Chronic Resuscitation After Trauma-Hemorrhage and Acute Fluid Replacement Improves Hepatocellular Function and Cardiac Output

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Objective
To determine whether prolonged (chronic) resuscitation has any beneficial effects on cardiac output and hepatocellular function after trauma-hemorrhage and acute fluid replacement.

Background Data
Acute fluid resuscitation after trauma-hemorrhage restores but does not maintain the depressed hepatocellular function and cardiac output.

Methods
Male Sprague-Dawley rats underwent a 5-cm laparotomy (i.e., trauma was induced) and were bled to and maintained at a mean arterial pressure of 40 mmHg until 40% of maximal bleed-out volume was returned in the form of Ringer's lactate (RL). The animals were acutely resuscitated with RL using 4 times the volume of maximum bleed-out over 60 minutes, followed by chronic resuscitation of 0, 5, or 10 mL/kg/hr RL for 20 hours. Hepatocellular function was determined by an in vivo indocyanine green clearance technique. Hepatic microvascular blood flow was assessed by laser Doppler flowmetry. Plasma levels of interleukin-6 (IL-6) were determined by bioassay.

Results
Chronic resuscitation with 5 mL/kg/hr RL, but not with 0 or 10 mL/kg/hr RL, restored cardiac output, hepatocellular function, and hepatic microvascular blood flow at 20 hours after hemorrhage. The regimen above also reduced plasma IL-6 levels.

Conclusion
Because chronic resuscitation with 5 mL/kg/hr RL after trauma-hemorrhage and acute fluid replacement restored hepatocellular function and hepatic microvascular blood flow and decreased plasma levels of IL-6, we propose that chronic fluid resuscitation in addition to acute fluid replacement should be routinely used in experimental studies of trauma-hemorrhage.
Severe trauma and blood loss continue to be the main cause of death in persons under age 35. Trauma and hemorrhage are also one of the most neglected serious health hazards in our society. Successful treatment of trauma victims who survive the initial injury includes control of ongoing hemorrhage and rapid restoration of intravascular volume to improve tissue perfusion. Clinically, this initial treatment is followed by continued fluid administration according to hemodynamic indices (e.g., cardiac output) and renal function. Depression of cardiac output after hemorrhage results in decreased organ blood flow and microcirculation, cellular dysfunction, and even cell death. Furthermore, after trauma or hemorrhage, significant immunologic alterations occur that are characterized by increased release of proinflammatory cytokines (e.g., interleukin-6 [IL-6], tumor necrosis factor [TNF]). These alterations persist despite acute fluid replacement. Although studies have shown that acute fluid resuscitation with Ringer’s lactate (RL) immediately after trauma-hemorrhage restores but does not maintain cardiac output and hepatocellular function, it is unknown whether continued fluid administration after acute resuscitation maintains hemodynamic stability and hepatocellular function. In addition, studies have shown that TNF and IL-6 levels remain elevated 24 hours after trauma-hemorrhage and acute resuscitation. However, it is unknown whether chronic resuscitation downregulates plasma IL-6 levels 1 day after the insult. Therefore, the aim of this study was to determine whether chronic resuscitation after trauma-hemorrhage and acute fluid replacement restores cardiovascular and hepatocellular function and decreases circulating proinflammatory cytokine levels.

**MATERIALS AND METHODS**

**Experimental Procedures**

A previously described nonheparinized model of trauma-hemorrhage in the rat was used, with minor modifications. Briefly, male Sprague-Dawley rats (275–325 g) were fasted overnight before the experiment but allowed water ad libitum. The rats were anesthetized by methoxyflurane inhalation before trauma was induced by a 5-cm midline laparotomy. The abdomen was then closed in layers, and catheters were placed in the right jugular vein at the level of the right atrium (silicone tubing, 0.03” ID, 0.065” OD; Baxter Health Care, McGaw Park, IL) and both femoral arteries (polyethylene 50 tubing; Becton Dickinson & Co., Sparks, MD). The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) to reduce postoperative pain. Rats were then bled to and maintained at a mean arterial pressure (MAP) of 40 mmHg until they could not maintain MAP of 40 mmHg unless extra fluid (Ringer’s lactate [RL]) was returned. This time was defined as maximum bleed-out, and the amount of withdrawn blood was noted. After this period, the rats were maintained at MAP of 40 mmHg until 40% of the withdrawn blood was given back in the form of RL. The animals were then resuscitated with 4 times the volume of the withdrawn blood (defined as acute resuscitation) over 60 minutes (approximately 44 mL/rat), and both femoral artery catheters were removed. The shed blood was not used for resuscitation. The animals then received additional fluid via the jugular vein by means of an infusion pump (pump 22; Harvard Apparatus, South Natick, MA) for 20 hours (defined as chronic resuscitation). The study groups received 0 (RL0), 5 (RL5), or 10 (RL10) mL/kg/hr of RL for 20 hours (6 or 7 rats per group). During this time, the rats were placed in metabolic cages. The venous catheter was connected to a custom-made swivel, allowing the animals to move freely in the cage. Water and food were given ad libitum during chronic resuscitation. At the end of the 20-hour chronic resuscitation period, cardiac output, hepatocellular function, and hepatic blood flow were measured under intravenous sodium pentobarbital anesthesia (total of 20 mg/kg) in each animal. Arterial blood samples were taken aseptically for determination of IL-6. In all groups, blood was withdrawn until the animal was exsanguinated. Abdominal and thoracic organs were taken for determination of water content.

All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital.

**Cardiac Output Measurements**

After sodium pentobarbital anesthesia, a polyethylene 50 catheter was placed into the right carotid artery to measure MAP. The arterial catheter was then replaced by a 2.4 French fiberoptic catheter (Hospex Fiberoptics, Chestnut Hill, MA), which was connected to an in vivo hemoflectometer (Schwarze, Picker International [now Pulsion], Munich, Germany). The fiberoptic catheter was positioned at the level of the aortic arch. Indocyanine green solution (ICG; Cardio Green; Becton Dickinson) was injected via the catheter in the jugular vein (1 mg/
mL aqueous solvent as a 50-μL bolus). Twenty ICG concentrations per second were recorded for approximately 30 seconds with the aid of a data acquisition program (Asystant+; Asyst Software, Rochester, NY). The area under the ICG dilution curve was determined according to a previous publication to calculate cardiac output. Cardiac output was then divided by the body weight to determine the cardiac index.

**Measurement of Hepatocellular Function**

Hepatocellular function was measured by the in vivo ICG clearance technique. ICG was administered by bolus injection (50 μL) of 1, 2, and 5 mg/mL ICG in aqueous solvent. The arterial concentration of ICG was recorded each second for 5 minutes. After this, the initial velocity of ICG clearance at time 0 for each dose was calculated after performing a nonlinear regression of the ICG clearance curves according to a second-order polynomial function. The initial velocities of ICG clearance were then plotted against the ICG doses according to the methods of Lineweaver-Burk. This results in a straight line, allowing determination of the maximum velocity of ICG clearance (Vmax) and the Michaelis-Menten constant (Km). In this active hepatocellular membrane transport system, Vmax represents the functional hepatocyte ICG receptors and Km represents the efficiency of the active transport process.

**Measurement of Hepatic Microvascular Blood Flow**

Hepatic microvascular blood flow was measured on the liver surface by laser Doppler flowmetry. Briefly, the abdomen was reopened and a flat flow probe, that was connected to a laser Doppler blood perfusion monitor (Laserflo, Model BPM 403A; TSI, St. Paul, MN), was placed on the liver surface. Hepatic microvascular blood flow represents the microvascular red blood cell flux in approximately 1 mm³ of surface organ tissue with a unit of mL/min/100 g tissue, as indicated by the manufacturer. Although this is a widely used technique for determining alterations of organ surface perfusion, the flow unit should be considered an arbitrary one.

**IL-6 Assay**

Blood samples were drawn aseptically from the carotid artery and immediately transferred to pyrogen-free microcentrifuge tubes (Sarstedt, Newton, NC) and spun down at 16,000 g in a refrigerated (4°C) centrifuge (MicroCentrifuge Model 235C; Fisher Scientific, Livonia, MI). The plasma was aliquoted into pyrogen-free microcentrifuge tubes and immediately frozen (−85°C) until the time of the assay. Plasma samples were diluted 1:15 (plasma:media) in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Biologos, Naperville, IL) and thereafter in threefold serial dilutions, with IL-6 activity determined by assessing the ability of the plasma to induce the proliferation of the 7TD1 B-cell hybridoma. The relative units of cytokine activity per milliliter of plasma were determined by comparing the curves produced from experimental plasma supernatant to standard curves produced from a recombinant human IL-6 standard (200 U/mL, Amgen, Thousand Oaks, CA), according to the methods of Mizel. The 7TD1 murine B-cell hybridoma, a gift from Dr. Jacques van Snick (Ludwig Institute of Cancer Research, Brussels, Belgium), was maintained as described by van Snick et al. This bioassay has been routinely used by our laboratory and was previously reported to react in a highly specific manner to IL-6. Supplementation of this bioassay with murine interleukin-2 through -4, TNF-α, or interferon-γ does not induce proliferation of the 7TD1 B-cell hybridoma cytotoxicity. However, these observations do not preclude the possibility that other agents (e.g., glucocorticoid, prostaglandin) may influence these assays. Nevertheless, IL-6 in this bioassay was completely inhibited by the addition of monoclonal IL-6 antibodies.

**Tissue Water Content Determination**

At the end of the experiment (20 hours after hemorrhage and acute fluid replacement), small pieces of the heart, lungs, liver, kidney, spleen, small intestine, and skin were excised, weighed, and dried for 24 hours at 95°C. Tissue water content (%, wt/wt) was calculated as (wt wt − dry wt)/(wet wt) × 100.

**Statistical Analysis**

Results are presented as mean ± standard error of the mean. The data were analyzed with one-way analysis of variance, followed by the Fisher’s least significant difference test as a post hoc test for multiple comparison. The differences were considered significant at p < 0.05.

**RESULTS**

**Hemorrhage Parameters**

The average time to reach the maximum bleed-out was 47 ± 1 minutes. The average bleed-out volume was 11 ± 0.1 mL per rat (approximately 59% of estimated circulating blood volume). The time until the end of the hemorrhage was 89 ± 1 minutes. There was no significant difference in these parameters between the three hemorrhage groups.
Chronic Resuscitation Improves Hepatocellular Function

Cardiac Index and Blood Pressure

The cardiac index decreased significantly at 20 hours after the end of acute resuscitation in RL0 and RL10 animals compared to sham-operated animals (Fig. 1). RL5 animals, however, showed no significant decrease compared to shams and a significantly improved cardiac index compared to the RL0 group (see Fig. 1). On the other hand, MAP decreased significantly in all hemorrhaged groups compared to sham-operated animals immediately, as well as 20 hours after the end of acute resuscitation (Fig. 2). Despite this, RL5 animals showed significantly higher MAP than RL0 animals at 20 hours after acute resuscitation (see Fig. 2).

Hepatocellular Function

$V_{\text{max}}$ values decreased significantly in RL0 and RL10 animals at 20 hours after acute resuscitation compared to sham-operated animals (Fig. 3A). The RL5 group showed significantly elevated $V_{\text{max}}$ values compared to RL0 animals, and the values were not significantly different from shams (see Fig. 3A). Furthermore, $K_m$ decreased signifi-
Hepatic Microvascular Blood Flow

The hepatic microvascular blood flow decreased significantly in RL0 and RL10 animals compared to sham-operated animals. The RL5 group showed a higher hepatic microvascular blood flow than did RL0 animals, and it was not significantly different from shams (Fig. 4A).

Plasma IL-6

Plasma IL-6 values increased significantly in RL0 animals compared to sham-operated animals. RL5 and RL10 animals showed significantly reduced plasma IL-6 values compared to RL0 animals (Fig. 4B). There was a significant negative linear correlation ($r = 0.56$) between IL-6 and $V_{max}$ values (Fig. 5).

Tissue Water Content

Almost every internal organ (except the spleen and the small intestine) showed a significant increase in tissue water content in the hemorrhaged groups compared to the sham group (Table 1). However, there was no significant difference in the tissue water content between the three hemorrhaged groups. Only the skin showed an increase in tissue water content, with the highest values in RL10 animals; these were significantly elevated compared to RL0 and RL5 animals.

Hematocrit and Urinary Output

In all hemorrhaged groups, the systemic hematocrit (%) was decreased significantly compared to sham-operated animals (sham, 46 ± 0.2; RL0, 18 ± 0.2 [p < 0.05 vs. sham]; RL5, 17 ± 0.3 [p < 0.05 vs. sham]; RL10, 16 ± 0.6 [p < 0.05 vs. sham and p < 0.05 vs. RL0]). RL10 animals showed a significant decrease in the systemic hematocrit compared to RL0 animals. The urinary output (mL/h) showed a tendency toward higher values in RL10 animals (sham, 1.0 ± 0.2; RL0, 0.7 ± 0.1; RL5, 1.1 ± 0.2; RL10, 1.7 ± 0.4). However, there was no significant difference between sham-operated and hemorrhaged animals, irrespective of the rate of chronic resuscitation.

DISCUSSION

The clinical course of trauma and hemorrhagic shock can be divided into three distinct physiologic stages. In phase I (from injury to control of bleeding), the patient suffers from low cardiac output, tachycardia, oliguria, and reduced organ perfusion. During this phase, compensa-
tory physiologic mechanisms are directed to re-establish and maintain plasma volume and organ blood flow. One of these mechanisms is the contraction of the interstitial cell matrix, leading to a fluid shift from the extravascular to the intravascular space.\(^{20}\) After control of bleeding and successful immediate resuscitation, a period of fluid sequestration occurs as the intracellular and extracellular spaces expand (phase II).\(^{19}\) Fluid therapy is used to maintain blood volume by continued fluid replacement. During phase III, mobilization of extravascular fluid occurs. This phase is also called the diuretic phase, and optimal therapy requires fluid restriction and support of diuresis.\(^{19}\)

Extensive studies have been conducted concerning optimization of initial fluid resuscitation with various types of fluids.\(^{6,7,21-23}\) Less attention has been directed to the events that follow successful acute resuscitation after experimental trauma and hemorrhagic shock. Studies indicate that despite successful resuscitation, disturbed organ function may persist for a prolonged time: defects in peripheral blood flow and oxygen use often can be demonstrated.\(^{24-26}\) In our laboratory, we have shown that aggressive fluid resuscitation immediately after trauma-hemorrhage restores but does not maintain cardiac output.\(^{6}\) In addition, our studies have indicated that acute resuscitation restores but fails to maintain hepatocellular function.\(^{7}\)

However, it is unknown if continued administration of crystalloid can maintain cardiac output and hepatocellular function after hemorrhagic shock and acute resuscitation. Our data indicate that continued fluid administration (5 mL/kg/hr RL) for 20 hours optimizes cardiac output, hepatic microvascular blood flow, and hepatocellular function. Furthermore, chronic resuscitation reduces IL-6 release. However, despite the improved cardiovascular and hepatocellular function in the group receiving chronic resuscitation with 5 mL/kg/hr RL, the reduced MAP was not restored. This was also associated with edema development, mainly in the skin. The significantly decreased MAP might be due to persistent endothelial dysfunction after hemorrhage, as previously reported from our laboratory.\(^{27}\) Those studies indicated that the endothelial dys-

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**Table 1. PERCENT CHANGES OF TISSUE WATER CONTENT IN VARIOUS ORGANS AT 20 HOURS AFTER HEMORRHAGE AND ACUTE RESUSCITATION**

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Small Intestine</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>78.1 ± 0.2</td>
<td>80.6 ± 0.3</td>
<td>70.3 ± 0.4</td>
<td>73.9 ± 0.8</td>
<td>76.3 ± 0.5</td>
<td>77.0 ± 0.1</td>
<td>58.2 ± 1.5</td>
</tr>
<tr>
<td>RL0</td>
<td>80.0 ± 0.2*</td>
<td>82.3 ± 0.6*</td>
<td>72.2 ± 0.2*</td>
<td>78.6 ± 0.7*</td>
<td>77.3 ± 0.2</td>
<td>77.1 ± 0.8</td>
<td>61.9 ± 0.8</td>
</tr>
<tr>
<td>RL5</td>
<td>79.6 ± 0.1*</td>
<td>81.4 ± 0.3</td>
<td>71.7 ± 0.3*</td>
<td>77.5 ± 1.3*</td>
<td>76.6 ± 0.4</td>
<td>78.1 ± 1.0</td>
<td>66.7 ± 3.4*</td>
</tr>
<tr>
<td>RL10</td>
<td>80.4 ± 0.5*</td>
<td>82.8 ± 0.7*</td>
<td>72.4 ± 0.4*</td>
<td>79.4 ± 0.9*</td>
<td>77.0 ± 0.5</td>
<td>79.1 ± 0.4</td>
<td>69.8 ± 3.6†</td>
</tr>
</tbody>
</table>

Tissue water content was calculated by the determination of the wet and dry tissue weights.

* \(p < 0.05\) vs. Sham.

† \(p < 0.05\) vs. RL0.

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function occurs as early as 1.5 hours after hemorrhage and persists despite acute fluid resuscitation.\(^{27}\) In the present study, MAP remained low even in the group with improved cardiac output. Therefore, it may be possible that chronic crystalloid resuscitation alone does not attenuate the endothelial dysfunction and fails to restore MAP after trauma-hemorrhage. Thus, pharmacologic agents may be necessary to restore MAP under such conditions. Nonetheless, our study clearly demonstrates that chronic resuscitation with 5 mL/kg/hr RL after trauma-hemorrhage and acute resuscitation restores cardiac output and hepatocellular function. We limited this study to 20 hours after hemorrhage because this seems to be a reasonable period of time to expect any salutary or deleterious effects of chronic fluid resuscitation after trauma-hemorrhage. Nonetheless, further studies are required to determine whether chronic resuscitation with 5 mL/kg/hr RL maintains cardiovascular and hepatocellular function for periods longer than 20 hours after hemorrhage and acute fluid replacement.

We used the ICG dilution technique to measure cardiac output and the ICG clearance technique to determine hepatocellular function. The ICG clearance technique has been used in different animal models and in the clinical setting to determine hepatocellular function\(^{28-31}\) and has been found to be a specific and extremely sensitive early indicator of hepatocellular dysfunction after various adverse circulatory conditions.\(^{12,29}\) The advantage of using ICG for measuring hepatocellular function is that this dye has no reported toxicity at low doses in humans and rats. It is cleared from the blood exclusively by an energy-dependent process in the liver, with negligible extrahepatic elimination.\(^{32}\) ICG can be measured in vivo by using a hemoreflectometer, which makes sampling of blood unnecessary.\(^{12}\) The determination of \(V_{\text{max}}\) and \(K_m\) with three different doses, as described previously, represents the hepatocellular function independent of the hepatic blood flow.\(^{18,33}\)

Hepatic microvascular blood flow was measured with laser Doppler flowmetry. This method has been shown to
correlate well with other techniques (e.g., the radiolabeled microsphere method), despite some limitations, such as the limited depth of the measurement in the tissue.13

We chose not to give blood for resuscitation. This kept the model simple and simulated the clinical situation in which a patient rejects blood products or blood is not given to avoid the risk of transmitting disease (e.g., AIDS, hepatitis). However, this raises the question of whether hemodilution (and the potential decrease of oxygen delivery) affects organ function after hemorrhagic shock. Recent studies in our laboratory have shown that acute hemodilution in sham-operated animals does not significantly affect hepatocellular function and MAP.7 Others have shown that hematocrit levels of 10% to 15% in animals subjected to acute hemodilution were well tolerated.34 Furthermore, in an intestinal ischemia model, Mesh and Gewertz25 showed that hemodilution has no adverse effects on oxygen consumption during hypotension and hypoperfusion. They also suggested that hemodilution may be beneficial during reperfusion after ischemia.35 Moreover, previous studies indicated that RL was as effective as blood in resuscitation after hemorrhagic shock in the rat.36 Thus, we suspect that hemodilution alone is not the major cause of organ dysfunction in this animal model of trauma-hemorrhage.

The animals in this study did not receive heparin before, during, and after trauma-hemorrhage or resuscitation because in the usual clinical situation, patients are not heparinized before or after a severe traumatic injury. Furthermore, we recently showed that the heparinization of animals before or after hemorrhage has beneficial effects on the microvascular blood flow and endothelial cell and hepatocellular function.37,38 Therefore, pre- or postheparinization of the animals would make it difficult to distinguish the effects of fluid administration versus heparin on cardiovascular responses under such conditions.

In RL10 animals, a decrease in cardiac output, liver surface blood flow, and hepatocellular function was observed comparable with RL0 animals. Increased edema formation, mainly in the skin, and a decreased systemic hematocrit compared to RL0 animals were also observed in RL10 animals. This indicates that RL10 animals were probably overresuscitated, which produced detrimental effects on these indices. RL0 animals, on the other hand, were most likely underresuscitated, because they showed a reduced microvascular blood flow, decreased cardiac output, and decreased hepatocellular function. The precise mechanisms responsible for the improved hepatocellular function, increased cardiac output, and hepatic microvascular blood flow in the RLS group remain unknown.

Studies in rats and phase II studies in humans have shown that IL-6 alone has no significant pathologic effect on the liver or kidneys but influences hematologic parameters.39,40 However, Preiser et al.41 found no significant effect of low-dose IL-6 administration on the hemodynamic and hematologic parameters in dogs. IL-6 might interact with other cytokines (e.g., TNF) and contribute to the tissue damage.42 In previous studies, we showed that IL-6 and TNF were elevated after hemorrhage, and both cytokines showed a comparable time course up to 24 hours after hemorrhage without chronic resuscitation.58 It is therefore likely that in the present study, TNF follows a pattern similar to that of IL-6. However, because TNF was not measured in the present experiments, further studies are required to determine its role in producing hepatocellular dysfunction after trauma-hemorrhage and acute resuscitation.

It could be argued that the decreased IL-6 level after chronic resuscitation is due to hemodilution. However, the systemic hematocrit was significantly lower only in the RL10 group compared to RL0. This indicates that there might be some dilutional effect in the RL10 group, causing lower IL-6 values in this group. Nonetheless, there was a significant negative correlation between IL-6 and Vmax. An additional multiple linear regression analysis demonstrated that there was no significant correlation between cardiac index or hepatic blood flow and Vmax (data not shown). The fact that chronic resuscitation with 5 mL/kg/hr RL improves hepatocellular function and decreases IL-6 release or production suggests that downregulation of this proinflammatory cytokine may be responsible for maintaining hepatocellular function under such conditions. The decreased hematocrit in the hemorrhaged animals does influence the ICG measurements: previous studies indicated that there is a negative correlation between the systemic hematocrit and blood ICG measurements.7 A 50% decrease in the hematocrit increases blood concentrations of cytokines by approximately 35%, as measured by in vivo hemoreflectometer versus spectrophotometer.7 Such an effect was taken into account in determining the hepatocellular function in this study.

In summary, we found that chronic resuscitation after trauma-hemorrhage and acute resuscitation with 5 mL/kg/hr but not 10 mL/kg/hr RL in the absence of blood resuscitation improves cardiac output, hepatocellular function, and microvascular blood flow. Although both rates of chronic resuscitation decrease circulating levels of IL-6, the decreased levels of this cytokine in the RL10 group may be due to dilutional effects. We therefore conclude that careful fluid optimization after trauma-hemorrhage is required for improving cardiovascular and hepatocellular function. Chronic fluid resuscitation should be included in long-term experiments of trauma-hemorrhage.

References

21. Behrman Starling


