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ORIGINAL ARTICLE

Hyperbaric Oxygen Therapy Effects on Tissue Lesions in Acute Pancreatitis. Experimental Study in Rats

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ABSTRACT

Objective To study the effects of hyperbaric oxygen therapy on tissue lesions in an experimental model of acute pancreatitis induced by pancreatic duct ligation.

Animals Forty-eight adult female Wistar rats were randomized into two groups (n=24): control group and hyperbaric oxygen therapy group.

Intervention The second group was treated with a two-hour daily session of hyperbaric oxygen therapy at 2.5 ATA started 6 hours after pancreatic duct ligation.

Setting The two groups were divided into 3 subgroups of 8 rats each undergoing euthanasia on days 1, 3, and 7 after the acute pancreatitis induction.

Main outcome measures The pancreas was evaluated according to the following histopathologic criteria: edema, hemorrhage, acinar necrosis and leukocyte infiltration.

Results Hyperbaric oxygen therapy was efficient in significantly reducing acinar necrosis on the first day (P=0.049) and the foci of hemorrhage on the seventh day (P=0.050). The edema and leukocyte infiltration did not show the expected reduction.

Conclusion The utilization of a daily session of hyperbaric oxygen therapy at 2.5 ATA is efficient in reducing the hemorrhage and acinar necrosis but is not sufficient to reduce edema and leukocyte infiltration.

INTRODUCTION

Acute pancreatitis is an inflammatory disease which affects the pancreas and the peripancreatic tissues, and may progress into multiple organ failure. The first intracellular events are observed with the activation of trypsinogen into trypsin inside the zymogen granules which is followed by the activation cascade of other enzymes, proteases, cytokines, oxygen free radicals and vasoactive molecules [1, 2, 3, 4, 5].

Pancreatic duct ligation at the duodenum is an experimental approach which promotes the progression of acute pancreatitis similarly to that observed in biliary acute pancreatitis in humans. Nevertheless, pancreatic duct ligation is not classified as a severe condition, as it allows survival of the animal for over 14 days and presents near-zero mortality figures [6, 7].

Hyperbaric oxygen therapy (HBO) is a technique which offers stressed cells what they need the most: oxygen (O_2). This is only possible because O_2 intake at atmospheric

pressure values above 1 absolute atmosphere (ATA), apart from saturating erythrocytes to 100%, also promotes the dilution of gas in plasma. As a whole, HBO leads to tissue and circulatory hyperoxia with a series of effects: i) a decrease in edema in consequence of the vasoconstrictor effect and elimination of tissue hypoxia which appears as a result of the edema and ischemia; ii) prevention and action against infections, by offering the O_2 levels that leukocytes need in order to play their oxidative role, apart from the generation of an inhospitable condition for microorganism growth; iii) the promotion of angiogenesis, for increasing collagen deposits and for producing the vascular endothelium growth factor by activated fibroblasts; iv) an increase in the production of antioxidant substances; and v) a decrease in leukocyte adherence to the vascular endothelium which improves microcirculation [8, 9]. HBO has been tested in diverse acute pancreatitis experimental models revealing an increase in the production of antioxidants and a decrease in oxidative stress parameters and in histopathological scores [10, 11, 12, 13, 14, 15].

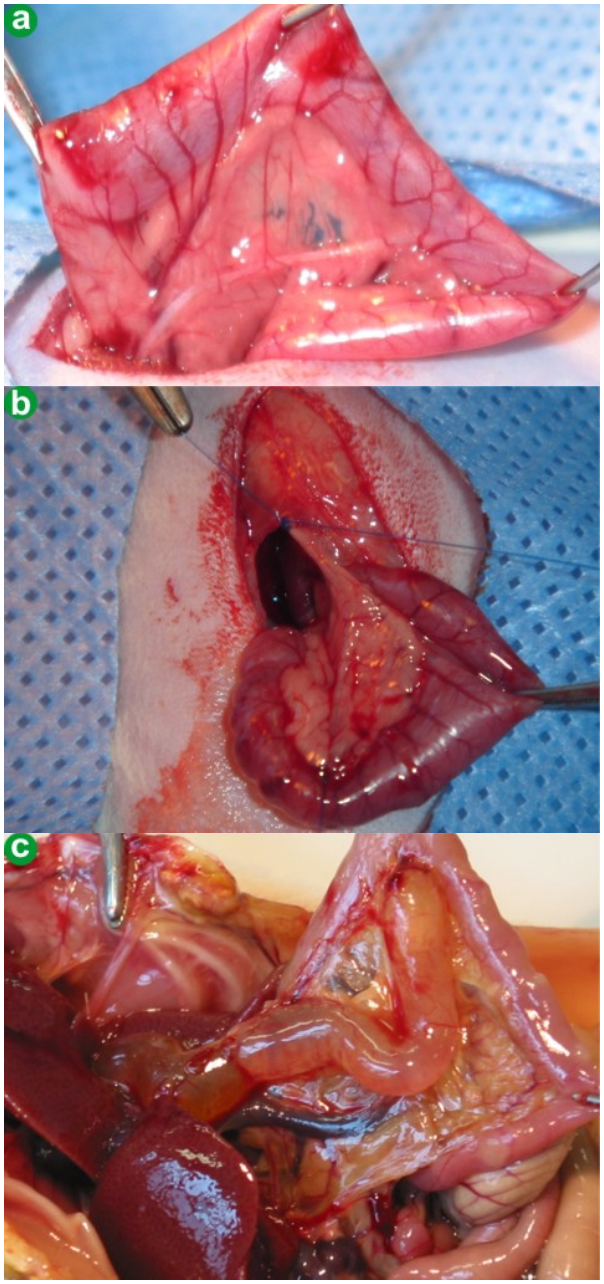

Figure 1. Pancreatic duct ligation (**a.**) and biliary duct ligation (**b.**). Aspect on the 7th postoperative day (**c.**).

The aim of the study was to assess the effects of HBO treatment in ligation-induced acute pancreatitis.

METHODS

Animals

Forty-eight adult female Wistar rats (*Rattus norvegicus albinus*) weighing between 200 and 280 g were used as the animal model in this study. All animals were kept in polypropylene boxes covered with metal screen grids. The boxes were lined with autoclave-sterilized sawdust which was replaced three times a week. The rats were given chow (Labina®, Purina, RS, Canoas, Brazil) and water *ad libitum*, and were kept in a 12 h circadian rhythm in a controlled environment throughout the experiment, as established by the Guide for Care and Use of Laboratory Animals [16].

Design

The rats were randomized to form two groups of 24 rats each: a control group and a HBO treatment group. These groups were then divided into 3 subgroups with 8 rats each (euthanasia after 1, 3, and 7 days from acute pancreatitis induction).

Acute Pancreatitis Induction

The model used to develop acute pancreatitis promoted by pancreatic duct ligation in rats was based on the study performed by Samuel *et al.* [6]. All animals underwent a 12 h fasting period and were then anesthetized by

sedation with halothane (Fluotane®, Astra-Zeneca, São Paulo, Brazil) in a campanula, to which a 0.1 mL intradermal injection of tiletamine chloridrate 125 mg and zolepan chloridrate 125 mg (Zoletil®, Virbac, São Paulo, Brazil) with 0.05 mL morphine for analgesia was added. The surgical procedure started with a 2-3 cm median laparotomy. Next, the duodenal loop was recognized and lifted to afford visualization of the biliopancreatic duct. The junction of the biliopancreatic duct and the duodenum was identified and characterized using a sharp polypropylene monofilament wire 5-0 (Premilene®, B-Braun Medical, Bogota, Colombia). Another ligation was carried out in this duct, at a point preceding the pancreatic duct and between 1.0 and 1.5 cm below the hepatic hilum (Figure 1ab).

Hyperbaric Oxygen Therapy (HBO)

The animals of the HBO group were coupled in 15x15x20 cm polypropylene boxes covered with metal screen grids. The boxes were designed so as to fit in the hyperbaric chamber (Figure 2). Pressurization was conducted at 100% O_2 upon reaching 2.5 ATA, after which point the boxes were kept in the chamber for 120 min, in a procedure hereafter called HBO session. These HBO sessions were repeated once a day, up to the time span defined for necropsy. The 1st day subgroup was the only group to be given two HBO sessions within 24 h: the first session 6 h after acute pancreatitis induction and the second HBO session the morning after, before euthanasia.

Figure 2. Hyperabric chamber for small animals.

Macroscopic Inspection

On the 1st, 3rd and 7th postoperative days, the 8 rats of each group were once again anesthetized and euthanized by halothane intoxication. The abdominal and thoracic cavities were opened and underwent a comprehensive examination, with all macroscopically visualized lesions being photographed. The diameters of the dilated pancreatic duct were measured.

Histopathological Analysis

The pancreas, duodenum and part of the stomach were fixed in buffered formalin 10%. The pancreas was then dissected and sectioned along its longitudinal axis and subsequently divided into three transversal sections as head, body and tail. This material was treated according to standard analytical techniques and stained with hematoxylin-eosin to produce slides for microscopic examination by a pathologist blinded to the study groups. Two slides were prepared for each animal, with the random selection of four fields on each transversal section for scoring purposes. Histopathological lesions were analyzed according to the following criteria (Figure 3):

- Edema: serous fluid spills between the acinar zones, especially the septae;
- Hemorrhage: the presence of erythrocytes outside the vascular bed;
- Acinar necrosis: the presence of cytoplasmatic changes in microvascularization, with scattered cell nucleus degeneration;

• Leukocyte infiltration: the presence of polymorphonuclear and mononuclear cells in edematous zones.

Amylase

Blood samples (3.0 mL) were taken by direct heart puncture from the rats euthanized on the postoperative 1st day in order to measure serum amylase levels (BioTécnica, Minas Gerais, Brazil; reference range for pilot study of the assay in rats without pancreatitis: 530-810 U/L). These data were used for the laboratory confirmation of the diagnosis of acute pancreatitis.

ETHICS

This study was approved by the Committee for Ethics in Research of the University of Caxias do Sul, Brazil. All animals received humane care according to the criteria outlined in the Guide for Care and Use of Laboratory Animals [16].

STATISTICS

Data are reported as mean and standard deviations (SD). The histological score was compared between the two groups using the Mann-Whitney U test, with significance level defined as two-tailed P value less than 0.05. We used the SPSS for Windows (Version 13.0) package for statistical data analysis.

RESULTS

Amylase

The laboratory acute pancreatitis diagnosis was confirmed in only six animals of each group on the 1st day. Amylase levels were not tested in the rats euthanized after 3 and 7 days because amylase levels fall in the days after pancreatitis induction. Amylase mean levels in rats sacrificed on the 1st day after pancreatic duct ligation were 3,517±1,754 U/L (range: 2,000-5,850 U/L) in the control group and 5,428±3,773 U/L (range: 2,000-10,230 U/L) in the HBO therapy group (P=0.415). All animals had amylase levels at least two times higher than the upper limit of the reference range and the criteria for diagnosis of pancreatitis were histologic.

Macroscopic Inspection

The macroscopic inspection of the abdominal cavity revealed the presence of an

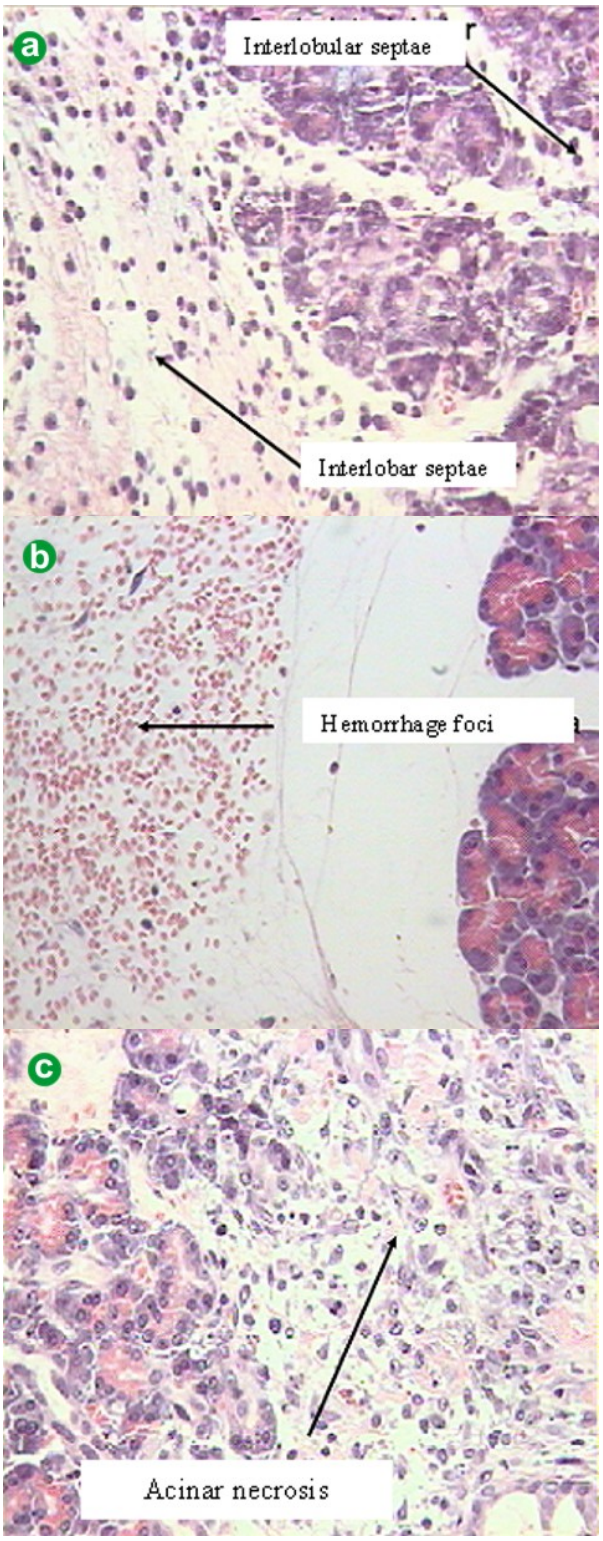

Figure 3. Edema and leukocyte infiltration (**a.**), hemorrhage (**b.**) and acinar necrosis (**c.**). (H&E, 400x magnification)

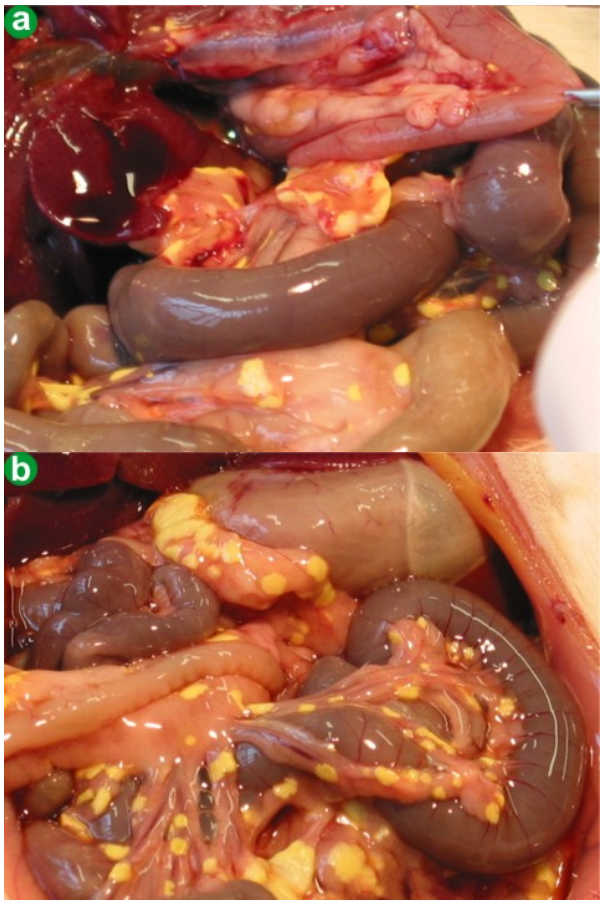

Figure 4. Macroscopic image of the HBO group (**a.**) presenting a pancreas with necrosis. Small intestinal loop with ischemia and steatonecrosis (**b.**).

inflammatory process in the pancreas, and foci of steatonecrosis (fat necrosis) scattered in the cavity, more prominent in the group euthanized on the 3rd day and almost undetectable in the 7th day specimens (Figure 4), except for one rat in the control group sacrificed on the 3rd day which presented a large amount of fat necrosis, apart from signs of stress and distension of the intestinal loops as is typically observed in intestinal ischemia processes. The rats sacrificed on the 7th day exhibited intense dilation of the biliary and pancreatic ducts as observed macroscopically. In 4 rats of the control group, the mean pancreatic duct diameter was 5.25±0.29 cm, while, for the 7 rats of the HBO group, this value went down to 4.21±1.60 cm (P=0.255) (Figure 1c). The mean biliary duct diameter was 6.88±0.85 cm and 7.71±2.94 cm in the control and HBO groups, respectively (P=0.849). The inflammatory response in the pancreas was minimal, and fat necrosis in the abdominal cavity was scarce.

Microscopic Investigation

One animal in the HBO group sacrificed on the 7th day was accidentally lost. By comparing the results of the histological scores of the control group and the HBO group, a statistically significant difference in the reduction of acinar necrosis in the pancreas was observed for the latter (P=0.049; Figure 5), for the first 24 h only. The use of a once daily HBO session for 7 days was efficient in decreasing the number of hemorrhagic sites (P=0.050; Figure 6). The comparison of the occurrence of edema (Figure 7) and pancreas inflammation (Figure 8) between the animals of the HBO and the control groups did not present statistically significant differences.

DISCUSSION

In consequence of the diverse manifestations of acute pancreatitis a comprehensive, realistic representation of its progression in

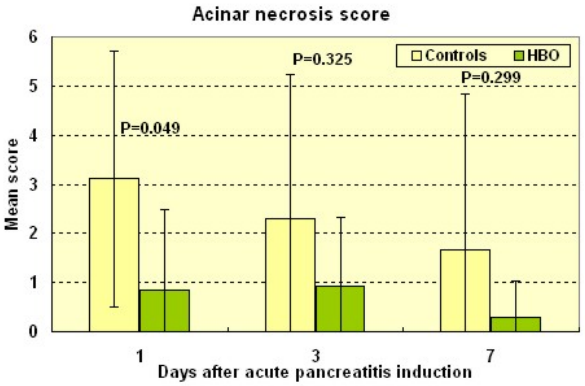

Figure 5. Mean histopathological scores of acinar necrosis.

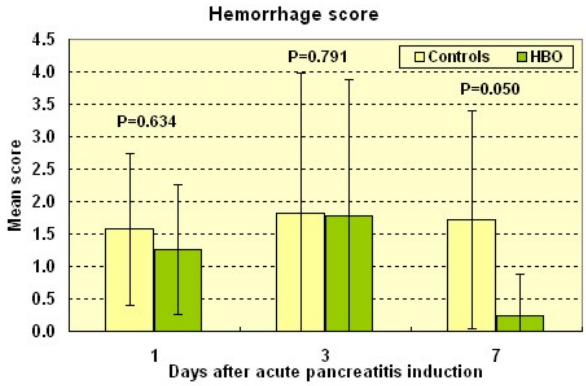

Figure 6. Mean histopathological scores of hemorrhage.

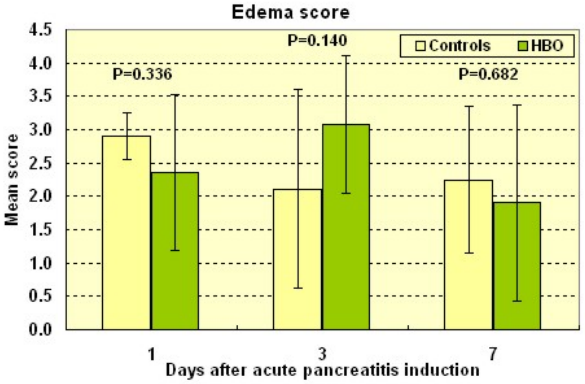

Figure 7. Mean histopathological scores of edema.

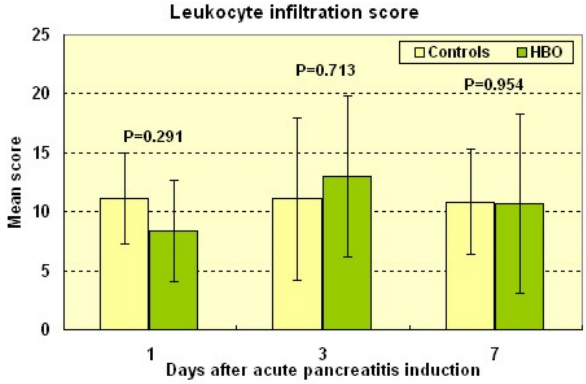

Figure 8. Mean histopathological scores of leukocyte infiltration.

humans becomes difficult. There is no such experimental model considered ideal for depicting acute pancreatitis, and the experimental approach adopted depends on the main objectives of the individual research papers [1]. Yet, the pancreatic duct ligation model is easy to conduct and reproduce, affording the study of histological lesions such as edema, necrosis, hemorrhage and leukocyte infiltration. Moreover, the method mimics biliary acute pancreatitis and imposes minimal mortality rates on the sample, since severe acute pancreatitis does not develop [6, 7]. The body of research published on the role of HBO in acute pancreatitis is very limited, both from the clinical and the experimental standpoints. MEDLINE/PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed) and LILACS (http://bases.bireme.br/cgi-bin/wxislind.exe/iah/online/?IsisScript=iah/iah.xis&base=LILACS&lang=i) databases do not indexed the publication of studies on HBO in a pancreatic duct ligation model, at least as of April, 2008.

In 1998, Chen *et al*. studied the effects of HBO on microcirculatory changes (the technique used was the vital microscopy technique of post-capillary venules), tissue lesions and malondialdehyde levels (an oxygen-free radical) produced during the first 12 h after acute pancreatitis induction by cerulein i.v. and intraductal glycodeoxycolic acid. The results showed that HBO improves the flow of erythrocytes and reduces leukocyte adhesion to the venular endothelium. Apart from this, the treatment improved the severity status of edema-related histological changes as well as inflammation, hemorrhage and necrosis, and it also led to lower malondialdehyde levels as compared to the control group [10]. Nikfarjam *et al*. studied pancreatitis induced by sodium taurocholate 4% and HBO treatment (2.5 ATA for 90 min, every 12 h, with up to 8 sessions), and showed a decrease in tissue lesion severity, a decline in necrosis incidence (from 54 to 40%, P=0.02), and an increase in survival after 7 days (from 27 to 40%) [12]. In the present study, the animals which received two HBO sessions [7] and were euthanized 24 h after acute pancreatitis induction presented a lower incidence of edema, inflammation, hemorrhage and necrosis (only the necrosis figures being statistically significant; P=0.049). These findings may be explained by the obstructions observed in the microcirculation in the pancreas and in other organs as a result of leukocyte adhesion to the vascular endothelium, which may ultimately increase the incidence of necrosis, inflammation and tissue edema. Furthermore, activated leukocytes are the main agents responsible for oxygen free radical production, developing oxidative stress which in turn is behind systemic inflammatory response and multiple organ failure [9].

In a study of HBO as a treatment for severe necrotizing acute pancreatitis in acute pancreatitis induced by sodium taurocholate 3% in rats, Yasar *et al*. used two daily sessions for five days. Malondialdehyde

levels, superoxide dismutase (SOD) and glutathione peroxidase activities were the parameters measured in the erythrocytes and the pancreatic tissue. HBO produced significant results in the reduction of oxidation levels in comparison with the control group, due to the decrease in malondialdehyde levels and to the increase in antioxidant activity by increasing SOD and glutathione peroxidase activities, both in the pancreatic tissue and in the erythrocytes. Lower mortality figures were also observed in the HBO group as compared to the control group [11]. Similar results were found in a study of these antioxidants in the lung parenchyma [13].

Zalaudeck *et al*. studied the effect of HBO on a necrotizing acute pancreatitis model which used pigs as the animal model, and observed that all animals (n=4) in the group which was not treated eventually died while, in the treated group, two out of 3 animals survived. These animals presented more clearly defined necrotic zones, and the conclusion was that HBO reduces mortality and improves the prognosis in necrotizing acute pancreatitis [17]. In the present study, as the experimental design did not include severe necrotizing acute pancreatitis and mortality was an undesired outcome, a once daily HBO session was adopted to establish HBO effects on tissual lesions throughout the seven days of treatment. This aspect of our experimental protocol is explained by the fact that one single HBO session a day is the approach normally adopted in the treatment of inflammatory and traumatic human diseases [8]. As far as we know, there are no studies which demonstrate the period in which the effects of hyperoxia are seen in the pancreatic tissues. Similarly, no knowledge of the ideal ATA pressure is indicated for the treatment of such cases [8, 9]. This explains why no statistically significant results were obtained for the group of rats euthanized on the 3rd day. Indeed, such a result came as a surprise, and proves the need for research protocols to study the ideal HBO regimens to be observed in pancreatic duct ligation-induced acute pancreatitis. Throughout the seven days of the treatment, HBO was shown to be capable of reducing all the histological parameters measured. Nevertheless, among the parameters investigated, only the reduction in hemorrhage sites was statistically significant, possibly due to the benefits of HBO to microcirculation. It may be hypothesized that the absence of statistical significance in the other parameters may be the consequence of the accidental loss of one animal of the 7th day HBO group. We did in fact consider the implications of adding a new animal to the group to compensate for this loss. However, this idea was rejected, because a statistical test revealed that no significant difference would be observed between the groups, even if the lost animal had been taken as presenting the top necrosis score.

CONCLUSION

The adoption of a once daily HBO session at 2.5 ATA efficiently reduces hemorrhage and acinar necrosis, although it is not enough to reduce edema and leukocyte infiltration.

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Keywords Hyperbaric Oxygenation; Pancreatitis

Abbreviations ATA (absolute atmosphere)

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Conflict of interest The authors have no potential conflicts of interest

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