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Pulmonary Protective Effects of Hyberbaric Oxygen and N-Acetylcysteine Treatment in Necrotizing Pancreatitis

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Summary

The purpose of this study is to analyze the protective effect of combining N-acetylcysteine (NAC) and hyberbaric oxygen (HBO) treatment in the lung tissue during acute pancreatitis. Sixty Sprague-Dawley male rats were randomly divided into five groups; Group I; Control group (n=12), Group II; pancreatitis group (n=12), Group III; pancreatitis + NAC treatment group (n=12), Group IV; pancreatitis + HBO treatment group (n=12), Group V; pancreatitis + HBO + NAC treatment group (n=12). HBO was applied postoperatively for 5 days, twice a day at 2.5 fold absolute atmospheric pressure for 90 min. Lung tissue was obtained for measuring malondialdehyde (MDA), superoxide dismutase (Cu/Zn-SOD) and glutathione peroxidase (GSH-Px) levels along with histopathological tissue examinations. This study showed that all three treated groups (HBO alone, NAC alone and combined HBO+NAC treatment) had pulmonary protective effects during acute necrotizing pancreatitis.

Key words

Acute necrotizing pancreatitis • Lung injury • Hyperbaric oxygen • Reactive oxygen species

Introduction

Despite the new diagnostic and therapeutic advancements, acute pancreatitis induced ARDS (Adult Respiratory Distress Syndrome) and respiratory failure still remains an important cause of morbidity and mortality in critical ill patients. Pulmonary complications of acute pancreatitis are characterized by widespread inflammation and tissue damage due to activation of pancreatic digestive enzymes, which are usually present in inactive form in the pancreas tissue (Renner *et al.* 1985). Necrotic pancreatic tissue infection occurs in 40-70 % of patients and this is considered to be the most important risk factor for pulmonary fatalities from acute pancreatitis (Bassi *et al.* 1997).

Pancreatic enzymes may activate oxygen radicals. These reactive oxygen species (ROS) and their derivates may be activated by direct or indirect routes in acute necrotizing pancreatitis resulting in the distribution of proenzymes following destruction of acinary cells. ROS have been considered as an important factor in the pathogenesis and progress of pancreatitis and pulmonary complications (Formela *et al.* 1995).

Approximately 95 % of molecular oxygen in

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biological systems undergoes controlled reduction through the addition of four electrons in the mitochondrial cytochrome oxidase system to form water under normal conditions. The residual molecular oxygen undergoes sequential and univalent reduction resulting in partially reduced intermediates, known as ROS such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical. Along with mitochondria, other important biological sources of ROS, including xanthine oxidase and leukocytes, appear to be major sources in clinical disease states (Parks 1989). It has been shown that 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. It has been well documented that reactive oxygen species (ROS) play an important role in the pathogenesis of acute pancreatitis induced by cerulein (Dabrowski *et al.* 2000). The histological picture of cerulein-induced pancreatitis is suggested to resemble the early phase of acute edematous pancreatitis in man.

ROS are scavenged by SOD, GSH-Px and catalase. N-acetylcysteine (NAC)-induced antioxidant activity seems to have two mechanisms: ROS scavenger activity and the capacity to support glutathione synthesis (Sala *et al.* 1993). The mechanism of direct inhibition of ROS in patients with acute pancreatitis is not clear, but there is evidence that the use of NAC in lung-injured patients, including ARDS, could improve the evolution of this disease (Bernard 1991, Gonzalez *et al.* 1996).

Hyperbaric oxygen (HBO) enhances oxygenation in the whole body. The increased tissue oxygenation promotes the growth of fibroblasts, collagen formation, angiogenesis, and the phagocytic capabilities of the hypoxic leukocytes which results in beneficial effects on wound healing. Inappropriate activation of leukocytes is responsible for the damage related to reperfusion injury. After an ischemic interval, the total injury from hypoxia, and indirect injury largely mediated by inappropriate leukocyte activation can be observed (Jain 1996). Indirect component of injury is reduced by HBO administration by preventing such activation (Oriani *et al*. 1996). The net effect is the sparing of marginal tissue that may otherwise be lost after ischemia-reperfusion injury (Chen *et al.* 1998).

This study was designed to evaluate the protective effects of combining HBO and NAC in pulmonary tissue during cerulein-induced acute necrotizing pancreatitis.

Methods

All experiments were performed according to protocols approved by the Institutional Animal Use and Care Committee of Gulhane Medical Academy and were performed in accordance with the National Institutes of Health guidelines for the care and handling of animals. Sixty male Sprague-Dawley rats weighing 280 to 330 g were obtained from Gulhane School of Medicine Research Center (Ankara, Turkey). Before the experiment, the animals were fed standard rat chow and given water *ad libitum* and housed in standard cages in a climate-controlled room with an ambient temperature of 23±2 °C and a 12-h light/dark cycles for at least 1 week.

After the stabilization period, the rats were randomly divided into five groups: Group I (Control group, n=12), Group II (Acute pancreatitis group without any treatment n=12), Group III (Acute pancreatitis group undergoing NAC treatment, n=12), Group IV (Acute pancreatitis group undergoing HBO treatment, n=12), Group V (Acute pancreatitis group undergoing HBO and NAC treatment, n=12) Anesthesia was induced with ether *via* a mask and maintained by an intraperitoneal injection of ketamine 40 mg/kg (Ketalar, Parke-Davis and Eczacıbası, Istanbul). Laparotomy was performed through a midline incision. A micro aneurysm clip was placed around a biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents of the duct. A 28-gauche 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, and 1 ml/kg of 3 % sodium taurocholate (Sigma, St Louis, MO, USA) was injected into the common biliopancreatic duct under steady manual pressure, as described by Liu *et al.* (1999). After the injection, the microclips were removed, and the abdomen was closed in two layers. All procedures were performed under sterile conditions. We administered HBO in a hyperbaric chamber, 6 h after induction of pancreatitis in group IV and V. HBO treatment lasted five days, 2 sessions per day (90 min) at 2.5 fold atmospheric pressure (Chen *et al.* 1998). Groups I, II, III were left under normal atmospheric pressure. On the day 5, surviving animals were sacrificed by an intracardiac injection of pentobarbital (200 mg/kg). Pulmonary tissue samples were obtained from each animal. Lung tissues were stored at -70 °C.

Morphometric studies of the lung

All lungs were examined grossly after sacrifice. Lung sections were then fixed in formalin for histologic examination. Hematoxylin and eosin staining was performed, and the stained sections were reviewed by staff pathologist who were uninformed as to the conditions of each animal. The specimens were evaluated for the presence of interstitial edema, alveolar edema, alveolar hemorrhage, and interstitial mononuclear infiltrate. Each lung specimen was given a score of 0 to 3 in each category, depending on whether the findings were absent: 0, mild: 1, moderate: 2 or severe: 3.

Tissue specimens were obtained from all animal groups for determination of MDA, SOD, GSH-Px. Blood for serum amylase determinations was obtained from all animals when they were sacrificed. Hitachi 917 autoanalyzer (Boehringer Manheim, 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 (Schoenberg *et al.* 1994). Lung MDA levels were determined on erythrocyte lyte obtained after centrifugation. After the reaction of thiobarbituric acid with MDA, the reaction product was extracted in butanol and was spectrofluorometrically (excitation 532 nm, emission 553 nm, slit 10 nm) evaluated. Tetramethoxypropane solution was used as standard. TBARS levels in the lung tissue were expressed as nmol/g.

Cu/Zn-SOD activity in pulmonary tissue was measured by the method described previously (Schoenberg *et al.* 1990). Each hemolyte was diluted to 1:400 with 10 mM phosphate buffer (pH 7.0). 25 µl of diluted hemolyte was mixed with 850 µl of substrate solution containing 0.05 mmol/l xanthine sodium and 0.025 mmol/l 2-(4-iodophenol)-(4-nitrophenol)-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 Ul) 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 concentrations in place of the sample were used as blank or standard determinations. Cu/Zn-SOD levels in the pulmonary tissue were expressed as U/g.

Glutathione peroxidase (GSH-Px) activity in the pulmonary tissue was measured by the method described previously (Schoenberg *et al.* 1990). The reaction mixture was 50 mmol/l tris buffer (pH 7.6) containing 1 mmol/l of Na_2EDTA, 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 H_2O_2 and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Enzyme activities were expressed as U/g in the lung tissue.

Results are expressed as the mean ± S.D., and the median. The significance of differences between groups were tested by Kruskal-Wallis test, Bonferroni adjusted Mann-Whitney U test and chi-square test. Differences were considered significant at $p<0.05$. Statistical analysis was performed by using the SPSS 10.0 Statistical Package Program for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

In our study, fifty-eight animals completed the experimental protocol. One animal died on the second day in group II (pancreatitis without treatment) and another in the NAC group died following pancreatitis induction. The overall results are presented in Tables 1 and 2. All lobes of the lungs were intact in all groups after 5 days. Using histopathological analysis we have observed that the lungs from groups II, III, IV, and V had alveolar edema, hemorrhage, alveolar distension and collapse and interstitial cell infiltration 5 days after injecting sodium taurocholate.

Amylase

On the 5th postoperative day, the levels of amylase in the group II (1625±420) and in the group III (1310±165), IV (1220±127), V (1200±150) were signify-cantly greater than in the control group (495±85) ($P<0.05$). The presence of acute pancreatitis in these groups was also confirmed by a substantial amount of fluid found in the abdomen. Pharmacological evaluation of the oxidative stress was evaluated by measuring SOD, MDA and GSH-Px activity in the lung tissue.

SOD activity in lungs

When SOD activity was measured in the lung tissues, we found that it was significantly lower in group II (pancreatitis without treatment) compared to groups

treated with NAC, HBO or HBO+NAC ($p<0.05$). SOD activity was not significantly different between the groups treated with NAC, HBO or HBO+NAC ($p>0.05$) (Table 1).

GSH-Px activity in lungs

In the lung tissue, GSH-Px was significantly higher in the NAC, HBO and NAC + HBO treated groups when compared to group II (pancreatitis without treatment) ($p<0.05$) (Table 1). In addition, GSH-Px activity of lung tissue in the HBO+NAC group was significantly higher than in the animals treated with NAC alone ($p<0.05$) but was not different from the HBO group (Table 1).

MDA activity in lungs

In the lung tissue, we observed significantly higher MDA levels in NAC, HBO and HBO+NAC groups when compared to group II (pancreatitis only) ($p<0.05$) (Table 1). Lung MDA activity significantly differed between HBO, NAC and HBO+NAC treatment groups ($p<0.05$) (Table 1).

Histopathology scoring

In the lung tissue, we observed significantly less edema, alveolar hemorrhage, interstitial infiltration and interstitial edema in HBO, NAC and HBO+NAC treatment groups compared to group II (pancreatitis without treatment) (for all $p<0.05$). Although all three treatments improved lung protection, we did not observe any statistical differences between the groups (Table 2).

Table 1. MDA, GSH-Px and SOD in the lung of rats in particular experimental groups.

Groups	MDA (nmol/g)		GSH-Px (U/g)		SOD (U/g)	
	Mean ± SD	Median	Mean ±SD	Median	Mean ±SD	Median
Group I	0.26±0.04	0.25	254.82±5.99	252	3220.27±289.75	3241
Group II	0.82±0.10	0.83	107.25±5.14	106	1878.50±188.10	1902
Group III	1.26±0.11	1.24	149.91±26.07	147	3043.00±866.10	3017
Group IV	1.46±0.07	1.47	168.55±31.74	171	3502.82±251.00	3421
Group V	1.55±0.09	1.58	175.17±24.70	176	3801.58±1512.6	3535
p (II vs V)	<0.001		<0.001		<0.001	
p (III vs V)	0.002		0.037		0.190	
p (IV vs V)	0.027		0.449		1.000	

MDA: malondialdehyde, SOD: superoxide dismutase, GSH-Px: glutathione peroxidase) Group I: Control group (n=11), Group II: Pancreatitis group (n=12), Group III: Pancreatitis + NAC treatment group (n=12), Group IV: Pancreatitis + HBO treatment group (n=12), Group V: Pancreatitis + HBO + NAC treatment group (n=12)

Table 2. Comparison of pathological results of lung tissue in particular experimental groups.

Lung tissue	Edema	Alveolar Hemorrhage	Interstitial Infiltration	Interstitial Edema	Alveolar Emphysema
p (II vs III)	0.002	0.008	0.010	0.025	0.174
p (II vs IV)	0.001	0.008	0.005	0.010	0.074
p (II vs V)	<0.001	0.002	0.003	0.016	0.110
p (III vs V)	0.501	0.558	0.549	0.750	0.265
p (IV vs V)	0.757	0.558	0.619	0.558	0.439
p (III vs IV)	0.721	1.000	0.478	0.330	0.842

Group I; Control group (n=12), Group II; Pancreatitis group (n=12), Group III; Pancreatitis + NAC treatment group (n=12), Group IV; Pancreatitis + HBO treatment group (n=12), Group V; Pancreatitis + HBO + NAC treatment group (n=12)

Discussion

Clinical acute pancreatitis can be present with varying degrees of severity and associated with several systemic complications. In adults, the disorder is frequently associated with acute lung injury, manifesting itself as the adult respiratory distress syndrome. Pathological findings are characterized as diffuse alveolar damage and may include alterations such as atelectasis and alveolar edema. These respiratory complications are similar to those of ARDS (Renner *et al*. 1985). In the progression of acute necrotizing hemorrhagic pancreatitis there is complemental activation followed by neutrophil recruitment, sequestration, and adherence to alveolar capillary endothelial cells. Lung injury appears to result from local endothelial cell injury secondary to neutrophil-generated ROS that may be myeloperoxidase-dependent (Guice *et al*. 1989). Therefore, protection against oxidant injury can be provided by preventing ROS generation or accumulation in the lungs, or by increasing the pulmonary antioxidant defense mechanisms.

An increasing number of animal studies indicates that NAC plays an important role in prevention and treatment of ROS-induced lung injury (Bernard *et al*. 1991). A protective effect of this agent against lung endothelial cell damage in a model of acute immunological alveolitis was shown in rats with lipopolysaccharide-induced pulmonary edema (Faggioni *et al*. 1994). Several authors showed that NAC prevented tissue edema and endothelial permeability in most organs and tissues, including lung and pancreas, in rat model of severe acute pancreatitis (Wang *et al.* 1995). However, Miller *et al*. (1994) did not show any improvement after the use of NAC. In agreement with these findings we have also observed that NAC treatment was effective in prevention of lung complications. In our study we showed that antioxidants such as GSH-PX and SOD increased significantly in HBO+NAC treated groups in the lungs.

Patients with ARDS show a deficiency in the reduced form of glutathione (GSH) and an increase in the oxidized form (GSSG) in the early phase of the disease (Gonzalez *et al*. 1996). NAC is a GSH precursor; it enhances intracellular glutathione by affecting the metabolism of cysteine, and therefore it increases the lung levels of this antioxidant molecule (Ortolani *et al.* 2000). In addition, NAC can also directly increase the scavenging of ROS produced by activated neutrophils such as $^{\bullet}OH$, H_2O_2, HOCl and $^{\bullet}O_2^-$ (Gonzalez *et al*. 1996).

Moine *et al.* (2000) showed an increased activation of NF-κB in alveolar macrophages of patients with ARDS, suggesting that it has an important role in this syndrome. Increased levels of proinflammatory cytokines, ROS, endotoxin, and complement fragments are present in ARDS and may contribute to NF-κB activation. Several antioxidants such as NAC seem to participate in the inhibition of NF-κB activation by a number of inducers. NAC also has antiapoptotic effects due to both direct action against ROS and/or the stimulated synthesis of GSH (Cotgreave 1997).

Leme *et al.* (2002) showed an important effect of NAC in preventing histological changes of acute lung injury induced by experimental necrohemorrhagic pancreatitits, measured by morphometric analysis of alveolar edema, hemorrhage, emphysema, interstitial edema and infiltrate.

O'Brien *et al.* (2005) investigated the effects of COX-2 inhibitors in pancreatitis-induced lung injury. They found that histological injury scores were improved by this treatment. Bhatia *et al.* (2005) showed that DL-propargylglycine decreased the histopathological findings of pancreatitis-induced lung injury. Consistent with these results, we also showed that in the treated groups, histopathological studies, such as edema, alveolar hemorrhage, interstitial infiltration, and interstitial edema were significantly decreased compared to the pancreatitis group. However, we did not observe any significant difference between the treated groups (Table 2).

Previous studies have shown that HBO is useful in the treatment of acute pancreatitis and accompanying complication of interstitial pneumonia (Chen *et al.* 1998). HBO significantly improved the pathological changes in the lung tissue.

We have confirmed by detecting increasing amylase activity that pancreatitis occurred in all groups except the control group. In the HBO-treated group, amylase activity was lower than in the pancreatitis group. This finding supports previous studies, which showed the protective effect of HBO in acute pancreatitis (Chen *et al*. 1998).

In our study, enhanced lipid peroxidation in terms of elevated MDA concentrations was present in the lung tissue of the pancreatitis group. GSH-Px and SOD levels were increased in the lung tissue of the HBO, NAC and HBO+NAC groups than in animals with pancreatitis only. These results suggest that pancreatitis induces an oxidative stress in the rat lung tissue. 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-induced lung injury. We observed that treatment of pancreatitis was further improved by HBO due to increased levels of SOD and GSH-Px.

Oxidative stress and resultant tissue damage are the hallmarks of cell death (Norman 1998). There is increasing evidence that in certain pathological states the increasing 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 (Mates *et al*. 1999).

In earlier studies, Yasar *et al.* (2003) demonstrated that treatment with HBO had a protective effect in pancreatitis. Leme *et al.* (2002) also showed that NAC treatment could be protective against lung injury in acute pancreatitis. This study supports the idea that both HBO and NAC and their combination provides an acceptable tissue protection.

We conclude that although NAC, HBO or HBO+NAC can protect against pancreatitis-induced acute lung injury, there is no additional benefit in combining HBO+NAC treatment when compared to NAC and HBO treatment alone.

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Reprint requests

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