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# Liver lipid peroxidation and antioxidant capacity in cerulein-induced acute pancreatitis

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## Abstract

The aim of this study was to evaluate the role of oxidative damage in pancreatitis-induced hepatic injury. Thirty-five rats were divided into five groups (each of 7 rats): control, cerulein (100 µg/kg body weight), cerulein and pentoxifylline (12 mg/kg body weight), cerulein plus L-NAME (10 mg/kg body weight) and cerulein plus L-arginine (160 mg/kg body weight). The degree of hepatic cell degeneration differed significantly between groups. Mean malondialdehyde levels were 7.00 ± 2.29, 20.89 ± 10.13, 11.52 ± 4.60, 18.69 ± 8.56, and 8.58 ± 3.68 nmol/mg protein for the control, cerulein, pentoxifylline, L-NAME, and L-arginine groups, respectively. Mean catalase activity was 3.20 ± 0.83, 1.09 ± 0.35, 2.05 ± 0.91, 1.70 ± 0.60, and 2.85 ± 0.47 U/mg protein for the control, cerulein, pentoxifylline, L-NAME, and L-arginine groups, respectively, and mean glutathione peroxidase activity was 0.72 ± 0.25, 0.33 ± 0.09, 0.37 ± 0.04, 0.34 ± 0.07 and 0.42 ± 0.1 U/mg protein for the control, cerulein, pentoxifylline, L-NAME, and L-arginine groups, respectively. Cerulein-induced liver damage was accompanied by a significant increase in tissue malondialdehyde levels ($P < 0.05$) and a significant decrease in catalase ($P < 0.05$) and GPx activities ($P < 0.05$). L-arginine and pentoxifylline, but not L-NAME, protected against this damage. Oxidative injury plays an important role not only in the pathogenesis of AP but also in pancreatitis-induced hepatic damage.

Key words: Acute pancreatitis; Liver injury; Oxidative damage; Pentoxifylline; L-NAME; L-arginine

## Introduction

Acute pancreatitis (AP) is an inflammatory disease with pathophysiological mechanisms that have not been thoroughly elucidated and few, if any, effective treatments. AP is characterized by edema, hemorrhage and necrosis of the pancreas related to tissue damage in the pancreas and its adjacent tissues, such as the liver. This damage is caused by the activation of normally inactive digestive enzymes owing to some etiologic factor (1).

There is strong evidence suggesting that oxidative stress occurs during the course of AP, as has been shown in different experimental models (2,3). Oxidative stress caused by free radicals has been proposed as a common mechanism for pancreatic injury in alcohol, gallstone and ischemic pancreatitis (4), as well as in other experimental models of acute pancreatitis (5). Schoenberg et al. (6) have shown that the concentration of malondialdehyde in the pancreas increases during edematous acute pancreatitis, indicating free radical-mediated lipid peroxidation. Nitric oxide (NO) is a free radical that is synthesized during the reaction of L-arginine with oxygen, a reaction that is catalyzed by NO synthase (NOS) enzymes. NO serves as an intracellular transmitter that regulates neuronal activity, thrombosis and blood flow in vertebrates. In the presence of a superoxide radical, excess NO is converted to an even more toxic molecule, peroxynitrite ($ONOO^-$), which directly damages proteins and decomposes to other toxic products, such as nitronium ion, nitrogen dioxide gas and hydroxyl radicals (7).

AP is a multi-system disease that causes alterations not only in the pancreas, but also in the liver, lungs and kidneys, potentially leading to distant organ dysfunction and death (8). Recently, a number of researchers have reported that pancreatitis-associated ascitic fluid plays a major role in inducing hepatocyte damage and apoptosis (9,10).

Experimental pancreatitis is induced by supramaximal doses of the cholecystokinin analogue, cerulein; the se-

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cretory block is subsequently followed by the lysosomal degradation of intercellular organelles within autophagic vacuoles in acinar cells and a marked interstitial edema. AP is considered to be an autodigestive disease. In addition to premature intracellular protease activation, other mechanisms such as oxidative stress have also been shown to be involved in the development of AP (11,12). In the present study, we investigated the possible protective effects of pentoxifylline, L-arginine and $N^G$-nitro-L-arginine methyl ester (L-NAME) against cerulein-induced acute pancreatitis.

## Material and Methods

### Animals and experimental protocols

Thirty-five female Wistar rats weighing 280-350 g were used. Animals received standard rat chow and tap water *ad libitum* and were maintained on a 12-h light/dark cycle at 2°C. AP was induced with two *ip* injections of cerulein (Sigma-Aldrich, Germany) at a total dose of 100 µg/kg body weight at 2-h intervals, with each injection containing 50% of the dose in the cerulein group (N = 7). The control group (N = 7) received 4 *ip* injections of 0.9% saline at 2-h intervals. To evaluate the effect of these agents, the rats were divided into three additional groups (N = 7): the first group was treated with cerulein (100 µg/kg body weight) and pentoxifylline (Sigma-Aldrich; 12 mg/kg body weight), the second group was treated with the same dose of cerulein plus L-arginine (Sigma-Aldrich; 160 mg/kg body weight), and the third group received the same dose of cerulein plus L-NAME (Sigma-Aldrich; 10 mg/kg body weight). All agents were dissolved in 0.9% saline and injected *ip* shortly before each injection of cerulein. The rats were sacrificed by decapitation 12 h after the last injection of cerulein.

For light microscopy, fragments from the right lobe of the liver were rapidly removed and divided into two pieces. The first part of the sample was placed in 10% buffered formalin and prepared for routine paraffin embedding and the other part was stored at -80°C for the determination of malondialdehyde (MDA), glutathione peroxidase (GPx), and catalase (CAT) activities.

Animal experiments were performed in accordance with the guidelines for animal research set by the National Institute of Health and approved by the Committee of Animal Research at Inonu University, Malatya, Turkey.

### Histological examination

Tissue sections were cut at 5 µm, mounted on slides, stained with hematoxylin-eosin and examined with a Leica DFC280 light microscope and Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd., UK). For each section, tissue alterations were assessed in 20 different fields by an experienced histologist who was unaware of the treatment.

Hepatic damage was scored (0-3) based on hepatocyte necrosis, hemorrhage, intracellular vacuolization, vascular congestion, sinusoidal dilatation, and inflammatory infiltration; the maximum score was 18.

### Preparation of tissue homogenates

Tissue was cut into small pieces on ice and homogenized in 1/5 (w/v) phosphate-buffered saline (PBS). Homogenates were divided into two portions. One part was immediately used for direct MDA measurement and the second part was sonicated four times for 30 s at 20-s intervals using a VWR Bronson scientific sonicator (VWR Int. Ltd. Merck House Pool, UK). The homogenates were then centrifuged at 20,000 *g* for 15 min in a Beckman L8-70M ultracentrifuge with an SW-28 rotor (Beckman, Germany). The supernatant solutions were separated and kept at -40°C throughout the preparation.

### Protein determination

Protein concentration in the supernatants was determined by the method of Lowry et al. (12) using BSA as a standard. A Shimadzu 1601 UV/VIS Spectrophotometer (Shimadzu, Japan) was employed for all spectrophotometric assays.

### Measurement of CAT activity

CAT activity in the supernatants was measured by the method of Luck (13). The decomposition of the $H_2O_2$ substrate was monitored spectrophotometrically at 240 nm. Specific activity was reported as $U \cdot min^{-1} \cdot mg\ protein^{-1}$.

### Measurement of GPx activity

GPx activity was measured by the method of Lawrence and Burk (14). One milliliter 50 nM PBS, pH 7.4, containing 5 mM EDTA, 2 µM NADPH, 20 µM GSH, 10 µM $NaN_3$, and 23 mU glutathione reductase was incubated at 37°C for 5 min. Next, 20 µL 0.25 mM $H_2O_2$ and 10 µL supernatant were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank containing all components except the supernatant was also monitored. One unit of GPx activity is reported as µmol NADPH consumed per min (i.e., µmol/min), using the appropriate molar absorptivity coefficient for NADPH (6220 M/cm). GPx specific activity is reported as $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ or U/mg protein.

### Measurement of tissue MDA

MDA equivalents in the liver tissue homogenate were determined by the method of Mihara and Uchiyama (15). Half a milliliter of homogenate was mixed with 3 mL $H_3PO_4$ solution (1% v/v), 1 mL thiobarbituric acid solution (0.67% w/v) was added and the mixture was heated in a water bath for 45 min at 95°C. The colored complex that was formed was extracted with n-butanol and the absorbance at 532 nm was measured. Tetramethoxypropane was used as a standard. The amounts of lipid peroxides were calculated as

thiobarbituric acid reactive substances of lipid peroxidation and are reported as nmol/mg protein.

**Statistical analysis**

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., USA). All data are reported as the arithmetic mean ± SEM. Biochemical data and histological scores were analyzed by the one sample Kolmogorov-Smirnov and Mann-Whitney U-tests. Values of $P < 0.05$ were regarded as significant.

## Results and Discussion

All animals survived until the end of the experiment. While the liver specimens from control animals presented no histological alterations (Figure 1), those from animals in the cerulein-treated group displayed necrosis, intracellular vacuolization, vascular congestion, and inflammatory infiltration (Figure 2A,B). The histopathological changes observed in the pentoxifylline, L-arginine and L-NAME groups are illustrated in Figures 3, 4, and 5A,B, respectively. The histopathological scores are summarized in Table 1 and the biochemical results are summarized in Table 2.

It is known that hyperstimulation of the exocrine pancreas causes an early activation of trypsinogen that leads to acute edematous pancreatitis (16). Recent studies have shown the involvement of the liver in the complex network of events triggering the multiorgan dysfunction associated with the disease. Once pancreatic mediators reach the liver, they activate Kupffer cells, increasing the release of cytokines into the bloodstream and thus contributing to the systemic manifestations of AP (17). Almost all pancreatic enzymes and mediators released from the inflamed pancreas into the bloodstream may pass through the liver (18). Kupffer cells, when activated by antigens or inflammatory stimuli, release superoxide anions, hydrogen peroxide, NO, hydrolytic enzymes, and other cytokines (19,20).

The present study was undertaken to confirm the role of free oxygen radicals in the pathogenesis of cerulein-induced acute pancreatitis and pancreatitis-induced hepatic damage. While the mechanism of AP is not fully known, it is believed that reactive oxygen species (ROS) play a major role in the pathogenesis of AP (21,22). Acinar cells have been shown to produce large amounts of ROS in the early stages of AP in rats (23). Reactive oxygen species, such as superoxide anion, $H_2O_2$ and the highly reactive hydroxyl radical, are believed to contribute to the etiology of aging

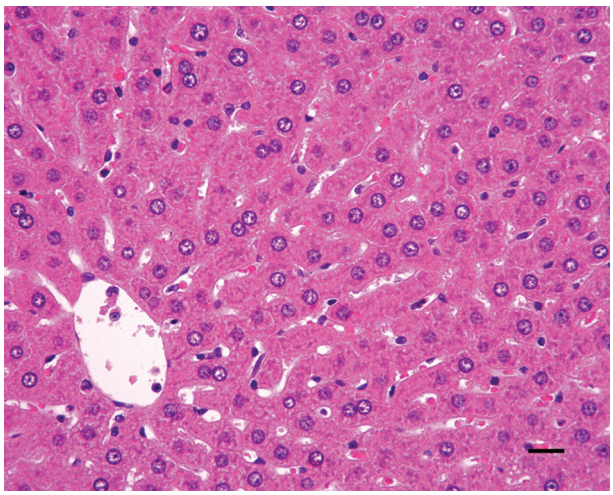

**Figure 1.** Liver specimen from the control group showing a normal appearance. H-E, 40X (bar = 20 µm).

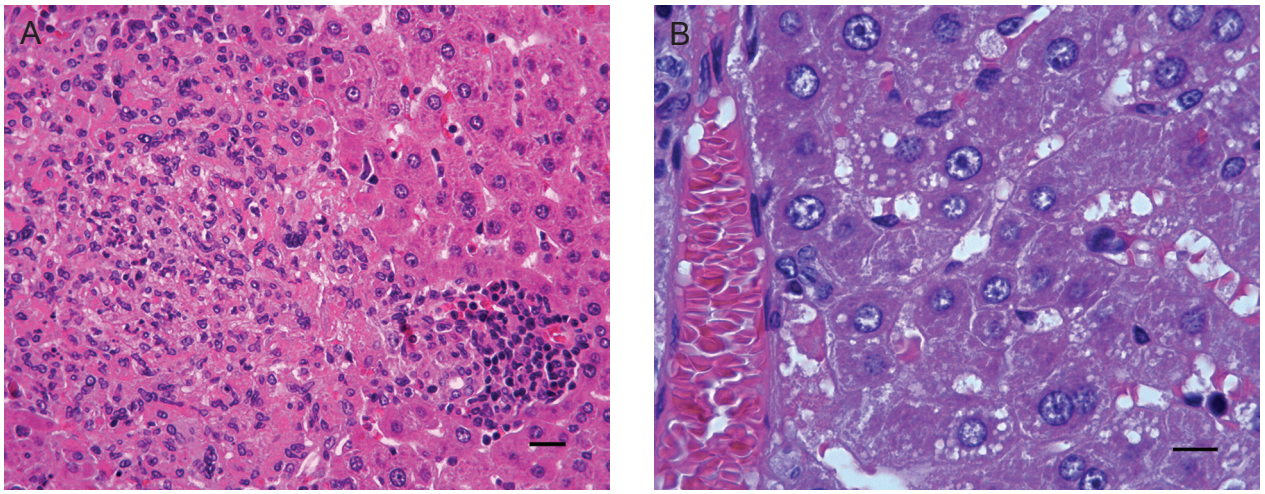


**Figure 2.** Liver specimen from the cerulein-treated group. *A*, Hepatocyte necrosis and inflammatory infiltration are visible. H-E, 40X (bar = 20 µm). *B*, Intracellular vacuolization and vascular congestion are shown. H-E, 100X (bar = 1 µm).

and the pathogenesis of a variety of diseases. Mammalian cells, however, have developed elaborate antioxidant defense systems to combat oxidative damage and facilitate survival in an aerobic environment. These mechanisms include enzymes like superoxide dismutase, CAT and GPx, as well as non-enzymatic antioxidant molecules. The increase in the rate of free radical generation and/or the decrease in antioxidant levels can lead to oxidative damage due to oxidative stress (24).

In AP, there is an excessive production of ROS due to the activation of leukocytes and the formation of pro-inflammatory interleukins (25). Several studies have reported an increase in MDA levels and a decrease in the activity of antioxidant enzymes (11,22-24). In the present study, we observed higher tissue MDA levels and lower CAT and GPx enzyme activity levels in the cerulein group than in the control group. MDA is an indicator of membrane lipid peroxidation. The destruction of the cell membrane structure and damage to cellular organization lead to a disruption in the intracellular transport of digestive enzymes, premature activation of these enzymes and damage to acinar cells (26). It is also possible that the breakdown of capillary permeability caused by oxidative damage is the origin of edema in AP.

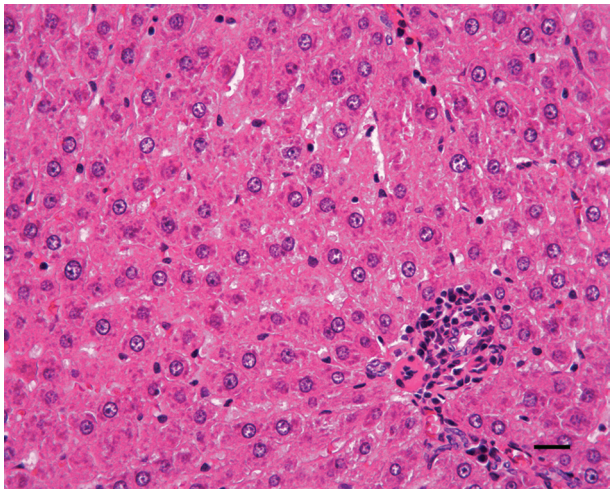

**Figure 3.** Liver specimen from the cerulein + pentoxifylline-treated group. A small area of cell infiltration in the portal area is evident. H-E, 40X (bar = 20 µm).

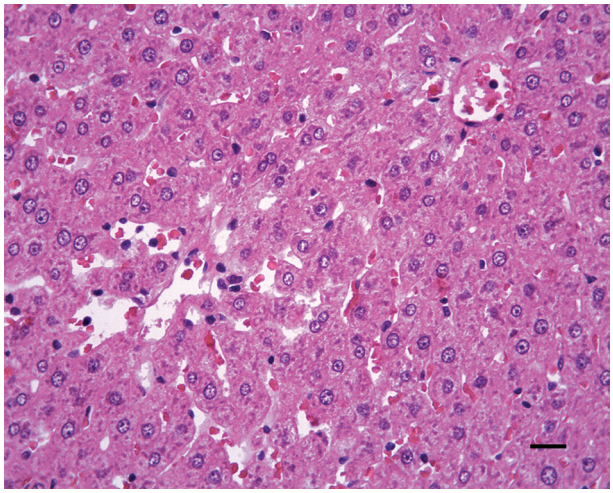

**Figure 4.** Liver specimen from the cerulein + L-arginine-treated group. The liver section is normal, except for mild sinusoidal dilatation and congestion. H-E, 40X (bar = 20 µm).

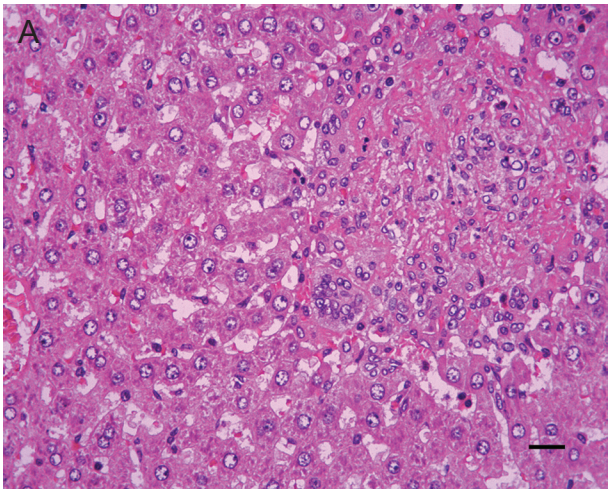


**Figure 5.** Liver specimen from the cerulein + L-NAME-treated group. *A*, Hepatocyte necrosis and sinusoidal dilatation are visible. H-E, 40X (bar = 20 µm). *B*, Diffuse hepatocellular degeneration, sinusoidal congestion and inflammatory infiltration are shown. H-E, 40X (bar = 20 µm).

We also obtained significantly low CAT and GPx activities. These enzymes are components of a cellular antioxidant enzyme system that detoxifies hydrogen peroxide and organic peroxides. Free radicals produced by active leukocytes may oxidize the active sites of these enzymes, thereby causing reduced activity. Also, premature protease activity plays a major role in the pathogenesis of AP. Trypsin inhibitor protein (TIP) inhibits the premature activation of trypsinogen to trypsin, and the modification of the structure of TIP by ROS may cause TIP inactivation. Hou et al. (27) have also reported on the antioxidant properties of trypsin inhibitor in plants.

NO generated from L-arginine by NOS is a potent vasodilator in various organs, including the pancreas (28). Here we have used L-arginine to stimulate NOS. The L-arginine substrate analogue L-NAME is a non-selective inhibitor of NOS that we used to inhibit NOS in the present study. Several studies have demonstrated the protective effect of NO on experimental pancreatitis (29-32). Similarly, in our study the results indicate that the stimulation of NOS by L-arginine to produce NO has a protective effect, which is probably mediated by an increase in the pancreatic and hepatic blood flow. L-arginine treatment showed beneficial effects and we found significantly decreased MDA levels and increased antioxidant enzyme levels ($P < 0.05$) when compared to the cerulein group. In contrast, L-NAME, a nonselective NOS inhibitor, did not inhibit the decrease of antioxidant enzyme activity levels and increase of MDA levels by cerulein. Takacs et al. (33) reported that rats with pancreatitis induced by injection of L-arginine had higher amylase levels, pancreatic weight/body weight ratios, inducible NOS activities, and pancreatic Evans blue dye concentrations than control rats; constitutive NOS activity was also altered in these animals. In addition, Cevikel et al. (34) reported that administration of two subcutaneous doses of L-arginine, the main substrate of NO, decreased both the inflammatory changes and bacterial translocation associated with cerulein-induced acute pancreatitis. Also, in the same study it was emphasized that rats that received cerulein plus L-NAME for two consecutive days had higher rates of bacterial translocation and greater pancreatic injury than control rats.

Pentoxifylline is a methylxanthine derivative that exhibits marked anti-inflammatory properties by inhibiting cytokine production. It inhibits lipopolysaccharide-induced production of tumor necrosis factor-α by monocytes and T cells and interleukin-2-induced adherence of leukocytes (33). We found decreased MDA levels and slightly increased CAT activity levels in the pentoxifylline group compared to the cerulein group. Pentoxifylline may possibly act as a radical scavenger and/or reduce the production of ROS from activated leukocytes. It also probably reduces the release of interferon c from the leukocytes by inhibiting leukocyte migration. Interferon c is partially responsible for the suppression of ROS production (34). The beneficial effects of pentoxifylline are partially dependent on its regulatory effects on microvascular blood flow. Pentoxifylline exhibits these regulatory effects by inhibiting platelet aggregation and reducing blood viscosity (35,36).

We conclude that ROS play a key pathogenic role not only in cerulein-induced acute pancreatitis, but also in pancreatitis-induced hepatic damage. Preventive agents such as L-arginine and pentoxifylline are capable of limiting hepatic damage produced during AP by restoring tissue antioxidant enzyme activities and preserving cell membrane lipids.

**Table 1.** Hepatic damage scores (hepatocyte necrosis, intracellular vacuolization, vascular congestion, hemorrhage, sinusoidal dilatation, and inflammatory infiltration with a maximum score of 18) for the groups studied.

| Groups | Hepatic damage score |
|---|---|
| Control | 0.28 ± 0.18 |
| Cerulein | 6.85 ± 0.26[a] |
| Pentoxifylline + cerulein | 2.42 ± 0.29[a,b] |
| L-arginine + cerulein | 2.00 ± 0.30[a,b] |
| L-NAME + cerulein | 7.57 ± 0.57[a] |

Data are reported as means ± SEM for 7 rats in each group. L-NAME = $N^G$-nitro-L-arginine methyl ester. [a]$P < 0.05$ compared to the control group; [b]$P < 0.05$ compared to the cerulein group (one sample Kolmogorov-Smirnov and Mann-Whitney U-tests).

**Table 2.** Malondialdehyde and antioxidant enzyme activity levels of cerulein-treated rats.

| Groups | MDA (nm/mg protein) | CAT (U/mg protein) | GPx (U/mg protein) |
|---|---|---|---|
| Control | 7.00 ± 2.29 | 3.20 ± 0.83 | 0.72 ± 0.25 |
| Cerulein | 20.89 ± 10.13[a] | 1.09 ± 0.35[a] | 0.33 ± 0.09[a] |
| Pentoxifylline + cerulein | 11.52 ± 4.60[b] | 2.05 ± 0.91 | 0.37 ± 0.04 |
| L-NAME + cerulein | 18.69 ± 8.56 | 1.70 ± 0.60 | 0.34 ± 0.07 |
| L-arginine + cerulein | 8.58 ± 3.68[b] | 2.85 ± 0.47[b] | 0.42 ± 0.10[b] |

Data are reported as means ± SEM for 7 rats in each group. In the cerulein-treated rats, malondialdehyde (MDA) levels were significantly higher ($P < 0.05$) and glutathione peroxidase (GPx) and catalase (CAT) activities were lower ($P < 0.05$) than in the control group. Apparently, pentoxifylline and L-arginine reduced lipid peroxidation. In addition, L-arginine prevented the loss of CAT and GPx enzyme activity. L-NAME = $N^G$-nitro-L-arginine methyl ester. [a]$P < 0.05$ compared to the control group; [b]$P < 0.05$ compared to the cerulein group (one sample Kolmogorov-Smirnov and Mann-Whitney U-tests).

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