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Changes in the morphology of the acinar cells of the rat pancreas in the oedematous and necrotic types of experimental acute pancreatitis

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Limited experimental models of the oedematous and necrotic types of acute pancreatitis provide some understanding of the pathophysiology of this disease. Wistar rats were treated with cerulein at 10 mg/kg of body weight or with L-arginine at 1.5 or 3 g/kg of body weight in order to induce the oedematous or necrotic type of acute pancreatitis. After the induction period we examined samples of pancreata with light and electron microscopes. Morphological examination showed profound changes in the histology of the pancreas and its acinar cells and subcellular structures, especially in the group of rats which received a higher dose of L-arginine, amounting to 3 g/kg body weight. These included parenchymal haemorrhage and widespread acinar cell necrotic changes. 4-OH-TEMPO successfully prevented morphological deterioration as well as amylase release, suggesting that the severity of the two types of disease strongly depends on the intensity of the oxidative stress. Our results lend support to the assumption that reactive oxygen species play an axial role in the pathogenesis of both types of acute pancreatitis.

Key words: cerulein, L-arginine, free radicals, 4-OH-TEMPO

INTRODUCTION

The oedematous form of acute pancreatitis (AP) is a relatively mild disease with a low 5% mortality rate. Necrotic APO, in contrast, exhibits a mortality rate as high as 35%, despite clinical efforts [1]. The pathophysiology of both types of disease and, in particular, the factors which trigger them still remain to be found. An involvement of free radicals in the pathophysiology of pancreatic necrosis has not yet been proved [2, 3]. The aim of this study was to determinate whether 4-OH-TEMPO, a non-peptidyl superoxide dismutase mimic and reactive nitrogen species scavenger, could exert a protective influence on free radical-induced morphological deterioration of the acinar cells in the two types of AP, the oedematous and the necrotic [4].

MATERIAL AND METHODS

All the procedures involving animals were approved by the Local Ethical Committee (LEC) and performed according to the instructions authorised by the LEC.

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A total of 64 male Wistar rats (weight 250–300 g) were divided randomly into 8 groups of 8 animals per group. In Group 1 only the solvent was administrated, Group 2 received 4-OH-TEMPO only, Group 3 received cerulein at 10 μg/kg of body weight and Group 4 cerulein plus 4-OH-TEMPO. Groups 5 to 8 received L-arginine, Groups 5 and 6 receiving 1.5 g/kg of body weight and Group 7 and 8 receiving 3 g/kg of body weight in 0.9% NaCl into the peritoneal cavity. In Groups 6 and 8 this was supplemented with 4-OH-TEMPO before induction of AP.

All the animals were kept at room temperature, in standard humidity and with a 12-hour dark/light cycle. They were fed with the standard diet for laboratory animals and water was given *ad libitum.*

The animals were anaesthetised with vaporised diethyl ether and ketamine (12 mg/kg b.w.) and xylazine (10 mg/kg b.w.) were given intraperitoneally.

The surgical procedure was as follows: the left and right jugular veins were cannulated with silastic tubes for obtaining blood samples and for drug administration. Both catheters were exteriorised at the nape of the neck through a subcutaneous tunnel. The catheters were flushed with 0.5 ml of heparinised saline (10 heparin units) after injection. The rats were allowed to recover for 24 hours with free access to water. Continuous i.v. infusion of physiological saline with or without cerulein (Sigma) 10 μg/kg/h was conducted using a peristaltic pump at a flow rate of 1 ml/h.

Blood samples from at least three cases were collected from the hearts to determine amylase activity, a BM-Hitaschi 917 Automatic Analyzer a-amylase system (Boehringer Mannheim) being used. For each experimental group descriptive statistics were calculated and analysed as mean values with standard deviation ($\pm$SD). The alpha-level was set at 0.05 for statistical significance. Each pancreas was quickly removed and weighed. Small tissue probes were excised from the pancreatic head for light and electron microscopic examinations. Pancreas oedema was measured by estimation of the ratio of organ weight per 100 g of body weight. For light microscopy tissue was fixed in 10% formalin solution and embedded in paraffin. Histological sections were stained with haematoxylin and eosin (H & E). For electron microscopy small specimens of pancreatic tissue were placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 hours and then post-fixed in 2% osmium tetroxide for 1 hour. After dehydration in ascending concentrations of alcohol and propylene oxide the specimens were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM 1200EX II electron microscope (Japan).

RESULTS

The present paper provides the morphological characterisation of the oedematous and necrotic types of experimental acute pancreatitis (AP) caused by cerulein and L-arginine injections respectively. Pancreata from control Group 1 and 4-OH-TEMPO Group 2 showed no pathomorphological changes in the acinar cells (Fig. 1A, 2A and B).

In Group 3, treated with cerulein, the characteristics of oedematous acute pancreatitis were observed microscopically. This type of AP included focal vacuolisation of the acinar cells and leucocyte infiltration of the stroma (Fig. 1B). The lobular structure of the pancreas was well preserved after a protective injection of 4-OH-TEMPO before cerulein (Fig. 1C). The electron microscopic observations revealed changes in the cytoplasm of the acinar cells such as a moderately dilated rough endoplasmic reticulum and the appearance of various vacuoles and megavacuoles with myelin figures inside them (Fig. 3A). In the mitochondria (Fig. 3B) the cristae were better preserved than those in the rats that received L-arginine. In Group 4 4-OH-TEMPO was found to have a highly protective effect on the ultrastructure of the acinar cells in AP (Fig. 4).

Because the inflammation process in the pancreas follows very fast, a smaller dose of L-arginine (1.5 g/kg b.w.) was used to inspect the course of morphological changes in the pancreas and its acinar cells (Group 5). The changes responded to focal necrosis. Microscopically the pancreata showed focal oedema and acinar cell vacuolisation (Fig. 1D) but normal structure of the acinar cells following the protective effect of 4-OH-TEMPO (Fig. 1E). The electron microscopic observations of the pancreatic cells revealed greater changes in the cellular organelles than in Group 3. The mitochondrial damage manifested by their swelling was associated with disintegration of the mitochondrial cristae (Fig. 5A). More advanced changes within the mitochondria included the formation of myelin figures from their cristae (Fig. 5B). The mitochondrial matrix was partially rinsed but the external mitochondrial membrane was usually well preserved (Fig. 6A). The rough endoplasmic reticulum was dilated in the form of vesicles of various sizes. Among these were found numerous free ribosomes and polyribosomes. Myelin figures were also formed from damaged cister-

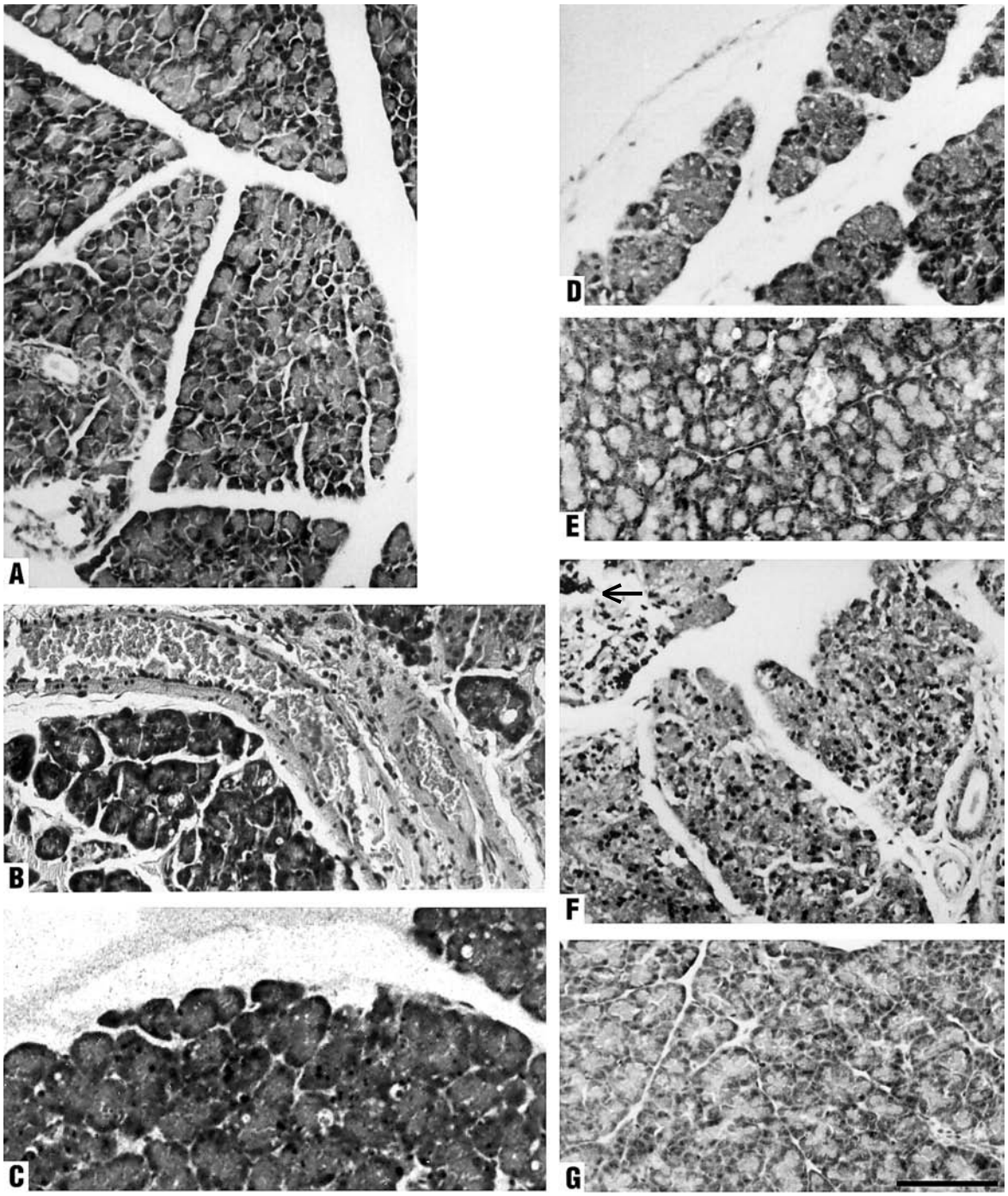

Figure 1. Microphotographs of the pancreatic tissue after intraperitoneal injection of: **A.** 0.9% NaCl (control group) or 4-OH-TEMPO. Normal structure of pancreatic tissue. **B.** 10 μg/kg b.w. of cerulein. Vacuolisation of acinar cells and leucocyte infiltration of stroma. **C.** 10 μg/kg b.w. of cerulein. Protective effect of 4-OH-TEMPO. Acinar cells are well preserved with a discreet vacuolisation. **D.** 1.5 g/kg b.w. of L-arginine. Vacuolisation of acinar cells is visible. **E.** 1.5 g/kg b.w. of L-arginine. Protective effect of 4-OH-TEMPO. Acinar cells are well preserved and without vacuolisation. **F.** 3 g/kg b.w. of L-arginine. Complete damage of lobular structure of pancreatic tissue with disintegration of the acinar cells. Arrow indicates focal necrosis. **G.** 3 g/kg b.w. of L-arginine. Protective effect of 4-OH-TEMPO. The structure of the pancreatic tissue is well preserved. H & E staining. Bar: 100 μm.

nae of the rough endoplasmic reticulum (Fig. 5C). We observed, in keeping with lysosome activation, the formation of autophagosomes from a fusion of zymogen granules with lysosomes (Fig. 6B, 7A). These structures contained fragments of cellular organelles and often myelin figures consisting of smooth membranes lying in a concentric formation. In their vicinity clusters of lipid vacuoles also appeared (Fig. 7A).

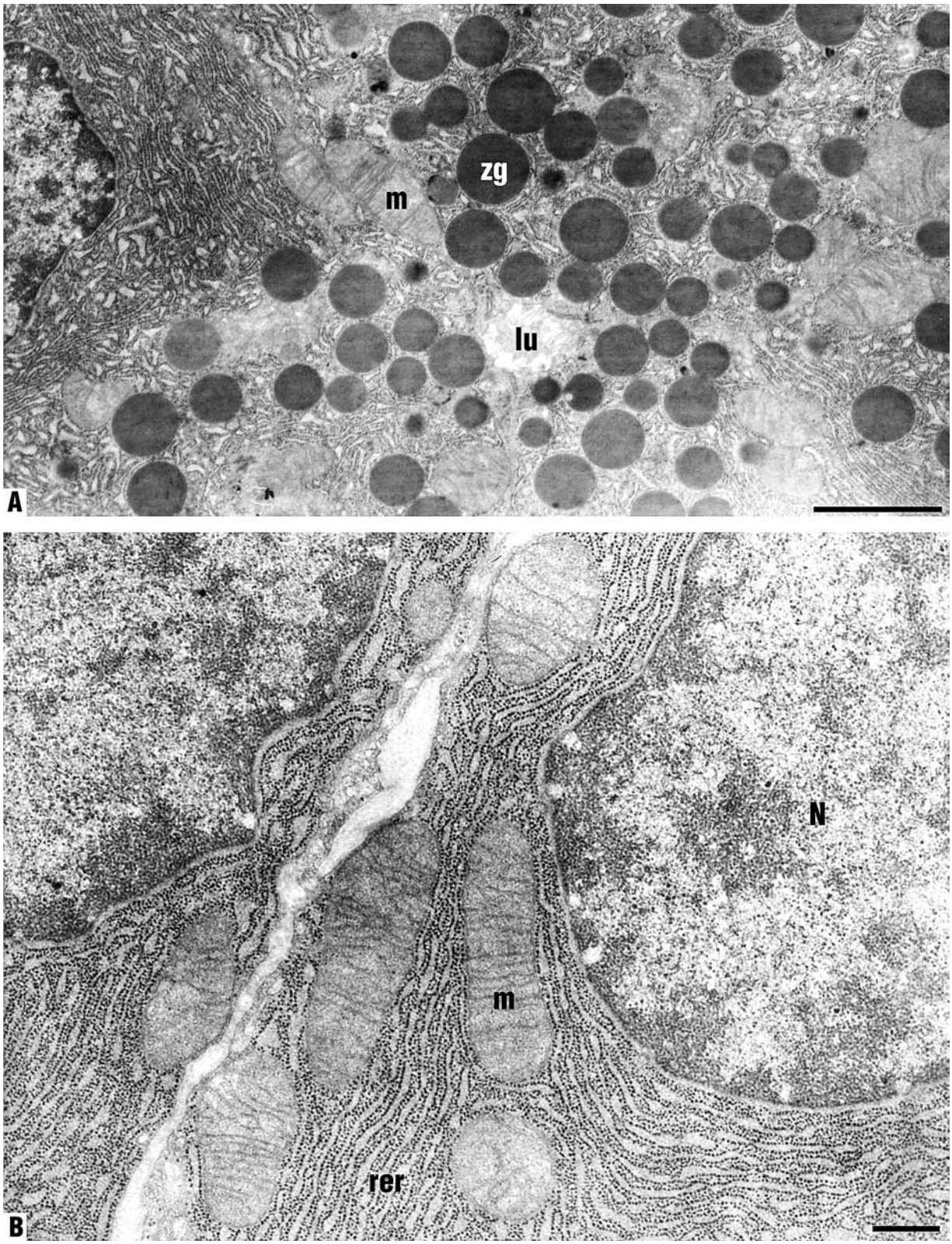

Figure 2. Electron micrographs from control rat: **A.** Numerous zymogen granules (zg) in association with mitochondria (m) are situated at the apical pole of the acinar cell. The lumen (lu) of a pancreatic canaliculus contains microvilli of acinar cells. Bar: 2 μm. **B.** Parts of two acinar cells separated by a narrow interstitial space. Normal appearance of rough endoplasmic reticulum (rer), mitochondria (m) and nucleus (N). Bar: 500 nm.

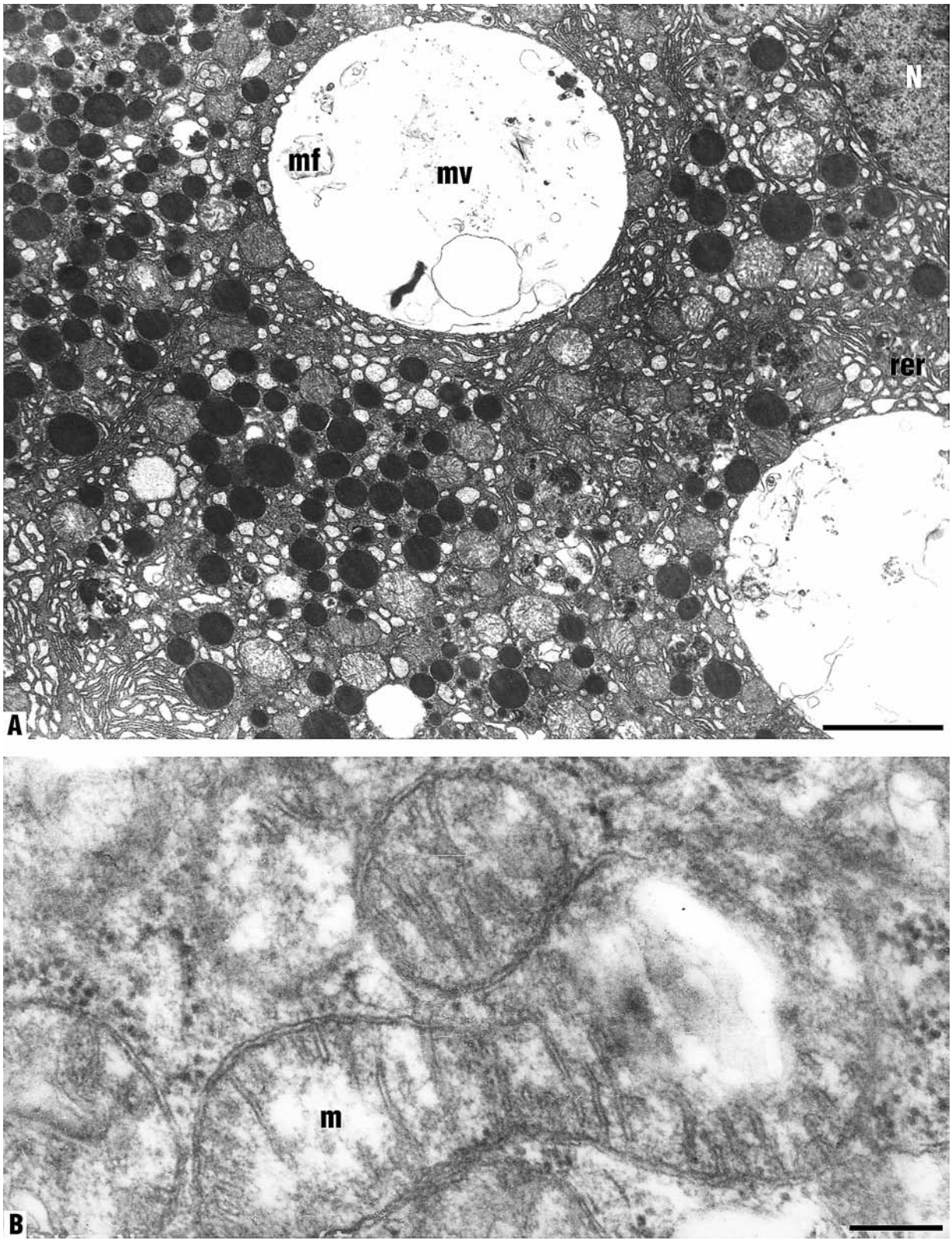

Figure 3. Electron micrographs of the acinar cells after 10 μg/kg b.w. of cerulein: **A.** Dilated cisternae of fragmented rough endoplasmic reticulum (rer) and megavacuoles (mv) with forming myelin figures (mf) inside them and numerous zymogen granules (zg); nucleus (N). Bar: 2 μm. **B.** Accumulation of mitochondria (m) with partially damaged cristae Bar: 500 nm.

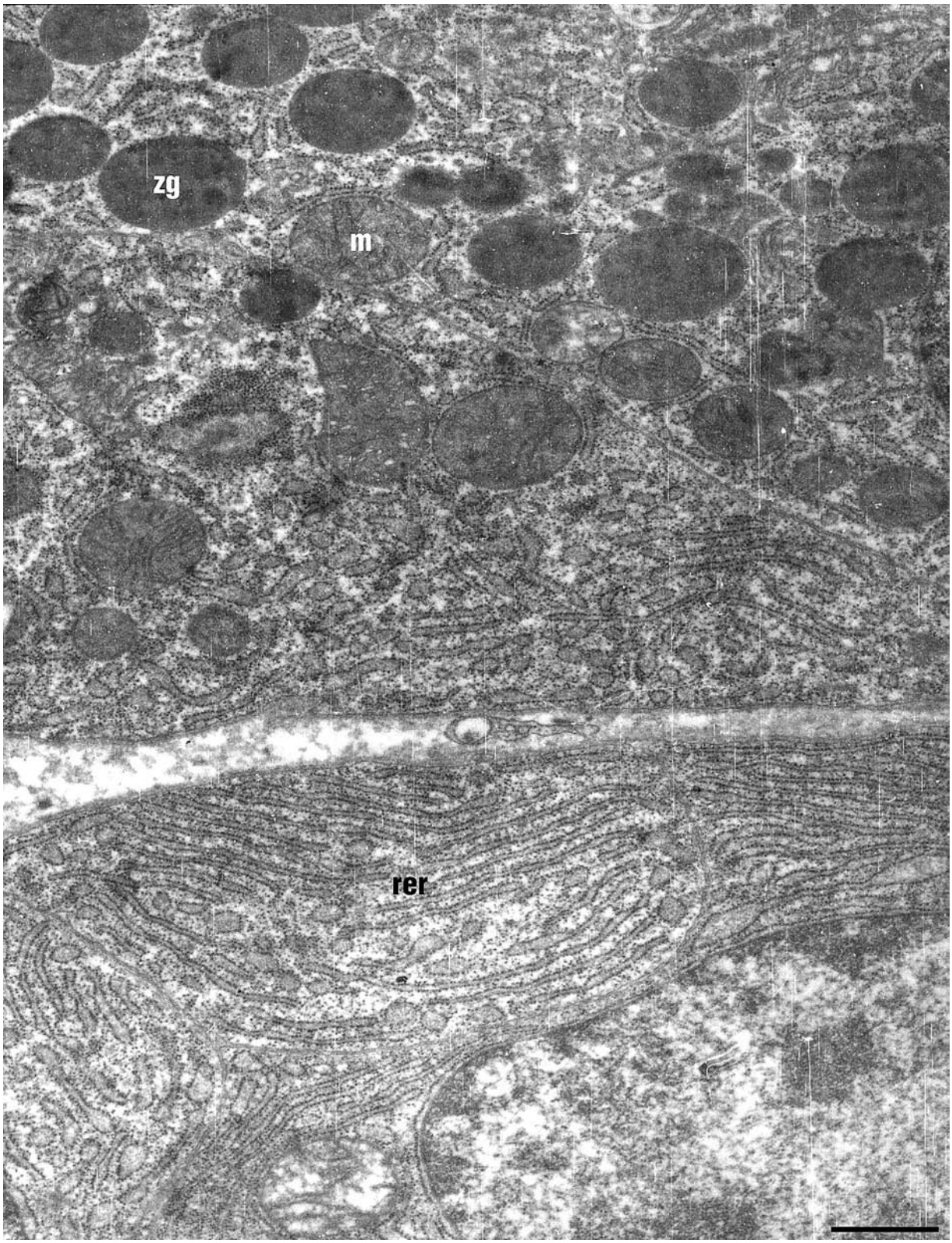

Figure 4. Rat receiving 10 μg/kg b.w. of cerulein. Protective effect of 4-OH-TEMPO. Well preserved rough endoplasmic reticulum (rer), mitochondria (m) with unchanged cristae; zymogen granules (zg). Bar: 1 μm.

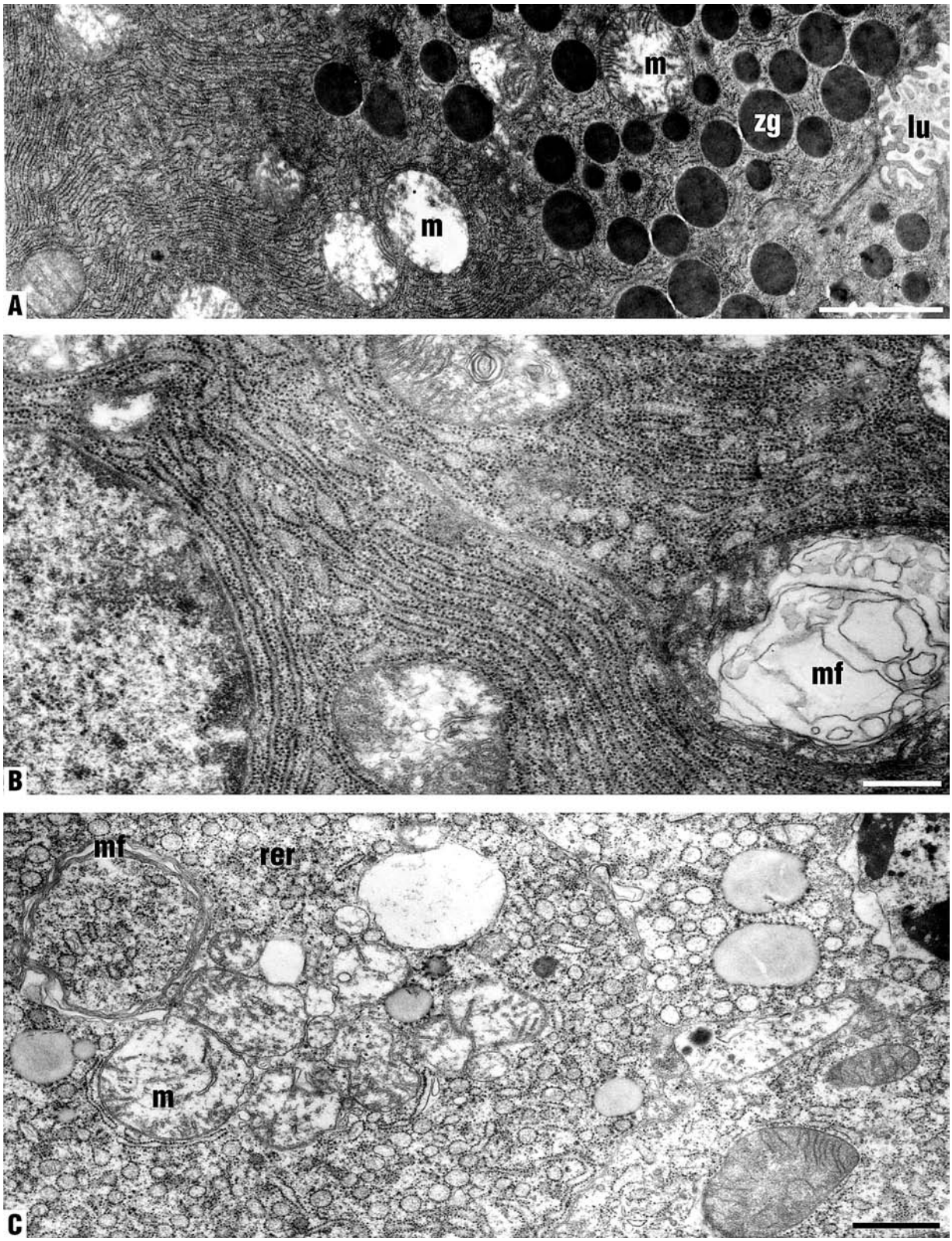

Figure 5. Rat receiving 1.5 g/kg b.w. of L-arginine: **A.** Various stages of mitochondria (m) damage: some have partly preserved cristae or their debris, others are devoided cristae; the matrix is rinsed. Numerous zymogen granules (zg) are near the pancreatic canaliculus. Note the microvilli in its lumen (lu) Bar: 2 μm. **B.** Various stages of myelin figure (mf) formation within mitochondrial matrix Bar: 500 nm. **C.** Myelin figure (mf) formation from damaged cisternae of rough endoplasmic reticulum (rer). In the vicinity an accumulation of damaged mitochondria (m). Bar: 1 μm.

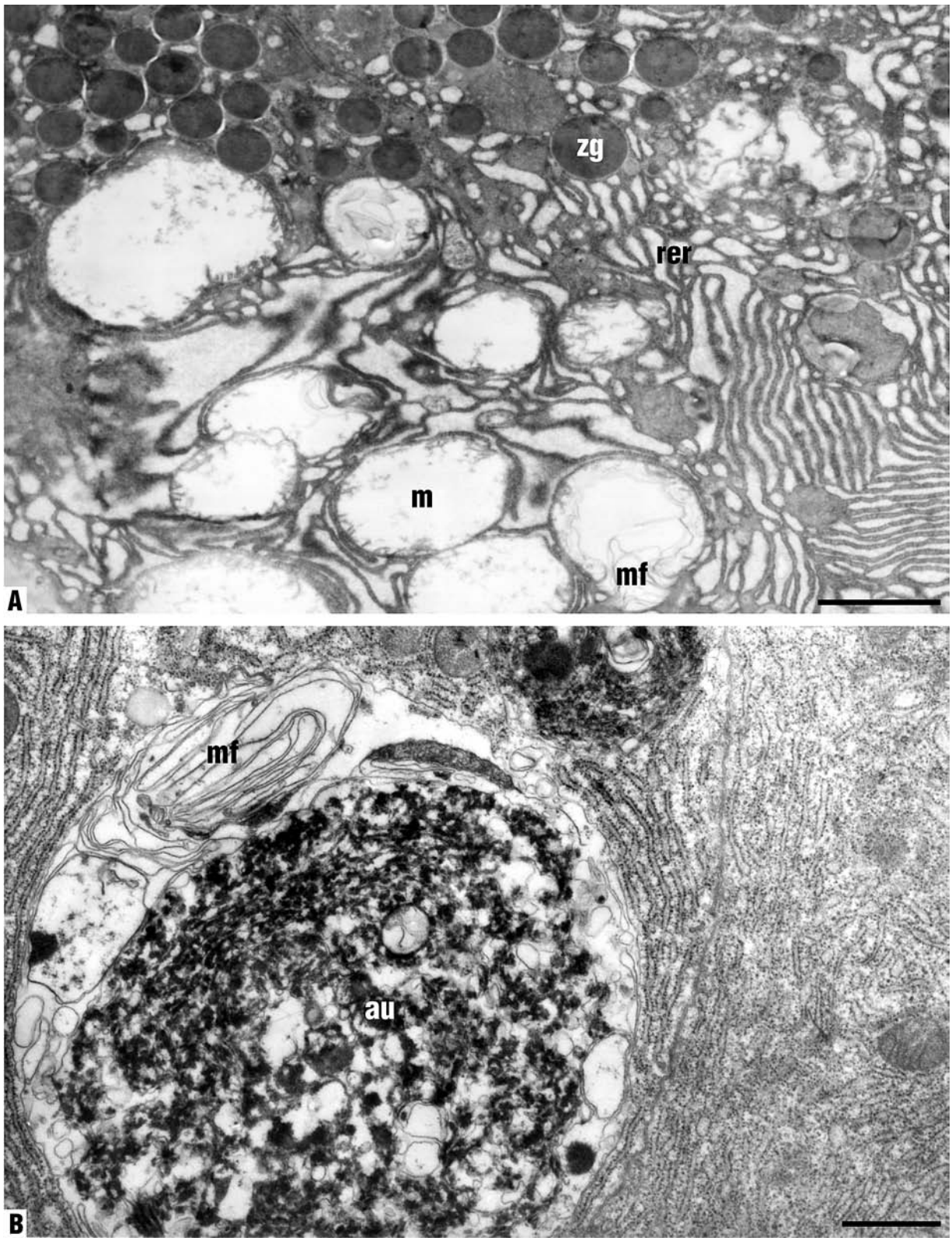

Figure 6. Rat receiving 1.5 g/kg b.w. of L-arginine: **A.** Rat receiving 1.5 g/kg b.w. of L-arginine. Intracellular lytic necrosis with accumulation of mitochondria (m) containing debris of mitochondrial cristae and myelin figures (mf) forming from them; disorganised rough endoplasmic reticulum (rer); zymogen granules (zg). Bar: 2 μm. **B.** Autophagosome (au) with myelin figure (mf) localised at its periphery. Bar: 1 μm.

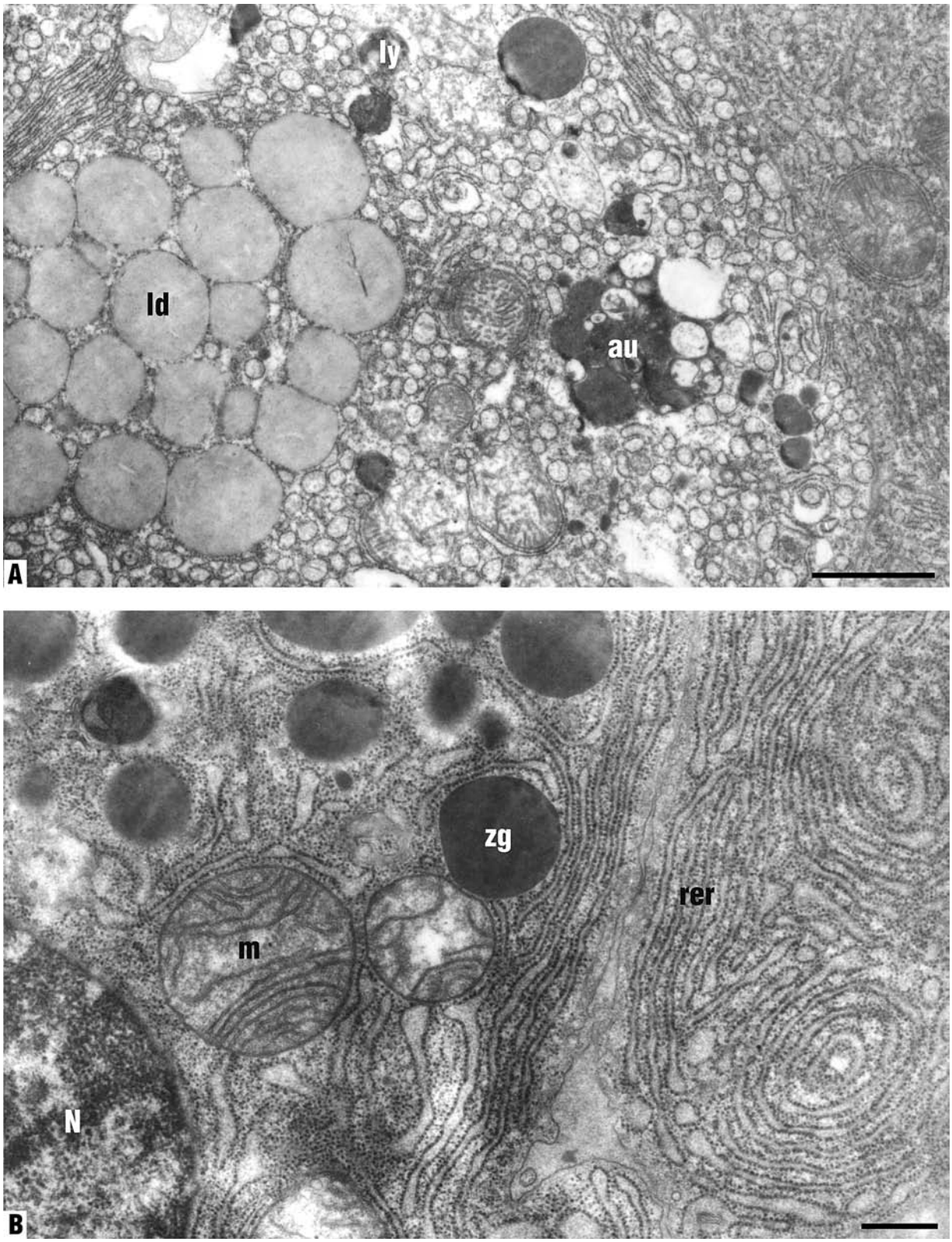

Figure 7. A. Rat receiving 1.5 g/kg b.w. of L-arginine. Earlier stage in the formation of the autophagosome (au). Lipid droplets (ld). Lysosome (ly). Bar: 500 nm. **B.** Rat receiving 1.5 g/kg b.w of L-arginine. Protective effect of 4-OH-TEMPO. Fragment of acinar cell filled with unchanged mitochondria (m), rough endoplasmic reticulum (rer), numerous zymogen granules (zg) and nucleus (N). Bar: 500 nm.

The alternated organelles were frequently separated by a membrane from the rest of the cytoplasm. These changes responded to focal necrosis. Rats receiving 4-OH-TEMPO before injection of L-arginine showed unchanged cytoplasmic organelles (Fig. 7B).

In Group 6, which received a higher dose of L-arginine (3 g/kg b.w.), the morphological changes in the pancreas were diffused and more advanced than in the animals who received smaller doses of L-arginine. They had the appearance of necrotising AP with wide destruction of the acinar cells (Fig. 1F). The protective effect of 4-OH-TEMPO was observed (Fig. 1G). Ultrastructurally the mitochondria were swollen with a loosened or rinsed matrix. The mitochondrial cristae were preserved only focally (Fig. 8A). The rough endoplasmic reticulum was completely disorganised. Their cisternae were distended and fragmented in the form of vesicles of various sizes (Fig. 8B). The number of free ribosomes increased between the changed cisternae of the reticulum. A characteristic feature of this type of necrosis was the appearance of numerous myelin figures in the

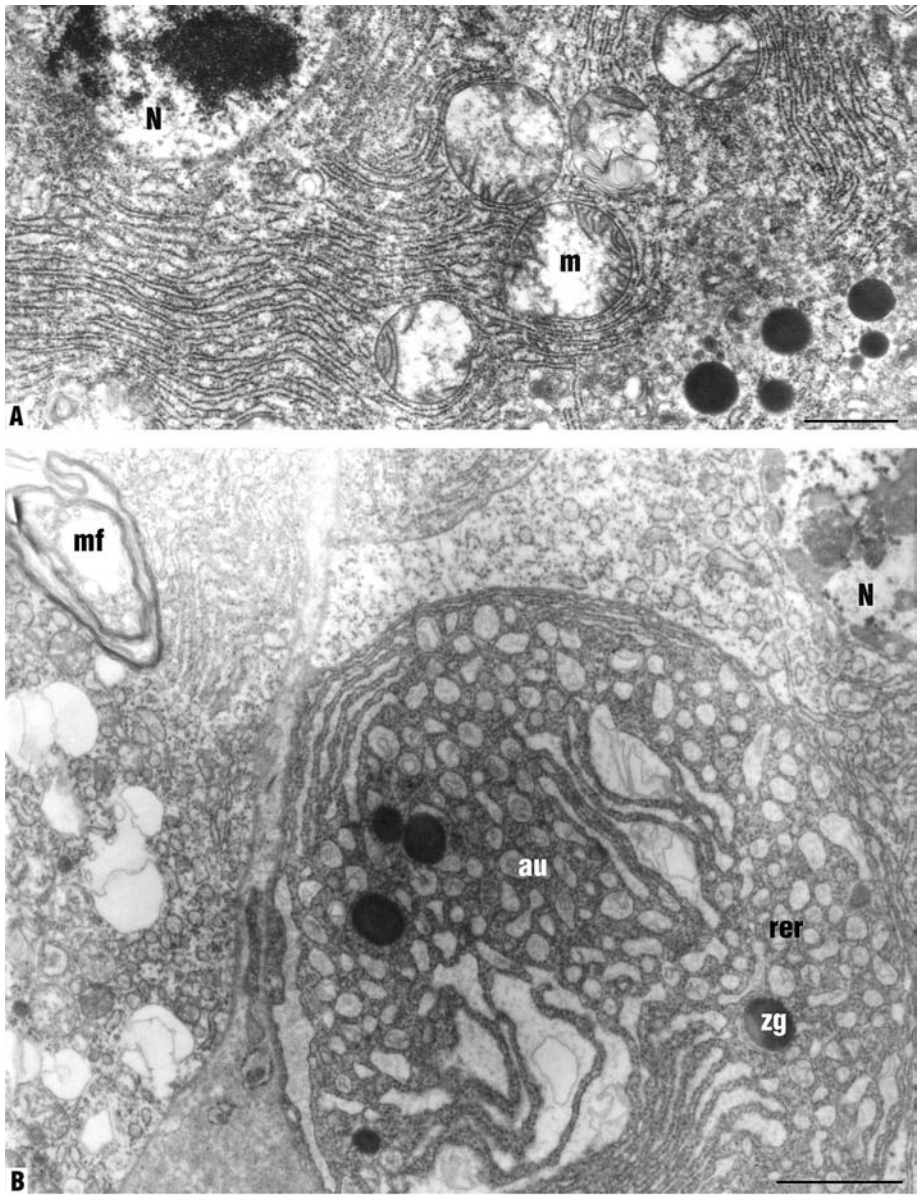

Figure 8. Electron micrographs from a rat receiving 3 g/kg b.w. of L-arginine: **A.** Cytoplasm of acinar cell containing mitochondria (m) with peripherally visible remnants of cristae and focally rinsed matrix. Part of the nucleus (N) containing clumps of condensed chromatin. Bar: 1 μm. **B.** Large autophagosome (au) filled with fragments of rough endoplasmic reticulum (rer) and single zymogen granules (zg). There is a typical myelin figure (mf) inside the bordering cell with lytic necrosis. Nucleus (N) with clumps of condensed heterochromatin and electron-dense granules. Bar: 2 μm.

cytoplasm of the changed cells (Fig. 8B) as well as in the karyoplasm. However, the complete destruction of cytoplasmic organelles predominated. These changes were linked with the activation of lysosomes and the formation of large autophagosomes (Figs. 8B, 9A). The nuclei were often deformed and shrunken with chromatin assembled below the nuclear envelope (Fig. 8A, 9B). After the protective injection of 4-OH-TEMPO well preserved rough endoplasmic reticulum, mitochondria with unchanged cristae and zymogen granules were observed (Fig. 9B).

Pancreatic morphology has been found to correlate with greater activity of serum amylase (Fig. 10). The activity of serum amylase was found to be 7 times greater in animals treated with L-arginine at a dose of 3 g/kg of body weight than in the control group. This clearly demonstrates lesion of the pancreatic cells. The lower dose of L-arginine, amounting to 1.5 g/kg of body weight, also increased the activity of serum amylase, but not as significantly as the previous dose. This suggests that the degree of lesion of the pancreas depends of the dose of L-arginine.

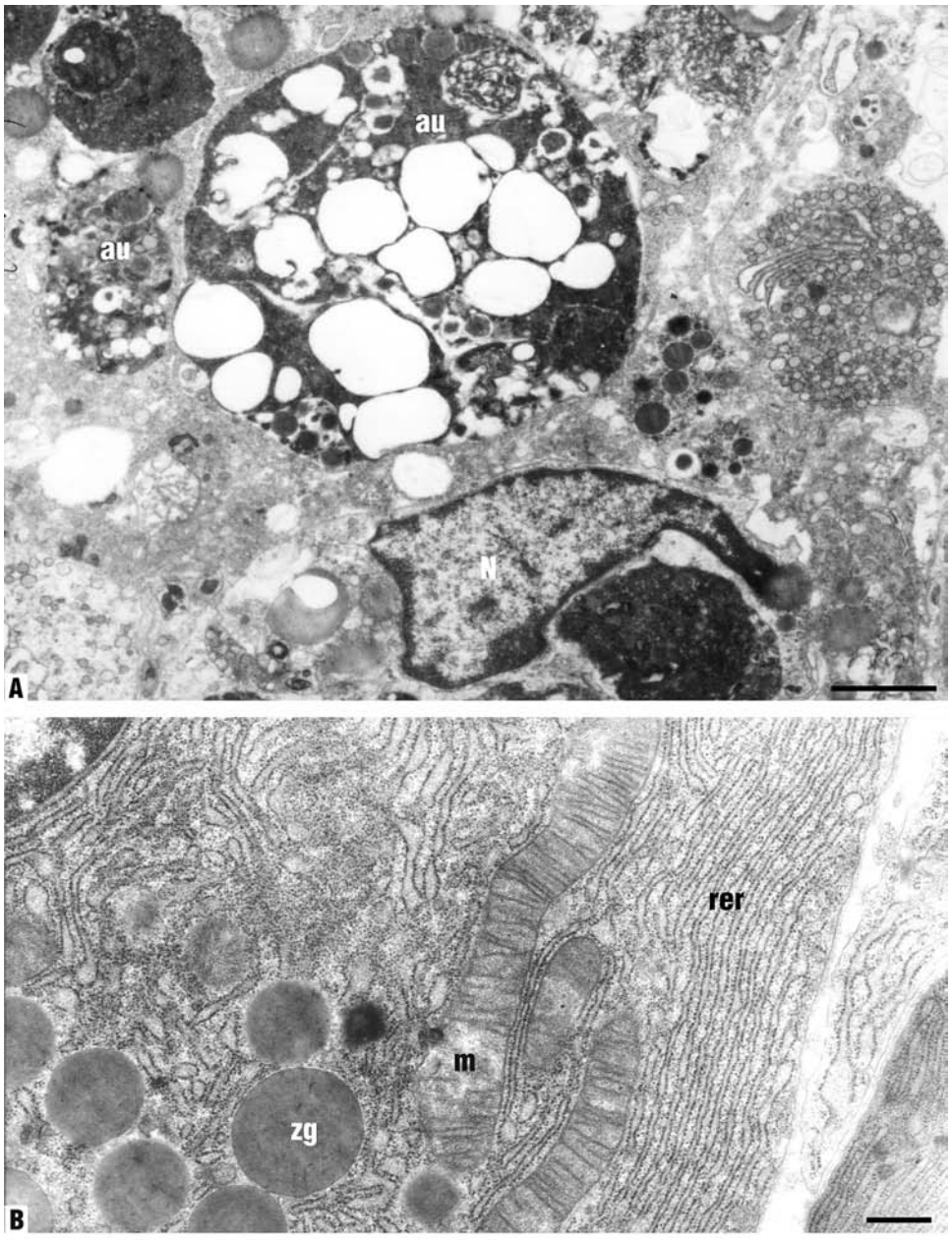

Figure 9. A. Rat receiving 3 g/kg b.w. of L-arginine: electron micrograph shows necrotic AP with forming autophagosomes (au). The structure of the cytoplasm is blurred. The nucleus (N) is deformed. Bar: 2 μm. **B.** Rat receiving 3 g/kg b.w. of L-arginine: Protective effect of 4-OH-TEMPO. Rough endoplasmic reticulum (rer), mitochondria (m) and zymogen granules (zg) show no significant changes. Bar: 500 nm.

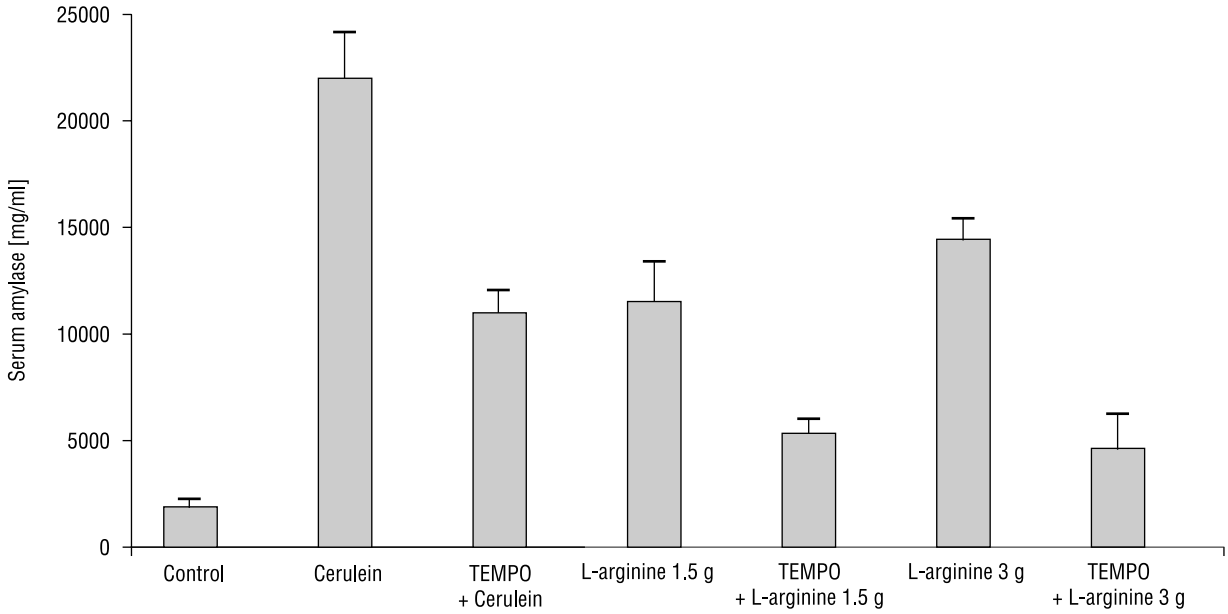

Figure 10. Activity of serum amylase in rats treated with cerulein or L-arginine (Mean ± SD).

Cerulein given intravenously greatly increases the activity. We can observe the protective effect of the scavenger, 4-OH-TEMPO, which is direct proof of free radical activity in the pathogenesis of acute pancreatitis in these two experimental models.

DISCUSSION

Free radicals are highly reactive short-lived species displaying an unpaired electron in their outer orbital. Most radicals of biological importance come from oxygen and nitrogen, giving rise to the terms "reactive oxygen" and "reactive nitrogen" species or intermediates (ROS/RNS or ROI/RNI) [5]. 4-OH-TEMPO, a low molecular weight non-peptidyl SOD mimic, has been found to prevent lipid hydroperoxide formation with mild cerulein pancreatitis [6]. Because the involvement of free radicals in the pathogenesis of pancreatic necrosis is still an open question, we decided to inspect carefully the morphological changes to the pancreatic acinar cells in rats exposed to cerulein as an inducer of oedematous pancreatitis and L-arginine as an inducer of pancreatic necrosis in order to establish the progression of morphological alternations of the pancreas during AP as well as the possible development of early pancreatic tissue damage when the well defined ROS and RNS scavenger 4-OH-TEMPO was administrated as a prophylactic and as a drug confirming free radical participation.

Our results confirm the participation of free radicals in the development of both the oedematous and the necrotic types of AP. 4-OH-TEMPO administered before induction of oedematous and necrotising acute pancreatitis exerts a beneficial effect, alleviates the course of the disease and is helpful in establishing their significant prognostic role during clinical acute pancreatitis.

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