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Title

Therapeutic effect of rupatadine against L-arginine-induced acute pancreatitis in rats: role of inflammation

Mervat Z. Mohamed^{a*}, Hanaa H. mohammed^b, Hanaa M. Khalaf^a

^aDepartment of Pharmacology, Faculty of Medicine, Minia University, 61511 Minia, Egypt.

^bDepartment of Histology, Faculty of Medicine, Minia University, 61511 Minia, Egypt.

***Corresponding author:**

Name: Mervat Zaki Mohamed

Address: Pharmacology Department, Faculty of Medicine, Minia University, 61511 Minia, Egypt.

E-mail: mervat.zedan@mu.edu.com

Telephone: (+2) 0100 6318305

Fax: 0020862342813

Abstract

Acute pancreatitis (AP) is an abrupt inflammatory disorder causing high morbidity and mortality. As AP is an insidious medical emergency, a curative modality is required instead of a preventive measure. Thus, we investigated the possible curative effect of rupatadine on a rat model of AP. Rupatadine is a potent histamine receptor 1 (H1R) and platelet-activating factor (PAF) blockers. We used four groups of six Wistar rats as follows: the control group received vehicle, the rupatadine control group received rupatadine 6 mg/kg orally, the AP group received L-arginine intraperitoneal, and the treatment group received rupatadine at 1, 6, and 24 h after L-arginine injection. The levels of serum amylase, pancreatic oxidative parameters, and pancreatic cytokines were measured. PAF, histamine, and myeloperoxidase levels were determined in the pancreas. Histopathological and immunohistochemical examinations were performed to determine nuclear factor kappa-B (NF- κ B) and caspase 3 expressions. Oxidative damage and severe inflammation were detected in the pancreas of the AP group. Rupatadine reduced the oxidative damage and the levels of proinflammatory cytokines, PAF, histamine, and myeloperoxidase and NF- κ B and caspase 3 expressions. It restored the pancreatic acini to almost the normal condition. Rupatadine induced important anti-inflammatory and antiapoptotic effects against L-arginine-induced AP.

Key words: L-arginine, acute pancreatitis, rupatadine, PAF, histamine

1. Introduction

Acute pancreatitis (AP) is an acute inflammatory disease that causes high morbidity and mortality. The mortality rate of AP ranges from 5% to 30% of patients according to the severity of the disease. AP is classified into mild and severe (Shah et al. 2018). Its presentation is initially confined to the pancreas, but the disease may progress beyond causing multiorgan failure. The primary leading causes of AP are gallstones and alcohol consumption (Liu and Xia 2006).

The pathophysiology of AP is initiated by the inappropriate release and activation of pancreatic enzymes, including α -amylase, into the pancreas and systemic circulation. Subsequently, the inflammation of the pancreas is initiated. Cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor α (TNF- α) as well as signaling molecules such as nuclear factor kappa-B (NF- κ B) play an essential role in establishing the inflammatory response of AP (Gao et al. 2020; Liu and Xia 2006; Pan et al. 2018; Wu et al. 2021). AP induces cell death through either apoptosis or necrosis according to the severity of the pancreatic injury as well as the species. Rat models of AP primarily display apoptosis, whereas mice AP models exhibit necrosis. TNF- α mediates the process of apoptosis in AP.

Platelet-activating factor (PAF) plays a critical role in the development of AP. It is a phospholipid mediator released because of pancreatic injury. PAF injection was able to induce experimental AP in animals. PAF induces leukocyte adhesion, neutrophil migration, and pancreatic mast cell degranulation with subsequent histamine release (Braganza 2000; Liu and Xia 2006). Mast cells are involved in the induction of experimental pancreatitis and pancreatitis-induced pulmonary injury, whereas mast cell stabilizers alleviate pancreatitis (Lopez-Font et al. 2010).

Although different antioxidant and anti-inflammatory agents were investigated in different models of experimental pancreatitis (Liang et al. 2020; Yang et al. 2020), the management of AP depends primarily on supportive measures such as fluid replacement and antibacterial usage to prevent the secondary bacterial infection (Shah et al. 2018). Thus, there is a critical need for exploring new pharmaceutical strategies to treat AP.

Rupatadine is an FDA-approved histamine receptor 1 (H1R) and PAF receptor blocker. Its maximum inhibitory effect on PAF has been obtained at higher doses (Shamizadeh et al. 2014). It induces an excellent anti-inflammatory and antioxidant effect on the respiratory system through its dual actions and by modulating mast cell degranulation (Kahveci and Kuzu 2021; Merlos et al. 1997; Mullol et al. 2008; Queralto et al. 2000; Shamizadeh et al. 2014). A recent study suggested using rupatadine for COVID-19 prophylaxis either alone or with other PAF inhibitors to dampen the release of proinflammatory mediators from mast cells in the lung (Demopoulos et al. 2020). In the present study, we investigated the therapeutic effect of rupatadine on experimental AP and pancreatic inflammation.

2. Material and methods

2.1. Chemicals and animals

Rupatadine was purchased from Pharmed Healthcare (Egypt). L-arginine and other chemicals used in the biochemical analysis were obtained from Sigma (USA) and were of commercial grade. The animals were obtained from the National Research Center, Cairo, Egypt. A total of 30 adult male albino rats (Wistar strain) weighing 200 ± 30 g were left freely under standard laboratory conditions including diet, light, and temperature for 10 days. The procedures were according to the Guidelines for Ethical Care of Experimental Animals, approved by the

Institutional Animal Care and Use Committee of the Faculty of Medicine, Minia University (approval number 281/9:2019), and compliant with the Canadian Council on Animal Care guidelines.

2.2. Experimental procedures

After acclimatization, the rats were randomly arranged into four groups each with six rats. The control group received vehicle alone, and the rupatadine control group received rupatadine 6 mg/kg suspended in carboxymethylcellulose by oral gavage (Hafez et al. 2020) for the last 2 days. In the AP group, fasting rats received L-arginine intraperitoneal (i.p.) at a dose of 400 mg/100 g body weight at two doses 1 h apart (Zhang et al. 2019). Each dose was freshly prepared in saline, with pH adjusted at exactly 7.4. The treatment group received rupatadine at 1, 6, and 24 h after L-arginine i.p injection. The dose of L-arginine was established after testing three different doses based on previous literature (Czakó et al. 2000; Kui et al. 2015; Zhang et al. 2019). The dose of rupatadine was also selected according to previous studies conducted on models of diabetic nephropathy and pulmonary fibrosis, respectively (Hafez et al. 2020; Lv et al. 2013), and our pilot studies. A preliminary study was conducted to evaluate the best preventive dose of rupatadine against L-arginine-induced AP (data not published).

2.3. Biochemical analysis

The animals were anesthetized and sacrificed after 48 h of the last L-arginine injection. Blood samples were collected, allowed to clot, and then centrifuged to obtain clear sera. Serum amylase level was measured colorimetrically using commercial kits. The pancreas was removed carefully, weighed, and then stored at -80°C . Parts of pancreatic tissue were soaked in 10% formalin. The pancreatic tissue at 1:10 was homogenized in cold potassium phosphate buffer (pH 7.4). Tissue

homogenates were centrifuged at -4°C to obtain the supernatant that was used for the colorimetric determination of peroxidation products, malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) activity. The levels of MDA, GSH, and SOD were determined using biochemical methods described previously, with slight modifications (Giustarini et al. 2016; Ramasarma et al. 2015; Zeb and Ullah 2016).

2.4. Nitrosative stress assessment

Nitric oxide overproduction is the mainstay in L-arginine-induced AP. Nitrite and nitrate are the stable products of NO. They were estimated by the conversion of nitrates to nitrites by cadmium granules, followed by the measurement of total nitrite concentration in the pancreatic supernatant (Sastry et al. 2002).

2.5. Cytokine analysis

The protein expressions of pancreatic TNF- α , IL-1 β , IL-6, and IL-10 were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (Glory Science Co., Ltd.) according to the manufacturer's instructions. The absorbance was plotted into concentrations using the obtained standard curve.

2.6. Myeloperoxidase and PAF determination

PAF and histamine levels were measured using ELISA kits (Elabscience, USA). MPO level was measured using ELISA kits (Glory Science Co., Ltd.).

2.7. Histological analysis and immunohistochemical studies

The pancreatic tissue, which was preserved in formalin, was processed for hematoxylin and eosin (H&E) staining. Sections of 5 to 6 μm thickness were examined under a light microscope. For immunohistochemical staining, paraffin sections were deparaffinized, hydrated, and stained with primary antibodies for NF- κB and caspase 3 obtained from Lab Vision Corp., USA, and

GeneTex, Inc., USA, respectively.

Photography: Olympus light microscopy (Olympus, Japan) was used for examining and capturing images of the histological and immunohistochemical sections. The slides were photographed using an Olympus digital camera (U.TV0.5XC-3), and the images were saved as jpg. Sections were scored according to the extent and severity of pancreatitis on a scale of 0–3 as shown in Table 1.

Morphometric analysis:

NF- κ B and caspase 3 immunoreactivity was examined under the 10 $\times$ objective and evaluated as area fraction using the image analysis software ImageJ (<http://rsbweb.nih.gov/ij/>; NIH, Bethesda).

2.8. Statistical analysis

Data analysis was performed using the Graph Pad Prism software (version 5). Results are expressed as mean $\pm$ S.E.M using the one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests.

3. Results

3.1. Ratio of pancreatic weight to body weight

The pancreatic weight (mg)/body weight (g) ratio showed a significant increase in the L-arginine-treated rats compared with that in the control rats. Rupatadine administration after L-arginine injection significantly reduced the degree of edema (Figure 1A).

3.2. Serum amylase

No significant change was observed in serum amylase levels among the groups (Table 2).

3.3. Pancreatic oxidant status

L-arginine injection induced a significant increase in pancreatic lipid peroxidation, a decrease in pancreatic glutathione content, and a decrease in SOD activity compared with those in the

control groups. Treatment with rupatadine after L-arginine injection significantly improved the oxidative parameters, MDA, GSH, and SOD (Table 2).

3.4. Pancreatic nitric oxide

Pancreatic nitrite is the footprint of NO overproduction and nitrosative stress. L-arginine injection into rats resulted in NO overproduction as indicated by marked and significant elevation of total nitrite levels in the pancreatic tissue. Rupatadine treatment successfully prevented the overproduction of NO and almost normalized the total nitrite level (Figure 1B).

3.5. Cytokine expression

TNF- α expression was increased in the pancreas in the AP group compared with that in the control groups. The rupatadine-treated group exhibited a decrease in TNF- α levels when compared with the AP group (Figure 1C). Similarly, IL-1 β levels were increased significantly in the AP group and then reduced with rupatadine treatment (Figure 1D). The anti-inflammatory cytokine IL-10 level showed a decrease in the AP group, which then improved with rupatadine treatment (Figure 1E).

3.6. Myeloperoxidase, PAF, and histamine protein levels

MPO, PAF, and histamine levels were markedly elevated in the AP group compared with those in the control groups. Their protein levels were almost normalized with rupatadine treatment (Figures 2A, B, C).

3.7. Histopathological changes

The control groups showed normal pancreatic architecture with basal basophilia and apical acidophilia of acinar cells, as well as regular islet morphology in the endocrine pancreas, whereas the AP group exhibited loss of the normal lobular architecture and marked inflammatory cellular infiltration. The rupatadine-treated group showed few inflammatory cells in the connective tissue septum and normal pancreatic acini with normal lobular architecture (Figure 3).

3.8. Protein expression of NF- κ B and caspase 3

Immunostaining of the pancreatic tissue for NF- κ B revealed minimal expression in the control groups. The AP group exhibited strong immunostaining that regressed significantly with rupatadine treatment (Figure 4). Caspase 3 expression was upregulated in the AP group when compared with that in the control groups. Rupatadine treatment significantly reduced the expression of caspase 3 when compared with that in the AP group (Figure 5).

4. Discussion

Rupatadine treatment at 6 mg/kg exerted an excellent curative effect against the experimentally induced AP. To our best knowledge, this study is the first to examine the role of dual H1R and PAF receptor blockers in experimental AP. Despite the large dose administered in the experiment, several studies have confirmed the efficacy and safety of rupatadine doses of up to 100 mg/kg in animals and up to 40 mg/kg in humans; however, high doses such as 80 mg/kg were found to induce cognitive and psychomotor impairment (Merlos et al. 1997; Shamizadeh et al. 2014). Rupatadine was found to antagonize PAF at a high dose of 40 mg/kg in humans, and it was found to be safe and effective in treating patients with acute dengue infection at high doses (Malavige et al. 2018).

As AP represents an inflammatory condition, a complex cascade of immunological reactions affects the disease pathogenesis and progression. L-arginine-induced AP is one of the most common models for the induction of AP. The toxic high dose of this basic amino acid induces the production of toxic reactive oxygen species in pancreatic acinar cells (Zhang et al. 2019). The histological findings of L-arginine-induced AP resemble those observed in human pancreatitis (Kui et al. 2015). In our study, the pancreatic pathology induced by the administration of 4 g/kg of L-arginine was similar to that reported in previous studies (Sadek and

Khattab 2017; Yang et al. 2020). Contrary to most studies, serum amylase levels showed no significant changes among the groups despite the obvious pathological profile and the significant changes in other biochemical parameters. This result may be explained by the normalization of amylase enzyme levels after 48 h of acute L-arginine-induced pancreatitis (Czakó et al. 2000). The presence of multiple cytoplasmic vacuoles in the pancreatic acinar cells of the L-arginine-treated group in this study was in agreement with another study (Vaccaro et al. 2003), in which the authors attributed the presence of these vacuoles to the existence of vacuole membrane protein 1. Acinar cells may be the first target of an arginine overdose, resulting in degradation or necrosis. The vascular congestion and edema detected by the pancreatic weight and the histological profile in this study could be explained by the increase of NO, which is involved in the regulation of the rate of perfusion of pancreatic microvessels. The excess NO induced by L-arginine results in a direct toxic effect on pancreatic acinar cells, increase of vascular congestion, permeability, and the occurrence of pancreatic edema, in consistency with other studies (Sadek and Khattab 2017; Vonlaufen et al. 2007).

The necrosis of pancreatic acini observed in the histopathology correlated with the disruptive oxidative parameters in the pancreatic tissue. The oxidative and nitrosative stress due to NO overproduction was suspected to be responsible for L-arginine-induced necrotizing pancreatitis (Yu and Kim 2014). In our study, the repeated administration of rupatadine restored the cellular antioxidant capacity, prevented lipid peroxidation, and dampened the nitrosative stress. This antioxidant effect of rupatadine recovered the architecture of the pancreatic acini. The antioxidant property of rupatadine has been demonstrated previously in rats with diabetic nephropathy and patients with allergic rhinitis (Hafez et al. 2020; Kahveci and Kuzu 2021).

224 NF- κ B activation is a basic event in the progression of AP. The present study demonstrated the
225 upregulation of pancreatic NF- κ B and its subsequent downstream signaling TNF- α and IL-1 β ,
226 which leads to the initiation and progression of AP in agreement with other studies (Gao et al.
227 2020; Yu and Kim 2014). Upregulation of the NF- κ B/TNF- α and IL-1 β pathway in the pancreas
228 has been confirmed to play a critical role in the severity and outcome of AP experimentally and
229 clinically in humans (Gao et al. 2020; Liu and Xia 2006; Pan et al. 2018). Inhibition of NF- κ B
230 activity has been reported to be effective in decreasing the fatal outcome of severe AP in rats
231 (Sato et al. 1999). Previous studies have also reported the ability of rupatadine to inhibit NF- κ B
232 activity and TNF- α expression (Mullol et al. 2008; Queralt et al. 2000). We assume that the
233 inhibition of the NF- κ B/TNF- α pathway mediates the therapeutic effect of rupatadine against AP
234 consistent with (Chen et al. 2019). The increased IL-1 β production in the AP group was
235 consistent with the finding of (Wu et al. 2021) who reported that IL-1 β production by activated
236 neutrophils causes more tissue damage during AP progression. The precursor of IL-1 β in AP is
237 converted into an active form through the NLRP3 inflammasome (Hoque et al. 2011), which is a
238 cytosolic multiprotein complex responsible for the initiation of the inflammatory response as a
239 part of the innate immune system. NF- κ B has been implicated in the activation of the NLRP3
240 inflammasome (Ferrero-Andrés et al. 2020).

241 Contrary to proinflammatory cytokines, IL-10 was found to have a potent anti-inflammatory
242 action in cerulein-induced AP. IL-10 inhibits the release of other cytokines from activated
243 macrophages during the induction of pancreatitis (Rongione et al. 1997). Consistent with the
244 previous study, IL-10 production was increased in the rupatadine-treated group after its reduction
245 in the AP group. IL-10 production was also found to be increased in the L-arginine-induced
246 model for the protection of neutrophil depletion in mice (Chen et al. 2015).

In the present study, PAF and its pivotal role were evident in neutrophil chemotaxis and the production of proinflammatory cytokines. Rupatadine exerted beneficial effects through the inhibition of PAF and histamine consistent with other studies (Braganza 2000; Liu and Xia 2006; Lopez-Font et al. 2010; Mullol et al. 2008). The elevated histamine level observed in our study may be explained by pancreatic mast cell activation during the inflammatory response in AP. Pancreatic mast cells are the major generators of local and remote organ inflammation during the early phases of AP (Lopez-Font et al. 2010). High histamine levels in AP may be another explanation for the increased vascular congestion, permeability, and subsequent neutrophil migration in the present study.

Our study showed neutrophil chemotaxis in the pancreas of the AP group as evident by the elevated MPO level and histopathology. Rupatadine treatment markedly reduced the MPO level and neutrophil infiltration. This result may be explained by its H1R-antagonistic effect that decreases the vascular permeability and neutrophil migration and activation. Consistent with our findings, rupatadine treatment was found to exhibit an ameliorating effect on histamine and TNF- α release from skin mast cells *in vitro* (Queralt et al. 2000). Importantly, the absence or depletion of neutrophils protects against L-arginine-induced AP. The depletion of neutrophils was found to reduce the levels of MPO and NF- κ B and the acinar cell apoptosis (Chen et al. 2015).

Apoptosis is a programmed cell death that differs from necrosis. Both processes occur in AP. In the present study, increased apoptosis was evident in the AP group in the immunostaining result of caspase 3, which was consistent with other studies (Abdelzaher et al. 2021; Chen et al. 2015; Siriviriyakul et al. 2019). Treatment with rupatadine ameliorates apoptosis that depends on the caspase 3 pathway. We postulate that the rupatadine induced antiapoptotic effect is secondary to

the reduction of neutrophil migration and activation in agreement with (Chen et al. 2015). Another study linked the induction of acinar cell apoptosis to inflammatory injury, showing protection against the AP model through regulatory proteins that reduce both apoptosis and inflammation (Tan et al. 2020). Contrary to our finding, other studies have reported enhanced apoptosis as a protective pathway. The shift in cell death from necrosis to apoptosis was found to be one of the beneficial strategies to overcome AP injury in those studies (Chen et al. 2019; Nakamura et al. 2010). However, we suggest that rupatadine provided protection during the earlier stages of AP pathogenesis. Rupatadine dampened the early spark in the inflammatory process that is considered as a trigger for apoptosis and necrosis.

5. Conclusion: Rupatadine treatment at a high dose exhibited a therapeutic effect on the L-arginine-induced AP model that was mediated by the amelioration of the inflammatory process, cytokine production, neutrophil infiltration, and apoptotic cell death. Both roles of H1R blockade and PAF antagonism are involved in the emerging curative effect. The present study provides a foundation for further research on the utility and applicability of rupatadine as a therapy for such an emergency condition.

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Figure legends

Figure 1: Effect of rupatadine on pancreatic weight/body weight (A), pancreatic nitric oxide (B), TNF- α (C), IL-1 β (D), and IL-10 (E) protein expressions after the administration of L-arginine in rats measured via ELISA. Animals were administered intraperitoneal L-arginine (4 g/kg) to induce acute pancreatitis (AP), followed by oral administration of rupatadine (6 mg/kg). Data are presented as mean $\pm$ SEM (n = 5–6). Significant differences at $P < 0.05$; *from control (received vehicle alone); #from AP (received L-arginine). One-way ANOVA and Tukey's test.

Figure 2: Effect of rupatadine on pancreatic myeloperoxidase (A), platelet-activating factor (B), and histamine (C) and protein expressions after the administration of L-arginine in rats measured via ELISA. Animals were administered intraperitoneal L-arginine (4 g/kg) to produce acute pancreatitis (AP), followed by oral administration of rupatadine (6 mg/kg). Data are presented as mean $\pm$ SEM (n = 5–6). Significant differences at $P < 0.05$; *from control (received vehicle alone); #from AP (received L-arginine). One-way ANOVA and Tukey's test.

Figure 3: A representative photomicrograph of the pancreas stained with H&E (400 $\times$ magnification). The control groups (A1 and A2) showing the normal pancreatic architecture, with the pancreatic acini (arrow) filling the pancreatic lobule and surrounding an islet of Langerhans (star). The acini are lined with large pyramidal cells with basal round vesicular nuclei. Basal basophilia and apical acidophilia of acinar cells are noticed. The affected group (B1, B2, and B3) showed loss of the normal lobular architecture. B1 shows dilated congested blood vessel (bv), marked inflammatory cellular infiltration (oval), necrotic widely separated acini (arrows), and neutrophils (curved arrow). B2 shows vacuolation in the islet of Langerhans (arrows) and disintegrated necrotic acini (oval). B3 shows edema (star), vacuolated acinar cells (arrows), and dark pyknotic nuclei of some acinar cells (red arrows). The treated group (C)

shows few inflammatory cells (arrows) in the connective tissue septum and normal pancreatic acini (black arrows).

Figure 4: Representative photomicrographs of the pancreatic tissue immunostained for NF- κ B (100 $\times$). The control groups (A1 and A2) show minimal expression (arrow). The AP group (B) shows strong expression (arrows). The treated group (C) shows less expression (arrows). (D) The area fraction of NF- κ B is presented as mean $\pm$ SEM (n = 5–6). Significant differences at $P < 0.05$; *from control (received vehicle alone); #from AP (received L-arginine). One-way ANOVA and Tukey’s test. AP: acute pancreatitis.

Figure 5: Representative photomicrographs of the pancreatic tissue immunostained for caspase 3 (100 $\times$). The control groups (A1 and A2) show mild to minimal staining, respectively (arrow). The AP group (B) shows strong expression (arrows). The treated group (C) shows less expression (arrows). (D) The area fraction of NF- κ B is presented as mean $\pm$ SEM (n = 5–6). Significant differences at $P < 0.05$; *from control (received vehicle alone); #from AP (received L-arginine). One-way ANOVA and Tukey’s test. AP: acute pancreatitis.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1: The criteria of pancreatic histological score.

Pathological Factors	Histological Score			
	0	1	2	3
Cell necrosis	none	<10% necrosis	< 40% necrosis	> 40% necrosis
Vacuolization	none	<20% acini with vacuoles	<50% acini	>50% acini
Inflammation	none	inflammatory cells present at interlobular areas	present at intralobular areas	present at interacini
Acinar edema	none	interlobular edema	intralobular edema	interacinar edema

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Table 2: Effect of rupatadine on pancreatic oxidant and antioxidant parameters, malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) on L-arginine-induced acute pancreatitis in rats

group	Serum α amylase (U/L)	Pancreatic MDA (nmol/g tissue)	Pancreatic GSH (nmol/g tissue)	Pancreatic SOD (U/mg tissue)
control	1305 $\pm$ 29.2	15.02 $\pm$ 0.49	482.8 $\pm$ 12.94	1064 $\pm$ 43.4
rupatadine	1302 $\pm$ 16.4	15.47 $\pm$ 0.59	400.2 $\pm$ 33.95	1030 $\pm$ 46.1
AP	1685 $\pm$ 51.5	22.93 $\pm$ 1.13*	269.7 $\pm$ 14.46*	409.5 $\pm$ 80.9*
AP+ rupatadine	1424 $\pm$ 29.7	18.51 $\pm$ 0.94 [#]	486.8 $\pm$ 14.53 [#]	887.2 $\pm$ 219.1 [#]

All parameters are expressed as means $\pm$ S.E.M. (n=5-6). *# Significantly different (at P< 0.05) from the control group, and AP group respectively. AP: acute pancreatitis.