and expression pattern of glutamine synthetase 1 (GluS-1), an enzyme exhibiting a highly zonal heterogeneity depending on hepatic partial pressure of oxygen.18

Materials and Methods

Oxygent, a lecithin-based emulsion (0.6 g perfluorocarbon/ml) of perfluorooctyl bromide (C8F17Br; 58%) and perfluorodecyl bromide (C10F21Br; 2%) in phosphate-buffered saline, was obtained from Alliance Pharmaceutical Corp., San Diego, CA. All other chemicals used were purchased from Sigma (München, Germany) if not specified otherwise. All chemicals were of the highest purity commercially available.

Animals

Male Sprague-Dawley rats (200–260 g body weight) were obtained from Charles River, Sulzfeld, Germany. Pellet food was withheld overnight before preparative surgery, whereas animals had free access to water. All experiments were performed in the laboratories of the University of the Saarland and in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 86-23, revised 1985) after approval of the protocol by the institutional review board (University of the Saarland, Homburg, Germany).

Experimental Design

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and anesthesia was maintained by additional boluses throughout the experiment. After onset of anesthesia, a tracheotomy was performed to facilitate spontaneous breathing. After blunt dissection and exposure of the left carotid artery, a thermistor-tip catheter (9490 E; Columbus Instruments, Columbus, OH) for measurement of cardiac output by transpulmonary thermodilution (Cardiotherm 500; Columbus Instruments) was inserted into the vessel and advanced to the aortic arch. The left femoral artery was cannulated to allow blood withdrawal and measurement of systemic arterial blood pressure with a standard pressure transducer (Medex Medical, Ratingen, Germany). The right jugular vein was cannulated for drug administration and fluid resuscitation. A continuous infusion of Ringer’s solution (10 ml · kg⁻¹ · h⁻¹) Ringer’s solution was supplied to compensate for fluid losses during preparative surgery.

Animals (n = 8 per group) were subjected to hemorrhagic shock by rapid (< 5 min) arterial blood withdrawal to achieve a MAP of 35–40 mmHg; hemorrhagic hypotension was maintained for 2 h in a modified hemi-free Wiggers model followed by fluid resuscitation over 4 h. At the end of hemorrhagic hypotension, resuscitation of intravascular volume was initiated by infusion of (1) stored whole rat blood; (2) pentastarch (6% hydroxy-ethyl starch 200/0.5; Fresenius, Bad Homburg, Germany) alone; or pentastarch together with (3) 2.7 g PFE/kg or (4) 5.4 g PFE/kg. Stored rat blood was obtained by cannulating the abdominal aorta of a donor animal under strictly aseptic conditions. The collected blood was stored for 21 days after addition of 0.14 ml citrate-phosphate-dextrose-adenine (CPDA)-1/ml blood (Baxter, Dreieich, Germany) as the anticoagulant. Thus, all animals received a volume of stored blood or colloids equalling 60% of the blood volume withdrawn. In addition, twice the shed blood volume was infused in the form of Ringer’s solution during the first hour of resuscitation. The infusion rate of Ringer’s solution was subsequently lowered to a volume equalling the maximal bleed-out volume for the second hour of resuscitation. Until the end of the reperfusion period a continuous infusion of 10 ml · kg⁻¹ · h⁻¹ Ringer’s solution was supplied. The fraction of inspired oxygen was increased with onset of resuscitation to 1.0 in all animals by connecting the tracheostomy tube to a reservoir with a constant flow of pure oxygen.

Sham-operated animals not subjected to hemorrhage in which the fraction of inspired oxygen was increased to 1.0 from 2 to 6 h after onset of the experiment served as time-matched controls. Liver biopsies were taken from all animals at the end of the experiment, i.e., at 6 h for assessment of ATP content and GluS-1 gene expression.

Hematocrit, blood gases, and acid–base status were monitored at baseline, end of shock, and end of the experiment in arterial blood samples (0.2 ml) using an automated analyzer (Nova Profil 5; Nova Biomedical GmbH, Rödermark, Germany).

Determination of Hepatic ATP Content

To quantify the ATP content in the liver, at the end of the experiment, the tissue was freeze-clamped and stored at −80°C until analysis. Frozen liver samples were rapidly homogenized in cold 3% sulfosalicylic acid with a Powergen 125 tissue homogenizer (Fisher Scientific, Pittsburgh, PA), and the ATP concentrations were determined enzymatically in the supernatant with Sigma test kit 366-UV. A standard curve with purified ATP was used to calculate tissue concentrations. Since phagocytosis of PFE particles is particularly important in Kupffer cells of the liver and may affect the organ weight, ATP concentrations were calculated for wet and dry tissue. To assess the wet-to-dry weight ratio, specimens of approximately 200 mg liver tissue were weighed on a precision scale (Sartorius Research, Sartorius GmbH, Göttingen, Germany) before and after incubation at 60°C for 72 h.

To semiquantitatively assess the regional ATP content in liver sections, a bioluminescence method based on
ATP-dependent luciferase reaction was used. At the end of the experiment, the left liver lobe was frozen immediately in liquid nitrogen and stored at −70°C until analysis. Frozen liver lobes were cryosectioned in a cryostat at 20 μm. Sections were freeze-dried for 24 h at −20°C, and subsequently, the sections were heated to 95°C to inactivate endogenous enzymes. Bioluminescence imaging of ATP was performed as described previously.19 Briefly, a solution was prepared for the substrate-specific bioluminescence reaction in the absence of ATP containing 12 ml basic buffer (200 mM hydroxypropyl-perizino-ethanesulfonic acid [HEPES] plus 100 mM arsenate, pH 7.6). Pulverized dried light organs (260 mg) from fireflies (Phontinus pyralis) were added. Following homogenization and centrifugation, the supernatant was mixed with 25 μl MgCl₂, 1 M. The solution was frozen and cut into 60-μm slices in a cryostat at −20°C. A freeze-dried and heat-inactivated liver section was then covered with a 60-μm section of frozen enzyme block and was placed onto a photographic film (Agfapan, 100 ASA; Agfa, Köln, Germany) for recording of bioluminescent light emitted from the section after warming to room temperature. Exposure time was 30 s. Quantification of the signal was performed by computer-assisted densitometry using an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

**Western Blot Analysis**

Approximately 150 mg liver tissue was homogenized in a hypotonic cell lysis buffer 1:10 (w/v), which contained 10 mM TRIS (pH 7.5), 10 mM NaCl, 10 μM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 0.02% sodium azide, and 1 mM phenylmethylsulfonylfuoride. Homogenates were clarified by centrifugation at 18,000 g for 5 min, and total soluble protein concentration was determined according to Bradford, using a commercially available dye reagent (Protein Assay Kit II; Bio Rad, Hercules, CA) with bovine serum albumin as a standard. Aliquots of protein (100 μg/lane) from total liver were fractionated by SDS-polyacrylamide gel electrophoresis under denaturing conditions using NuPAGE MOPS SDS Running Buffer (Novex, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Westram, Schleicher & Schuell, Dassel, Germany) and stored at 4°C until detection of GluS-1 immunoreactive protein within 24 h. Nonspecific binding sites were blocked by preincubation of the membrane with 5% nonfat dry milk in Tris-buffered saline/Tween (TBST: 20 mM Tris [pH 7.5], 0.5 mM NaCl, 0.1% Tween 20) followed by incubation of the membrane with a monoclonal mouse antirat GluS-1 primary antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing the membrane with TBST, a horse antimouse antibody was used as secondary antibody (dilution 1:10,000). After repeated washes of the membrane with TBST, detection of the antigen–antibody conjugate was achieved by an enhanced chemiluminescent reaction (Lumi-Light Western Blotting Substrate; Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. All incubations were performed at room temperature. The signal was detected by short exposure to a blue light-sensitive autoradiography film (Hyperfilm ECL; Amersham Pharmacia Biotech, Braunschweig, Germany).20

**Immunohistochemical Staining for GluS-1**

Formalin-fixed, paraffin-embedded, dewaxed liver sections were used to assess the spatial and cell-type-specific expression pattern of GluS-1. Sections were subjected to an antigen retrieval using microwave irradiation. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂–methanol. After subsequent treatment with normal horse serum, slides were incubated at 37°C for 1 h with the identical monoclonal antibody used in Western blot analysis at a dilution of 1:200. As secondary antibody, a biotinylated horse antimouse antibody was used for streptavidine-biotin complex peroxidase staining. 3,3′Diaminobenzidine and 3% CoCl₂ were used as chromogens, and slides were counterstained with hematoxylin.

**Quantitative Determination of Liver Enzymes**

Blood samples were taken at baseline and at the end of the experiment. Serum was prepared, and aliquots thereof were stored at −70°C until analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glutamate dehydrogenase (GLDH) were analyzed with commercially available kits (Roche Diagnostics).

**Statistical Analysis**

Data are presented as mean ± SD. Criteria for parametric testing, i.e., normal distribution and equal variance, were tested by Kolmogorov-Smirnov and Levene-Median test, and data were log-transformed when appropriate. Differences were evaluated by analysis of variance followed by post hoc multiple comparison according to the Student–Newman–Keuls method using the SigmaStat software package (Jandel Scientific, San Rafael, CA). P < 0.05 was considered significant.

**Results**

**Macrobemodynamic Response to Hemorrhage and Resuscitation**

The mean volume of shed blood withdrawn during the shock period to achieve and maintain a MAP of 35–40 mmHg for 2 h varied from 50.5 to 52.9 ml/kg body weight in the four treatment groups without any statistical significance between the groups. Hemodynamic changes were similar in the four experimental groups during the shock period. MAP recovered on resuscitation with stored blood, whereas it remained de-
pressed when pentastarch was used for resuscitation. Addition of PFE was paralleled by a better recovery of MAP as compared to pentastarch alone. However, there was no difference between the two doses of PFE with respect to recovery of MAP (fig. 1). Cardiac output increased to values exceeding baseline approximately by 50% when stored blood was used for resuscitation (fig. 2). All asanguineous regimens were paralleled by an even greater increase in cardiac output immediately on resuscitation to approximately twice the baseline. Although cardiac output steadily decreased in animals receiving pentastarch alone during the 4-h observation period finally approximating baseline values, the hemodynamic circulation was sustained in animals receiving PFE. As observed for MAP, there was no difference between the two doses of PFE with respect to recovery of cardiac output (fig. 2). None of the animals showed any adverse reactions on infusion of the resuscitation fluids. Time-matched sham-operated controls displayed stable macrohemodynamics throughout the experiment (data not shown).

**Hematocrit, Blood Gas, and Acid-Base Status**

Hematocrit slightly decreased during hemorrhagic hypotension and recovered partially on resuscitation with stored blood. The hematocrit concentrations after resuscitation with stored blood were in the range of 20–29% (table 1) to mimic the clinical standard of care. In contrast to resuscitation with stored blood, a further decline in hematocrit was observed with all asanguineous resuscitation regimens.

PaO₂ significantly decreased, whereas PaCO₂ moderately increased at the end of hemorrhagic hypotension reflecting hyperventilation. PaO₂ further substantially increased during resuscitation in response to the raised fraction of inspired oxygen. PaCO₂ decreased at the end of hemorrhagic hypotension and increased again on resuscitation in all groups (table 1). No significant differences with respect to PaO₂ and PaCO₂ between the different treatment groups were observed.

Base deficit as an indicator of ischemic injury was comparable at the end of hemorrhagic hypotension in all groups. Base deficit recovered with all resuscitation regimens applied almost to control levels. There were no differences between the shock groups with respect to recovery of base deficit. However, base deficit was significantly higher at the end of the experiment after resuscitation with stored blood as compared to pentastarch (table 1). Changes in pH were less pronounced, not reaching statistical significance, reflecting respiratory compensation of the metabolic acidosis associated with hemorrhage (table 1).

**Effect of Fluid Resuscitation after Hemorrhagic Shock on Hepatic ATP Concentrations**

The effects of fluid resuscitation with stored blood or pentastarch with or without PFE following prolonged periods of hemorrhagic shock on tissue ATP concentrations as measured at the end of the experiment are summarized in figures 3 and 4. The ATP content measured enzymatically in liver homogenates failed to recover when stored blood or pentastarch was used for resuscitation. In contrast, PFE at a dose of 2.7 g/kg restored hepatic ATP content to control, whereas PFE at 5.4 g/kg even increased hepatic ATP above control (fig. 3). To assess local heterogeneity of ATP concentrations, a hallmark of prolonged ischemia and reperfusion, regional
Table 1. Hematocrit and Acid–Base Status

<table>
<thead>
<tr>
<th>Parameter/Time Point</th>
<th>Control</th>
<th>SB</th>
<th>Pentastarch</th>
<th>PFE 2.7 g/kg</th>
<th>PFE 5.4 g/kg</th>
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<tr>
<td>Hematocrit (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>39 ± 2.3</td>
<td>37 ± 2.9</td>
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<td>36 ± 2.1</td>
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<td>17 ± 2.6*</td>
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<td>6 h</td>
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<td>26 ± 2.7*</td>
<td>15 ± 2.3*†</td>
<td>17 ± 3.7*†</td>
<td>16 ± 4.3*†</td>
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<td>PaO₂ (mmHg)</td>
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<td>80 ± 13</td>
<td>81 ± 7</td>
<td>81 ± 10</td>
<td>70 ± 8</td>
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<td>111 ± 44*</td>
<td>122 ± 15*</td>
<td>113 ± 15*</td>
<td>111 ± 17*</td>
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<td>6 h</td>
<td>396 ± 32</td>
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<td>409 ± 82</td>
<td>385 ± 90</td>
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<td>PaCO₂ (mmHg)</td>
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<td></td>
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<tr>
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<td>43 ± 4.3</td>
<td>38 ± 3.9</td>
<td>40 ± 5.6</td>
<td>37 ± 3.0</td>
<td>41 ± 4.2</td>
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<tr>
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<td>17 ± 3.6*</td>
<td>18 ± 3.0*</td>
<td>18 ± 7.5*</td>
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<tr>
<td>6 h</td>
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<td>32 ± 4.5</td>
<td>36 ± 4.6</td>
<td>34 ± 6.6</td>
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<td>BE (mM)</td>
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<td>pH</td>
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<td></td>
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<tr>
<td>Baseline</td>
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<td>7.41 ± 0.03</td>
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<td>7.47 ± 0.05</td>
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</table>

Hematocrit PaO₂, PaCO₂ and base excess (BE) and pH were measured at baseline, at the end of shock (2 h; 35–40 mmHg) or time-matched sham operation (control) and at the end of the experiment (6 h); resuscitation was achieved by transfusion of stored blood (SB) or by infusion of pentastarch with or without addition of perflubron emulsion (PFE) in a dose-dependent fashion (2.7 or 5.4 g/kg body weight). Data are presented as mean ± SD for n = 8 animals per group.

* P < 0.05 compared to control. † P < 0.05 compared to stored blood (SB).

distribution of hepatic ATP was studied in cross-sections of liver lobes by ATP bioluminescence. Resuscitation using stored blood or pentastarch without addition of PFE was paralleled by a substantial heterogeneity of the ATP content reflecting persistence of patchy areas of substantial ATP depletion, whereas other parts of the studied lobes displayed recovery of the ATP content.

Fig. 3. Adenosine triphosphate (ATP) concentrations in liver homogenates after shock and resuscitation. ATP content in liver homogenates was assessed enzymatically at the end of each experiment for wet (solid bar) and dry (hatched bar) tissue. ATP concentrations remained significantly below controls after resuscitation with stored blood or pentastarch. ATP concentrations completely recovered in animals after resuscitation with low-dose perflubron emulsion (PFE) and were significantly increased after administration of high-dose PFE. Data are mean ± SD for n = 8 animals per group. * P < 0.05 compared to control; † P < 0.05 compared to 5.4 g/kg PFE. SB = stored blood.

Fig. 4. Regional distribution of hepatic adenosine triphosphate (ATP) content after shock and resuscitation. Regional distribution of hepatic ATP concentrations after shock and resuscitation was studied in cross sections of liver lobes obtained at the end of the experiment by ATP bioluminescence. Representative liver sections of n = 4 are shown. Livers from sham-operated control rats (A) showed homogenous distribution of ATP over the whole liver lobe. Resuscitation with stored blood (B) or pentastarch (C) was associated with a heterogenous distribution of ATP with areas of substantial ATP depletion and areas with normal ATP content. Resuscitation with low-dose perflubron emulsion (PFE) led to an almost homogenous distribution (D) comparable with controls, whereas resuscitation with high-dose PFE (E) led to ATP concentrations substantially above control over the whole liver lobe.
The low dose of PFE studied largely prevented persistence of areas exhibiting substantial depression of energy metabolism despite resuscitation. The high dose of PFE homogenously increased local ATP concentration above control (fig. 4).

**Gene Expression of GluS-1 after Different Resuscitation Regimen**

An approximately twofold increase of GluS-1 immunoreactive protein in the liver was observed after 2 h of hemorrhagic hypotension and subsequent resuscitation with either stored blood or pentastarch as assessed by Western blot, which was completely prevented by resuscitation with either dose of perflubron emulsion (PFE). Data are mean ± SD. *P < 0.05 compared to control; #P < 0.05 compared to 2.7 g/kg PFE; §P < 0.05 compared to 5.4 g/kg PFE.

The low dose of PFE studied largely prevented persistence of areas exhibiting substantial depression of energy metabolism despite resuscitation. The high dose of PFE homogenously increased local ATP concentration above control (fig. 4).

**Hepatocellular Injury after Prolonged Hemorrhage and Resuscitation**

Hepatocellular injury was estimated by measurement of serum enzyme concentrations of ALT, AST, and GLDH. Prolonged hemorrhagic shock and subsequent fluid resuscitation with either stored blood or pentastarch alone led to induction of GluS-1 immunoreactive protein in pericentral hepatocytes 4 h after the end of hemorrhage. In contrast, 2 h of hemorrhage and subsequent fluid resuscitation with 2.7 g perflubron emulsion (PFE)/kg or 5.4 g PFE/kg resulted in a restoration of the physiologic expression pattern of GluS-1 immunoreactive protein comparable with that of time-matched sham-operated controls.

**Fig. 5. Expression of glutamine synthetase 1 (GluS-1) immunoreactive protein in liver homogenates.** Gene expression of GluS-1 was assessed by Western blot analysis in livers harvested at the end of the experiment. The upper panel shows a Western blot of two representative samples for each condition. The lower panel represents the densitometric analysis of GluS-1 protein expression of n = 6 animals per group. Hemorrhagic shock and resuscitation with stored blood (SB) or pentastarch led to a significant induction of GluS-1 protein over control, which was completely prevented by resuscitation with either dose of perflubron emulsion (PFE). Data are mean ± SD. *P < 0.05 compared to control; #P < 0.05 compared to 2.7 g/kg PFE; §P < 0.05 compared to 5.4 g/kg PFE.

**Fig. 6. Spatial expression pattern of glutamine synthetase 1 (GluS-1) protein after hemorrhagic shock and resuscitation in the liver.** GluS-1 immunoreactive protein was restricted to one layer of hepatocytes surrounding the central vein after sham operation (A). Fluid resuscitation with either stored blood (B) or pentastarch alone (C) led to induction of GluS-1 immunoreactive protein in pericentral hepatocytes 4 h after the end of hemorrhage. In contrast, 2 h of hemorrhage and subsequent fluid resuscitation with 2.7 g perflubron emulsion (PFE)/kg (D) or 5.4 g PFE/kg (E) resulted in a restoration of the physiologic expression pattern of GluS-1 immunoreactive protein, i.e., in these animals, expression of GluS-1 was restricted to the immediate surroundings of the central vein.
resuscitation led to a significant increase in ALT, AST, and GLDH in all groups compared to respective time-matched sham-operated controls. AST and ALT concentrations were not significantly different between the treatment groups. In contrast, resuscitation with pentastarch and both doses of PFE led to significantly lower GLDH concentrations compared to resuscitation with stored blood (fig. 7).

Discussion

In the current study, we investigated whether PFE, a second-generation oxygen carrier, may improve oxygen supply to the liver in severe prolonged hemorrhagic shock as compared with the standard of care. Addition of PFE restored or even increased hepatic ATP content above control in a dose-dependent manner, whereas resuscitation with either stored blood or pentastarch resulted in a persisting depression of hepatic energy metabolism. In addition to the restoration of hepatic ATP, administration of PFE normalized expression of GluS-1, a hypoxia-inducible gene, which was substantially up-regulated on resuscitation with stored blood or pentastarch. However, improved oxygen availability by the use of PFE failed to affect early hepatocellular injury in our model.

In addition to early control of the sources of bleeding the restoration of the circulating volume is considered as a pivotal measure in the management of hemorrhagic-traumatic shock, whereas restoration of the oxygen content, e.g., by the administration of erythrocytes, is a secondary priority. Moreover, the usefulness of stored blood to improve tissue oxygenation has been questioned in experimental and clinical studies and may depend on the duration of storage and the used additives. The failure of stored blood to improve tissue oxygenation is multifactorial and may reflect a lower p50 due to depletion of 2,3-diphosphoglycerate and the parallel increase in the affinity of hemoglobin for oxygen as well as impaired deformability and, thus, limited access to the microcirculation. The latter mechanism may be particularly significant after prolonged hemorrhage where ischemia and reperfusion injury increase hydraulic resistance of capillaries due to endothelial cell swelling. Moreover, the functional capillary density is reduced essentially in all organs including the liver under...
these conditions as a result of the no-reflow phenomenon.\textsuperscript{7,18,25} These characteristic changes of the nutritive perfusion of vital organs are likely underlying the observed lack of erythrocyte transfusion to increase oxygen consumption of the whole organism or to improve parameters of regional oxygen availability.\textsuperscript{9} Furthermore, massive transfusions of stored blood represent a significant metabolic burden at a time when metabolic energy sources are extremely limited.\textsuperscript{20} Consistent with this concept, the beneficial effects of blood transfusion have been debated in experimental and clinical studies with respect to tissue oxygenation and survival.\textsuperscript{27,28} As a result, a fairly restrictive transfusion policy has been advocated lately.\textsuperscript{29} Nevertheless, the current standard of care frequently fails to restore energy metabolism in vital organs,\textsuperscript{7} which has been shown to correlate with significant organ dysfunction under experimental conditions\textsuperscript{4,5} and is thought to contribute to the development of multiple organ failure.\textsuperscript{2} Under these conditions, PFE along with an increased inspiratory oxygen fraction may help to improve oxygen availability to tissues \textit{via} increased oxygen content and/or recruitment of microvessels failing to conduct corpuscular flow.\textsuperscript{30,31} Consistent with this concept, addition of PFE to the resuscitation regimen in the current study restored hepatic energy metabolism as reflected by the hepatic ATP content, suggesting substantial improvement of oxygen availability to hepatic cells.

In addition to its role in hepatocellular energy metabolism, oxygen subserves an important role for the regulation of gene expression underlying the substantial heterogeneity of the metabolic capacity of periportal and perivenous hepatocytes.\textsuperscript{18} Key enzymes for glucose output, urea synthesis, and bile formation are primarily restricted to the periportal area, where oxygen is abundantly present. In contrast, genes encoding for key enzymes of glucose uptake and glutamine formation are primarily expressed in the perivenous zone, which is experiencing low \(P_{O2}\) values already under physiologic conditions (fig. 8). Thus, persistently lower \(P_{O2}\) values in the perivenous zone as a result of anemia or hypoxemia are likely to increase gene expression of metabolic key enzymes regulated by low \(P_{O2}\). Consistent with this concept, stored blood and pentastarch not only failed to restore ATP, but also failed to restore the physiologic expression pattern of GluS-1. In contrast, improved oxygen delivery by the use of PFE normalized gene expression of GluS-1. Thus, restoration of hepatic ATP and physiologic expression pattern of GluS-1 by increasing the oxygen content \textit{via} addition of PFE along with a fraction of inspired oxygen of 1.0 strongly suggest that an impairment of oxygen availability rather than disturbances in oxygen utilization ("cytopathic hypoxia")\textsuperscript{32} is responsible for the persisting depression of hepatic energy metabolism when stored blood or pentastarch is used for resuscitation.

There is evidence from clinical studies that PFE is more effective under conditions of elective surgery than stored autologous blood in improving oxygen supply to the tissues as indicated by the reversing of physiologic transfusion triggers.\textsuperscript{17} Although PFE improved hepatocellular oxygen availability in the current investigation, this was not reflected in a decrease in the early hepatocellular injury as assessed by serum enzyme concentrations of ALT, AST, and GLDH as compared to pentastarch alone. These seemingly discrepant findings may reflect a variety of underlying mechanisms. For instance, the early injury may primarily result from the prolonged ischemic insult in this model, which is identical for all studied treatments since PFE was given on resuscitation. In addition, improved microvascular flow may result in a more efficient washout of these enzymes from the liver\textsuperscript{33} or from aggravation of the reperfusion injury due to the so-called "reflow paradox."\textsuperscript{34} Alternatively, an increase of plasma enzyme concentrations has been reported for PFE, which may mask any early beneficial effect due to improved oxygen supply. Nevertheless, PFE was clearly superior to stored whole blood with respect to restoration of oxygen availability to hepatocytes and was associated with a trend to lower serum liver enzyme concentrations as compared to stored blood, which currently reflects the standard therapy to improve oxygen content. These unfavorable effects of stored blood may result from accumulation of noxious factors during storage, e.g., activated leukocytes, as well as from the metabolic burden resulting from the clearance of metabolites and preservatives, and may further enhance detrimental effects associated with transfusion. Nevertheless, there is evidence to suggest that these detrimental effects of allogeneic transfusion are less accentuated when
packed erythrocytes rather than stored whole blood is used as it currently reflects the standard of care in the clinical setting.55

In conclusion, our results demonstrate that PFE is able to improve oxygen availability to vital organs as compared to asanguineous resuscitation or stored blood. The observed beneficial effects on hepatic energy metabolism and oxygen-dependent gene expression may contribute to the salutary effects of PFE with respect to survival56 in models of severe or prolonged hemorrhage.

References