Free Radical Research, April 2008; 42(4): 289–296

informa
healthcare

The contradictory effects of nitric oxide in caerulein-induced acute pancreatitis in rats

FERAL OZTURK[1], MEHMET GUL[1], MUKADDES ESREFOGLU[1], & BURHAN ATES[2]

[1]*Department of Histology and Embriyology, and* [2]*Department of Chemistry, Inonu University Science and Art Faculty, 44280 Malatya, Turkey*

Accepted by Professor F. Kelly

(Received 17 July 2007; in revised form 28 December 2007)

Abstract
This study was planned to observe the effects of nitric oxide synthesis on the antioxidative defense enzymes and pancreatic tissue histology in caerulein-induced acute pancreatitis. Acute pancreatitis was induced by intraperitoneal injections of 50 μg/kg caerulein, L-arginine used for NO induction and N^{ω}-nitro-L-arginine methyl ester (L-NAME) used for NO inhibition. In the caerulein group acinar cell degeneration, interstitial inflammation, oedema and haemorrhage were detected. Pancreatic damage scores were decreased with both NO induction and inhibition ($p < 0.05$). MDA, GSH-Px, CAT, GSH and SOD activities were significantly changed in the caerulein group and indicated increased oxidative stress. Both NO induction and inhibition decreased this oxidative stress. It is concluded that both nitric oxide induction and inhibition ameliorated caerulein-induced acute pancreatitis. The findings indicate that a certain amount of NO production has beneficial effects in experimental acute pancreatitis, but uncontrolled over-production of NO may be detrimental.

Keywords: *Acute pancreatitis, nitric oxide, oxidative stress, light, electron microscopy*

Introduction

Acute pancreatitis is an inflammatory disease resulting from the activation of proteolytic and lipolytic pancreatic enzymes and their liberation into the pancreatic interstitium. Normally the pancreas is protected from self-destruction. However, a variety of causes such as alcohol, drugs or virusus can lead to aciner cell injury and spoil this protection [1,2]. The severity of pancreatitis varies from mild to severe and incapacitating. Many of the systemic features of severe acute pancreatitis can be attributed to the release of proteolytic enzymes, cytotoxic and inflammatory substances, reactive oxygen species, cytokines and other mediators into the circulation and explosive activation of the systemic inflammatory response [1,3–7].

Nitric oxide (NO) is a soluble gas and an important chemical mediator generated by endothelial cells, macrophages, neurons and other cell types. NO acts in a paracrine manner on target cells. It can act as a free radical and exert beneficial effects on the body. However, high amounts of NO can react with concomitantly produced superoxide anions, which thereby generate highly toxic compounds, such as peroxynitrate and hydroxyl radicals. NO is synthesized from L-arginine, by the enzyme nitric oxide synthase (NOS). There are three different types of NOS, endothelial (eNOS), neuronal (nNOS) and cytokine inducible (iNOS) [8,9]. eNOS and nNOS, also called constitutive NOS (cNOS), are constantly present and synthesize small amounts of NO in response to physical or receptor stimulation. iNOS in contrast is induced when macrophages are

Correspondence: Feral Ozturk MD, Department of Histology and Embriyology, Inonu University Medical Faculty, 44280 Malatya, Turkey. Tel: +90-422-3410660/1305. Fax: +90-422-3410036. Email: fozturk@inonu.edu.tr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760801930730

activated by cytokines such as tumour necrosis factor α (TNF-α), interferon γ (IFN-γ) or other agents and generates large amounts of NO in a sustained and largely uncontrolled manner [8–11]. An enhanced formation of NO due to the induction of iNOS has been implicated in the pathogenesis of inflammation [8,11]. The role of NO in pathogenesis of acute pancreatitis remains controversial. Some studies suggest that NO produced by iNOS from activated macrophages acts as toxic reactive oxygen species when combined with superoxide anion to form peroxynitrate [12,13], whereas others report that NO acts as a biological scavenger and inactivates reactive oxygen species, it protects pancreatic acinar cells [14,15] and has also beneficial effects by increasing pancreatic microvascular blood flow [16].

This study was planned to investigate the role of NO and its contradictory effects in caerulein-induced acute pancreatitis. For this purpose both NO induction and inhibition were applied to the caerulein-treated rats. Pancreas histology and the oxidative stress markers of the pancreatic tissue were examined.

Materials and methods

Animals and experimental groups

Twenty eight adult female Sprague-Dawley rats weighing 230–280 g were used in the study. Animals were fed with standard rat chow and tap water *ad libitum* for 10 days and randomly divided into four groups of seven each. The groups were as follows: control group, caerulein group, caerulein + L-arginine group and caerulein + L-NAME group. Caerulein, L-arginine and L-NAME were dissolved in 0.3 ml of 0.9% saline and all injections were performed intraperitoneally. Control group rats received two injections of 0.3 ml of 0.9% saline at 2-h intervals. Caerulein group rats were injected with 50 μg/kg caerulein twice at 2-h intervals to induce acute pancreatitis. In the caerulein + L-arginine group, NO induction was performed with 160 mg/kg L-arginine 15 min before the induction of acute pancreatitis with caerulein. N^{ω}-nitro-L-arginine methyl ester (L-NAME) was used for NO inhibition; 10 mg/kg L-NAME injected to caerulein + L-NAME group rats 15 min before caerulein injections. The rats were killed by decapitation 12 h after the last injections. All chemicals were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany).

After decapitation, the trunk blood was collected and centrifuged. An automated analyser (Olympus AU 600, Diamond Diagnostics, Holliston, MA) was used for the spectrophotometric measurements of serum amylase and lipase levels.

The pancreas was rapidly removed and divided into three pieces. One group of samples were placed in 10% phosphate buffered formalin and processed for light microscopic examination. The second group of samples were processed for ultrastructural examination. The last group of pancreas samples were stored at -80°C for the determination of malondialdehyde (MDA), glutathione peroxidase (GPx), catalase (CAT), total glutathione (GSH) and superoxide dismutase (SOD).

All experiments in this study were performed in accordance with the guidelines for animal research from the National Institute of Health and approved by the Comittee on Animal Research at Inonu University, Malatya.

Histologic examinations

Paraffin embedded specimens were cut into 5 μm sections and stained with haematoxylin-eosin (H-E) and Masson's trichrome stainings. Sections were examined by a Leica DFC 280 light microscope and Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, UK) by an experienced observer unaware of the animal treatment groups. Ten sections for each rat and four fields for each section were examined. All grading procedures were carried out according to Um et al. [11]. Histologic grading of interstitial inflammation was scaled from 0–3 (minimal to maximal, respectively). The grading of haemorrhage and acinar cell necrosis was based on the percentage contained in the examined area. The samples were graded as follows: absence of lesion = 0; involvement of 1–10% = 1; 11–25% = 2; 26–50% = 3; > 50% = 4.

For transmission electron microscopic evaluation the samples were placed into 3% glutaraldehyde buffered with 0.2 M $NAH_2PO_4 + NaHPO_4$ (pH 7.2–7.3) and post-fixed with 2% osmium tetroxide (OsO_4) and embedded in Araldite CY 212. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss Libra 120 (Carl Zeiss NTS GmBH. Oberkochen, Germany) transmission electron microscope.

Biochemical determination

Pancreas samples stored for enzyme analysis were homogenized in phosphate buffered saline (1/5)(w/v). Homogenates were divided into two pieces. One piece was directly used for MDA measurement. The second was sonicated four times for 30 s with 20 s intervals between each sonication period, using a VWR Branson Scientific sonicator (VWR Int. Ltd, Merch House Pool, UK) and centrifuged at 17 000 rpm ($r = 6.88$ cm) for 15 min using a Beckman L8-70M ultracentrifuge. The supernatants were seperated and kept at -40°C until the enzyme activity measurements were performed. The temperature was kept at 4°C throughout the preparation of homogenates and supernatants. A Shimadzu 1201 UV spectrophotometer was used for all spectrophotometric assays. GSH-Px activity was measured

according to Lawrence and Burk [17], CAT activity was measured in supernatants by the method of Luck [18], SOD activity was measured in the supernatant fraction using xanthine oxidase/cytochrome c method according to McCord and Fridovich [19]. GSH was determined by utilizing a commercial GSH as standard.

The level of MDA in tissue homogenates was determined using the method of Uchiyama and Mihara [20].

Statistical analysis

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., Chicago, IL). Results were expressed as arithmetic mean ± standard deviation. Results were statistically analysed by the Kruskal Wallis H-test and were considered significant when $p < 0.05$. The differences between the groups were evaluated by Mann-Whitney U-test.

Results

The pancreatic tissue of the control group rats presented normal histology (Figure 1A). The pancreas sections of the caerulein group showed histologic alterations such as oedema and interstitial inflammation (Figure 1B), haemorrhage (Figure 1C) and acinar cell degeneration (Figure 1D). All changes were widespread and severe.

In the caerulein + L-arginine and caerulein + L-NAME groups, pancreas sections showed histologic alterations. Pancreatic damage scores are summarized in Table I. In the caerulein + L-arginine group acinar cell degeneration were sparse and few acinus were involved. Interstitial inflammation and oedema were rarely detected. There wasn't any evidence of haemorrhage (Figure 1E).

Caerulein + L-NAME group pancreas sections showed rare acinar cell degeneration, inflammation, oedema and haemorrhage (Figure 1F). All the changes were limited in localized areas. Endocrine pancreas showed normal histology in all groups.

The ultrastructure of the acinar cells was not altered in the control group. The acinar cells in the caerulein group displayed dilated and irregular arranged granular endoplasmic reticulum (GER) cisterns. Golgi apparatus cisterns were also dilated. Many mitochondria were swelled and showed myelin figures. Some mitochondria showed partial or complete crista loss (Figure 2A and B). Numerous and large autophagosomes containing zymogen granules and cytoplasmic elements were present in the cytoplasm (Figure 2C). In the caerulein + L-arginine group most of the acinar cells showed normal GER and Golgi apparatus structure. Rarely acinar cells with dilated and irregular GER cisterns were observed. Some of the cells showed mitochondrial swelling, crista loss and myelin figures. Autophagosomes were small and sparse (Figure 2D). In the caerulein + L-NAME group the ultrastructure of acinar cells were close to control group. Only mitochondrial changes were detected such as partial crista loss and slight swelling (Figure 2E).

The results of the statistical evaluation of pancreatic tissue MDA, GSH-Px, CAT, GSH and SOD activities (means ± SD) are shown in Table II. In the caerulein-treated group MDA levels were increased ($p < 0.05$), GSH-Px, CAT, GSH and SOD activities were decreased significantly ($p < 0.05$) when compared to the control group. MDA levels were decreased in the caerulein + L-arginine and caerulein + L-NAME groups when compared to the caerulein group ($p < 0.05$). GSH-Px, CAT, GSH and SOD activities were significantly increased in the caerulein + L-arginine group when compared to the caerulein group ($p < 0.05$). In the caerulein + L-NAME group, GSH-Px, GSH and SOD activities were significantly increased when compared to the caerulein group ($p < 0.05$). CAT didn't show significant difference between caerulein and caerulein + L-NAME groups.

Caerulein administration resulted in a significant increase in serum amylase ($p < 0.05$) and lipase ($p < 0.05$) levels. NO inhibition and NO induction reduced amylase and lipase values (Table III).

Discussion

In this study, we first demonstrated the effects of caerulein on pancreatic tissue. Caerulein is the analogue of cholecystokinin and can stimulate the acinar cells to synthesize large amounts of digestive zymogens and pancreatic fluid, resulting in oedematous pancreatitis characterized by interstitial oedema, leukocyte infiltration and the vacuolization of acinar cells [4,10]. In this study we have observed changes in several parameters indicative of acute pancreatitis. Acinar cell degeneration, interstitial inflammation, oedema and haemorrhage were detected. Pancreatic damage was significant when compared to the control group ($p < 0.05$) (Table I). The acinar cells showed ultrastructural changes. Mitochondrial alterations such as swelling, crista loss and myelin figure formation may reflect growing energy demand and ATP deficiency during acute pancreatitis. The lack of sufficient energy supply may induce the morphological alterations, such as dilation of GER and Golgi apparatus [6,15]. Our histologic findings are concordant with some previous investigations [10,15].

There are experimental data indicating that oxidative stress plays a role in the pathogenesis of caerulein-induced acute pancreatitis [12,21]. We detected significant changes in MDA and antioxidant enzymes in the caerulein-treated group. MDA is a product of lipid peroxidation and CAT, SOD,

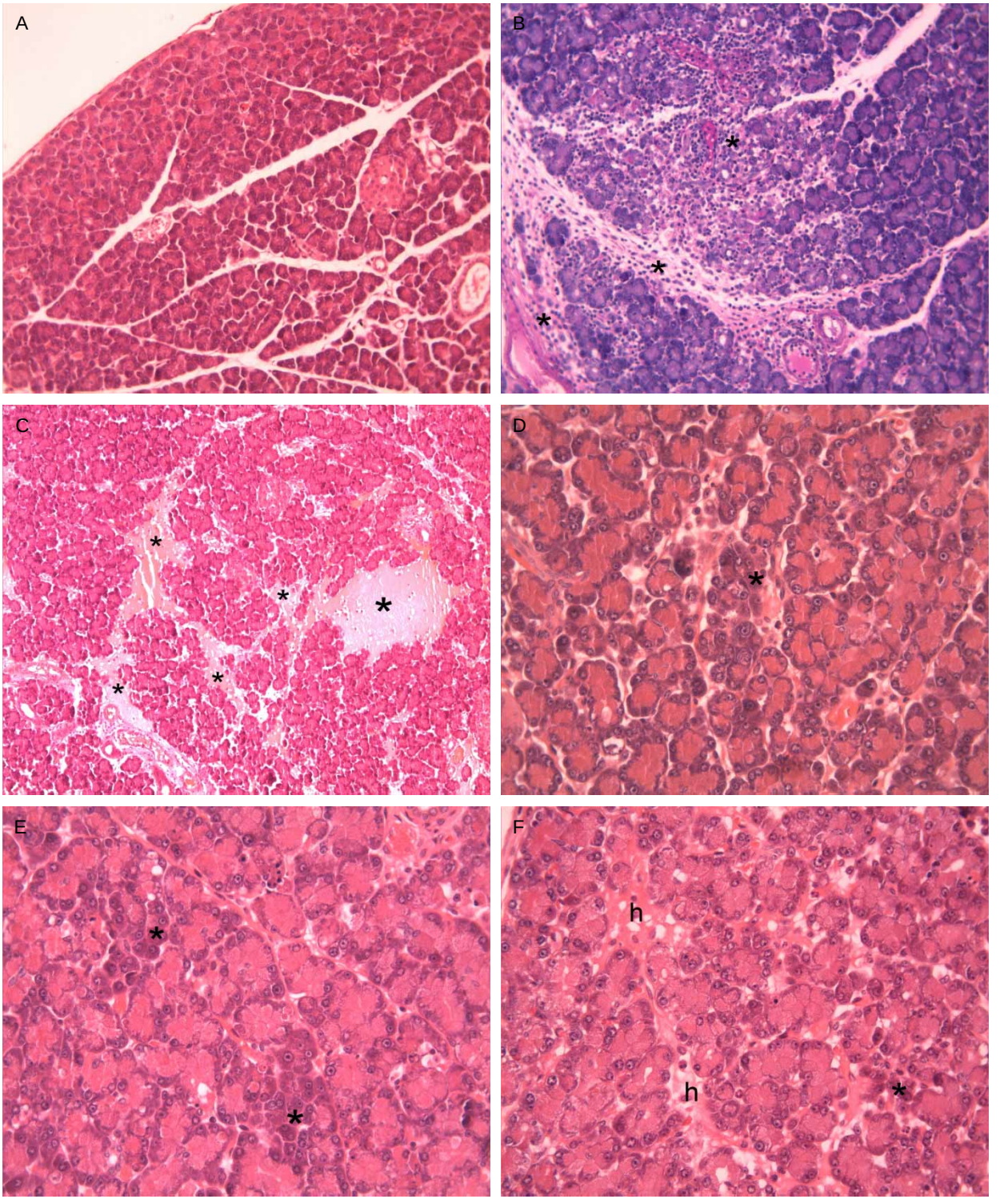

Figure 1. Photomicrographs of pancreatic tissue of rats. In the control group (A, X66) pancreatic tissue appear normal. In the caerulein group (B–D) prominent oedema and interstitial inflammation (asterisks) (B, Masson's trichromeX66), haemorrhage (asterisks) (C, H-EX33) and acinar cell degeneration (asterisk) (D, H-EX132) are visible. In the caerulein+L-arginine group (E, H-EX132) acinar cell degeneration were sparse (asterisks). Rare haemorrhage (h) and acinar cell degeneration (asterisk) are seen (F, H-EX132) in the caerulein+L-NAME group.

GSH-Px and GSH are intrinsic scavengers. The increased levels of MDA and the decreased levels of the antioxidative enzymes probably occured as a result of the reactive oxygen species (ROS) production and the excessive use of the enzymes. When ROS were produced, such as superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), the primary defence is provided by enzymes, SOD, CAT and GSH-Px [12]. If these radicals are produced in amounts that exceed the

Table I. Effect of NO induction and inhibition on histologic changes in caerulein-induced pancreatitis. The groups treated with L-arginin and L-NAME show significant reduction of interstitial inflammation, haemorrhage and necrosis. Data are represented as means ± SD.

Groups	Interstitial inflammation	Haemorrhage	Necrosis
Control ($n=7$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Caerulein ($n=7$)	2.5 ± 0.78^{a}	3.0 ± 0.81^{a}	1.5 ± 0.53^{a}
Caerulein + L-arginine ($n=7$)	$1.42 \pm 0.53^{a,b}$	$1.71 \pm 0.53^{a,b}$	0.42 ± 0.53^{b}
Caerulein + L-NAME ($n=7$)	$1.71 \pm 0.48^{a,b}$	$1.85 \pm 0.69^{a,b}$	$0.71 \pm 0.48^{a,b}$

[a]Significant difference ($p < 0.05$) vs control, [b]significant difference ($p < 0.05$) vs caerulein.

capacity of intrinsic scavengers, they can cause membrane damage. The large autophagosomes that we detected in the cytoplasms of the acinar cell probably appear as a result of focal cytoplasm degradation and damage to cell organelle membranes [15]. The membrane damage results in leakage of excess free radicals, pancreatic enzymes and cellular debris into the interstitium. These events are then propagated by the production of additional free radicals and injury to other cellular membranes, such as capillaries [12]. We detected widespread and severe haemorrhage in pancreatic tissue of the caerulein-induced pancreatitis.

The release of proinflammatory cytokines such as TNF-α or interleukin-6 from the inflamed pancreas can activate the production of iNOS, resulting in over-production of NO [7,10,11,22]. Um et al. [11], have shown that the administration of a supramaximal dose of caerulein induced iNOS expression in pancreatic tissue and significantly increased plasma concentrations of NO metabolites. They also observed that over-production of NO arising from the iNOS was associated with the development of acute pancreatitis as shown by increased pancreas water content and histologically extensive acinar cell vacuolization. Cuzzocrea et al. [23] used wild type mice and iNOS-deficient mice to induce acute pancreatitis by caerulein. Wild type mice developed acute necrotising pancreatitis but pancreatic tissue of iNOS deficient mice showed normal histology. They concluded that NO contribute to the pathophysiology of acute pancreatitis.

There is evidence that some of the cytotoxic effects of NO are due to formation of peroxynitrate, a reactive oxidant formed by the rapid reaction of NO with superoxide anions [11,22]. Peroxynitrate is formed by a diffusion-limited reaction between superoxide ($O_2^{-\bullet}$) and NO. Peroxynitrate may have considerably greater toxicity than hydroxyl radical ($HO^{\bullet}$) [13].

We used L-NAME for NO inhibition. L-NAME treatment showed beneficial effects in caerulein-induced acute pancratitis. MDA levels decreased and antioxidant enzyme levels except CAT increased significantly ($p < 0.05$) when compared to the caerulein group. The production of reactive oxygen species (ROS) causes excessive use of antioxidant enzymes [12]. As high amounts of NO can react with superoxide anions and generate highly toxic peroxynitrate and hydroxyl radicals, the inhibition of NO production may also inhibit the production of these radicals. Thus, the increase in antioxidant enzyme levels in the L-NAME group—when compared to the caerulein group (Table II)—probably occured as a result of the decreased levels of peroxynitrate and hydroxyl radicals. In the L-NAME group CAT was also increased, but this increase was not significant. This finding might show that in the L-NAME group hydrogen peroxide (H_2O_2), which was the substrate of CAT, was high in level.

Pancreas histology and acinar cell ultrastructure were also better preserved in the L-NAME group than the caerulein group. Um et al. [11] observed that L-NAME reduced the severity of caerulein-induced acute pancreatitis and decreased plasma concentrations of NO metabolites. Sandstrom et al. [24] detected that highly selective inhibition of iNOS ameliorates experimental acute pancreatitis. On the other hand, some investigations point to the detrimental effects of NOS inhibition. Andrzejewska and Jurkowska [15] reported that NO induction with L-arginine reversed the effects of caerulein. In our study we observed that L-arginine ameliorated the effects of caerulein. Acinar cell degeneration, interstitial inflammation and oedema were rare. There wasn't any evidence of haemorrhage. Ultrastructural features were also diminished. Most of the acinar cells showed normal GER and Golgi apparatus structure. Autophagosomes were small and sparse. Mitochondrial swelling, crista loss and myelin figures were present but not frequent. Antioxidant enzyme levels increased and MDA levels decreased significantly ($p < 0.05$) when compared to the caerulein group. Our findings about NOS inhibition were not in concordance with Andrzejewska and Jurkowska [15]. They found that NOS inhibition increased inflammatory infiltration and enhanced ultrastructural acinar cell damage. However, we found that pancreas histology and acinar cell ultrastructure were better preserved with NOS inhibition. In our study this morphologic data also supported increased levels of antioxidant enzymes and decreased level of MDA.

According to our results both NO induction and inhibition ameliorated the effects of caerulein on pancreatic tissue. This puzzling observation could have two reasons; the type of NOS that leads to NO

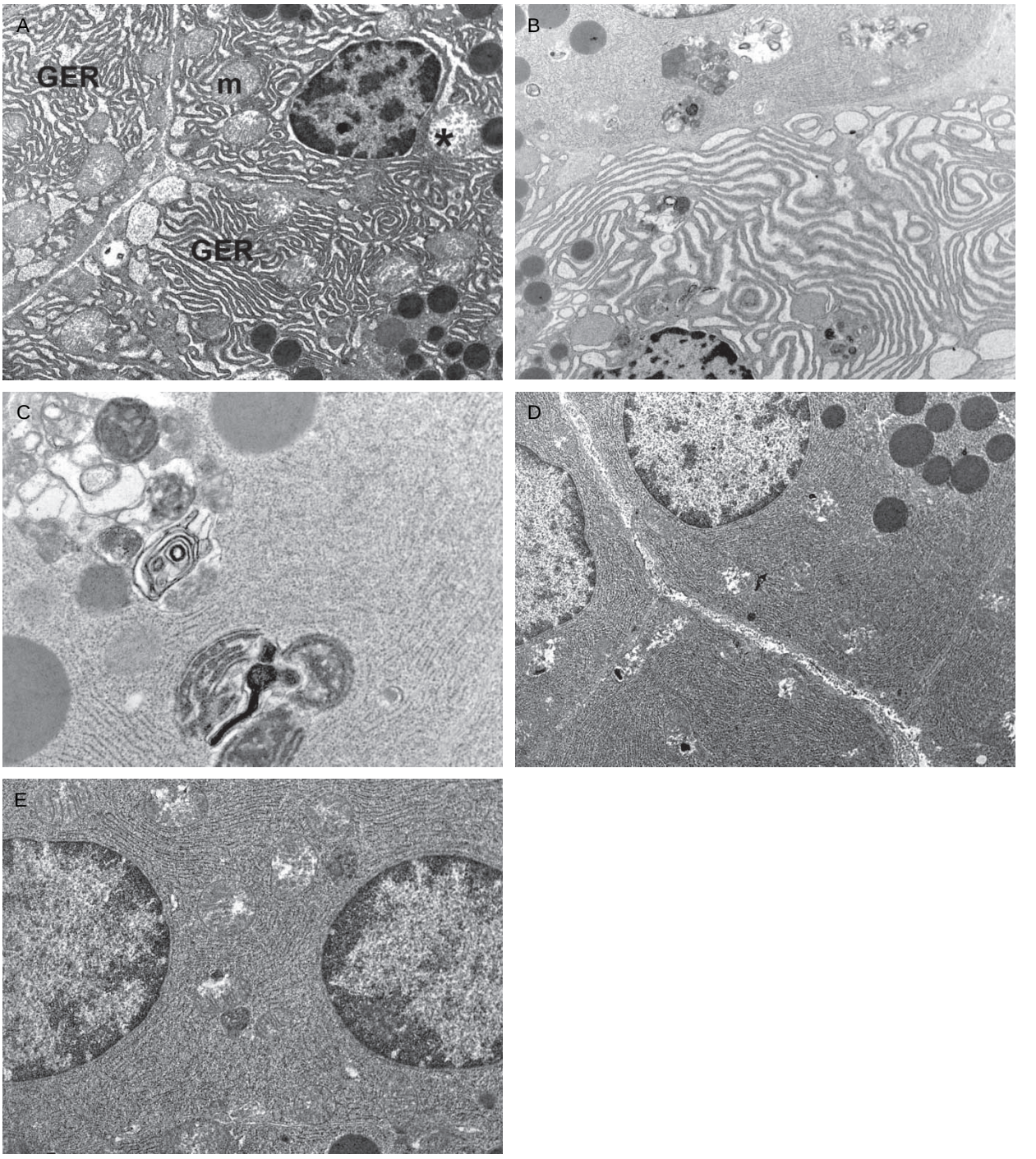

Figure 2. Electron photomicrographs of pancreatic tissue of rats. In the caerulein group (A–C) granular endoplasmic reticulum (GER) cisterna dilations, mitochondrial changes such as swelling (asteriks) and crista loss (m) (A, X6300), myelin figure formation (arrow) (B, X10000) and numerous large autophagosomes (C, X20000) are seen. In the caerulein+L-arginine (D, X6300) and caerulein+L-NAME (E, X10000) groups ultrastructure of the acinar cells are nearly normal except some mitchondrial changes such as swelling, partial crista loss and rare myelin figures.

synthase and the amount of NO. Endogenous NO originates from L-arginine due to the activity of cNOS and iNOS [12]. Endothelial cells possess multiple mechanisms for NO production via cNOS and high output iNOS after inflammatory activation by cytokines. NO produced by cNOS in vascular endothelium may play a protective role in acute pancreatitis through its influence on pancreatic microcirculation and inhibition of leukocyte adhesion and migration into pancreatic tissue [25]. DiMagno et al. [16] showed a protective effect of eNOS in the initiation of caerulein-induced acute pancreatitis. iNOS has detrimental effects by the production of peroxynitrate as we mentioned before. The factors initiating iNOS are proposed to also inhibit NO production from nNOS [24]. Al-Mufti et al. [22]

Table II. The effects of caerulein, NO induction and NO inhibition on pancreas tissue MDA, GSH-Px, CAT, GSH and SOD activities (means ± SD).

Groups	MDA (nmol/ml)	GSH-Px (Umol/ml)	CAT (μmol/mg)	GSH (nmol/mg)	SOD (ng/ml)
Control ($n=7$)	14.82 ± 4.32	0.58 ± 0.13	10.41 ± 1.95	3.81 ± 0.51	22.27 ± 4.17
Caerulein ($n=7$)	28.69 ± 1.79[a]	0.11 ± 0.02[b]	2.66 ± 0.77[b]	2.08 ± 0.44[b]	2.16 ± 0.47[b]
Caerulein + L-arginine ($n=7$)	16.97 ± 3.22[c]	0.33 ± 0.02[b,d]	8.13 ± 3.35[d]	3.96 ± 1.31[d]	4.70 ± 2.15[b,d]
Caerulein + L-NAME ($n=7$)	19.38 ± 6.36[c]	0.38 ± 0.21[d]	5.47 ± 2.8[b]	6.36 ± 1.6[d]	3.7 ± 1.32[b,d]

[a]Significant increase ($p<0.05$) vs control, [b]significant decrease ($p<0.05$) vs control, [c]significant decrease ($p<0.05$) vs caerulein, [d]significant increase ($p<0.05$) vs caerulein.

Table III. The effects of caerulein, NO induction and NO inhibition on serum amylase and lipase levels (means ± SD).

Groups	Amylase (*U*/L)	Lipase (*U*/L)
Control ($n=7$)	383.00 ± 44.26	23.33 ± 1.86
Caerulein ($n=7$)	2068.33 ± 713.62[a]	128.16 ± 86.01[a]
Caerulein + L-arginine ($n=7$)	840.5 ± 467.86[a,b]	54.83 ± 31.12[a,b]
Caerulein + L-NAME ($n=7$)	831.16 ± 379.8[a,b]	42.83 ± 20.31[a,b]

[a]Significant increase ($p<0.05$) vs control, [b]significant decrease ($p<0.05$) vs caerulein.

observed induction of iNOS with down-regulation of cNOS in caerulein-induced acute pancreatitis. NO induction by L-arginine probably showed beneficial effects by induction of cNOS and NO inhibition with L-NAME probably showed beneficial effects by inhibiting iNOS production.

Our findings indicate that a certain amount of NO production via cNOS has beneficial effects in experimental acute pancreatitis, but excessive production of NO via iNOS may be detrimental. The optimum NO levels can minimize the negative effects and maximize the beneficial effects of NO in acute pancreatitis.

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