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Hyperbaric Oxygen Therapy Reduces Severity and Improves Survival in Severe Acute Pancreatitis

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Abstract Severe acute pancreatitis is characterized by pancreatic necrosis, resulting in local and systemic inflammation. Hyperbaric oxygen (HBO) therapy modulates inflammation, but has not been extensively studied in pancreatitis. This study investigates the effects of HBO in a rat model of severe acute pancreatitis. Sixty-four rats were induced with severe pancreatitis using 4% sodium taurocholate and randomized to HBO treatment or control. HBO was commenced 6 h after induction (100% oxygen at 2.5 atmospheres for 90 min) and continued every 12 h for a maximum of eight treatment episodes. Surviving animals were killed at 7 days. Severity of pancreatitis was graded macroscopically and microscopically. Lung edema was calculated using wet and dry lung weights. Macroscopic and microscopic severity scores (mean ± SE) of HBO-treated animals with pancreatitis (8.3±0.7; 9.6±0.4) were lower than those of controls (10.5±0.5; 11.1±0.4) (p=0.02 and p=0.03, respectively). The HBO-treated group had reduced pancreatic necrosis compared to controls (40±4% vs. 54±4%; p=0.003). There was no difference in pulmonary edema between the groups. Median survival in the HBO-treatment group was 51 h, compared to 26 h in controls. Day-7 survival was significantly improved in the HBO-treated animals compared to controls (40% vs. 27%; p=0.04). HBO therapy reduces overall severity, decreases the extent of necrosis, and improves survival in severe acute pancreatitis.

Keywords Severe acute pancreatitis · Necrosis · Hyperbaric oxygen therapy

Introduction

Acute pancreatitis is a common condition with an annual incidence ranging from 5 to 80 cases per 100,000 population.[1,2] Despite advances in the supportive management of this condition, the mortality rate of severe acute pancreatitis still approaches 30% in some series.[3] The key feature of severe disease is pancreatic tissue necrosis, leading to both local and systemic inflammatory responses.[4]

There is increasing evidence that microcirculatory alterations play a major role in the pathogenesis of acute pancreatitis.[5–12] Changes in vascular morphology and flow lead to decreased tissue oxygenation and worsening of pancreatitis severity. Apart from systemic antibiotics, administration of pharmacological agents does not significantly alter patient outcomes in severe acute pancreatitis.[13] Treatment has generally focused on modification of specific pathways involved in oxidative stress, ischemia reperfusion injury, and stabilization of the microcirculation.

A potential approach in the management of severe acute pancreatitis is the administration of hyperbaric oxygen (HBO). Beneficial effects of HBO therapy include increased tissue oxygenation, inhibition of ischemia reperfusion injury, and stimulation of angiogenesis.[14] HBO therapy also modifies neutrophil function, impairs bacterial replication, and has an overall antioxidant and antiedema effect.[14–16] Its potential role in modifying the pathophysiological effects of

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severe acute pancreatitis has not been fully elucidated. HBO therapy may simultaneously modify several pathways involved in the local and systemic inflammatory response and microcirculatory changes in acute pancreatitis and potentially reduce disease severity and mortality.[15,16]

The aim of this study is to evaluate the effects of HBO treatment on severity and mortality in a rat model of severe acute pancreatitis.

Methods

Animals

Experiments were conducted with the approval of the Austin Health Animal Ethics Committee. Male Wistar albino rats (280–320 g) were used. Animals were housed two per cage prior to surgery, with access to food and water ad libitum, and exposed to a 12-h light–dark cycle. Food was withdrawn from animals 12 h prior to the experiments.

Experimental Model of Severe Acute Pancreatitis

Rats were anesthetized by intraperitoneal injection of ketamine hydrochloride 100 mg/kg (Parke Davis, Melbourne, Victoria, Australia) and xylazine 10 mg/kg (Bayer, Melbourne, Victoria, Australia). Carprofen 5 mg/kg (Pfizer, Melbourne, Victoria, Australia) was administered subcutaneously for analgesia. Pancreatitis was induced by intraductal infusion of 4% sodium taurocholate (Sigma, Melbourne, Victoria, Australia) by modifications of methods previously described.[17]

A midline laparotomy was performed. A 27-gauge blunt needle was introduced into the distal end of the biliopancreatic duct through a duodenotomy. The proximal bile duct was temporarily occluded at the porta hepatis by a small vascular clamp. Sodium taurocholate (4% solution, 0.1 mL per 100 g) was infused at a pressure of 20 mm Hg controlled by a sphygmomanometer. At the end of the infusion, the needle was withdrawn and the clamps removed. The duodenum was closed with 6/0 prolene sutures and animals were hydrated by instillation of 5 mL 0.09% saline into the peritoneal cavity. Abdominal wounds were closed in two layers using 2/0 prolene sutures and animals recovered on heat pads.

Study Design

At the end of each operative session, animals with pancreatitis were randomly assigned to either HBO treatment or control. Four to eight animals were induced with pancreatitis in any one operative session. Thirty-two animals were allocated to each group.

HBO therapy [2.5 atmospheres (atm) 90 min] was commenced in the treatment group 6 h after induction of pancreatitis and administered every 12 h for a maximum of 4 days. Control animals received no treatment. Animals were assessed in a blinded manner at 6-h intervals following induction of pancreatitis based on an animal health scoring criteria and euthanized accordingly.[18] Animals classified as being in *very poor* health were killed immediately. Animals in *poor* health were administered 5 mL of 0.09% saline via subcutaneous injection when exhibiting signs of dehydration and carprofen 5 mg/kg subcutaneously for analgesia. Animals were then reassessed 2 h later and killed if there was no improvement in their condition. All animals surviving to day 7 were killed.

Blood was taken by tail vein bleed at 24 h to assess the early effects of HBO therapy compared to control and at day 7 in surviving animals. All HBO-treated animals at 24 h had completed two treatment sessions. The liver, pancreas, and lung were removed for analysis at the time of euthanasia in all animals following macroscopic assessment of disease severity.

HBO Administration

HBO was administered using a purpose-built animal hyperbaric chamber (Fink Engineering, Melbourne, Victoria, Australia) in assigned animals 6 h following induction of pancreatitis. Two to four animals were given HBO at one time. Treatment consisted of administration of 100% oxygen at 2.5 atm for 90 min, with a compression time of 10 min and a decompression time of 15 min. Protocols employed were according to recommendations used clinically for treatment of severe necrotizing infections.[14] HBO therapy was administered at 12-h intervals following initial treatment for a maximum of 4 days.

Assessment

Overall comparisons were made between HBO-treated and control animals with severe pancreatitis. Subgroup analysis of animals surviving to 7 days was performed.

Blood Tests

Serum was taken from animals for amylase and lipase measurement prior to induction of pancreatitis and at 24 h. Amylase and lipase was also measured again in those animals surviving to 7 days.

Severity Score

Laparotomy was performed on all animals at death or euthanasia. Macroscopic severity of pancreatitis was based

on a previously described scoring system.[19] Up to three points are allocated for each of ascites, extrapancreatic fat necrosis, pancreatic edema, hemorrhage, and necrosis. The maximum score by this method was.[15] Microscopic severity of pancreatitis was scored on hematoxylin and eosin (H&E)-stained sections by a scoring system modified from Yotsumoto et al.[20,21] The maximum possible score is 17 (Table 1). Sections in which no viable acinar tissue was seen were excluded from the comparison. All assessments were performed in a blinded manner, with the assessor unaware of the treatment group and the time of euthanasia or death.

Pancreatic Necrosis

Pancreatic tissue was sectioned longitudinally, formalin-fixed, and paraffin-embedded for histology. Cut sections were stained by H&E. Photomicrographs of the entire section were taken using an Olympus digital microscope (Coolscope, Nikon, Tokyo, Japan). A minimum of 12 images per pancreas were obtained from this micrograph at magnification ×100. These images were used for histological assessment using image analysis software and performed in a blinded manner (Image Pro-Plus version 4.5.1, Media Cybernetics, Bethesda, MD, USA). The area of pancreatic glandular tissue and pancreatic necrosis represented in each slide was measured. The percentage necrosis in each section was determined and an overall percentage of necrosis was determined using the calculation

$$N_{\text{total}}=[(N_1 \times A_1) + (N_2 \times A_2) + \ldots(N_n \times A_n)]/(A_1 + A_2 + \ldots A_n),$$

where N is the percentage necrosis and A is the area.

Table 1 Criteria for Microscopic Assessment of Pancreatitis Severity, Modified from Yotsumoto et al.[20,21]

Histological parameters	Score	Assessment
Edema	0	No edema
	1	Mild—interlobular septa expanded
	2	Moderate—interacinar septa expanded
	3	Severe—individual acini separated
Acinar necrosis	0	No necrosis
	3	Mild—<20%
	5	Moderate—21–50%
	7	Severe—>50%
Hemorrhage	0	No hemorrhage
	3	Mild—1–2 foci/slide
	5	Moderate—3–5 foci/slide
	7	Severe—>5 foci/slide

Lung Edema

The right lung of animals surviving to day 7 and those euthanized or found immediately after death were removed arbitrarily for analysis. A wet weight measurement of the lung, minus any connective tissue was taken using an analytical balance (AG204, DeltaRange®, Greifensee, Switzerland). The lung was then placed in containers and incubated in 37°C oven for 48 h. The desiccated lung tissue was reweighed and percentage fluid was calculated by the following formula:

$$\text{Relative water content}(\%)=(\text{wetweight} - \text{dryweight})/\text{dryweight} \times 100\%.$$

Survival

All animals surviving to 7 days postpancreatitis were considered to be long-term survivors. Animals were excluded from the study if there was a failure to induce pancreatitis based on changes noted in the pancreas at the time of induction or lack of elevations in amylase and lipase levels at 24 h compared to baseline. Animals were included in the study as censored data when the cause of death was unrelated to pancreatitis or treatment.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 11.5®, Chicago, IL, USA). All continuous variables and severity scores between control and treated animals were expressed as mean ± SEM but compared by the Mann–Whitney U test. Animal survival was determined using the Kaplan Meier method. Comparisons in survival were by the Breslow–Wilcoxon method, where losses occurring early are counted more heavily, and a constant hazard ratio is not assumed. A p value of less than 0.05 was considered statistically significant. All data are expressed as mean and SE, unless otherwise specified.

Animal numbers were based on calculations to achieve a power of 0.8 and p value of less than 0.05. Thirty-two animals were required in each group to detect a 30% improvement in survival at 7 days postoperatively from an expected survival of 20% in untreated animals.

Results

A total of 64 animals were used in this study. In two animals, acute pancreatitis was unable to be induced due to technical difficulties related to bile duct cannulation and one animal failed to recover following an anesthesia. These

animals were excluded from analysis. In three animals with established pancreatitis, the cause of death was related to operative complications and they were considered as censored data. Overall, euthanasia was implemented according to health scoring criteria in 40% of control animals with pancreatitis, compared to 36% in the HBO-treated group.

Blood Results

Baseline amylase and lipase levels in animals were 1,027±54 and 28±5 U/L, respectively. At 24 h, all animals induced with pancreatitis had significant elevations in serum amylase compared to baseline ($p<0.001$). The entire HBO group had undergone two treatment sessions by 24 h. Compared to controls, serum amylase (4,901±1,215 vs. 5,596±907 U/L $p=0.143$) and lipase levels (303±61 vs. 1,000±386 U/L, $p=0.089$) were not significantly different at 24 h. In surviving animals with pancreatitis, no differences were observed between HBO-treated and control groups at 7 days in serum amylase (3,981±1,444 vs. 8,996±3,705 U/L, $p=0.698$). There was also no statistically significant difference in serum lipase levels (418±199 vs. 1,505±524 U/L, $p=0.190$).

Severity Score

Pancreatic ascites, edema, necrosis, hemorrhage, and extrapancreatic fat necrosis were features of all animals induced with pancreatitis that did not survive beyond 72 h. Based on macroscopic scores, the overall severity of disease in the HBO-treated animals was significantly less than control animals with pancreatitis (8.3±0.7 vs. 10.5±0.5, $p=0.02$) (Fig. 1a). On histological examination, acute pancreatitis was characterized by necrosis, leukocyte infiltration, edema, and hemorrhage. The overall microscopic severity was also significantly reduced in animals undergoing HBO therapy, compared to the controls with pancreatitis (9.6±0.4 vs. 11.1±0.4, $p=0.03$) (Fig. 1b).

In surviving animals at 7 days, the macroscopic severity score of HBO-treated animals (4.8±0.5) was significantly less than that of controls with pancreatitis (8.1±0.5, $p=0.002$) (Fig. 1a). In animals surviving to 7 days, there was, however, no significant difference in the microscopic severity score (HBO: 10.0±0.8 vs. control: 11.8±0.9, $p=0.13$) (Fig. 1b).

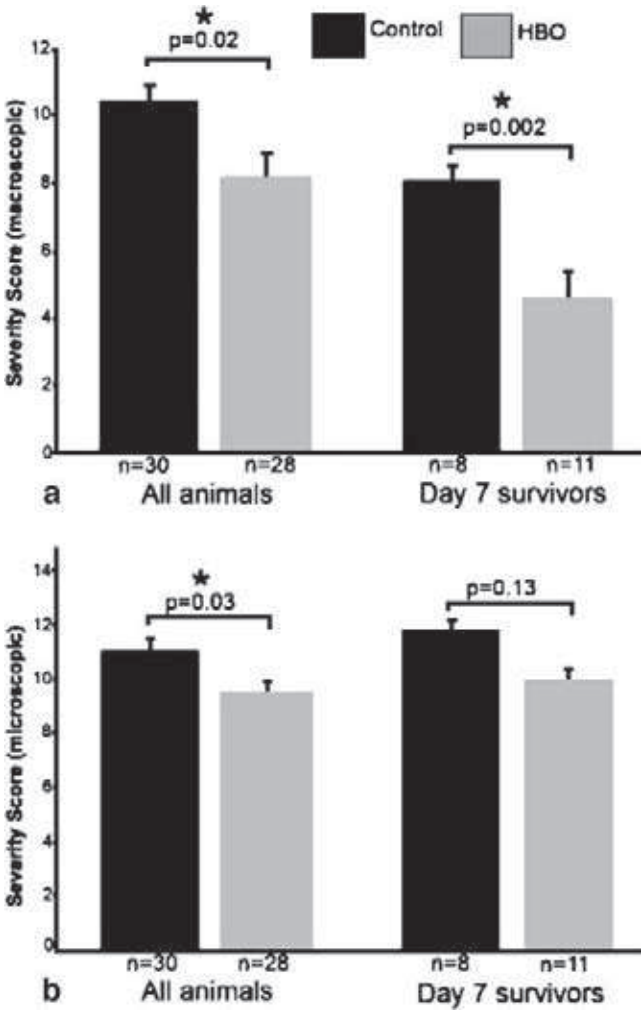

Figure 1 **a** Mean macroscopic pancreatitis severity score (as per Schulz et al.[19]), comparing control animals with pancreatitis to those with pancreatitis undergoing HBO therapy. Subgroup analysis of animals surviving to 7 days is shown. **b** Mean microscopic severity score of control vs. HBO-treatment groups (Mann–Whitney U test).

Pancreatic Necrosis

Features of pancreatic acinar necrosis were prominent in all animals examined, confirming the development of severe necrotizing pancreatitis. Pancreatic hemorrhage was observed more commonly in animals not surviving beyond 72 h. Pancreatic acinar necrosis was observed throughout the gland at all time points. In both HBO and control groups, the head of the pancreas was affected significantly more than the tail (61±4 vs. 47±4%, $p<0.001$, paired samples T test). The overall pancreatic necrosis in HBO-treated animals was significantly less than the necrosis in control animals (40±4 vs. 54±4% $p=0.046$) (Fig. 2). In animals surviving to 7 days, the extent of pancreatic necrosis was similarly significantly lower in HBO-treated animals compared to controls with pancreatitis (46±6 vs. 70±7% $p=0.046$).

Lung Edema

Edema and pulmonary hemorrhage were observed on macroscopic assessment of lungs in some animals with pancreatitis. Overall, relative water content of the lungs in

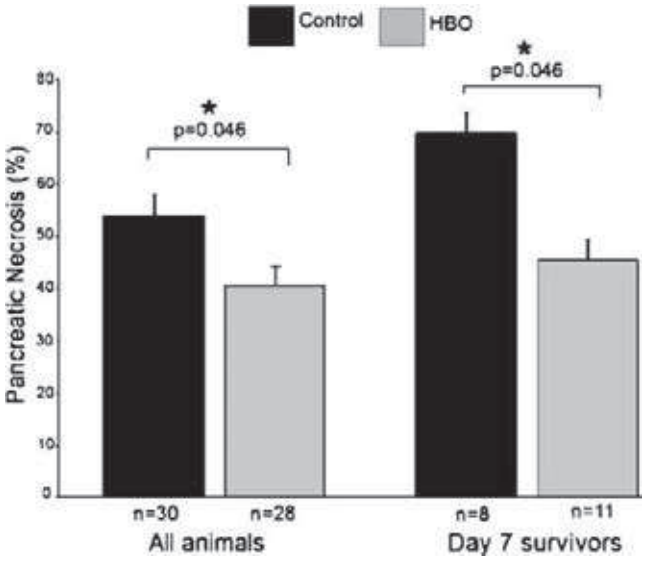

Figure 2 Mean pancreatic necrosis in control and HBO-treated animals. Subgroup analysis of animals surviving to 7 days is shown (Mann–Whitney U test).

HBO-treated animals was not significantly different to controls (76±1 vs. 76±1% p=0.737). Similarly, in animals surviving to 7 days, the relative water content in the HBO-treated group was not significantly different to control animals with pancreatitis (79±1 vs. 76±1% p=0.069).

Survival

One animal in the HBO group developed respiratory distress immediately following HBO therapy, 4 days post-disease onset, and died shortly thereafter. It was included as an uncensored death as part of the analysis. There was collapse and hemorrhage into both lungs and death was possibly the result of therapy or delayed complications of severe pancreatitis. HBO treatment was otherwise well tolerated without any apparent complications. The mortality rate in animals with severe pancreatitis was greatest within the first 72 h following induction of pancreatitis. In HBO-treated animals, the median and 7-day survival were 51± 44 h and 40±9%, respectively. In control animals the median and 7-day survival were 26±2 h and 27±8%, respectively. The overall survival was significantly greater in the HBO-treated animals than controls with pancreatitis (p=0.04) (Fig. 3).

Discussion

Acute pancreatitis is a common disorder with an incidences of 79.8 per 100,000 in the USA.[1] The incidence and etiology of the disease varies in different regions of the world, reflecting patterns of alcohol intake and gallstone prevalence.[1,22] Approximately 10 to 15% of patients have severe disease, with a fulminant course of pancreatic necrosis and multiorgan failure.[2,23] The mortality in this patient group generally ranges from 10 to 30%.[1,3,24] Antibiotics, early enteral feeding, and therapeutic endoscopy are advocated in the treatment of specific cases of severe pancreatitis. There is no single therapy to date, however, that consistently improves outcomes in this condition.[23]

The underlying processes involved in severe acute pancreatitis are complex and not fully elucidated. The critical initiating event appears to be activation of trypsinogen within pancreatic acinar cells and destruction of the duct and acinar cell cytoskeleton. This produces activation of an inflammatory cascade, mediated by various cytokines, immunocytes, and the complement system, leading to a systemic inflammatory response syndrome.[13] Pancreatic necrosis is the key feature in severe disease and results from impairment of the pancreatic microcirculation, free oxygen radical production, and ischemia reperfusion injury.[25–28] Inflammatory mediators play a critical role in the local and systemic manifestations of severe pancreatitis. These mediators include platelet activating factors, interleukins, bradykinin, and endothelins, all of which exacerbate microcirculatory disturbances within the pancreas, leading to hypoxia and tissue necrosis.[29,30]

Experimental studies in the treatment of severe pancreatitis generally include antagonists to specific mediators involved in the inflammatory process. IL-1β and TNF-α blocking commenced shortly following induction of pancreatitis reduces severity and improves survival in rat models.[30,31] In a study of 3% taurocholate and cerulein induced pancreatitis in rats, monoclonal antibody to TNF-α administered shortly following induction of disease ameliorated both parenchyma and fatty tissue necrosis caused by pancreatitis.[32] Antioxidant treatment in animal models of severe hemorrhagic pancreatitis has also shown promising results in reducing tissue damage and improving survival.[33] Anti-TNF-α and anti-IL-1β therapies in the clinical setting have failed to reproduce the same beneficial effects seen in experimental models.[34]

A potential alternative approach to modify the various pathophysiological pathways involved in severe pancreatitis is the administration of HBO. Beneficial effects of HBO therapy include increased tissue oxygenation by improved blood rheology and reduced shunting of blood from hypoperfused tissue.[14,35] The oxygen diffusion distance from the perfused capillaries is increased approximately fourfold with HBO administration.[36] HBO therapy can increase arterial oxygen tensions to 2,000 mm Hg and achieves partial pressure of oxygen in tissues in the order of 500 mm Hg.[37] Tissue oxygenation is increased to levels that are sufficient to support resting tissues without a contribution from hemoglobin, resulting in a reversal in

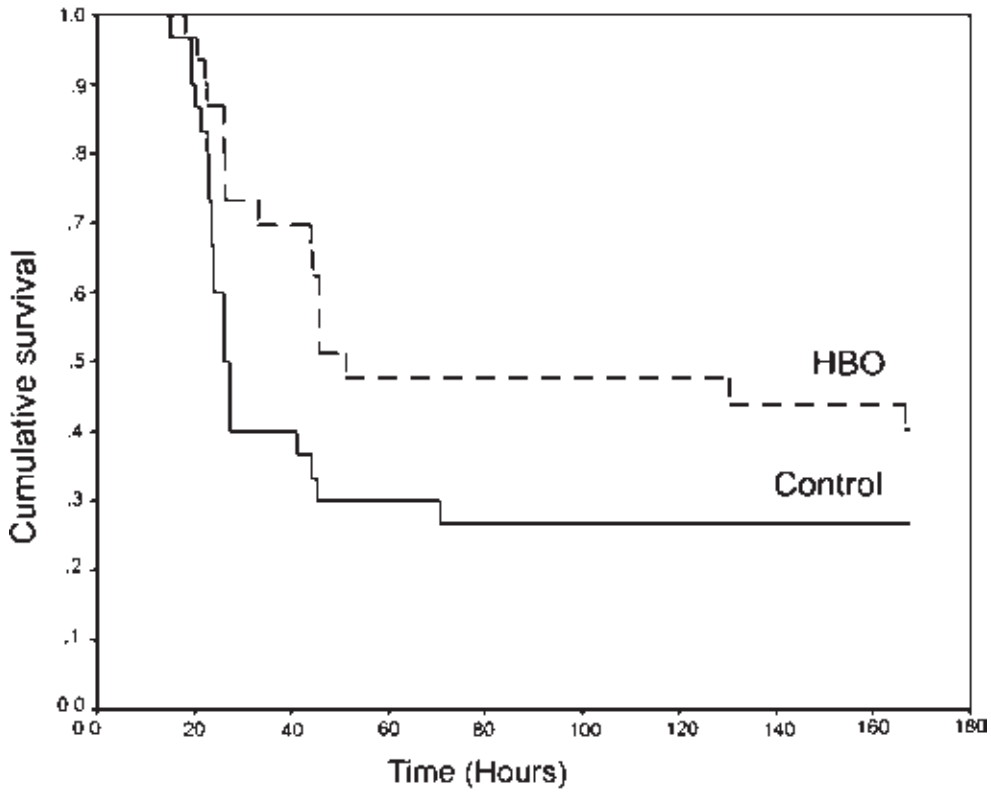

Figure 3 Kaplan Meier survival curve of rats with acute pancreatitis treated with HBO, compared to control (p=0.04, Breslow–Wilcoxon test).

hypoxia for the duration of the treatment and for a significant time following therapy.[38,39] It also inhibits ischemia reperfusion injury, stimulates collagen matrix formation needed for angiogenesis, modifies neutrophil function, and impairs bacterial replication and toxin production, with an overall antioxidant and antiedema effect.[15,16] HBO more specifically suppresses TNF-α and anti-IL-1β in response to lipopolysaccharide[40] and down-regulates ICAM-1 expression on endothelial cells to reduce polymorphonuclear leukocyte adhesion.[41] Its general anti-inflammatory effects are clearly demonstrated in "zymosan" models of shock, in which HBO attenuates the inflammatory response and reduces mortality.[42–44] All these features may significantly improve outcomes in severe pancreatitis.

Most available data pertaining to HBO and pancreatitis in a clinical setting are in the treatment of infective complications.[45] In a study of 12 patients with peripancreatic sepsis and abscess formation due to severe pancreatitis, there was significant clinical improvement, and surgical debridement was avoided in four of five patients assigned to HBO therapy (three to seven sessions) at 2.8 atm.[45] This was compared to seven patients with peripancreatic sepsis who had conventional treatment, with three deaths following surgical debridement. Although encouraging, the study was not randomized and the utility of HBO therapy in actually preventing complications was not defined.

In a rat model of severe pancreatitis, HBO therapy with either 40 or 100% oxygen up to 4 atm reduced the severity of pulmonary edema and improved pancreatic microcirculation.[46] HBO was well tolerated in treated animals and compared to normobaric hyperoxia and control. However, animals were not recovered following anesthesia to assess the impact of therapy on survival. In addition, the effect of only a single HBO treatment session on severe pancreatitis was tested. In a more recent study in rats with pancreatitis, HBO therapy for 48 h reduced histopathological findings (nonquantitative assessment) compared to controls with pancreatitis.[47] In two rat models of severe pancreatitis, oxidative injury was significantly reduced with HBO.[47,48] In a porcine model of severe acute necrotizing pancreatitis, HBO therapy combined with surgery resulted in improved survival. All animals without HBO died (five of five), while only two of five animals with HBO therapy died as a consequence of pancreatitis.[49] However, there are few studies to date with sufficient treated animal numbers to demonstrate a reduction in morbidity and mortality of severe acute pancreatitis with HBO therapy.

Our study was powered to detect a 30% reduction in mortality in severe pancreatitis following HBO therapy. The survival rate at day 7 in the HBO-treated animals in our study was 40% compared to 27% in control animals with pancreatitis, representing a 48% relative increase in survival (p=0.04). Features of the study design mean this survival advantage is relevant to the human situation. HBO treatment was commenced well after the induction of pancreatitis, when pancreatic injury and systemic inflammatory

effects were well established. Delays in presentation and diagnosis frequently occur in patients with pancreatitis. In addition, HBO therapy would rarely be available *immediately* upon patient presentation. We speculate that treatment at 6 h post severe pancreatitis in this model is more relevant to the clinical situation[50] than models where treatment is commenced immediately upon induction of pancreatitis.[46]

Our model produces significant pancreatitic necrosis in both HBO-treated and control animals (40±4 and 54±4%, respectively). There was a clear reduction in percentage necrosis in HBO-treated animals, both overall and at 7 days following therapy. Overall, there was a 14% absolute reduction in pancreatic necrosis in HBO-treated animals compared to controls (p=0.046), representing a 25% relative decrease in the extent of necrosis. When only animals surviving to 7 days were compared, the absolute reduction in necrosis was 24%, with a relative decrease of 34%. In this study, groups of animals were not killed at defined earlier time points for comparisons. The findings of our study contrast a previously published study, in which a single session of HBO failed to reduce the percentage necrosis following severe pancreatitis.[46] However, histological assessment in that study was at 9 h after a single HBO treatment session. Histological assessment in our study was performed at later time points, which may potentially explain the difference in detected effect.

In our study, there were also significant reductions in macroscopic severity grading of pancreatitis in HBO-treated animals compared to control animals with pancreatitis. Although the absolute changes in both microscopic and macroscopic pancreatitis severity following HBO treatment were small, it appeared to result in significant improvements in overall mortality. The microscopic scoring scale utilized was weighted toward acute features of pancreatitis, which may account for a nonsignificant difference in tissue taken at 7 days.

The pulmonary water content was similar between HBO-treated animals and controls overall and in those surviving to 7 days following therapy in our study. This is in contrast to a study by Chen et al.,[46] who reported reduced pulmonary edema following HBO treatment, assessed within 24 h of disease onset in a mild–moderate pancreatitis model. Our study was designed to determine differences in pulmonary edema at 7 days post disease onset. It is likely that the degree of pulmonary edema partly resolves by this time point. To demonstrate potential HBO pulmonary effects in severe acute pancreatitis, assessment of lung edema, histology, and blood gases at specified earlier time points is required.

There are some concerns regarding potential deterioration of respiratory function during HBO therapy.[51] In our study, one animal had potential respiratory complications related to HBO therapy. Treatment was uncomplicated in the remaining 31 animals. Based on published literature, intermittent therapy, ranging from 2 to 3 atm, generally has minimal pulmonary side effects.[52] However, careful monitoring of respiratory function, particularly in unwell patients, would appear prudent.

In conclusion, HBO therapy significantly reduces morbidity and mortality when administered to rats with established severe acute necrotizing pancreatitis. The exact mechanisms of HBO-induced reduction in mortality of severe pancreatitis require further investigation, but are almost certainly multifactorial.[14] Further evaluation of the action of HBO therapy in severe acute pancreatitis is warranted to improve treatment results, possibly by development of synergistic therapies.

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