



Alcohol-Related Cirrhosis with Pancreatitis. The Role of Oxidative Stress in the Progression of the Disease

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Abstract. To assess the level of oxidative stress, measured as prooxidant-antioxidant imbalance in the blood of patients with alcohol-related injury of the liver and pancreas, we determined superoxide ion ($O_2^{\cdot-}$) production by neutrophils isolated from the peripheral blood of 3 groups of patients. Patients with compensated alcoholic liver cirrhosis (n=16), with alcoholic chronic pancreatitis (n=20), and with concomitant cirrhosis and pancreatitis (n=10) were included in this study. All patients had consumed at least 70 g of pure alcohol per day over 5 years. They had not abstained before admission to hospital. The control group consisted of 16 healthy non-alcohol-abusive subjects. As antioxidative enzymes (AOE) present in sera play a very important role in the regulation of plasma reactive oxygen species (ROS) levels and in the protection of plasma compounds against ROS action, we also examined the serum activity of catalase (CAT), superoxide dismutase (SOD), total activity, and the glutathione peroxidase (GPx) serum concentration. Neutrophils of patients with concomitant alcoholic liver cirrhosis and pancreatitis exhibited, similarly to the neutrophils of patients with chronic alcoholic pancreatitis, an enhanced ability to produce superoxide anions *in vitro*. In contrast, neutrophils of patients with alcoholic liver cirrhosis exhibited a defect in resting and PMA-induced superoxide anion production. The AOE activity in the sera of patients was also significantly changed. Total SOD activity was enhanced in all groups of patients with alcoholic liver cirrhosis, chronic pancreatitis and with concomitant injury of both organs. CAT activity was only increased in the sera of patients with liver cirrhosis or pancreatitis, but not in the patients with concomitant cirrhosis and pancreatitis. GPx concentration was only diminished in the patients with chronic pancreatitis. It seems likely that oxidative stress, defined as the imbalance between prooxidant and antioxidant activity, is highest in the blood of patients with chronic pancreatitis and, especially, in patients with concomitant liver cirrhosis and pancreatitis.

Key words: alcohol; cirrhosis; pancreatitis; superoxide anion; catalase; glutathione peroxidase; superoxide dismutase.

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Introduction

Investigations have provided strong evidence that reactive oxygen species (ROS) are important in the genesis of alcoholic liver cirrhosis and pancreatitis because they cause membrane lipid peroxidation^{3, 21}.

In the liver, ethanol can be oxidized to a free radical intermediate, the 1-hydroxyethyl radical (HER), by microsomes¹⁸. Moreover, chronic alcohol consumption increases rates of microsomal HER generation by the induction of cytochrome P-450E1^{24, 31}. It was shown experimentally that liver microsomes from alcohol-fed rats metabolize ethanol to HER at higher rates than those of controls not fed with ethanol³⁰.

In alcoholic pancreatitis some evidence suggests that metabolism of ethanol via cytochrome P-450E1 may contribute to oxidative stress in the pancreas during chronic alcohol consumption^{19, 24}. It has been reported that ethanol reduces the pancreatic content of glutathione (GSH) and that the metabolism of acetaldehyde via aldehyde oxidase might also be responsible for oxidative changes in pancreatic tissue³. However, xanthine oxidase and activated neutrophils appear to be the main sources of ROS in pancreatic tissue during the process of pancreatitis⁹. It has been showed that, among the pancreatic enzymes, elastase plays a role in the development of pancreatitis by enhancing ROS production in neutrophils⁴⁰.

ROS interact with lipoproteins and polyunsaturated fatty acids at the cell membrane level, resulting in the formation of several oxygenated compounds, the lipoperoxides. In the plasma of patients with alcoholic liver disease (ALD), increased levels of the oxidized form of ubiquinone 10, the lipoperoxide hydrolysis product malondialdehyde (MDA), and F₂-isoprostanes or 4-hydroxynonenal as peroxidation products of polyunsaturated fatty acids were detected^{2, 4, 44}. Moreover, MDA plasma concentrations were the highest in the group of cirrhotic patients with a very high alcohol intake. In contrast to the high lipid peroxidation products, reduced levels of ascorbate, low vitamin E, and low concentrations of serum trace elements such as zinc and selenium in cirrhotic patients were detected^{11, 22, 44}. However, in alcoholic liver cirrhosis, besides ROS generated during ethanol metabolism, also cytokine-activated blood leukocytes can participate in ROS generation.

Mammalian cells are protected against oxidative damage by natural antioxidant factors, notably glutathione, and diverse antioxidative enzymes (AOE), such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD catalyzes the dis-

mutation of superoxide ion ($O_2^{\cdot-}$) to H_2O_2 and O_2 , whereas GPx and CAT scavenge H_2O_2 . Mammalian cells have extracellular SOD (EC SOD) and two forms of intracellular SOD of which one, the manganese SOD (Mn SOD), is found in the mitochondrial matrix and the other, the copper-zinc SOD (CuZn SOD), in cytosol. Synthesis of CuZn SOD is constitutive, whereas Mn SOD is inducible, for example by TNF- α , irradiation and hypoxia^{14, 34, 36}.

A large dose of ethanol can change the AOE activity. A significant increase in SOD with normal levels of CAT and GPx in the liver of rats treated with large doses of ethanol has been described⁵. Also, a significant increase in Mn SOD in the sera of alcohol-dependent patients has been observed^{38, 39}. In alcoholic hepatitis and in cirrhosis, changes in the expression of CuZn SOD in the liver and plasma CAT activity has been described^{13, 45}. Also, GPx and CAT activity in erythrocytes of patients with alcoholic liver cirrhosis was detected to be significantly decreased¹¹.

Elevated levels of Mn SOD were also detected in sera of patients with acute pancreatitis, which correlated with peripheral plasma markers of peroxidation³⁵. Also, CAT activity was heightened in plasma, especially in the necrotic form of acute pancreatitis¹². In patients with chronic pancreatitis, CuZn SOD was present at elevated concentrations in serum and in pancreatic juice¹⁵. In contrast, low blood levels of many antioxidative factors, such as hemoglobin, vitamin E, vitamin A, selenium and plasma GPx, were detected⁴².

To assess the level of oxidative stress, defined as prooxidant-antioxidant imbalance in the blood of patients with alcohol-related liver cirrhosis, chronic pancreatitis and concomitant cirrhosis and pancreatitis, we measured superoxide ion ($O_2^{\cdot-}$) production by neutrophils isolated from the peripheral blood of 3 groups of patients and a control group. As the AOE present in sera play a very important role in the regulation of plasma ROS levels and the protection of plasma compounds against ROS action, we also examined the serum activity of CAT, SOD (total activity) and GPx serum concentration.

Materials and Methods

Patients. Patients with compensated alcoholic liver cirrhosis (n=16), with alcoholic chronic pancreatitis (n=20) and patients with concomitant liver cirrhosis and pancreatitis (n=10) were included in this study. All patients had consumed at least 70 g of pure alcohol per

day for over 5 years. They had not abstained before admission to hospital.

The diagnosis of alcoholic cirrhosis was based on clinical history, clinical examination, laboratory findings, gastroscopy, ultrasonography and, in half of the patients with compensated liver cirrhosis, also on liver biopsy with the finding of nodules of liver cells separated by a dense and broad band of fibrosis. None of the cirrhotic patients had been diagnosed as having acute alcoholic hepatitis before the moment of entering the study nor had received a blood transfusion nor was under treatment with steroids or immunosuppressive therapy.

The diagnosis of alcoholic pancreatitis was based on clinical history, clinical examination, laboratory findings, ultrasonography and retrograde cholangiopancreatography (EPC). None of the patients examined had chronic B or C viral hepatitis or HIV infection. Blood tests, including complete blood pictures, electrolytes, urea, creatinine, glucose, albumin, gamma globulins, bilirubin and prothrombin time were performed. Lipase, α -amylase, AST and ALT activity were performed on admission and several times if deemed necessary. A blood sample was obtained from each patient on admission to hospital.

The control group consisted of 16 age- and sex-matched healthy university employees receiving an annual health examination. They were not alcohol abusive, confirmed to be free of major cardiopulmonary, gastrointestinal and hepatobiliary-pancreatic diseases. None of them had drunk alcohol for at least 3 weeks. Blood samples were obtained and ROS production by neutrophils as well as serum activity of AOE were measured to provide local reference material for this study. There were no significant differences in the ages and sexes between both groups of patients and controls. The characteristics of the subjects of each group are presented in Table 1. Approval for this study was

granted by the Clinical Ethics Committee and informed consent was obtained from each of the patients.

Granulocyte separation. Blood (8 ml) was taken into heparinized tubes (20 U/ml, Heparinum-Polfa). Granulocytes were separated according to the Sigma procedure: a gradient was formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (both from Sigma, St.Louis, MO, USA). The whole blood was carefully layered onto the upper Histopaque-1077. The tubes were then centrifuged at $700 \times g$ for 30 min. Granulocytes were isolated from the Histopaque-1077/1119 interphase and washed three times with HBSS (Hanks' Balanced Salt Solution), centrifuged ($350 \times g$ for 15 min) and resuspended in HBSS.

Measurement of superoxide production by nitroblue tetrazolium (NBT) reduction assay. Neutrophils (4×10^6 /ml HBSS) distributed into wells on a 96-well microplate (100 μ l/well) were covered with 100 μ l/well of NBT (Sigma) solution (2 mg/ml) in phenol red-free HBSS containing the stimulants of oxidative burst phorbol 12-myristate-13-acetate (PMA, Sigma, final concentration 1 μ g/ml)²⁸. Eight vertical wells served as blanks, and in these wells the cells were preincubated for 10 min at 37°C with 100 μ l/well of 10 mM iodoacetamide (Sigma) in HBSS. After incubation, the iodoacetamide solution was removed and replaced by a solution containing both 1 mg/ml NBT and 10 mM iodoacetamide. Samples were incubated at 37°C for 30 min. The amounts of formazan were quantified in a microplate reader (Molecular Devices, Menlo Park, CA, USA) compared with the blanks (with iodoacetamide) at 570 nm. The results were expressed as optical density (OD) per well after blank subtraction.

Serum CAT activity assay. Serum was separated by centrifugation of unheparinized blood and kept at -20°C before measurement of enzyme activity. The assay was

Table 1. Clinical characteristic of patients and controls

Parameter	Healthy controls n = 16	Compensated cirrhosis n = 16	Chronic pancreatis n = 20	Pancreatitis and cirrhosis n = 10
Age	53.9 $\pm$ 17.5	51.4 $\pm$ 15.4	48.1 $\pm$ 8.4	41.6 $\pm$ 7.1
Sex (M/F)	14/2	14/2	18/2	8/2
AST (U/ml)	<50	118.5 $\pm$ 102.1	52.6 $\pm$ 46.5	181.2 $\pm$ 152.1
ALT (U/ml)	<50	75.8 $\pm$ 50.1	38.4 $\pm$ 28.6	64.2 $\pm$ 46.8
α -Amylase (U/ml)	<32	–	191 $\pm$ 228	276.7 $\pm$ 247.7
Lipase (U/ml)	<3	–	15 $\pm$ 20	28.0 $\pm$ 18.0
T-bilirubin (mg/dl)	<1	3.1 $\pm$ 5.2	1.12 $\pm$ 0.66	7.6 $\pm$ 11.3
Albumin (% of total serum protein)	54–60	53.4 $\pm$ 5.7	60.8 $\pm$ 8.5	46.5 $\pm$ 3.67
Cholesterol (mg/dl)	200–250	173 $\pm$ 61	200.6 $\pm$ 93.7	121 $\pm$ 50
GGT (U/ml)	10–100	43.6 $\pm$ 38.6	69.1 $\pm$ 165.5	41.9 $\pm$ 34.2
Leukocyte ($\times 10^9$ /l)	4.5–10.0	11.5 $\pm$ 6.6	11.3 $\pm$ 5.6	11.7 $\pm$ 6.3

performed according to the method described by PIFFERI et al.²⁹ in the NOWAK et al.²⁶ modification. The reaction mixture, prepared in an Eppendorf tube consisted of 500 μ l of 0.05 M phosphate buffer pH 7.0, 300 μ l of distilled water, 50 μ l of 1.1 mM H_2O_2 in distilled water and 50 μ l of serum sample (or 50 μ l of distilled water as a blank). After 5 min of incubation at 25°C, 100 μ l of 50% trichloroacetic acid (TCA, Sigma) was added to each tube and the tubes were centrifuged ($1000 \times g$ for 5 min). Next, to each tube 10 μ l of titanium(IV) reagent was added and 200 μ l of supernatant was transferred into wells on a 96-well microplate. The absorbance was read at 405 nm in the microplate reader. The results were expressed as CAT activity in U/ml of serum after comparison with the standard curve, which was prepared by plotting the absorbance (OD) at 405 nm (ordinate) as a function of standard CAT (Sigma) concentration (abscissa) between 0 to 33 U/ml. One unit of CAT decompose of 1.0 μ mole of H_2O_2 per min at pH 7.0 and 25°C.

Serum SOD activity assay. To measure the SOD activity, we modified the method described by OBERLEY and SPITZ²⁷. Briefly, to each well on a 96-well microplate, 160 μ l of the reaction mixture, consisting of DETAPAC (diethylenetriamine pentaacetic acid, Sigma, final concentration 1 mM in 0.05 M phosphate buffer, pH 7.8), MTT (thiazyl blue, Sigma, final concentration 1 mg/ml in phosphate buffer) and xantine (Sigma, 1.8 mM in buffer), was added. 20 μ l of serum or 20 μ l of phosphate buffer (blank) was added to the wells. Reaction was started by adding 20 μ l of xantine oxidase (final concentration 0.01 U/ml of phosphate buffer). The microplate was incubated at 25°C for 60 min and the absorbance of samples and blank was read at 570

nm in the microplate reader. The results were expressed in units of SOD/ml of serum after comparison with the standard curve, which was prepared by diluting standard SOD (Sigma) in the range from 0 to 0.935 U/ml of phosphate buffer. One unit of SOD inhibited the rate of MTT reduction by 50% generated by the xantine/xantine oxidase system at 25°C, pH 7.8.

Serum GPx assay. Serum concentration of GPx was measured by using Bioxytech pl-GPx Enzyme Immunoassay (Oxis International Inc., Portland, OR, USA) according to the manufacturer's method. Briefly, samples of serum were incubated in the wells (96-well microplate) which were coated with polyclonal antibodies specific for human plasma GPx. The presence of GPx was detected by means of a biotinylated-polyclonal second antibody to pl-GPx. The final step of the assay was based on amplification by biotin-streptavidin coupling with streptavidin covalently linked to alkaline phosphatase. The amounts of pl-GPx were measured after enzymatic revelation with para-nitrophenyl-phosphate (pNPP). The absorbance was read at 405 nm in the microplate reader. The detection limit was 2.5 ng/ml.

Data analysis. Data were expressed as mean $\pm$ SD. A statistical analysis was performed using the two-tailed Mann-Whitney U test. Statistical significance was set at $p < 0.05$.

Results and Discussion

As can be seen from Fig. 1., neutrophils isolated from the blood of patients with liver cirrhosis exhibited significantly decreased resting (not induced *in vitro*)

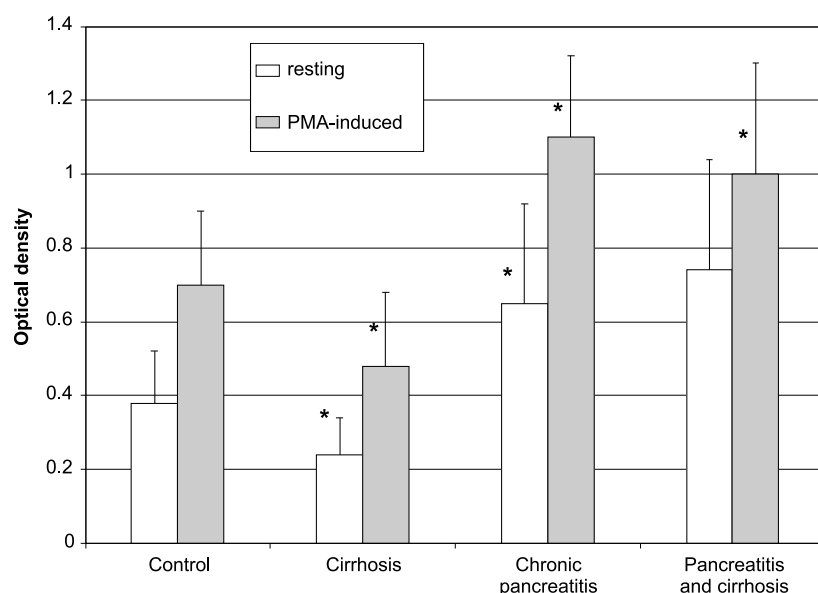


Fig. 1. Superoxide anion production by blood neutrophils of patients with alcoholic liver cirrhosis, chronic pancreatitis and concomitant cirrhosis and pancreatitis. Neutrophils isolated from peripheral blood were incubated at 37°C without any induction (resting activity) or PMA-induced, and the amounts of superoxide anion were estimated by the NBT method as described in Materials and Methods. *Statistically significant difference in comparison with control at $p < 0.05$ in the Mann-Whitney U test

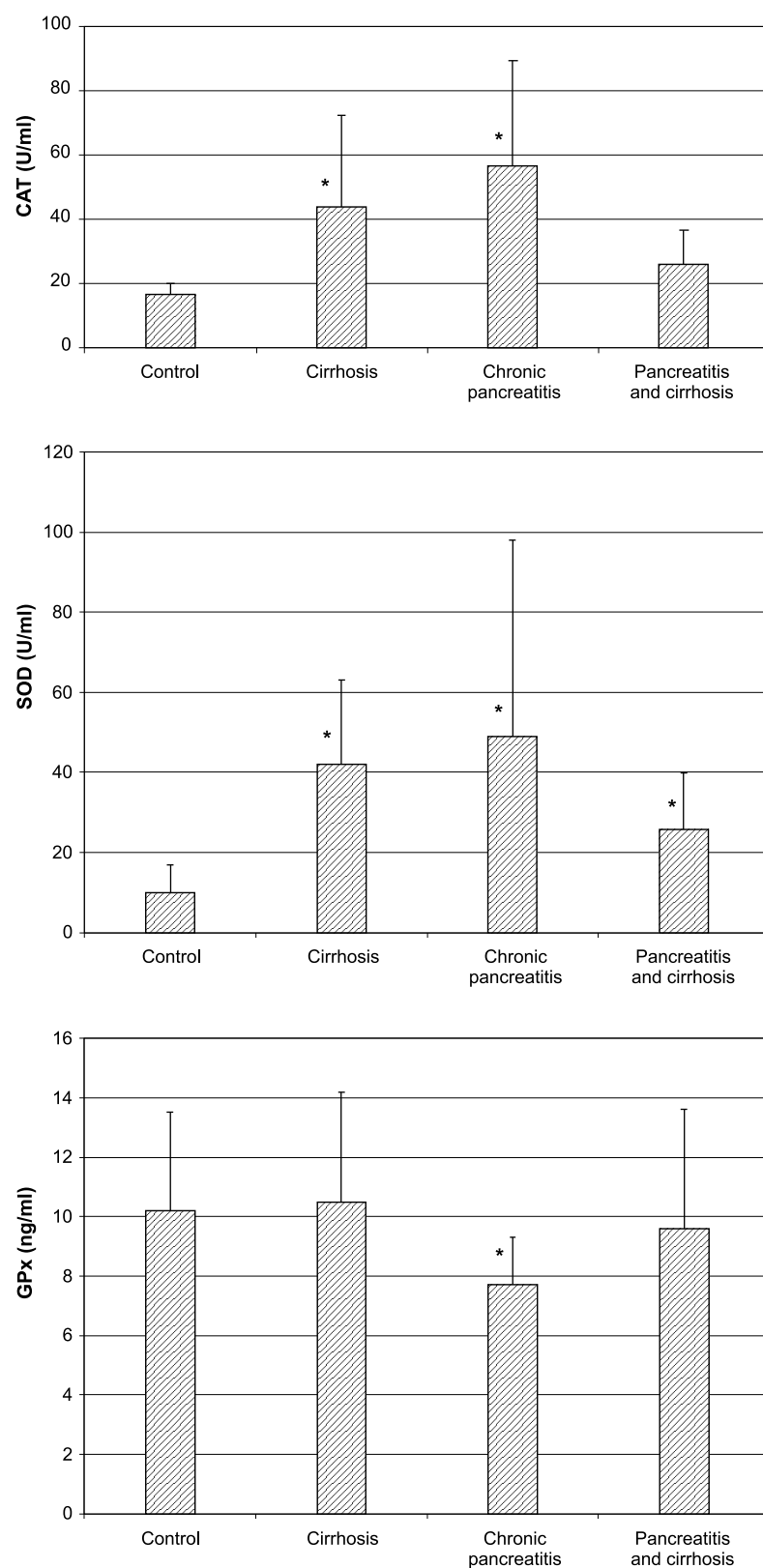


Fig. 2. Antioxidative enzyme activity in sera of patients with alcoholic liver cirrhosis, pancreatitis and concomitant cirrhosis and pancreatitis. *Statistically significant difference in comparison with control at $p < 0.05$ in the Mann-Whitney U test

and PMA-induced $O_2^{\cdot-}$ production. In contrast, the neutrophils of patients with chronic pancreatitis and with concomitant cirrhosis and pancreatitis produced more superoxide anions spontaneously and after PMA induc-

tion. These results confirmed the observations of other authors that, in liver cirrhosis, blood leukocytes exhibited a defect in superoxide anion production after treatment with PMA and that this effect did not depend

on the cirrhosis etiology, viral or alcoholic. This defect was considered responsible for the increased susceptibility of cirrhotic patients to viral and bacterial infections^{10, 17, 20, 23, 41}.

The increased production of superoxide anion by the blood neutrophils of patients with pancreatitis observed in our study is also in agreement with the observations of other authors indicating that the leukocytes of patients with pancreatitis are preactivated *in vivo* probably by cytokines or enzymes, such as elastase, released by injured pancreatic tissue, and *in vitro* exhibit an enhanced ability to ROS production. Excessive amounts of ROS produced by neutrophils can, in addition to the ROS generated by ethanol in pancreatic tissue, participate in pancreatitis progression and also in the systemic manifestation of the disease, particularly in the lungs, liver and blood^{1, 6, 8, 16, 25, 32, 33, 35, 43}. Considering the results of our study, we would like to extend the above-mentioned observations on the patients with concomitant alcoholic cirrhosis and pancreatitis. We detected that their blood neutrophils, similarly to patients with pancreatitis, spontaneously produced *in vitro* more superoxide anion and after induction with PMA. It seems likely that also an *in vivo* excessive production of ROS can participate in tissue injury.

In our study we also measured the total activities of SOD, CAT and GPx concentration in the sera of patients with alcoholic liver cirrhosis, chronic pancreatitis and concomitant cirrhosis and pancreatitis. As shown in Fig. 2, total SOD activity was highest in the sera of patients with chronic pancreatitis and in patients with liver cirrhosis. In the sera of patients with concomitant cirrhosis and pancreatitis, SOD activity was a little lower, but still significantly higher in comparison with the healthy controls. As SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 , but CAT and GPx catalyze degradation of H_2O_2 to water and O_2 , we also measured the serum activity of CAT and detected a statistically significant increase in CAT activity in the sera of patients with alcoholic cirrhosis or chronic pancreatitis, but not in patients with concomitant cirrhosis and pancreatitis. GPx concentration was comparable to the controls in patients with liver cirrhosis and concomitant cirrhosis and pancreatitis, but significantly lower in patients with chronic pancreatitis. It seems likely that, in patients with chronic pancreatitis and concomitant cirrhosis and pancreatitis, the imbalance between prooxidant/antioxidant factors is the highest.

The role of ROS in the etiopathology of pancreatitis, independent of its origin (gallstones, idiopathic or alcohol), was confirmed by experiments in which a significant elevation of AOE in plasma was detected. For

example, increased CAT¹² and Mn SOD³⁵ activities were observed in patients with acute pancreatitis, which correlated with peripheral plasma markers of peroxidation. Also, in chronic pancreatitis, increased CuZn SOD in plasma was detected¹⁵. In contrast, patients with alcohol-related chronic pancreatitis had low levels of many antioxidant factors, such as vitamin E, vitamin A, selenium and plasma GPx⁴². In our experiments we examined the total activity of CAT, which was characterized by its activity in the decomposition of hydrogen peroxide to form H_2O and O_2 . This method has been used by other authors¹². In fact, we confirmed that in patients with pancreatitis, serum CAT activity was higher than in healthy persons. However, in contrast to the papers mentioned above concerning the increase of CuZn SOD and Mn SOD activity in pancreatitis, in our experiments we measured the total SOD activity. There are 3 types of SOD: CuZn SOD (SOD1), mainly intracellular, mitochondrial Mn SOD (SOD2), and extracellular SOD (SOD3), being the glycosylated form of SOD1⁴⁵. While our method did not distinguish these different types, plasma SOD can be a mixture of SOD3 as well as other types released from injured cells, such as duct and islet pancreatic cells or endothelial cells induced by cytokines^{15, 35}. Such SOD release from pancreatic tissue can effect, as observed by other authors, a significant decrease of SOD activity in the pancreas accompanied by a high level of SOD in the pancreatic juice^{8, 15, 35}.

In the case of GPx, we used the immunoenzymatic method, which allowed us to detect only one form of this enzyme, extracellular human plasma GPx, which differs from the other GPx by its primary sequence and glycosylation³⁷. Despite the method used, our results are also in agreement with the results of other authors who in experimental L-arginine-induced pancreatitis, detected a significant reduction of GPx activity in pancreatic tissue⁷, and with results indicating a low GPx activity in the sera of patients with alcohol-related chronic pancreatitis⁴². It seems likely that, independent of the etiology of pancreatitis, the reduction of intracellular and extracellular GPx activity is a general phenomenon.

Our results indicating an increased total SOD activity in the sera of patients with alcoholic cirrhosis partially confirm the observations of other authors that, in chronic alcoholics the serum activity of Mn SOD significantly increases^{38, 39}.

However, our results indicating serum concentrations of GPx in patients with cirrhosis comparable to the control are not in agreement with the observations of other authors who detected a decreased GPx activity

in the erythrocytes of patients with cirrhosis¹¹. It is possible that the intracellular and extracellular forms of GPx are differentially regulated and by different factors in the organisms of patients with alcoholic cirrhosis, and the in erythrocytes the activity of GPx can be decreased, but in sera could be normal.

We also detected a significantly increased CAT activity in the sera of patients with liver cirrhosis. Our results are not in agreement with a paper¹³ in which no significant changes in serum activity of CAT was observed in patients with cirrhosis. However, its authors did not discriminate between compensated and decompensated cirrhosis. In our experiments only patients with the compensated form of cirrhosis, in which the AOE system was still able to react to increased liver production of ROS during metabolism of alcohol were included in the study. It should be noted that patients in our study had not abstained before admission to hospital.

Summing up, neutrophils of patients with concomitant alcoholic liver cirrhosis and pancreatitis exhibited, similarly to those of patients with chronic alcoholic pancreatitis, an enhanced ability to produce superoxide anions *in vitro*. It is possible that excessive ROS production by neutrophils can participate in the injury of pancreatic tissue in such patients. We detected that antioxidant enzyme activity was also significantly changed. Total SOD activity was enhanced in all 3 groups of patients with alcoholic liver cirrhosis, chronic pancreatitis and concomitant injury of both organs. CAT activity was only increased in the sera of patients with liver cirrhosis or pancreatitis, but not in the patients with concomitant cirrhosis and pancreatitis. GPx concentration was only diminished in the group of patients with chronic pancreatitis. It seems likely that oxidative stress, defined as imbalance between prooxidant and antioxidant activity, is strongest in patients with chronic pancreatitis and especially, in patients with concomitant cirrhosis and pancreatitis.

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