

Antioxidative effect of melatonin, ascorbic acid and *N*-acetylcysteine on caerulein-induced pancreatitis and associated liver injury in rats

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Abstract

AIM: To investigate the role of oxidative injury in pancreatitis-induced hepatic damage and the effect of antioxidant agents such as melatonin, ascorbic acid and *N*-acetyl cysteine on caerulein-induced pancreatitis and associated liver injury in rats.

METHODS: Thirty-eight female Wistar rats were used. Acute pancreatitis (AP) was induced by two i.p. injections of caerulein at 2-h intervals (at a total dose of 100 µg/kg b.wt). The other two groups received additional melatonin (20 mg/kg b.wt) or an antioxidant mixture containing L(+)-ascorbic acid (14.3 mg/kg b.wt.) and *N*-acetyl cysteine (181 mg/kg b.wt.) i.p. shortly before each injection of caerulein. The rats were sacrificed by decapitation 12 h after the last injection of caerulein. Pancreatic and hepatic oxidative stress markers were evaluated by changes in the amount of lipid peroxides measured as malondialdehyde (MDA) and changes in tissue antioxidant enzyme levels, catalase (CAT) and glutathione peroxidase (GPx). Histopathological examination was performed using scoring systems.

RESULTS: The degree of hepatic cell degeneration, intracellular vacuolization, vascular congestion, sinusoidal dilatation and inflammatory infiltration showed a significant difference between caerulein and caerulein+melatonin ($P=0.001$), and caerulein and caerulein+L(+)-ascorbic acid+*N*-acetyl cysteine groups ($P=0.002$). The degree of aciner cell degeneration, pancreatic edema, intracellular vacuolization and inflammatory infiltration showed a significant difference between caerulein and

caerulein+melatonin ($P=0.004$), and caerulein and caerulein+L(+)-ascorbic acid+*N*-acetyl cysteine groups ($P=0.002$). Caerulein-induced pancreatic and liver damage was accompanied with a significant increase in tissue MDA levels ($P=0.01$, $P=0.003$, respectively) whereas a significant decrease in CAT ($P=0.002$, $P=0.003$, respectively) and GPx activities ($P=0.002$, $P=0.03$, respectively). Melatonin and L(+)-ascorbic acid+*N*-acetyl cysteine administration significantly decreased MDA levels in pancreas ($P=0.03$, $P=0.002$, respectively) and liver ($P=0.007$, $P=0.01$, respectively). Administration of these agents increased pancreatic and hepatic CAT and GPx activities. Melatonin significantly increased pancreatic and hepatic CAT ($P=0.002$, $P=0.001$, respectively) and GPx activities ($P=0.002$, $P=0.001$). Additionally, L(+)-ascorbic acid+*N*-acetyl cysteine significantly increased pancreatic GPx ($P=0.002$) and hepatic CAT and GPx activities ($P=0.001$, $P=0.007$, respectively).

CONCLUSION: Oxidative injury plays an important role not only in the pathogenesis of AP but also in pancreatitis-induced hepatic damage. Antioxidant agents such as melatonin and ascorbic acid+*N*-acetyl cysteine, are capable of limiting pancreatic and hepatic damage produced during AP via restoring tissue antioxidant enzyme activities.

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Key words: Caerulein; Liver; Melatonin; Oxidative stress; Pancreatitis

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease characterized by tissue edema, acinar necrosis, hemorrhage and fat necrosis as well as inflammation and perivascular infiltration in the pancreas^[1,2]. In experimental pancreatitis induced by supramaximal doses of the cholecystokinin (CCK) analogue, caerulein, the secretory block is subse-

quently followed by lysosomal degradation of intercellular organelles within autophagic vacuoles in acinar cells and a marked interstitial edema^[3-6]. AP is usually considered as an autodigestive disease, in which in addition to premature intracellular protease activation^[7], other mechanisms such as oxidative stress have also been shown to be involved in the development of AP^[6,8-11].

AP is a multi-system disease with alterations not only in the pancreas but also in liver, lungs and kidneys, which may lead to distant organ dysfunction and death^[12,13]. The liver is a critical organ for metabolic homeostasis and toxic substance clearance and plays an important role in the systemic response to critical illness^[14]. Recently, a number of studies have suggested that pancreatitis-associated ascitic fluid (PAAF) plays a critical role in inducing hepatocyte injury by inducing hepatocyte apoptosis^[14-16]. PAAF induces liver injury by direct hepatocyte injury and death independent from locally produced Kupffer-cell-derived cytokines and PAAF-induced liver injury is mediated by heat-stable factors other than pancreatic enzymes^[16]. Though the role of oxidative stress in AP has been studied in several animal models^[2,6,11,17] and in humans^[18], few data are available regarding AP-induced hepatic oxidative stress^[19].

Previous reports have indicated that antioxidant agents such as melatonin, retinol, ascorbic acid and *N*-acetyl cysteine have beneficial effects in the treatment of caerulein-induced AP^[18,20-22]. This study was undertaken to examine the role of oxidative stress in the pathogenesis of AP and AP-induced hepatic damage as well as the protective effect of antioxidant agents against caerulein-induced pancreatic and liver injury.

MATERIALS AND METHODS

Animals and experimental protocol

Thirty-eight female Wistar rats weighing 280-350 g were used. Animals were fed with standard rat chow and tap water *ad libitum*. They were maintained in a 12 h light/12 h dark cycle at 21°C.

AP was induced by two i.p. injections of caerulein (Sigma-Aldrich Co., Taufkirchen, Germany) at a total dose of 100 µg/kg b.wt. at 2-h intervals, each injection containing 50 % of the dose. A control group received four i.p. injections of 0.9% saline at 2-h intervals. To evaluate the effect of antioxidant agents, the rats were divided into two additional groups. One group was treated with caerulein (100 µg/kg b.wt) and melatonin (Sigma, St Louis, MO, USA) (20 mg/kg b.wt) and the other group was treated with the same dose of caerulein and an antioxidant mixture containing L(+)-ascorbic acid (Mallinckrodt Baker B.V., Deventer, Holland) (14.3 mg/kg b.wt.) and *N*-acetyl cysteine (Sigma, St. Louis MO, USA) (181 mg/kg b.wt.). Melatonin was dissolved in absolute ethanol and further diluted in saline, with 1 % final concentration of ethanol. Therapeutic agents were administered i.p. shortly before each injection of caerulein. The rats were sacrificed by decapitation 12 h after the last injection of caerulein.

For light microscopy, pieces from central part of the pancreas and right lobe of the liver were rapidly removed and divided into two pieces. The first part of

tissue samples was placed in 10% buffered formalin and prepared for routine paraffin embedding. The other part of tissue samples was stored at -80°C for determination of MDA, GPx and CAT.

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

Histological examination

Five-µm thick sections of tissues were cut, mounted on slides, stained with hematoxylin-eosin (H-E) and examined under a Lyca DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K). Assessment of tissue alterations in 20 different fields for each section was conducted by an experienced histologist who was unaware of the treatment. Pancreatic damage was scored by grading acinar cell degeneration, interstitial inflammation, edema and hemorrhage with a maximum score of 12. Schmidt's standards^[1] were modified as follows. Grading for edema was scaled as 0: absent or rare; 1: edema in the interlobular space; 2: edema in the intralobular space; 3: isolated-island shape of pancreatic acinus. Inflammation was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Acinar cell degeneration was scaled as 0: absent; 1: focal (<5 %); 2: and/or sublobular (<20 %); 3: and/or lobular (>20 %). Parenchyma hemorrhage was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Hepatic damage was scored (0 to 3) by grading hepatocyte necrosis, intracellular vacuolization, vascular congestion and sinusoidal dilatation. The maximum score for inflammatory infiltration was 12.

Biochemical determination

After decapitation, the trunk blood was collected and centrifuged for measurements of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), total and direct bilirubin. Amylase. Lipase levels were determined spectrophotometrically using an automated analyzer (Olympus AU 600, Diamond Diagnostic, Holliston, USA). All chemicals were obtained from Sigma (Sigma, St Louis MO, USA).

Preparation of tissue homogenates

After being cut into small pieces on ice, tissues were homogenized in 1/5 (w/v) phosphate-buffered saline. Homogenates were divided into two portions. One part was directly used for immediate MDA measurement. The other part was sonicated four times for 30 s at 20 s intervals using a VWR Bronson scientific sonicator (VWR Int. Ltd. Merch House Pool, UK). Then, homogenates were centrifuged at 20000 g for 15 min in Beckman L-8-70M ultracentrifuge (Rotor SW-28; Beckman L8-70M Ultracentrifuge, München, Germany). Supernatants were separated and kept at -40°C for enzyme activity measurement. Care was taken to keep the temperature at -4°C throughout the preparation of homogenates and supernatants.

Determination of protein concentration

Protein determination in supernatants was done according to Lowry and Rosebrough^[23] using BSA as standard. A

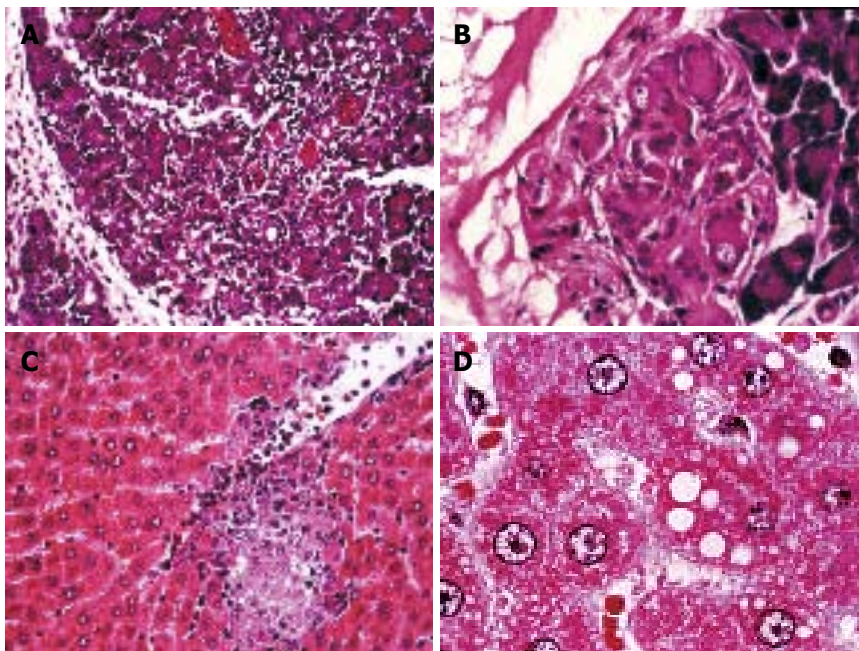


Figure 1 Histopathological changes in pancreatic and hepatic specimens

A: Obvious acinar cell degeneration, edema and inflammation in caerulein group.

B: Acinar cell degeneration and fat necrosis in caerulein group.

C: Hepatocyte necrosis, vascular congestion, sinusoidal dilatation and inflammatory infiltration in caerulein group.

D: Prominent intracellular vacuolization in hepatic specimens from caerulein group.

Table 1 Scores for pancreatic and hepatic damage in different groups (mean \pm SE)

Group	Scores for pancreatic damage	Scores for hepatic damage
Control	0.00 \pm 0.00	0.00 \pm 0.00
Caerulein	6.16 \pm 0.67 ^a	6.28 \pm 0.56 ^a
Caerulein + melatonin	2.14 \pm 0.70 ^c	1.14 \pm 0.26 ^c
Caerulein + antioxidant mixture	1.85 \pm 0.45 ^c	1.42 \pm 0.36 ^c

^a $P < 0.005$ vs control; ^c $P < 0.005$ vs caerulein.

Shimadzu 1601 UV/VIS spectrophotometer (Shimadzu, Kiyoto, Japan) with a connected PC and a Grand LTD 6G thermo stability unit adjusted to $37 \pm 0.1^\circ\text{C}$ was employed for all spectrophotometric assays.

Assay of CAT activity

CAT activity was measured in supernatants by the method of Luck^[24]. The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as micromole substrate decomposed per minute per milligram of protein (i.e. U/mg protein). CAT levels were expressed as a micromole per milligram of protein (U/mg/protein).

Assay of GPx activity

GPx activity was measured according to Lawrence and Burk^[25]. In brief, 1.0 mL of 50 nmol/L PBS solution (pH 7.4) including 5 mmol/L EDTA, 2 $\mu\text{mol/L}$ NADPH, 20 $\mu\text{mol/L}$ GSH, 10 $\mu\text{mol/L}$ NaN_3 and 23 mU of glutathione reductase was incubated at 37°C for 5 min. Then 20 μL of 0.25 mmol/L H_2O_2 solution and 10 μL of supernatant were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank with all ingredients except for supernatants was also monitored. Specific activity was calculated as micromole NADPH consumed

per minute per milligram of protein (i.e. U/mg protein) using an appropriate molar absorptivity coefficient ($6220 \text{ M}^{-1} \text{ cm}^{-1}$). GPx levels were expressed as a micromole per milligram of protein (U/mg protein).

Measurement of tissue MDA levels

The level of MDA in tissue homogenate was determined using the method of Uchiyama and Mihara^[26]. Half a milliliter of homogenate was mixed with 3 mL H_3PO_4 solution (1 % v/v) followed by addition of 1 mL thiobarbituric acid solution (0.67 % w/v). Then the mixture was heated in water bath for 45 min. The colored complex was extracted into n-butanol and absorption at 532 nm was measured using tetramethoxypropane as standard. MDA levels were expressed as a nanomol per milligram of protein (nm/mg protein).

Statistical analysis

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., Chicago, IL, USA). All data were expressed as mean \pm SE. For the analysis of histological scores and enzyme levels Mann Whitney-U test and *t* test were used. $P < 0.05$ was considered statistically significant.

RESULTS

All animals survived until the end of the experiment. Animals from control group presented no histological alterations. The pancreatic specimens from the caerulein treated group showed some histopathological changes such as acinar cell degeneration, edema and inflammation (Figure 1A, Figure 1B). The liver specimens from this group also showed histopathological alterations such as hepatocyte necrosis and intracellular vacuolization vascular congestion, sinusoidal dilatation and inflammatory infiltration (Figure 1C, Figure 1D). Histopathological scores of the groups are summarized in Table 1. In the groups treated

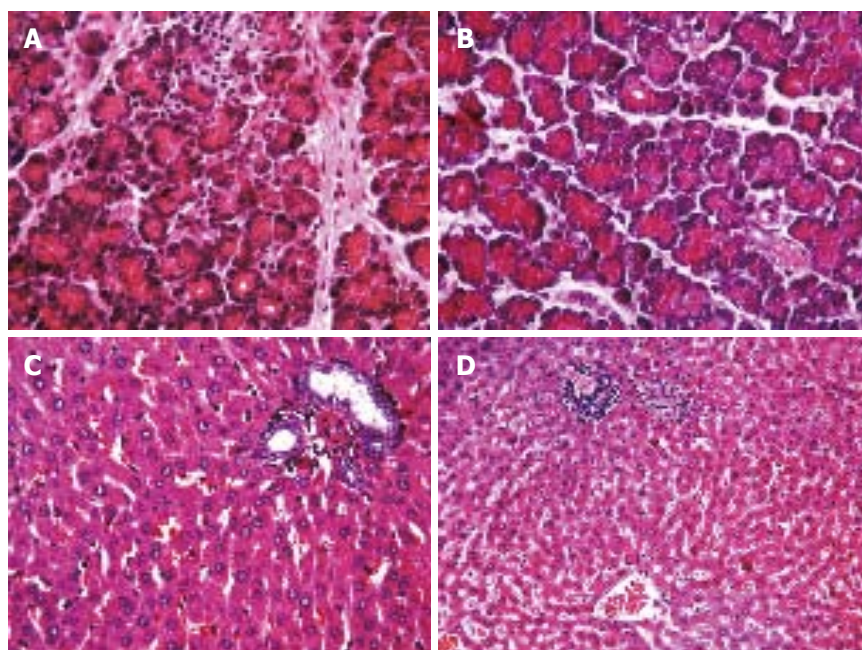


Figure 2 Histopathological evidence of pancreatic and hepatic damage

A: Normal histological appearance except for minimal infiltration in caerulein + melatonin group.

B: Normal histological appearance except for minimal edema in caerulein + L(+)-ascorbic acid + N-acetyl cysteine group.

C: Normal histological appearance in caerulein + melatonin group.

D: Two small areas of necrosis and cell infiltration in caerulein + L(+)-ascorbic acid + N-acetyl cysteine group.

Table 2 MDA, CAT and after treatment GPx levels in pancreatic tissues of different groups after treatment (mean \pm SE)

Groups	MDA (nmol/mg pr)	CAT (U/mg pr)	GPx (U/mg pr)
Control	1.74 \pm 0.23	10.29 \pm 0.68	0.59 \pm 0.05
Caerulein	2.63 \pm 0.22 ^a	2.78 \pm 0.28 ^b	0.10 \pm 0.00 ^b
Caerulein + melatonin	1.90 \pm 0.17 ^c	15.63 \pm 0.67 ^e	1.72 \pm 0.29 ^e
Caerulein + antioxidant mixture	1.30 \pm 0.15 ^e	7.02 \pm 1.84	1.15 \pm 0.18

^a*P* < 0.05 vs control; ^c*P* < 0.05 vs caerulein; ^b*P* < 0.005 vs caerulein; ^e*P* < 0.005 vs control.

with caerulein + melatonin and caerulein + L(+)-ascorbic acid + N-acetyl cysteine, histopathological evidence of pancreatic and hepatic damage was markedly reduced (Figure 2). The degree of hepatic cell degeneration, intracellular vacuolization, vascular congestion, sinusoidal dilatation and inflammatory infiltration showed a significant difference between groups treated with caerulein and caerulein + melatonin (*P* = 0.001), caerulein and caerulein + L(+)-ascorbic acid + N-acetyl cysteine (*P* = 0.002). The degree of acinar cell degeneration, pancreatic edema, intracellular vacuolization and inflammatory infiltration showed a significant difference between groups treated with caerulein and caerulein + melatonin (*P* = 0.004), caerulein and caerulein + L(+)-ascorbic acid + N-acetyl cysteine (*P* = 0.002). Caerulein-induced pancreatitis and liver damage were accompanied with a significant increase in tissue MDA levels (*P* = 0.01, *P* = 0.003, respectively) whereas a significant decrease in CAT (*P* = 0.002, *P* = 0.003, respectively) and GPx activities (*P* = 0.002, *P* = 0.03, respectively). MDA, CAT and GPx levels measured in pancreatic and hepatic tissues of all groups are summarized in Tables 2 and 3 respectively. Administration of melatonin and L(+)-ascorbic acid + N-acetyl cysteine significantly decreased

Table 3 MDA, CAT and GPx levels in liver tissues of different groups after treatment (mean \pm SE)

Group	MDA (nmol/mg pr)	CAT (U/mg pr)	GPx (U/mg pr)
Control	7.00 \pm 2.29	3.20 \pm 0.83	0.72 \pm 0.25
Caerulein	20.89 \pm 10.13 ^a	1.09 \pm 0.35 ^a	0.33 \pm 0.09 ^b
	9.66 \pm 3.65 ^c	3.69 \pm 1.35 ^e	0.50 \pm 0.12 ^c
Caerulein + antioxidant mixture	10.49 \pm 3.03 ^c	3.12 \pm 1.02 ^e	0.61 \pm 0.26 ^c

^a*P* < 0.005 vs control; ^c*P* < 0.05 vs caerulein; ^b*P* < 0.005 vs caerulein; ^e*P* < 0.05 vs control.

MDA levels in pancreas (*P* = 0.03, *P* = 0.002, respectively) and liver (*P* = 0.007, *P* = 0.01, respectively) while increased pancreatic and hepatic CAT and GPx activities. Melatonin significantly increased pancreatic and hepatic CAT (*P* = 0.002, *P* = 0.001, respectively) and GPx activities (*P* = 0.002, *P* = 0.001). Additionally, L(+)-ascorbic acid + N-acetyl cysteine significantly increased pancreatic GPx (*P* = 0.002) and hepatic CAT and GPx activities (*P* = 0.001, *P* = 0.007, respectively).

Caerulein administration resulted in a significant increase in serum AST (*P* < 0.02), ALT (*P* = 0.009), LDH (*P* = 0.002), total bilirubin (*P* = 0.001), direct bilirubin (*P* = 0.001), amylase (*P* = 0.02) and lipase (*P* = 0.002) levels (Tables 4 and 5). Both melatonin and ascorbic acid + N-acetyl cysteine reduced these levels. Ascorbic acid + N-acetyl cysteine caused a significant decrease in ALT level (*P* = 0.01). Additional administration of melatonin and ascorbic acid + N-acetyl cysteine resulted in a significant decrease in both total and direct bilirubin (*P* = 0.001)

DISCUSSION

The present study was undertaken to confirm the role of reactive oxygen species (ROS) in the pathogenesis

Table 4 AST, ALT, LDH, total and direct bilirubin levels in different groups after treatment (mean ± SE)

Group	AST (U/L)	ALT (U/L)	LDH (U/L)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)
Control	186.85±19.5	53.71±2.39	4088.43±510.5	0.02±0.02	0.01±0.01
Caerulein	259.57±18.60 ^a	68.14±4.04 ^a	7631.14±281.3 ^c	6.88±0.12 ^c	3.06±0.09 ^c
Caerulein + mel	193.28±26.64	47.71±6.98	5065.71±508.10	0.04±0.01 ^e	0.00±0.01 ^e
Caerulein + antioxidant mixture	274.71±17.16	70.14±4.84 ^e	6592.71±1065.41	0.05±0.02 ^e	0.04±0.02 ^e

^aP < 0.05 vs control; ^cP < 0.005 vs control; ^eP < 0.05 vs caerulein+mel; ^eP < 0.005 vs caerulein.

Table 5 Serum amylase and lipase levels in different groups after treatment (mean ± SE)

Group	Amylase (U/L)	Lipase (U/L)
Control	379.28±15.71	22.71±0.68
Caerulein	2182.29±563.92 ^a	117.0±7.56 ^c
Caerulein + mel	1406.71±169.44	65.0±10.81
Caerulein + antioxidant mixture	1881.57±414.20	70.71±8.72

^aP < 0.05 vs control; ^cP < 0.005 vs control.

of caerulein-induced acute pancreatitis and pancreatitis-induced hepatic damage. The exact pathogenesis of AP is not fully understood, but a large body of evidence suggests that pancreatic damage depends upon the toxic effect of ROS^[18,27]. However, few data are available regarding AP-induced hepatic oxidative stress^[19].

It is shown that acinar cells produce large amounts of ROS at early stage of AP in rats^[17]. Highly reactive ROS directly attacks lipids, proteins in the biological membranes and cause their dysfunction^[28]. Under normal conditions, a natural system of scavengers and antioxidants counteracts the cytotoxicity of ROS produced from molecular oxygen in the mitochondria. When the production of ROS is increased in AP by activated leukocytes, the capacity of intrinsic defense mechanism leads to alteration in cytoskeleton of acinar cells and damage of cell membranes^[27]. Degradation of polyunsaturated fatty acids in cell membranes by ROS results in the destruction of membranes and formation of MDA, which is an indicator of ROS generation^[29]. Disruption of the cytoskeleton leads to disturbance of intracellular transport of digestive enzymes and their premature activation and damage in acinar cells^[30]. Large amounts of ROS and activated pancreatic enzymes leaked from the broken cells injure capillary endothelium. Increased capillary permeability contributes to edema^[27]. It is suggested that PPAA contributes to hepatocyte injury by inducing hepatocyte apoptosis during AP. Barlas *et al*^[19] reported that ROS plays an import role in the pathogenesis of pancreatitis-induced hepatic damage.

In this study we measured the levels of MDA, a product of lipid peroxidation, and endogenous scavengers CAT and GPx in pancreatic and hepatic tissues. Caerulein-induced pancreatitis and liver damage were accompanied with a significant increase in tissue MDA levels ($P < 0.05$)

whereas a significant decrease in CAT and GPx activities ($P < 0.05$). These findings demonstrate that lipid peroxidation is not only dramatically enhanced within pancreatic tissue but also within hepatic tissue. Elevated MDA as an offense system and lowered CAT and GPx activities as a defense system play an important role in the development of pancreatic and liver injury.

Previous reports indicate that treatment with antioxidant agents such as melatonin^[19,20,22], N-acetylcysteine, ascorbic acid^[21], SOD, CAT and deferoxamine^[31], has beneficial effects on caerulein-induced AP. Melatonin can directly neutralize a number of free radicals and reactive oxygen and nitrogen species^[32-34] and stimulate several antioxidative enzymes (superoxide dismutase, glutathione peroxidase)^[32]. Free radical scavengers and antioxidants neutralize and/or metabolically remove reactive species from cells before they carry out their destructive activities^[33]. While the antioxidative actions of most molecules are limited by their specific intercellular distribution, antioxidative actions of melatonin include the protection of lipids in cell membranes, proteins in cytosol and DNA in nuclei. Furthermore, melatonin can crossover all morphophysiological barriers and enter all cells in the organism^[35]. In the present study, treatment with melatonin and N-acetylcysteine + ascorbic acid mixture significantly decreased histopathological damage score in both pancreas and liver. Additionally these therapies decreased tissue MDA levels but increased tissue CAT and GPx activities. Histopathological changes correlated with biochemical changes.

In conclusion, oxidative injury plays an important role not only in the pathogenesis of AP but also in pancreatitis-induced hepatic damage. Antioxidant agents such as melatonin and ascorbic acid + N-acetyl cysteine, are capable of limiting pancreatic and hepatic damage produced during AP *via* restoring tissue antioxidant enzyme activities.

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