

The Effect of Hyperbaric Oxygen Treatment on Oxidative Stress in Experimental Acute Necrotizing Pancreatitis

M. YASAR, S. YILDIZ¹, R. MAS², K. DUNDAR¹, A. YILDIRIM, A. KORKMAZ³, C. AKAY⁴, N. KAYMAKCIOGLU⁵, T. OZISIK, D. SEN⁵

Department of Emergency Medicine, Division of Surgery, ¹Department of Underwater Medicine, ²Department of Internal Medicine, ³Department of Physiology, ⁴Department of Toxicology, ⁵Department of Surgery, Gülhane Military Medical Academy, Gülhane School of Medicine, Ankara, Turkey

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Summary

Various protocols may be used for acute pancreatitis treatment. Recently, the benefit of hyperbaric oxygen (HBO) has been demonstrated. To clarify the mechanism of HBO on the process of the acute pancreatitis, we determined the levels of antioxidant enzymes in an acute pancreatitis model. Forty-five Sprague-Dawley rats were randomly divided into three groups: Group I: sham group (n=15), Group II: pancreatitis group (n=15), Group III: pancreatitis group undergoing HBO therapy (n=15). HBO was applied postoperatively for 5 days, two sessions per day at 2.5 fold absolute atmospheric pressure (ATA) for 90 min. Superoxide dismutase (Cu/Zn-SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH Px) activity were measured in pancreatic tissue and erythrocyte lysate. MDA and GSH Px were also determined in plasma. In addition, amylase levels were measured in the serum. While serum amylase levels and MDA values in erythrocyte, plasma and pancreatic tissue were decreased, the levels of GSH Px and SOD were found to be significantly increased in the Group III as compared to those of the Group II. The findings of our study suggest that HBO has beneficial effects on the course of acute pancreatitis and this effect may occur through the antioxidant systems.

Key words

Acute pancreatitis • Hyperbaric oxygen • Antioxidant enzymes

Introduction

Despite the array of new diagnostic and therapeutic tools, acute pancreatitis remains a critical condition with a high rate of morbidity and mortality. In patients with severe acute pancreatitis, organ failure is common and often occurs in the absence of infection (Tenner *et al.* 1997, Karne and Gorelick 1999). In the

natural course of the disease, infection of necrotic pancreatic tissue occurs in 40 % to 70 % of patients and it has become the most important risk factor of death from severe acute pancreatitis (Gerzof *et al.* 1987, Beger *et al.* 1997, Bassi *et al.* 1997). Reactive oxygen species (ROS) have been implicated as an important factor in the pathogenesis and progress of this disease (Koster and Slee 1983, Yamamoto 1985).

Hyperbaric oxygen provides more oxygenation in the whole body. The increased tissue oxygen enhances the growth of fibroblasts, collagen formation, angiogenesis, and the phagocytic capabilities of the hypoxic leukocytes so that it has beneficial effects on wound healing. Thus much of the damage associated with reperfusion is caused by the inappropriate activation of leukocytes. Following an ischemic interval, the total injury pattern is the result of two components: a direct irreversible injury component from hypoxia, and an indirect injury, which is largely mediated by the inappropriate activation of leukocytes. HBO reduces the indirect component of injury by preventing such activation. The net effect is the preservation of marginal tissues that may otherwise be lost following ischemia-reperfusion injury (Jain 1996, Oriani *et al.* 1996, Chen *et al.* 1998a,b). This study was designed to evaluate the effects of HBO on animals with induced acute pancreatitis.

Methods

Forty-five male Sprague-Dawley rats weighing 320-380 g were obtained from Gülhane School of Medicine Research Center (Ankara, Turkey). Before experiment, the animals were fed a standard rat chow, drank water *ad libitum* and were housed in metabolic cages under controlled temperature and 12-hour light/dark cycles for at least one week. The study was approved by the Institutional Animal Care and Use Committee, and all experiments were done in accordance with the National Institutes of Health guidelines.

The rats were randomly divided into three groups: Group I: sham group (n=15), Group II: pancreatitis group (n=15), Group III: pancreatitis group undergoing HBO therapy (n=15). Anesthesia was induced with vaporized ether and maintained by an intraperitoneal injection of ketamin 40 g/kg (Ketalar, Parke-Davis and Eczacibasi, Istanbul). Laparotomy was performed through a midline incision. A microaneurysm clip was placed around a biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. A 28-Gauge, ½-inch, micro-fine intravenous needle attached to a 1-ml U-40 insulin syringe (B. Braun Medical, S.A., Barcelona, Spain) was introduced into the biliopancreatic duct. Induction of pancreatitis was performed in the group II and III; 1 ml/kg of 3 % sodium taurocholate (Sigma, St. Louis, MO, U.S.A) was injected into the biliopancreatic duct under steady manual pressure as described by Liu *et al.* (1999) and Simsek *et*

al. (2001), whereas 1 ml/kg saline was injected in the group I. Once the injection was finished, the microclips were removed and the abdomen was closed at two layers. All procedures were performed using sterile techniques.

The first HBO treatment session was performed 6 h after induction of pancreatitis in the group III, using the animal hyperbaric chamber. HBO treatment lasted five days, 2 sessions per day (90 min) at 2.5 fold AAP (Chen *et al.* 1998a). Groups I and II were left under normal atmospheric pressure. On the day 5, surviving animals were killed by an intracardiac injection of pentobarbital (200 mg/kg). Blood samples were drawn into the tubes containing EDTA. Each blood sample was centrifuged for 10 min at 4000 g and 4 °C. After removal of plasma and buffy coats, erythrocytes were washed three times with two volumes of isotonic saline. Then, erythrocytes were lysed with cold distilled water, stored in refrigerator at 4 °C for 15 min and the cell debris were removed by centrifugation (2000 g for 10 min). Pancreatic tissue samples were obtained from each rat's pancreatic head. Plasma samples, erythrocyte lysates, and pancreatic tissues were stored at -70 °C.

Blood for serum amylase determination was withdrawn from all animals when they were killed. Hitachi 917 autoanalyzer (Boehringer Mannheim, Germany) was used for the amylase assay. Amylase activity was expressed in U/l.

Plasma thiobarbituric acid reactive substance (TBARS) levels were determined by the method described previously (Jain 1989, Schoenberg *et al.* 1990, Orhan *et al.* 1999). Erythrocyte and pancreas MDA levels were determined on erythrocyte lysate obtained after centrifugation. After the reaction of thiobarbituric acid with MDA, the reaction product was extracted in butanol and was measured spectrofluorometrically (excitation: 532 nm, emission: 553 nm, slit 10 nm). Tetramethoxy propane solution was used as standard. TBARS levels in plasma and erythrocytes were expressed as nmol/ml and in the pancreatic tissue as nmol/g.

Cu/Zn-SOD activity in erythrocyte lysate and pancreatic tissue was measured by the method described previously (Schoenberg *et al.* 1990, Fitzgerald *et al.* 1992, Bulucu *et al.* 2000, Demirkaya *et al.* 2001). Each hemolysate was diluted to 1:400 with 10 mM phosphate buffer, pH 7.00. 25 µl of diluted hemolysate was mixed with 850 µl of substrate solution containing 0.05 mmol/l xanthine sodium and 0.025 mmol/l 2-(4-iodophenyl)-(4-nitrophenyl)-5-n-phenyltetrazolium chloride (INT) in a buffer solution containing 50 mmol/l CAPS and 0.94 mmol/l EDTA pH 10.2. Then, 125 µl of xanthine oxidase

(80 U/l) was added to the mixture and absorbance was followed at 505 nm for 3 min against air. 25 µl of phosphate buffer or 25 µl of various standard concentration in place of the sample were used as blank or standard determinations. Cu/Zn-SOD levels of erythrocyte were expressed as U/ml, and in the pancreatic tissue as U/g.

Glutathione peroxidase (GSH-Px) activity in erythrocyte lysate, pancreatic tissue and plasma were measured by the method described previously (Schoenberg *et al.* 1990, Bulucu *et al.* 2000). The reaction mixture was 50 mmol/l tris buffer, pH 7.6 containing 1 mmol/l of Na₂EDTA, 2 mmol/l of reduced glutathione (GSH), 0.2 mmol/l of NADPH, 4 mmol/l of sodium azide, and 1000 U of glutathione reductase (GR). 50 µl of plasma and 950 µl of reaction mixture, or 20 µl of erythrocyte lysate and 980 µl of reaction mixture were mixed and incubated for 5 min. at 37 °C. Then the reaction was initiated with 8.8 mmol/l H₂O₂ and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Enzyme activities in erythrocyte lysate and

plasma were expressed as U/ml and in the pancreatic tissue as U/g.

Statistical analysis: Results were expressed as mean ± S.E.M. Randomization was done with sealed envelopes. The significance of difference between values was assessed by Mann Whitney-U test. P<0.05 values were considered significant.

Results

No complications related to surgical method and HBO were detected. However, four rats on the second day and one rat on the third day died following pancreatitis induction in group II. In group III, only two rats died on the third day of the study. The overall results are presented in Tables 1, 2 and 3.

Amylase: On the postoperative 5th day, the levels of amylase in the group II (1727±522) and in the group III (1214±161) were significantly greater than those of the group I (449±110; P<0.05). However, the levels in the group III were significantly lower than in the group II (P<0.05).

Table 1. MDA, GSH-Px and SOD values in pancreatic tissue of the three groups.

| Group | MDA (nmol/g) | GSH-Px (U/g) | SOD (U/g) |
|------------------|--------------|--------------|---------------|
| Group I (n=15) | 4.40 ± 1.4 | 246.4 ± 74.7 | 581.6 ± 134.1 |
| Group II (n=10) | 16 ± 1.9 | 157.0 ± 7.5 | 212.6 ± 9.5 |
| Group III (n=13) | 9.9 ± 1.8 | 223.6 ± 16.4 | 280.7 ± 16.4 |
| P< (I vs II) | 0.0001 | 0.001 | 0.0001 |
| P< (I vs III) | 0.0001 | NS | 0.0001 |
| P< (II vs III) | 0.0001 | 0.0001 | 0.0001 |

Table 2. MDA, GSH-Px and SOD values in erythrocyte of the three groups.

| Group | MDA (nmol/ml) | GSH-Px (U/ml) | SOD (U/ml) |
|------------------|---------------|---------------|--------------|
| Group I (n=15) | 2.7 ± 0.9 | 61.0 ± 5.8 | 359.0 ± 5.9 |
| Group II (n=10) | 6.1 ± 1.3 | 43.0 ± 13.0 | 210.2 ± 7.0 |
| Group III (n=13) | 3.7 ± 1.2 | 57.2 ± 8.3 | 331.2 ± 30.4 |
| P< (I vs II) | 0.0001 | 0.0001 | 0.0001 |
| P< (I vs III) | 0.029 | NS | 0.006 |
| P< (II vs III) | 0.0001 | 0.01 | 0.0001 |

MDA (TBARS) levels: In pancreatic tissue and erythrocytes, the levels of MDA in the groups II and III were higher compared to the group I. However, there was also a significant difference between the groups II and III ($P < 0.05$) (Tables 1 and 2). MDA levels in plasma were also higher in the groups II and III than the levels of the group I, and lower in the group III than in the group II ($P < 0.05$). There was no significant difference in plasma MDA values between the groups I and III (Table 3).

SOD activity: In pancreatic tissue and erythrocytes, the levels of SOD in the groups II and III were lower compared to the group I. However, there was also statistically significant difference between the groups II and III ($P < 0.05$). The levels in the group III were higher than the levels of the group II (Tables 1 and 2).

GSH-Px activity: In pancreatic tissue, erythrocytes and plasma, the activities of GSH-Px in the group II were lower compared to the groups I and III ($P < 0.05$). However, there was no significant difference in plasma GSH-Px values between the groups I and III (Tables 1-3).

Table 3. Plasma MDA and GSH-Px in the three groups.

| Group | MDA (nmol/ml) | GSH-Px (U/ml) |
|----------------------------|---------------|---------------|
| Group I (n=15) | 0.9 ± 0.5 | 35.8 ± 7.0 |
| Group II (n=10) | 2.2 ± 0.8 | 24.2 ± 6.6 |
| Group III (n=13) | 1.1 ± 0.3 | 34.2 ± 7.6 |
| $P < (I \text{ vs } II)$ | 0.0001 | 0.001 |
| $P < (I \text{ vs } III)$ | NS | NS |
| $P < (II \text{ vs } III)$ | 0.001 | 0.004 |

Discussion

Previous studies have shown that HBO is useful in the treatment of acute pancreatitis. Chen *et al.* (1998a) designed a study to investigate HBO as a treatment for managing animals with induced acute pancreatitis. Severe acute pancreatitis model was established. Pathology, serum amylase levels, pancreatic MDA levels and water content of the lungs and the pancreas were used to evaluate the severity of the disease. HBO was delivered in three regimens, i.e. 100 % oxygen at 2.5 ATA, 40 % oxygen at 2.5 ATA, and 100 % oxygen at 1 ATA, 6 h after the onset of acute pancreatitis induction. All animals

survived until the end of the experiments. HBO significantly improved the pathologic conditions and pancreatic MDA levels. The results support the findings that HBO has a beneficial effect on pancreatic microcirculation in rats (Knoefel *et al.* 1994). Izawa *et al.* (1993) treated five patients with peripancreatic abscesses associated with severe acute pancreatitis by HBO. In three patients, peripancreatic abscesses complicated the course after surgical mobilization of the pancreas and drainage of the pancreas bed. HBO was conducted under the pressure of 2.8 atmospheres for 2 h daily. Four of the 5 patients showed a progressive improvement of their condition. The main effects of HBO were the improvement of white blood cell count and serum amylase levels, and the reduction of the abscess size. They supposed that HBO was a successful treatment for peripancreatic abscess associated with severe acute pancreatitis and better results were obtained than in cases that did not receive HBO. Zalaudek *et al.* (1982) studied the effect of HBO in hemorrhagic necrotizing pancreatitis. While majority of patients died in consequence of pancreatitis shock, HBO improved all hypoxic circulation situations. Its value was also proved in the treatment of experimental necrotizing pancreatitis in pigs. With HBO, liquid sequestration was diminished and total protein loss prevented significantly, and foremost survival time was significantly prolonged. In consequence demarcation of necrosis with a connective tissue was possible, but an operative treatment remains essential. All pigs without HBO and only two pigs with HBO died in consequence of necrotizing pancreatitis. It was demonstrated that HBO as additive therapy could improve the prognosis of necrotizing pancreatitis.

Amylase activity increased in all pancreatitis groups compared to the sham-operated group. Therefore, it was accepted that pancreatitis occurred in both groups. In the HBO group, amylase activity was lower than in the pancreatitis group. Enhanced lipid peroxidation in terms of elevated MDA concentrations was present in all rats of the pancreatitis group. However, MDA concentration was significantly decreased in all measurements in the HBO group. GSH-Px and SOD levels were lower in the pancreatitis group than in the sham-operated group, while it was increased in the HBO group. These results suggest that pancreatitis induces an oxidative stress within rat tissues. The change in SOD activity may be regarded as an indicator of increased ROS production occurring during the inflammatory period and may reflect the pathophysiological process of the pancreatitis. SODs are

specific antioxidant enzymes that dismutate O_2^- , forming H_2O_2 , which is scavenged by peroxisomal catalase or GSH-Px. Three SODs, copper/zinc SOD (cytosolic SOD), manganese SOD (mitochondrial SOD), and extracellular SOD (ECSOD) are major antioxidant enzymes based on cellular distribution and localization. We observed that treatment of pancreatitis was further improved by HBO due to decreased levels of amylase MDA and increased levels of SOD and GSH-Px in HBO group.

As highly reactive biochemical species, ROS exert their pathophysiological effects by direct attacking lipids (Yamamoto 1985) and proteins (Koster and Slee 1983) in the biological membranes at the local site of generation and cause their dysfunction (De Groot and Littauer 1989, Farber *et al.* 1990). These ROS are scavenged by SOD, GSH-Px and catalase (CAT). MDA is the breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids and thus serves as a reliable marker of oxidative stress. Oxidative stress and resultant tissue damage are the hallmarks of cell death (Gloor and Reber 1998, Norman 1998). There is increasing evidence that in certain pathological states the increased production and/or ineffective scavenging of such reactive oxygen species

may play a crucial role in tissue injury. The levels of intermediate reduction products of oxygen metabolism (i.e. superoxide, hydroxyl radical, and hydrogen peroxide) are controlled by various cellular defense mechanisms consisting of enzymatic SOD, CAT, GSH-Px and non-enzymatic scavenger components (Wohaieb and Godin 1987, Mates *et al.* 1999). The present study has shown that experimental procedure caused an increase of lipid peroxidation in pancreas. The oxidative stress can stem from increased ROS production, and/or from decreased ROS scavenging capability. In pancreas endogenous free radical scavengers seem to fail to prevent the pancreatitis.

In conclusion, we found lower MDA levels and amylase activity in the HBO group than in the pancreatitis group but higher GSH-Px and SOD activity in the HBO group than in the pancreatitis group. These findings have suggested that it is possible to improve the treatment of pancreatitis by adding HBO therapy.

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Reprint requests

Mehmet Yasar, MD., Gulhane Askeri Tip Fak, GATA Loj. Serter Apt. No. 15, 06018 Etlik, Ankara, Turkey, fax: +90 312 352 81 81, e-mail: myasar45@yahoo.com