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BASIC RESEARCH

Effect of melatonin on the severity of L-arginine-induced experimental acute pancreatitis in rats

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Abstract

AIM: To determine the effect of melatonin pre- and post-treatment on the severity of L-arginine (L-Arg) -induced experimental pancreatitis in rats.

METHODS: Male Wistar rats (25) were divided into five groups. Those in group A received two injections of 3.2 g/kg body weight L-Arg i.p. at an interval of 1 h. In group MA, the rats were treated with 50 mg/kg body weight melatonin i.p. 30 min prior to L-Arg administration. In group AM, the rats received the same dose of melatonin 1 h after L-Arg was given. In group M, a single dose of melatonin was administered as described previously. In group C the control animals received physiological saline injections i.p. All rats were exsanguinated 24 h after the second L-Arg injection.

RESULTS: L-Arg administration caused severe necrotizing pancreatitis confirmed by the significant elevations in the serum amylase level, the pancreatic weight/body weight ratio (pw/bw), the pancreatic IL-6 content and the myeloperoxidase activity, relative to the control values. Elevation of the serum amylase level was significantly reduced in rats given melatonin following L-Arg compared to rats injected with L-Arg only. The activities of the pancreatic antioxidant enzymes (Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase (CAT)) were significantly increased 24 h after pancreatitis induction. Melatonin given in advance of L-Arg significantly reduced the pancreatic CAT activity relative to that in the rats treated with L-Arg alone. In the liver, L-Arg significantly increased the lipid peroxidation level, and the glutathione peroxidase and Cu/Zn-SOD activities, whereas the Mn-SOD activity was reduced as compared to the control rats. Melatonin pre-treatment prevented these changes.

CONCLUSION: Melatonin is an antioxidant that is able to counteract some of the L-Arg-induced changes during acute pancreatitis, and may therefore be helpful in the supportive therapy of patients with acute necrotizing pancreatitis.

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Key words: Acute pancreatitis; Melatonin; Scavengers

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INTRODUCTION

Acute necrotizing pancreatitis is a disease with a high mortality and no efficient treatment is available for it at present. Oxygen- and nitrogen-derived free radicals (FRs) and lipid peroxidation play an important role in the development of local inflammation and systemic complications during acute pancreatitis[1-6]. They damage the lipid membranes, structural and enzymatic proteins and DNA of the cells. The major target of FRs is polyunsaturated fatty acids of the lipid-rich membranes. Lipid peroxidation results in loss of the membrane fluidity and integrity, leading to cell death. Numerous antioxidants have recently been examined for their protective properties against oxidative damage in acute pancreatitis and have been shown to moderate the changes in several parameters of the disease[7-10]. We presumed that a compound with strong antioxidant properties and also anti-inflammatory features might exert a beneficial effect on the outcome of severe experimental pancreatitis.

The pineal product, melatonin, is known to play a role

in the regulation of circadian rhythm and in the seasonal reproduction of certain species. Melatonin is also important as regards the physiology of the retina and the immune system[11]. The antioxidant activity of melatonin has recently received significant attention. Although its level is low in the blood, a strong correlation has been observed between this level and the antioxidant capacity of serum both *in vitro* and *in vivo*[12, 13]. Since melatonin is strongly lipophilic, its intracellular concentration may be several times higher than that in serum[14]. Various body fluids likewise contain melatonin levels that are orders of magnitude higher than those measured in blood[15].

Melatonin can detoxify OH, ONOO-, NO and peroxyl radicals directly by electron donation, stimulate the activities of scavenger enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and protect these proteins from inactivation by the above-mentioned radicals[11, 16-18].The inhibition of nitrogen monoxide synthase (NOS) by melatonin leads to a reduction in the amount of NO generated[19, 20]. Moreover, melatonin exerts an anti-inflammatory effect by inhibiting nuclear factor kappa B (NF-κB) [21], a transcription factor with a central role in the development of inflammatory diseases. By blocking the activation of NF-κB, melatonin depresses the synthesis of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha) and adhesion molecules.

The protective effects of melatonin have been documented in experimental models of numerous diseases where oxidative damage is a component. In the central nervous system, melatonin is protective in experimental models of stroke, Alzheimer, Parkinson and Huntington diseases, and fetal brain injury, and improves the outcome of hypoxia/reperfusion-induced heart, liver, retina and gut injuries[22-27]. Melatonin additionally contributes to the improvement of inflammatory diseases, including endotoxic and circulatory shock[23, 28].

In an earlier study, Qi and colleagues[29] showed that in mild cerulein-induced edematous pancreatitis pharmacological doses of melatonin protect against the injury caused by FRs. Melatonin decreased the edema in the pancreas and stomach, and also the extent of lipid peroxidation in the pancreas. Jaworek *et al*[30]. demonstrated that even the circadian changes in physiological levels of melatonin reduce the severity of experimental pancreatitis[30].

L-Arg-induced pancreatitis is an experimental model of severe necrotizing acute pancreatitis. Twenty-four h after intraperitoneal (i.p.) injection of L-Arg, inflammation of the tissue is confirmed by histology and characteristic changes of the laboratory parameters. The model is highly reproducible, noninvasive and produces dose-dependent acinar necrosis, and is therefore ideal for studying the pathogenesis of acute pancreatitis[31-35].

The protective effect of melatonin in severe L-Arg-induced pancreatitis in rats has not been investigated to date. We hypothesized that administration of pharmacological doses of melatonin might improve the outcome of L-Arg-induced necrotizing pancreatitis in rats. To test this, we measured a variety of parameters related to pancreatic damage by L-Arg when melatonin was given before or after L-Arg.

Table 1 Agents used in the 5 groups of rats

Group	Time: 0 h	Time: 0,5 h	Time: 1,5 h	Time: 2,5 h	Time: 25,5 h
C	P.s. 1 mL	P.s. 1 mL	P.s. 1 mL		exsanguination
A	P.s. 1 mL	Arginine 3.2 g/kg	Arginine 3.2 g/kg		exsanguination
AM	Arginine 3,2 g/kg	Arginine 3.2 g/kg		Melatonin 50 mg/kg	exsanguination
MA	Melatonin 50 mg/kg	Arginine 3.2 g/kg	Arginine 3.2 g/kg		exsanguination
M	Melatonin 50 mg/kg	P.s. 1mL	P.s. 1 mL		exsanguination

MATERIALS AND METHODS

Experimental protocol

Male Wistar rats (weighing 200-250 g) were kept at constant room temperature (25 °C) in a 12h light-dark cycle with free access to water and standard laboratory chow (Biofarm, Zagyvaszárszó, Hungary). The study was approved by the Ethical Committee on Animal Experiments at the University of Szeged.

After one week of acclimatization, the rats were divided into five groups ($n = 5$ per group). In group A, pancreatitis was induced with 3.2 g/kg body weight L-Arg (Sigma-Aldrich, Budapest, Hungary) i.p. twice at an interval of 1 h. Rats in group MA were treated with a single dose of 50 mg/kg body weight melatonin (Helsinn, Biasca, Switzerland) i.p. 30 min prior to L-Arg administration. Rats in group AM received the same dose of melatonin 1 h after the second injection of L-Arg. In group M, a single dose of melatonin was administered. Rats in group C served as control animals and received i.p. injections of physiological saline. Twenty-four h after the last L-Arg injection, the rats were anesthetized with 44 mg/kg pentobarbital (Sanofi Phylaxia, Budapest, Hungary) and exsanguinated through the abdominal aorta. The pancreas, liver and lungs were quickly removed and frozen at -70 °C until use (Table 1).

Assays

The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema. For serum amylase activity, blood samples were centrifuged for 20 min at 2500 r/min. Serum amylase activities were determined by a colorimetric kinetic method (Dialab, Vienna, Austria). Concentration of the lipid peroxidation marker malonyl dialdehyde (MDA) was measured after the reaction with thiobarbituric acid as previously described [36]. Total SOD activity was determined on the basis of the inhibition of epinephrine-adenochrome autoxidation[37]. Mn-SOD activity was measured via the extent of autoxidation in the presence of 5×10^{-3} mol/L KCN[38]. Cu/Zn-SOD activity was obtained by deducting the Mn-SOD from the total SOD activity. CAT activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer [39] and expressed in Bergmeyer units (BU) (1 BU - decomposition of 1 g H_2O_2/min at 25ºC). GPx activity was determined by the "chemical" method using cumene

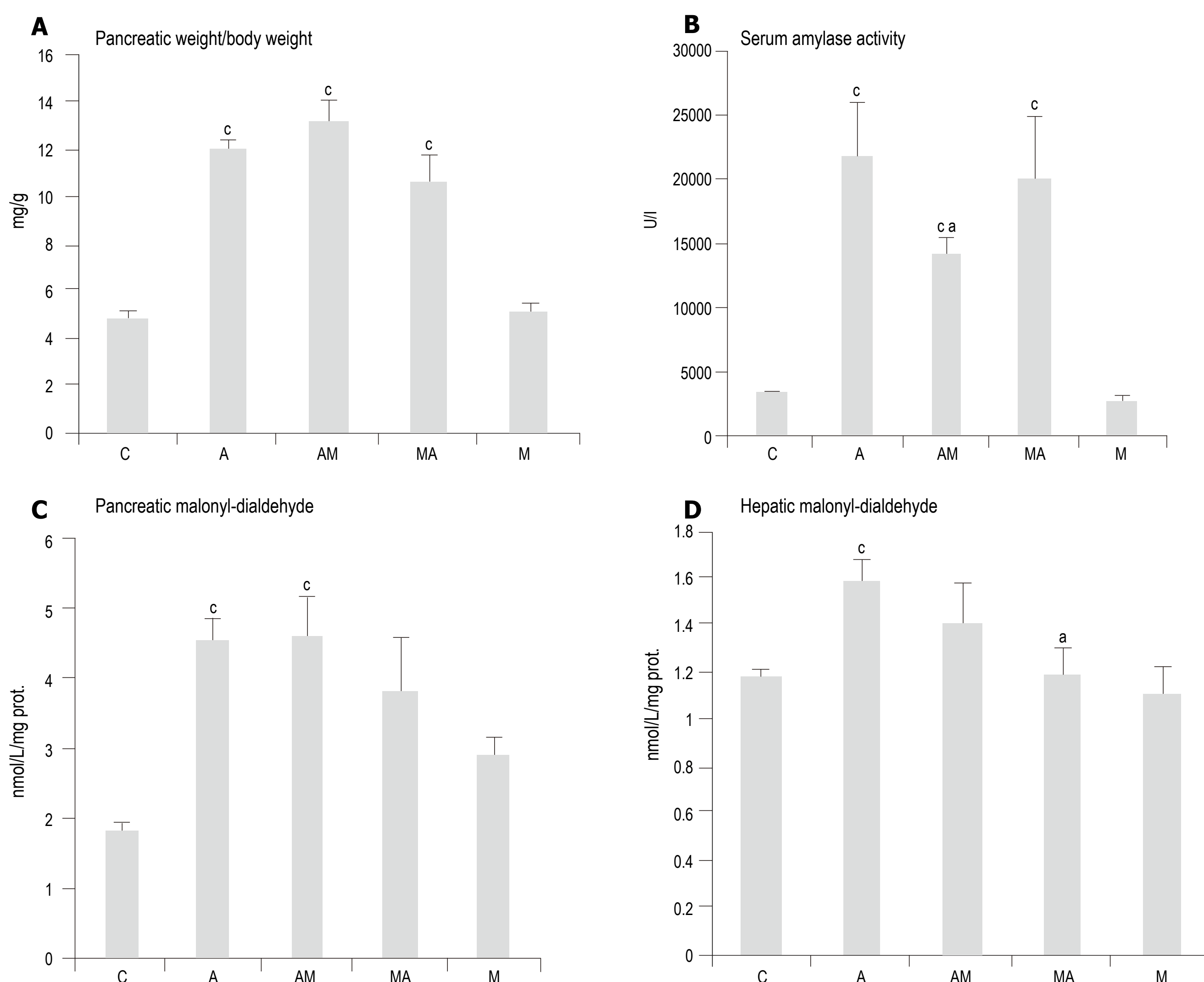

Figure 1 Effects of melatonin (50 mg/kg) treatment on the pancreatic weight/body weight ratio (p.w./b.w.) (**A**), serum amylase activity (**B**), amount of malonyl-dialdehyde in the pancreas (**C**) and liver (**D**) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means ± SE of results on 5 animals in each group are shown. $^a P$<0.05 vs group A; $^c P$<0.05 vs group C.

hydroperoxide and reduced glutathione as substrates of GPx[40]. Total glutathione (GSH) was measured spectrophotometrically with Ellman's reagent[41]. The level of leukocyte infiltration into the tissue was quantified by measurement of pancreatic myeloperoxidase (MPO) activities by the method of Kuebler *et al*[42].

Preparation of cytosolic fraction

The cytosolic and nuclear fractions were separated by the method of Dignam *et al*[43]. In brief, 250-300 mg of pancreatic tissue was lysed on ice in a hypotonic buffer in a Dounce homogenizer. The buffer contained 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$ 10 mmol/L KCl and was supplemented before use with 1mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L benzamidine, 100 IU/mL aprotinin and 1 mmol/L dithiothreitol (DTT). After incubation for 25 min on ice, Nonidet P-40 was added to a final concentration of 0.3-0.4% (v/v). The samples were vortexed and incubated on ice for 2 min. The homogenates were centrifuged at 13000 r/min for 50 s and the supernatants (cytosolic fraction) were collected for determination of protein and IL-6 concentrations.

Pancreatic cytokine concentration

Pancreatic IL-6 concentration in the cytosolic fraction was measured with an ELISA kit (Bender Medsystem, Vienna, Austria) according to the manufacturer's instructions and corrected for the protein content of the tissue. Protein concentration in the tissues was determined by the method of Lowry *et al*[44].

Histological examination

A portion of the pancreas was fixed in 8% neutral formaldehyde solution and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin and examined under light microscope. Slices were coded and examined blind by a pathologist for the grading of histological alterations.

Statistical analysis

One-way ANOVA and LSD *post hoc* analysis were performed to test for significant differences among the five experimental groups. Results were expressed as mean ± SE. P < 0.05 was considered statistically significant.

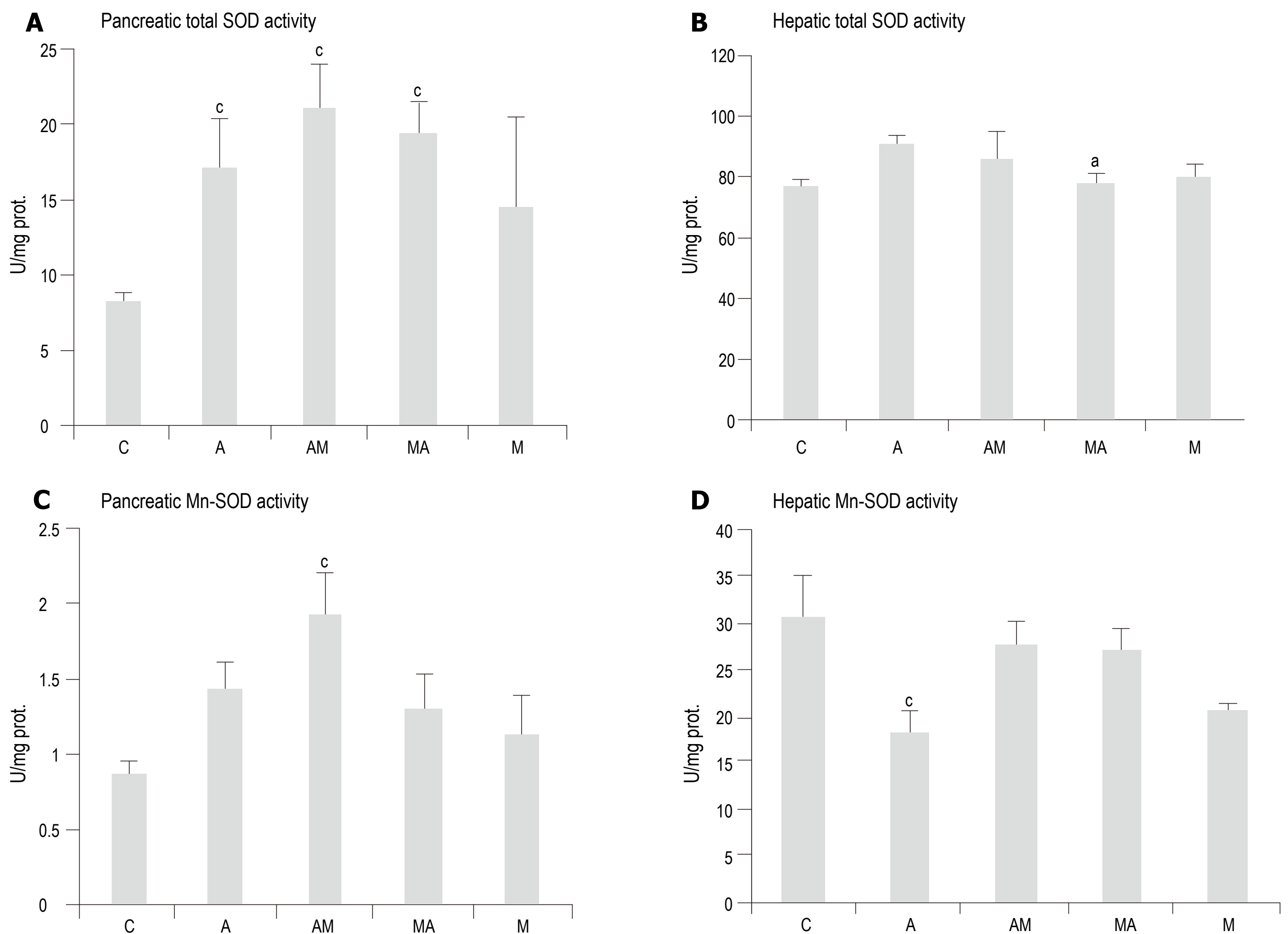

Figure 2 Effects of melatonin (50 mg/kg) treatment on the total SOD and Mn-SOD activities of the pancreas (**A, C**) and liver (**B, D**) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means ± SE of results on 5 animals in each group are shown. [a]*P*<0.05 vs group A; [c]*P*<0.05 vs group C.

RESULTS

Melatonin treatment alone caused no significant alterations in the measured parameters as compared to those in the control rats.

The pancreatic weight/body weight ratio was significantly higher in the L-Arg-treated rats than in the control group. Melatonin given before or after L-Arg, did not influence the degree of edema (Figure 1A).

The serum amylase activity in all L-Arg-injected rats was significantly elevated relative to the control. Melatonin posttreatment significantly reduced the amylase activity as compared to the treatment only with L-Arg (Figure 1B).

The concentration of lipid peroxidation product MDA in the pancreas was increased in the rats treated with L-Arg or with L-Arg followed by melatonin. When melatonin was given before L-Arg, the MDA level was not significantly changed compared to that in the control group (Figure 1C). The amount of MDA in the liver of animals given L-Arg was significantly increased compared to that in the control group. Melatonin pretreatment significantly reduced the concentration of the lipid peroxidation product (Figure 1D).

The pancreatic total SOD activity was significantly increased as a result of L-Arg administration and the effect was not modified by pre- or post-treatment with melatonin (Figure 2A). The hepatic total SOD activities in the L-Arg-treated groups were not significantly different from those in the control rats, but the L-Arg-treated rats having previously received melatonin exhibited a significantly lower SOD activity than those injected only with L-Arg (Figure 2B).

Relative to the level in the control rats, the pancreatic Mn-SOD activity was significantly elevated in animals given L-Arg followed by melatonin (Figure 2C). The Mn-SOD activity in the liver was significantly decreased as a consequence of L-Arg injections. Melatonin given before or after L-Arg prevented the reduction of Mn-SOD activity (Figure 2D).

As compared to the levels in the control rats, the pancreatic Cu/Zn-SOD activities were significantly elevated in all L-Arg-treated groups (Figure 3A). Relative to the control levels, the hepatic Cu/Zn-SOD activity was significantly increased in rats that received only L-Arg, this change was prevented by melatonin given before or after the L-Arg injections (Figure 3B).

The pancreatic CAT activity was significantly increased in the rats given only L-Arg injections and also in those given L-Arg followed by melatonin, relative to that in the control rats. However, in the rats pretreated with melatonin the changes in CAT activity were prevented (Figure 3C). As compared to the level in the control rats, the hepatic

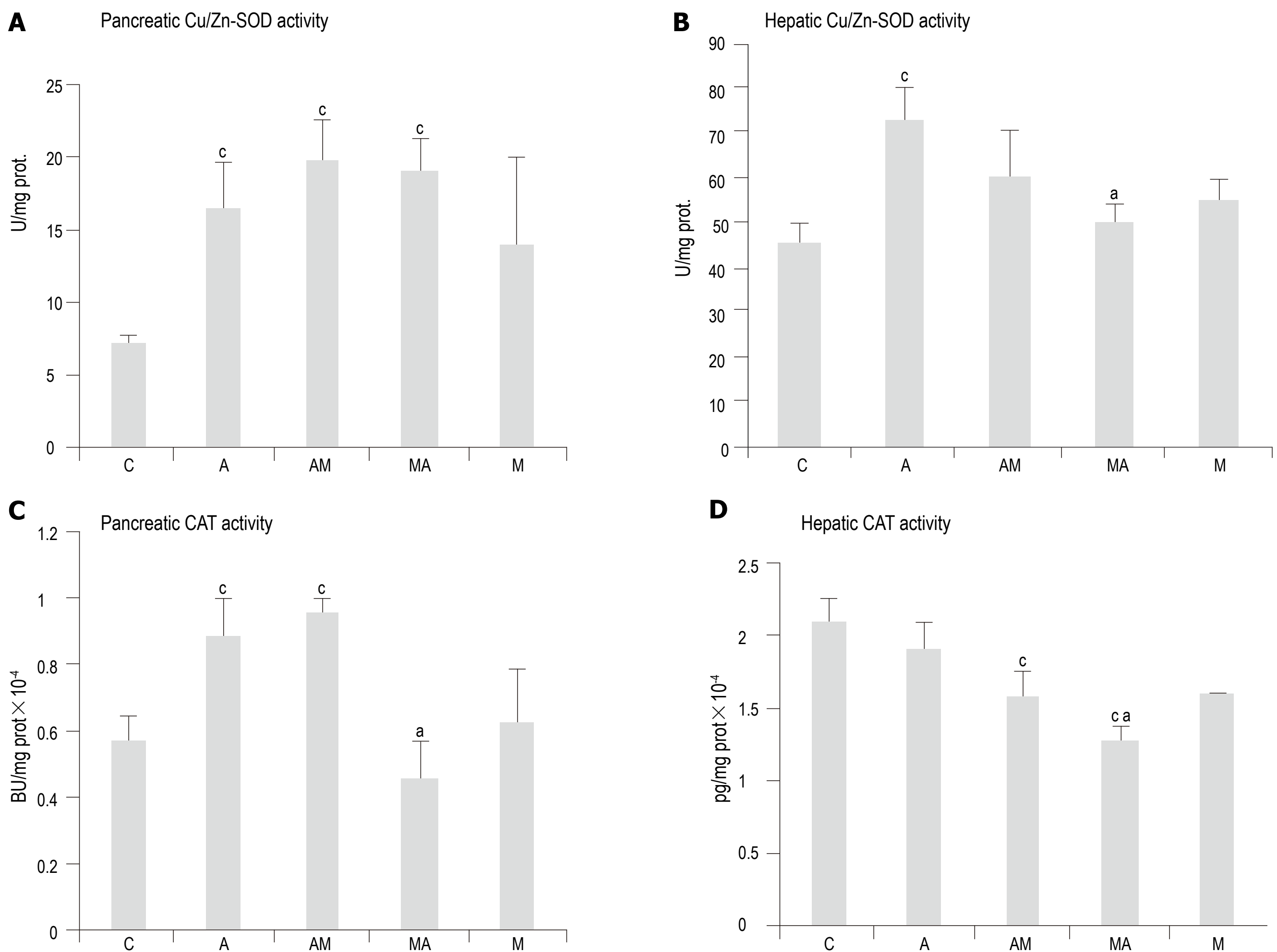

Figure 3 Effects of melatonin (50 mg/kg) treatment on the Cu/Zn-SOD and CAT activities of the pancreas (**A, C**) and liver (**B, D**) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means ± SE of results on 5 animals in each group are shown. ^{a}*P*<0.05 vs group A; ^{c}*P*<0.05 vs group C.

CAT activities were significantly reduced in the rats treated with melatonin before or after L-Arg, whereas in the rats given only L-Arg the CAT activity was unchanged (Figure 3D).

The pancreatic GSH level was not significantly influenced by any of the treatments (Figure 4A). GPx activity was not detectable in the pancreas of the rats in this study. In comparison with the level in the control rats, hepatic GPx activities were significantly elevated in the rats treated only with L-Arg and in the rats given melatonin following L-Arg, but not in the rats pretreated with melatonin (Figure 4B).

The pancreatic MPO activity was significantly increased in the L-Arg-treated rats compared to that in the control rats. This response was reduced by melatonin given before or after L-Arg injections (Figure 4C).

Relative to the level in the control rats, the pancreatic IL-6 concentration was significantly elevated in the L-Arg-injected animals (Figure 4D).

Histological investigation confirmed the development of severe necrotizing pancreatitis in all rats given L-Arg, with no discernible differences between the groups.

DISCUSSION

This study demonstrated the antioxidant effect of melatonin during L-Arg-induced severe necrotizing pancreatitis. The dose of 50 mg/kg melatonin was chosen according to literary data and a pilot-study investigating the effect of different doses of melatonin on the pancreatic weight/body weight ratio and the serum amylase activity [22,28-29]. In contrast to Qi and colleagues who used repeated injections of melatonin, we could detect the beneficial effect of the drug after administration of a single dose in the same order of magnitude[29]. Melatonin beneficially influenced the serum amylase activity, the free radical scavenger enzyme activity in the liver and pancreas as well as the lipid peroxidation level in the liver.

L-Arg-induced pancreatitis is a slowly-developing experimental model in which characteristic laboratory changes are observed 24 h after induction of the disease. By this time, administration of high doses of L-Arg can cause severe necrotizing pancreatitis, as observed in the current study and confirmed by the significant elevation of the serum amylase activity, edema of the pancreas and the increased level of lipid peroxidation marker MDA. The significantly higher MPO activity and the increased amount of pro-inflammatory cytokine IL-6 in the pancreas document the initiation of an inflammatory process in the pancreas.

Elevation of the serum amylase activity is one of the characteristic parameters of acute pancreatitis. The activ-

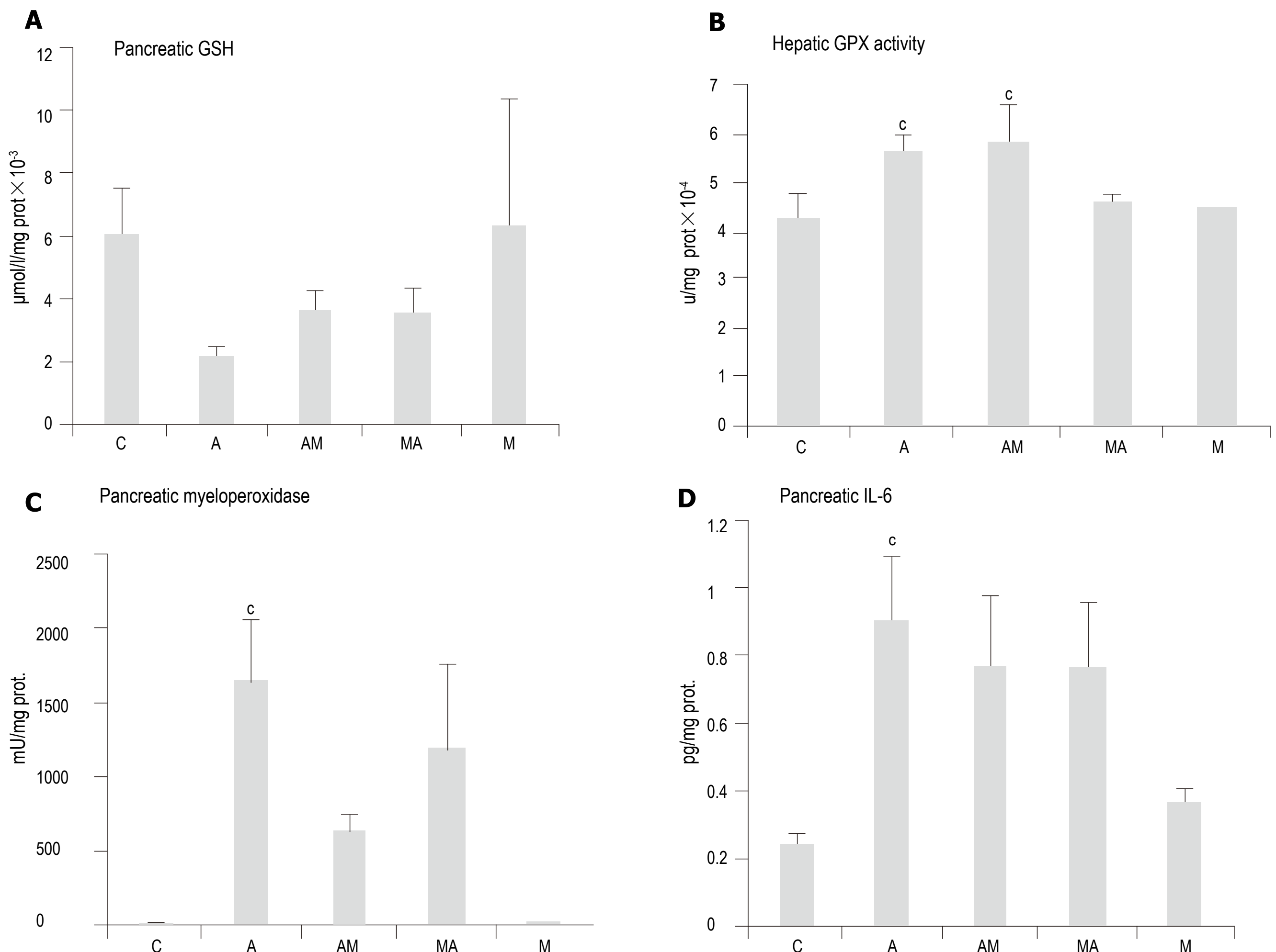

Figure 4 Effects of melatonin (50 mg/kg) treatment on the pancreatic GSH content (**A**), liver GPx activity (**B**), pancreatic myeloperoxidase activity (**C**) and pancreatic IL-6 content (**D**) in L-arginine-induced (2 x 3,2 g/kg) acute pancreatitis. Means ± SE of results on 5 animals in each group are shown. $^aP<0.05$ vs group A; $^cP<0.05$ vs group C.

ity of this enzyme begins to rise 12 h after administration of L-Arg and peaks at around 24 h. At this point, the level of serum amylase activity correlates with the severity of acinar destruction. In our experiment administration of melatonin following the induction of pancreatitis can significantly reduce the activity of this enzyme in serum.

Infiltrating leukocytes and mediators released by these cells are known to play a pivotal role in the amplification of the inflammatory process. One of the inflammatory cytokines that is important in the development of L-Arg-induced pancreatitis is IL-6. In the present study, the level of IL-6 and the MPO activity proved to be significantly elevated in the L-Arg-treated rats. However, the previously reported anti-inflammatory effect of melatonin could not be clearly demonstrated in severe necrotizing pancreatitis [45].

Lipid peroxidation in the pancreas and distant organs is a process mediated by free radicals. Detection of these radicals is difficult because of their high reactivity and short half-life. Accordingly, we measured the activities of free radical scavengers and the degree of lipid peroxidation in order to assess the extent of free radical damage during this inflammatory process. In our experimental setting we did not focus on the detection of early events of acute pancreatitis, such as decreased scavenger enzyme activities and enzyme-activating effects of melatonin, which took place during the first 6-12 h of the disease. We rather wanted to demonstrate the long-lasting effect of a single dose of melatonin on the fully developed illness.

The current study revealed that melatonin pretreatment significantly attenuated the lipid peroxidation in the liver of the rats. Changes in Cu/Zn-SOD, Mn-SOD and GPx activities in the liver were reduced by melatonin, whereas in the pancreas the beneficial effect was less pronounced and manifested only in the changes of CAT activity. In the pancreas, H_2O_2 generated by SOD can serve as a substrate of GPx or CAT. This explains the CAT activity elevation and the lack of GPx activity in the pancreas. The differences observed between the examined organs can be explained by the fact that the basal activities of scavenger enzymes are 10-fold higher in the liver than in the pancreas. The antioxidant effect of melatonin is therefore easier to demonstrate in the liver of animals. These differences in scavenger activities explain why melatonin pretreatment can reduce the lipid peroxidation in the liver, but not in the pancreas. The low scavenger activities are probably one reason for the high mortality associated with acute pancreatitis.

In conclusion, melatonin is able to counteract some of the L-Arg-induced changes in laboratory parameters of

acute pancreatitis. Even a single injection of melatonin can beneficially influence serum amylase activity and lipid peroxidation level in the liver, but can not prevent histological damage to the pancreas. Since melatonin has a very short half-life, repeated injections or continuous infusion may be necessary to develop its full effect. Multiple organ failure is the main reason for pancreatitis-associated mortality. As melatonin reduces hepatic damage caused by L-Arg, it is possible that continuous infusion of the substance may be beneficial in preventing multiple organ damage as a complication of acute necrotizing pancreatitis.

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