# Microcirculatory Function and Tissue Damage Is Improved After Therapeutic Injection of Bovine Hemoglobin in Severe Acute Rodent Pancreatitis

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**Objectives:** Stasis of the pancreatic microcirculation initiates and aggravates acute pancreatitis. Bovine hemoglobin has been shown to improve microcirculation in acute pancreatitis if prophylactically infused 15 minutes after initiation of acute pancreatitis. The purpose of this study was to evaluate the therapeutic effectiveness of bovine hemoglobin on pancreatic microcirculation and tissue damage later in the course of experimental acute rodent pancreatitis.

**Methods:** In Wistar rats, severe acute pancreatitis was induced by administration of glyco-deoxycholic-acid intraductally and cerulein intravenously. Pancreatic microcirculation was continuously monitored by intravital microscopy. Three hours after the initiation of acute pancreatitis, animals received either 0.8 mL bovine hemoglobin (Oxyglobin), hydroxyethyl starch (HES), or 2.4 mL 0.9% NaCl intravenously at random. After 6 hours, animals were killed, and histopathological damage of the pancreas was assessed using a validated histology score.

**Results:** Pancreatic microcirculation assessed by leukocyte adherence was significantly improved by the administration of bovine hemoglobin in comparison with normal saline over time (mean difference, 51.6 ± 9.2; $P < 0.001$) and HES (mean difference, 24.1 ± 9.2; $P = 0.037$). This result was paralleled by decreased tissue damage in the bovine hemoglobin group as opposed to NaCl (6.75 vs. 12; range, 5.25–7.75 vs. 8.25–14; $P < 0.001$) and HES (6.75 vs. 9; range, 5.25–7.75 vs. 7.5–10.75; $P < 0.001$).

**Conclusion:** Therapeutic intravenous infusion of bovine hemoglobin improves pancreatic microcirculation and reduces pancreatic tissue damage in severe acute rodent pancreatitis but is not as effective as early (prophylactic) administration.

**Key Words:** acute pancreatitis, bovine hemoglobin, microcirculation

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## INTRODUCTION

Acute pancreatitis is still an important medical and surgical problem when it progresses from the self-limiting mild edematous to the severe hemorrhagic necrotizing form. Mortality may be as high as 40% if severe necrotizing injury occurs.[1]

Microcirculatory dysfunction of the pancreas is a consequence of released proinflammatory cytokines and vasoactive mediators attracting leukocytes and activating vascular endothelium. Free oxygen radicals and the expression of adhesion molecules leads to stasis in small vessels.[2] Disturbed microcirculation is a crucial step in the cascade from self-limiting disease (preserved microcirculation-mild edematous pancreatitis) to progression to the often fatal form of acute hemorrhagic necrotizing pancreatitis.[3] Once the initiation of acute pancreatitis has begun, preservation or normalization of pancreatic microcirculation could possibly prevent further progression, tissue necrosis, and complications,[4,5] thus leading to lower morbidity and mortality.

Purified bovine hemoglobin as a hemoglobin-based oxygen carrier (HBOC-200, Oxyglobin; Biopure, Cambridge, MA) is a safe blood substitute and improves poststenotic cardiac and skeletal muscular oxygenation.[6–8] Besides its colloidal effect, HBOC-200 provides additional oxygen (noncorpuscular cell-free oxygen carrier). This therapeutic effect may be beneficial in a disease that is characterized by critical perfusion states, and in the severe form, by decreased hemoglobin oxygen saturation in the pancreas.[3,9] A single intravenous prophylactic intervention of bovine hemoglobin has been proven to be effective early in the course of severe acute pancreatitis.[10] Because therapeutic intervention has higher clinical relevance, the aim of this study was to evaluate the therapeutic effect of bovine hemoglobin on pancreatic microcirculation in rats suffering from acute pancreatitis. We hypothesized that, 3 hours after induction of severe acute pancreatitis, the treatment with HBOC-200 is more effective than treatment with HES or Ringer solution in terms of pancreatic microcirculation and histologic damage.

## METHODS

This study was approved by the Ethics Committee of the Hamburg Federal Board of Veterinary Medicine and Animal Care. Female Wistar rats with a body weight (BW) of 200 to

230 g were fasted overnight with free access to water containing 20% glucose. The experimental protocol is similar to a previously published protocol that can be referred to for further details,[10] with the exception that the therapeutic intervention started 3 hours after the initiation of severe acute pancreatitis instead of 15 minutes.

## Experimental Protocol

Thirty animals were randomly allocated to 3 groups (groups 1–3; n = 10 each). Ringer lactate was infused for fluid resuscitation to keep central venous pressure (CVP) between 4 and 6 mm Hg and heart rate (HR) and mean arterial pressure (MAP) within 10% of baseline values (before and after intervention) throughout the experiment. Acute pancreatitis was induced using a standardized model as described below.

## Operative Procedure

The animals were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg BW) and ketamine (10 mg/kg BW), allowing spontaneous breathing. MAP, HR, and CVP, as well as arterial oxygen saturation ($aSO_2$), were measured continuously (Datex AS/3 monitoring system; Hoyer, Bremen, Germany).

## Induction of Acute Pancreatitis

In animals subjected to acute pancreatitis, a midline laparotomy was performed, and the duodenal loop was mobilized. The pancreatic duct was cannulated transduodenally through the papilla of Vater with plastic tubing. The common bile duct was ligated close to the liver. The head and the body of the pancreas were placed on a plastic stage. The duodenal loop was fixed on the stage with tissue glue (Histoacryl, B. Melsungen, Germany). The pancreas was covered by a polyethylene film (Flexithen; Plastica, Renningen, Germany). After surgery, animals were allowed to reach a steady state within 15 minutes. Acute pancreatitis was induced by the combination of intravenous cerulein infusion (5 μg/kg per hour; Takus, Pharmacia, Erlangen, Germany) at a rate of 1 mL/h over 6 hours and intraductal infusion of glycodeoxycholic acid (GDOC: 10 mmol/L; 1.0 mL/kg for 300 seconds; infusion pressure 25 to 30 mm Hg).[2,10]

## Test Substances

Bovine hemoglobin glutamer-200 (Oxyglobin) is a hemoglobin-based oxygen carrier with an intravascular half-life of 30 to 40 hours.

Animals in group 2 received 6% hydroxyethyl starch (HES) 70,000/0.5 (osmolality, 310 mOsm/kg; Rheohes; B. Melsungen).

## Blood Samples

Three hours after the initiation of acute pancreatitis, 0.8 mL of central venous blood was drawn for measurement of blood gases and plasma hemoglobin concentration (ABL 725; Radiometer, Copenhagen, Denmark).

At the end of the observation period, all remaining blood was drawn from the vena cava by puncture. Again, a complete blood gas analysis, free hemoglobin and trypsinogen-activating peptide (TAP) were determined.

## TAP

TAP concentrations were measured in a blinded fashion by an ELISA (TAP EIA, no. BIO75TAP; Biotrin, Dublin, Ireland). TAP concentrations in serum are expressed as nanomoles per liter.

## Therapeutic Experiments

Three hours after the initiation of acute pancreatitis, animals were randomly allocated to 3 groups. Animals in group 1 were given 0.8 mL of bovine hemoglobin, ensuring 0.8 to 1.2 mg/dL of free hemoglobin. Animals in group 2 received 0.8 mL of HES, whereas animals in group 3 received 2.4 mL of normal saline (0.9% NaCl) to ensure normovolemic substitution compared with groups 1 and 2.[11] Blood was drawn, and test substances were given by a different researcher than the one observing pancreatic microcirculation to best ensure a blinded set-up. All other variables were measured by a blinded observer.

## Target Parameters

### Intravital microscopy

Intravital microscopy was performed with an epiluminescent microscope (Olympus BH-12, Olympus, Tokyo, Japan). Acridine orange (1%, 1.2 mL/kg BW, injection within 30 seconds; Sigma Chemicals, St. Louis, MO) was injected intravenously to label leukocytes. The experiments were recorded on videotape with an attached video line (camera: AV-1001; AVT, Germany; monitor: TC-1100 SDN, and recorder: NV-180; Panasonic, Osaka, Japan). The evaluation of microcirculatory data was done off-line after completion of the whole set of experiments in a blinded fashion.

Each pancreas was subjected to a thorough examination. Impaired flow or intrapancreatic hemorrhage at baseline examination led to exclusion of the animal from further evaluation. The head of the pancreas was defined to be the region of interest. One arterial, 1 venular, and 3 capillary sites were studied at each time of examination (TP 0, −15 minutes; TP 1, +180 minutes; TP 2, +210 minutes; TP 3, +240 minutes; TP 4, +270 minutes; TP 5, +300 minutes; TP 6, 330 minutes; TP 7, 360 minutes; where 0 minutes is defined as the intraductal injection of GDOC and start of intravenous cerulein infusion). Six hours after induction of pancreatitis, all animal were killed.

### Functional capillary density

The number of perfused capillaries per observation site was counted and related to the total number of capillaries.[12] This measurement was assessed using screen-analysis software (KS 300; Kontron, Eching, Germany), which was calibrated with an objective scale for length measurement at the beginning of the analysis. Over the observed pancreatic lobule, a virtual net of 4 lines (2 horizontal and 2 vertical) of defined length was set. The crossings between lines and total numbers of capillaries and perfused capillaries were counted. The relation between crossings of total capillaries and perfused capillaries was defined as the functional capillary density.

### Leukocyte adherence

A leukocyte was considered adherent to the postcapillary venular endothelial wall of the interlobular venules if it did not move for at least 30 seconds. Leukocyte adherence was expressed as the area of adherent leukocytes in a percentage of the vein cross-section.[4]

## Histopathologic Scoring

The specimens were fixed in neutral phosphate-buffered 3.5% formalin, routinely processed, and paraffin embedded. Two 5-μm slices were taken from each specimen. The slices were stained with hematoxylin and eosin. The slides were examined in a blinded fashion using light microscopy. The histopathologic findings were scored as previously described quantifying acinar necrosis, fat necrosis and hemorrhage, leukocyte infiltration, and edema, with a maximum score of 16.[13]

## Statistical Analysis

Primary endpoints were functional capillary density and leukocyte adherence. Secondary endpoints were defined as histopathologic scoring and TAP levels. Descriptive analysis of parametric data was expressed as means and SD. Ordinal data were expressed as medians and range. Differences were statistically evaluated using Mann-Whitney $U$ test for nonnormally distributed data and unpaired Student's $t$ test for normally distributed data.

For repeated measurements, group differences and interactions between group and time were analyzed with repeated-measure ANOVA. Additionally, the area under the curve (AUC) was computed (starting from time-point 1) and compared using 1-way ANOVA and post hoc testing according to Tukey honestly significantly difference (HSD).

For the primary endpoints (functional capillary density, leukocyte adherence), the significance level was set at $P \leq 0.05$; for all secondary endpoints, $P$ values were interpreted for purely descriptive purposes according to explorative data analysis. All analyses were performed with SPSS version 10.0.

# RESULTS

After having performed at least 50 operations (ie, catheter placement, laparotomy, and preparation for the initiation of acute pancreatitis), researchers were allowed to participate in the experimental setting. Only 3 researchers did the initial instrumentation (T.S., H.K., and S.R.). In this series, 3 animals had to be excluded from evaluation before initiation of acute pancreatitis because of low blood pressure paralleled by low central venous pressure and a result of insufficient volume treatment. In addition, 1 animal had to be excluded because of intrapancreatic hemorrhage at baseline.

## Mortality

In group 1 (HBOC-200), 2 animals died in the course of the observation period ($t$ = 210 and 240 minutes) and were excluded from the final evaluation. In group 2 (HES), 4 animals died ($t$ = 200, 230, 240, and 280 minutes), and 5 animals did not survive the observational period in group 3 (NaCl; $t$ = 190, 210, 210, 240, and 280 minutes). Two animals died before randomization (<180 minutes). All animals that died during the observation period were excluded from final calculation. Additional animals were operated and randomized to have n = 10 animals in each group that completed the observation period. There was no statistical difference regarding mortality between the 3 groups.

## Functional Capillary Density

Using ANOVA for repeated measurements, a group effect (repeated-measure ANOVA; $P < 0.001$) and a time by treatment interaction ($P < 0.001$) were detected (see Fig. 1).

Additionally, the AUC (starting from randomization) for each animal as a measure for change of capillary density over time was computed. Referring to AUC values, we could detect significant differences between the 3 groups (ANOVA $F$ test, $P < 0.001$). Subsequent groupwise comparisons (Tukey HSD) showed significant improvement of pancreatic microcirculation by the administration of bovine hemoglobin in comparison with normal saline. In comparison with group 3, functional capillary density was significantly higher in group 1 over the observational period (mean difference, 95.2 ± 12.4 [SE]; 1-way ANOVA, $P < 0.001$; Fig. 1).

This beneficial effect was observed after HES treatment as well. The difference was significant over the observational period (mean difference, 75.7 ± 12.4 [SE]; $P < 0.001$; Fig. 1).

Over the 6-hour period, the mean difference in functional capillary density between HBOC-200– and HES-treated animals was 19.4 ± 12.4 ($P = 0.279$).

## Leukocyte Adherence

Using ANOVA for repeated measurements, a group effect (repeated-measure ANOVA; $P < 0.001$) and a time by treatment interaction ($P < 0.001$) were detected (see Fig. 2).

Analogous to functional capillary density, the AUC (starting from randomization) for each animal was computed. Referring to AUC values, we could detect significant differences between the 3 groups (ANOVA $F$ test, $P < 0.001$). Subsequent groupwise comparisons (Tukey HSD) showed that

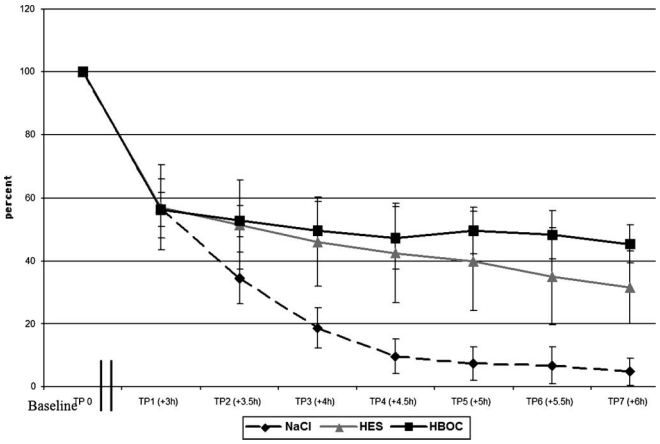


**FIGURE 1.** Functional capillary density over time (0 [baseline] = before initiation of acute pancreatitis; 1–7 = 3–6 hours after the initiation of acute pancreatitis; values are mean, SD, and 95% confidence interval [CI]). Area under the curve—HBOC-200 group: 149 ± 20.4; 95% CI, 134.4, 163.6 ($P < 0.05$ vs. NaCl); HES group: 129.5 ± 42.5; 95% CI, 99.1, 160 ($P < 0.05$ vs. NaCl); NaCl group: 53.8 ± 9.7; 95% CI, 46.9, 60.8.

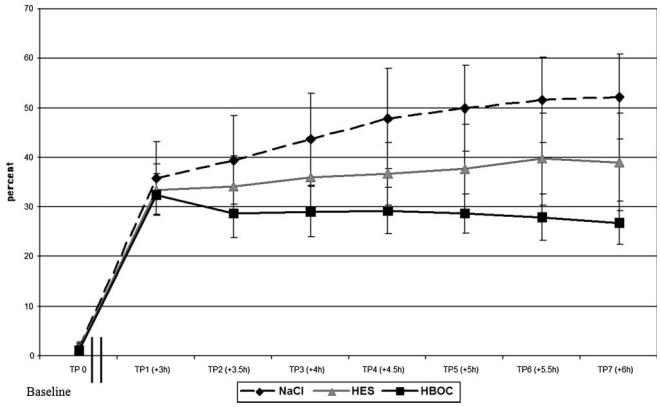


**FIGURE 2.** Leukocyte adherence over time (0 [baseline] = before initiation of acute pancreatitis; 1–7 = 3–6 hours after the initiation of acute pancreatitis; values are mean, SD, and 95% confidence interval [CI]). Area under the curve—HBOC-200 group: 86.6 ± 11.9; 95% CI, 78.1, 122.8 ($P < 0.05$ vs. HES, NaCl); HES group: 110.8 ± 22.2; 95% CI, 94.9, 126.7 ($P < 0.05$ vs. NaCl); NaCl group: 138.2 ± 25.2; 95% CI, 120.2, 156.3.

leukocyte adherence was significantly diminished by the administration of bovine hemoglobin in comparison with normal saline and HES. In comparison with group 3, leukocyte adherence was significantly lower in group 1 over the observational period (mean difference, 51.6 ± 9.2 [SE]; 1-way ANOVA $P < 0.001$; Fig. 1). Also, in comparison with group 2, leukocyte adherence improved over time in the HBOC-200 group (mean difference, 24.1 ± 9.2 [SE]; $P = 0.037$).

Comparing HES-treated animals with the NaCl group, less leukocyte adherence was found in group 2 (mean difference, 27.4 ± 9.2 [SE]; $P = 0.016$).

## Histology

Median histology score revealed less tissue damage in group 1 versus group 3 (6.75 vs. 12; range, 5.25–7.75 vs. 8.25–14; Mann-Whitney *U* test; $P < 0.001$), which was reflected in all subgroups.

Histology score improved in HBOC-200–treated animals in comparison with HES-treated animals (6.75 vs. 9; range, 5.25–7.75 vs. 7.5–10.75; $P < 0.001$). This was also reflected in all subgroups except for edema in the HBOC-200–treated animals.

In group 2, histology score improved in all subgroups except for fatty necrosis and hemorrhage compared with group 3 (see Table 1).

## TAP

After 6 hours, TAP levels in the HBOC-200 group were lower compared with the NaCl group (7.8 ± 4.2 vs. 17.8 ± 3.3 nmol/L [SD]; Student's *t* test $P < 0.001$).

There were no differences regarding TAP (15.1 ± 6.7 vs. 17.8 ± 3.3 nmol/L [SD]) levels between groups 2 and 3. Comparing HBOC-200–treated animals with HES-treated animals, there were higher TAP levels in the HES group (7.8 ± 4.2 vs. 15.1 ± 6.7 nmol/L [SD]; $P = 0.009$).

No differences between hematocrit levels were detected between the groups (data not shown).

# DISCUSSION

In severe acute experimental pancreatitis, bovine hemoglobin effectively improved pancreatic microcirculation and diminished tissue damage to the pancreas as assessed by a histology score. This novel therapeutic approach, consequently, deserves further study to potentially mitigate the devastating course of acute severe clinical pancreatitis.

A multitude of initiating factors can induce acute pancreatitis. Nonetheless, a common pathway including a cascade of cytokine activation[14] and free oxygen radicals[15] results not only in a systemic inflammatory response syndrome, but also in pancreatic microcirculatory dysfunction.[3,16] Therefore, in theory, a very promising approach is the effort to improve microcirculation irrespective of the underlying initiating factor, thereby treating acute pancreatitis irrespective of the initiating factor.

To parallel the clinical situation, we chose this standardized animal model, which represents an in vivo model of severe acute pancreatitis, severe enough that several animals died before and after randomization (mortality was not a study endpoint and animals were replaced). As opposed to hemorrhagic and ischemic experimental models of acute pancreatitis,[17,18] this is probably the most (patho)-physiological set-up,[2,19] because it resembles a situation with increased biliary intraductal reflux and exocrine hyperstimulation, both important factors in the development of severe human pancreatitis.[16]

A novel idea in the therapy for acute pancreatitis is the use of plasma oxygen carriers.[17] HBOC-200 is a noncorpuscular oxygen carrier with a low oxygen affinity dissociation

**TABLE 1.** Histology Score (Range, 0–16)[13] of the Pancreatic Head After 6 Hours

| Groups 1–3 (n = 10) | Acinar Necrosis (1–2: $P = 0.0171$; 1–3: $P = 0.0004$; 2–3: $P = 0.0028$) | Fatty Necrosis and Hemorrhage (1–2: $P = 0.0021$; 1–3: $P = 0.0013$; 2–3: $P = 0.1894$) | Leukocyte Infiltration (1–2: $P = 0.0028$; 1–3: $P = 0.0002$; 2–3: $P = 0.0091$) | Edema (1–2: $P = 0.0654$; 1–3: $P = 0.0002$; 2–3: $P = 0.0132$) | Total Score (1–2: $P = 0.0003$; 1–3: $P = 0.0002$; 2–3: $P = 0.0035$) |
|---|---|---|---|---|---|
| (1) HBOC-200 | 2 (1.5–2.75) | 1 (0.5–2) | 1.375 (0.5–2) | 2 (2–2.5) | 6.75 (5.25–7.75) |
| (2) HES | 2.5 (2–3) | 2 (1.5–3) | 2.25 (1.5–2.75) | 2.5 (1.25–3) | 9 (7.5–10.75) |
| (3) NaCl | 3.5 (2–3.5) | 3 (1.5–3.5) | 3 (2–4) | 3 (2.5–3.5) | 12 (8.25–14) |

Values are given as median and range.

curve that is shifted to the right ($P_{50}$ 36 mm Hg) as opposed to cellular human hemoglobin ($P_{50}$ 26 mm Hg). It may reach areas currently not perfused by erythrocytes and easily release oxygen to the damaged cells. In addition, bovine hemoglobin exerts a pronounced Haldane and Bohr effect that enhances oxygen release in acidotic tissue.[20] Furthermore, oxygen release from HBOC-200 is facilitated by the independent regulation of $O_2$ affinity in contrast to regular hemoglobin. In HBOC-200, chloride ions substitute for 2,3-diphosphoglycerate. These are readily available in plasma to enhance oxygen release from bovine hemoglobin.

We recently presented data evaluating the effect of early intervention with HBOC-200 on microcirculation and histologic damage in rodent severe acute pancreatitis.[10] These preliminary data reported on an early intervention, 15 minutes after the initiation of acute pancreatitis, in the same experimental model as in this study. Although this is an animal model that progresses about 5 times faster than clinical severe pancreatitis, treatment would still be very early in the course of severe acute pancreatitis, so that the preliminary data published before[10] reflected a prophylactic use of HBOC-200. This was the rationale to study an intervention at a later point in time, where microcirculatory breakdown has not passed a point of no return. This point of no return, however, is ill defined, because even after no therapeutic intervention, some animals (<10%) show at least some remaining pancreatic microcirculation after 6 hours, whereas in all other animals, pancreatic microcirculation is completely broken down (data not shown). Therefore, an intervention after 3 hours was a prudent point of intervention because marked microcirculatory effects were already visible, but intervention with HBOC stabilized this partial breakdown. Looking at microcirculatory data, the graphs show a significant difference regarding both parameters (leukocyte adherence and functional capillary density) in favor of the HBOC-200–treated animals. This was not the case in the early intervention study, where HBOC-200 was superior only regarding functional capillary density as opposed to NaCl. One potential explanation could be the doubling of measurements, because we doubled the observations (every 30 minutes instead of every 60 minutes as described previously). Another possible explanation is the difference in time of intervention. Very early in the course, the direct effect on the microcirculation (HBOC-200 and HES both were beneficial) may be of paramount importance. The additional capacity of HBOC-200 to deliver oxygen to an area that is less perfused and lacks oxyhemoglobin[10] 3 hours after induction may become more conspicuous if microcirculatory breakdown was not prevented as in our previous study.

The most pronounced effect in this study was seen in the improvement of leukocyte adherence compared with HES- and NaCl-treated animals. This effect can probably be explained by the colloid properties of bovine hemoglobin. However, it is remarkable that this effect was still present despite the well-known vasoconstrictor effect of bovine hemoglobin.[20,21] Oxyhemoglobin binds to NO, which leads to a decreased concentration of free NO, which in return, is associated with an increased vascular tone of the larger vessels but not at the microcirculatory level.[20]

Somewhat surprising is the fact that HBOC-200 improved microcirculation despite its described effect of hyperaggregability, which might lead to leukocyte activation and adherence.[22,23] In contrast, this study is in accordance to previous studies that showed no increased expression of adherence receptors.[24]

The clinical potential is also reflected by histologic assessment. HBOC-200–treated animals displayed less tissue damage both after early and late intervention compared with HES and NaCl. The median histology score in the HBOC-200 group reached only 50% of the NaCl score and 75% of the HES score. Thus, it seems that improved tissue oxygenation, caused by the effects outlined above, are the reason for less tissue damage found in animals treated with HBOC-200. Microcirculation was improved to a certain degree in the HES group also. In parallel, this led only to partial improvement in the histology score. However, it is still of notice that HES displayed a significant beneficial effect on pancreatic microcirculation compared with saline in terms of microcirculation and histologic damage. This effect probably reflects the colloidal property of HES compared with saline and is in accordance with the observation from other groups, who described the superiority of colloidal fluids in severe acute pancreatitis compared with saline.[25]

One hint toward a more effective treatment is that only 2 animals died during the observational period, whereas 4 and 5 animals died during the same period in groups 2 and 3, respectively. Although this study was not designed to be a survival study (mortality was not an endpoint and animals dying during the observational period were replaced), this trend is of some notice. However, the study would have been underpowered if the difference between 20% and 40% mortality was a real one, because in this instance, 91 animals would have been needed in each group (80% power, 5% $\alpha$[26]). A study with the respective power calculation should be considered to answer the clinically important question if a treatment regimen with HBOC-200 could eventually reduce mortality in severe acute pancreatitis.

In summary, this report on the therapeutic application of HBOC-200 later in the course of acute experimental pancreatitis proves its beneficial effect in an experimental setting of severe acute pancreatitis. The further combination of a plasmatic oxygen carrier with the benefit of hemodilution and improved rheology on the outcome of severe acute pancreatitis seems a logical consequence. The main problem with rodents in this setting will be to perform precise hemodilution and to calculate substitution with HBOC-200 because of a low total blood volume. Therefore, a trial using a canine or porcine model deserves consideration to evaluate the effect of combining these 2 complementary strategies and to assess their potential in human acute pancreatitis.

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