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A Study on the Effect of Calcium Channel Antagonist in Acute Pancreatitis

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Abstract: **Acute pancreatitis (AP) is one of the most common acute conditions affecting gastrointestinal tract with increasing incidences of morbidity and mortality. In AP, pancreatic enzymes are activated and cause digestion, edema, hemorrhage, and even necrosis of the pancreatic tissue. The actual molecular mechanism of AP is poorly understood. Based on the literature, oxidative stress and activation of inflammatory cascades are majorly involved in the pathophysiology of AP.**

Aim of the work: The aim of the current study was to clarify the promising anti-inflammatory action of calcium channel antagonist in treating induced AP in rats, expressed by morphological, morphometric changes and biochemical results.

Methods: Twenty- two Sprague Dawley rats were divided randomly into: Group I (Control group; n. 6). Group II ((AP) group; n.8): which was induced by injection of 2 gm/ kg body weight by intraperitoneal (IP). Group III (AP treated with verapamil): treated with verapamil by dose of (1.25mg/kg body weight) by IP injection 12 hours after L-arginine induction. Serological assessment of serum lipase and amylase, histological, histochemical, immunohistochemical and morphometric studies were done. Biochemical Study for Malondialdehyde (MDA), catalase. All studies were followed by statistical analysis.

Results: Group II (AP group) showed morphological changes indicating inflammation and degeneration that obviously regressed in groups III (AP treated with verapamil). Morphometric, serological and biochemical quantitative values were confirmative.

Conclusions: Verapamil therapy showed significant treating role; thus, verapamil can control experimentally induced AP in rats.

Keywords: **Pancreatitis, Calcium channel receptors, MDA.**

1. INTRODUCTION

Acute pancreatitis (AP) is an inflammatory potentially life-threatening disorder of the pancreas, that is associated with major morbidity and mortality. Recognized causes of AP; gallstones, alcohol, infections, hypercalcemia, endoscopic retrograde cholangio-pancreatography (ERCP) and different drugs trigger AP **[1].**

The occurrence of AP has increased worldwide in the last years, most patients with AP, who are discharged within days or weeks, have a mild and self-limited course. However, approximately 30 percent (%) of patients will develop severe AP with extensive pancreatic necrosis and multiple organ failure, leading to mortality rates as high as 30% [2].

Acute pancreatitis is diagnosed by typical abdominal pain; serum amylase and/or lipase elevation three times more than the normal level; and imaging findings consistent with acute pancreatitis [3].

Pathological elevation of calcium ion (Ca^{+2}) concentration in pancreatic acinar cells leads to premature trypsinogen activation into trypsin causes auto-digestion of the acinar cells, inflammation and finally causes acinar cell necrosis [4].

Although the protective mechanism of calcium channel antagonist is not clearly known, it is suggested that it is related to the stabilization of the membrane as increased pancreatic zymogen granule fragility is reported in conditions associated with pancreatic injury in the rats [5].

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Also, calcium channel antagonists have anti-inflammatory and anti-fibrotic activity thus can be used in the treatment of inflammatory disorders resulting in remodeling of connective tissue into fibrotic plaque [6].

The aim of current study was to clarify the promising anti-inflammatory action of calcium channel antagonist in treating induced AP in rats.

2. MATERIALS AND METHODS

Materials

Drugs:

1. L-arginine powder (L-Arginine – reagent grade, ≥ 98%) manufactured by Otto Chemie PVT Ltd, India in the form of powder 25 gm. The drug was dissolved in 0.9 % saline.

2. Verapamil was purchased from Abbott Egypt Company, New Cairo, Egypt, as ampoules of 5 mg / 2 ml.

Animals:

Twenty-two adult male albino Sprague Dawley rats 12-weeks old were included in this study, with average body weight 200 gm. They were housed in the Animal House of Kasr Al-Ainy, Faculty of Medicine after their local approval, under a 12-hour light/dark cycle and controlled temperature of 22 ± 2°C. Each subgroup was kept in a separate wire cage at room temperature, fed ad libitum and allowed for free water supply. Conventional size of cages was large enough to provide enough space for normal social behavior of each rat. Appropriate number of animals per cage was not exceeding 6 rats per cage with ambient humidity, ventilation and free access to food (Grains). All procedures were done in accordance with ethical guidelines approved by the Animal Ethics Committee of Cairo University.

Experimental Design

The rats were divided randomly into:

Group I (Control group; n. 6):

Each rat received 1.5 ml of 0.9% normal saline intraperitoneal (IP).

Group II (Acute pancreatitis (AP) group; n.8): IP injection of a dose of 2gm/kg body weight of L-arginine (Otto Chemie PVT Ltd, India). A fresh solution was prepared on the day of injection and each rat received the dose in a 1.5 ml saline by IP injection using insulin syringe [7].

Group III (AP treated with Verapamil) (Abbott Egypt Company, New Cairo, Egypt, ampoules)**:** rats were treated with verapamil in a dose of 1.25mg /kg body weight by IP injection using insulin syringe 12 hour after L-arginine induction [5].

Twenty-four hours following L-arginine injection, the rats were sacrificed by cervical dislocation [8] under anesthesia by IP injection of pentobarbital at dose of 50mg/Kg body weight [9]. They were subjected to the following studies:

A) Biochemical Study:

1- Fasting blood samples were collected by cardiac puncture into plain tubes, and tubes containing ethylene diamine tetra-acetic acid (EDTA); for assessment of serum pancreatic amylase and lipase [10]. They were determined by enzyme-colorimetric method using Automated Hitachi Analyzer (Diagnostic Product Corporation, California, USA).

2- The pancreas was immediately dissected out and subjected to the following studies:

Half of pancreatic specimens were homogenized for assessment of: Malondialdehyde (MDA) as an oxidative enzyme, catalase as antioxidant enzyme [11]. (Sigma Chemical CO., P.O. Box 14508 St. Louis, USA).

B) Histological Study:

Half of the pancreatic specimens were fixed in 10 % formol saline. Sections of 5 µm thickness were subjected to:

1. Hematoxylin and Eosin (H&E) staining [12].

2. Immunohistochemical staining for cyclooxygenase2 (COX2) inflammation marker [13]. It's a rabbit monoclonal IgG antibody SP21 (Invitrogen, Thermo Fisher, USA, MA5-14568).

3. Morphometric assessment: Using Leica Quin 500 LTD image analyzer system to assess: Area of dark nuclei and area of interstitial zymogen granules. Area % of COX 2 immunoexpress ion (IE). Optical density of COX 2 immunoreactivity (IR).

Statistical Study

Statistical study was carried out using the statistical package of social science (SPSS) version 25 (SPSS Inc., USA). All data were reported as mean ± standard deviation (SD) using (ANOVA) and Post hoc tests. p values ≤ 0.05 were regarded significant (sig) [14].

3. RESULTS

3 rats showed signs of distress, were immediately euthanized and compensated. Lethargy was noticed in AP group that improved in verapamil and dantrolene groups.

A) Biochemical Results:

1- Mean values of serum amylase and lipase as following:

The mean values of serum amylase and serum lipase were shown in (Table 1). The values indicated a significant (sig) increase in AP group compared to the control and the treated group. On the other hand, a sig decrease was found in verapamil group versus AP (Histogram 1).

2- The mean MDA values in nanomole (nM)/gram (gm) ± SD and Catalase values in unit/gm (U/gm) were (24.04±3.20) and (33.65±2.93) in group I, (87.08±5.32) and (8.97±0.98) in group II, (39.12±5.18) and (30.51±5.11) in group III respectively. The previous values indicated sig (P≤0.05) increase in MDA and decrease in Catalase in group II compared to control and treated group. While sig decrease in MDA and increase Catalase were noticed in group III compared to group II (Table 2, Histogram 2).

B) Histological Results:

1. Hematoxylin and Eosin (H&E) Stained Sections:

Group I, revealed normal histological architecture, demonstrating pale stained islets surrounded by darkly stained acini, central acidophilic zymogen granules (ZGs), basal basophilia and pale basal nuclei in acini (Fig.1). **Group II** showed destruction of the normal pancreatic architecture, many cells appear apoptotic with darkly stained pyknotic nuclei and deeply stained acidophilic cytoplasm, while other cells contain vacuolations in their cytoplasm, Inflammatory cell infiltration and blood vessels and duct are seen (Fig.2). **Group III** demonstrated apparently normal acini in many fields, pale nuclei, apical acidophilia, basal basophilia of acini with apparently normal islets, few fields infiltrated by inflammatory cells and some acini containing few dark nuclei (Fig.3).

2. Immunohistochemical stained sections using COX2:

Group I showed negative immunoexpression (–ve IE) (Fig.4), in group II, strong positive (+ve) IE was seen among multiple acini in most fields, among fewer acini in other fields and dense + ve IR was evident among few acini (Fig.5). Group III showed weak + ve IE among few acinar cells (Fig.6).

3. Morphometric Results:

Mean area of dark nuclei indicated sig (P<0.05) increase in group II versus group III. Mean area of interstitial ZGs denoted sig increase in group II versus group III (Table 3, Histogram3). Mean area% of IE and the optical density of COX2 IR reported sig increase in group II versus group III (Table 4, Histogram 4).

4. DISCUSSION

In the current study, mean serum amylase and lipase values indicated sig increase in acute pancreatitis (AP) group. On contrary, sig decrease was found in AP treated with verapamil group versus AP group. In agreement, the levels of serum amylase and lipase were detected to evaluate whether the AP model was successfully constructed.[15] Similarly, AP patients show elevated values of serum lipase or amylase three times the normal limit.[16]

As regard the mean MDA values indicated sig increase in AP group versus AP treated with verapamil while the mean catalase values indicated sig decrease in AP group versus AP treated with verapamil. Similarly, L-arginine induced AP in a rat model produced a high increment in MDA content. [17] Verapamil was proved as an efficient therapeutic agent for neuroprotection during cell oxidation stress as proved by Jangholi et al [18] who recorded sig increase in catalase activity and dramatic inhibition of increased MDA.

Acute pancreatitis group showed multiple congested vessels, homogenous material in vessels and interstitium, also, inflammatory cellular infiltration. The previous results confirmed the incidence of AP. In accordance, congested vessels were found among the parenchyma in AP rat model.[19] Pancreatic tissues showing exudation and inflammatory cell infiltration proved that AP model rats were successfully established.[20]

Widespread vacuolation and multiple dark nuclei were seen among acinar cells, confirmed by sig increase in mean area of dark nuclei in AP group. The previous findings indicated L-arginine induced inflammation that can progress to degeneration. In support, it was confirmed that the previous changes were found in AP rat model after nine hours. [21]

Acute pancreatitis group revealed multiple disorganized acini and multiple ZGs were seen, confirmed by sig increase in area of interstitial ZGs. The previous findings may precipitate intrapancreatic activation of digestive enzymes. Going with, acinar cell autophagy was proved experimentally [22], acinar cell loss [23] and intrapancreatic trypsin activity [24] were recorded in AP mouse models.

Acute pancreatitis treated with verapamil group demonstrated apparently normal acini, few dark nuclei and sig decrease in mean area of dark nuclei. The previous findings denoted obvious amelioration of morphological changes, indicating inflammation and degeneration, that developed in AP. Ghandi et al [25] added that renal tubulointerstitial inflammation and degeneration were downregulated by verapamil therapy. Recently, verapamil exerted definite antioxidant activity. [26]

In AP group, +ve IE was detected among multiple acini and dense + ve IR was obvious among few acini, AP treated with verapamil group showed + ve IE among few acinar cells. Area % of IE and optical density of IR of COX2 values reported a sig increase in AP group versus AP treated with verapamil group.

Higher expression of COX2 (important for prostaglandin synthesis) in brain of copper-treated rats proved its vital role in inflammation.[27] Accumulated prostaglandins stimulate inflammatory cells to release proinflammatory cytokines such as TNF-α. [28] Slight repressive effects of verapamil lead to strong TNF-α suppression, indicating anti-inflammatory effects. [29]

It can be concluded that morphological findings confirmed by morphometric, serological and biochemical quantitative values proved definite therapeutic effect of verapamil in controlling experimentally induced AP in rats.

No funding

Availability of data and materials

Data and materials are available from the corresponding author upon request.

Ethics approval and Consent to participate

The housing and handling of rats follow ethical guidelines approved by the Animal Ethics Committee of Faculty of Medicine- Cairo University. Animals are monitored daily for infection and other illnesses by trained animal technicians. Only trained laboratory personnel and animal technicians were allowed to handle laboratory animals.

Competing interests

The author does not declare any competing interests.

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APPENDICES - A

Legend of Figures

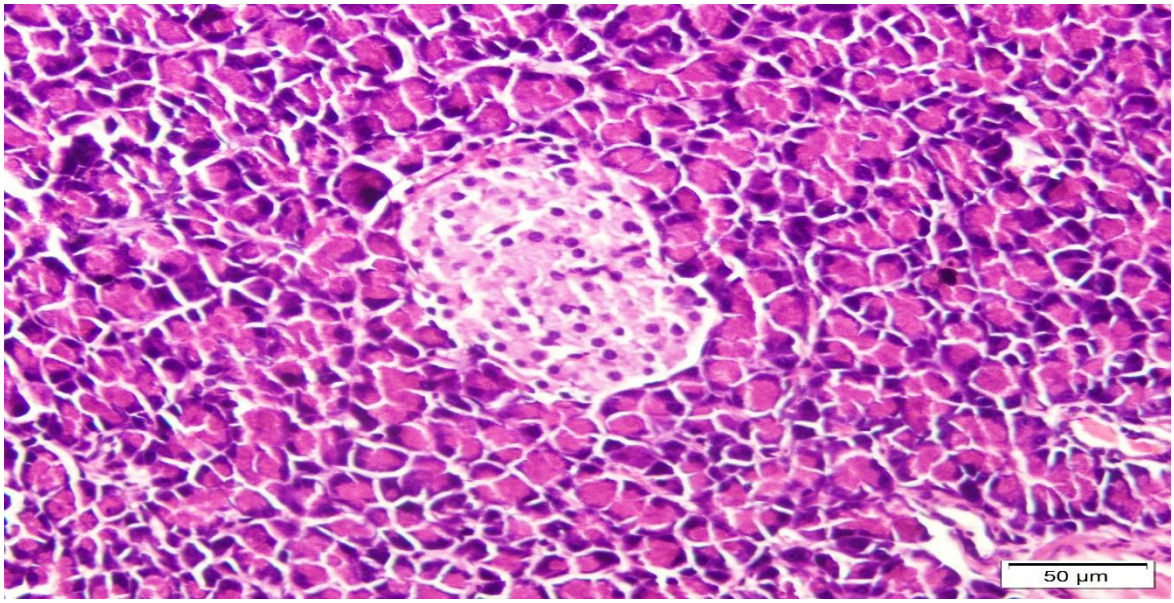

Figure 1: pancreatic section from control group showing a pale stained islet surrounded by deeply stained pancreatic acini. The acini have basal nuclei surrounded by basophilic cytoplasm with apical acidophilic granules (x 200).

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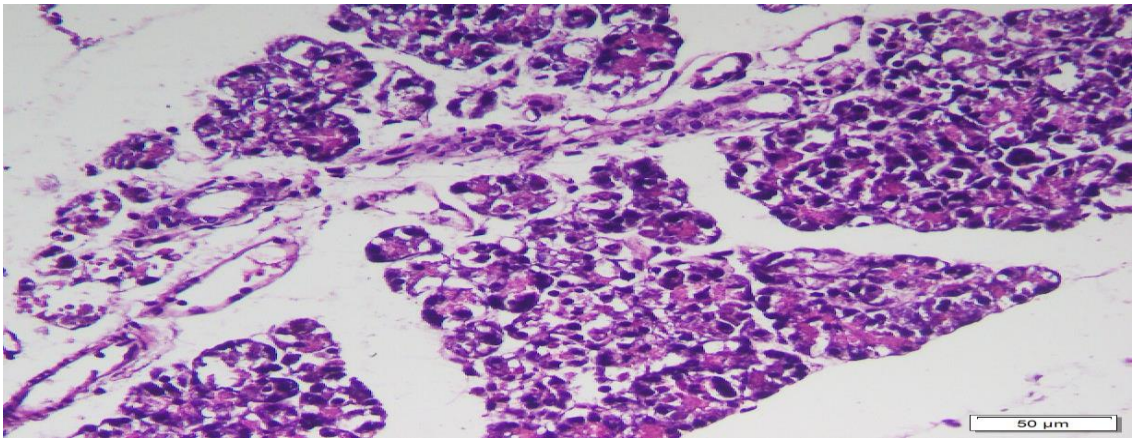

Figure 2: Pancreatic section from AP group showing destruction of the normal pancreatic architecture. Many cells appear apoptotic with darkly stained pyknotic nuclei and deeply stained acidophilic cytoplasm, while other cells contain vacuolations in their cytoplasm. Inflammatory cell infiltration, blood vessels and duct are seen. (H&E x 200).

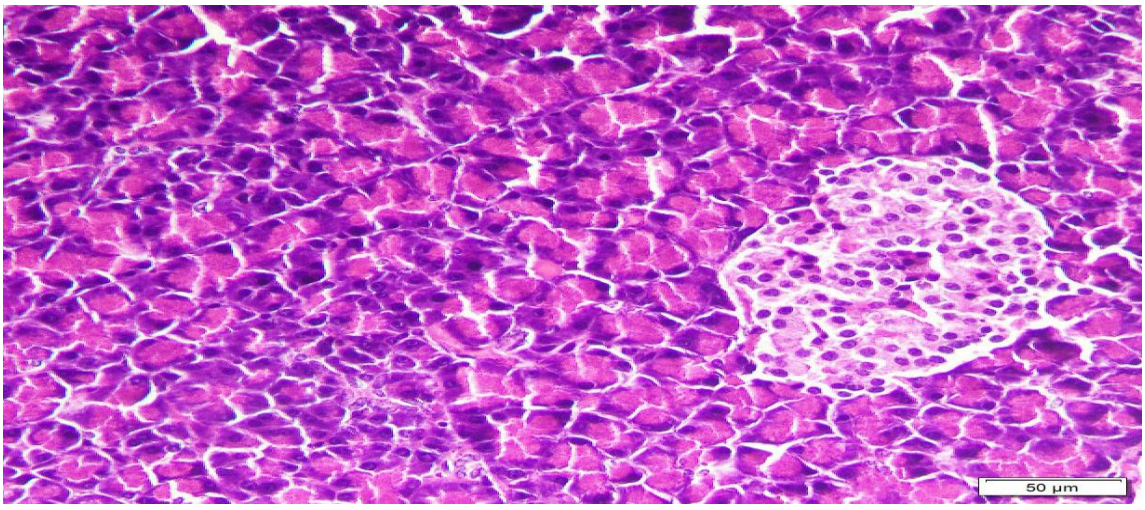

Figure 3: Section in the pancreas of a rat in AP treated with verapamil group showing apparently normal acini in many fields, pale nuclei, apical acidophilia, basal basophilia of acini with apparently normal islets, few fields infiltrated by inflammatory cells and some acini containing few dark nuclei (H&E x 200).

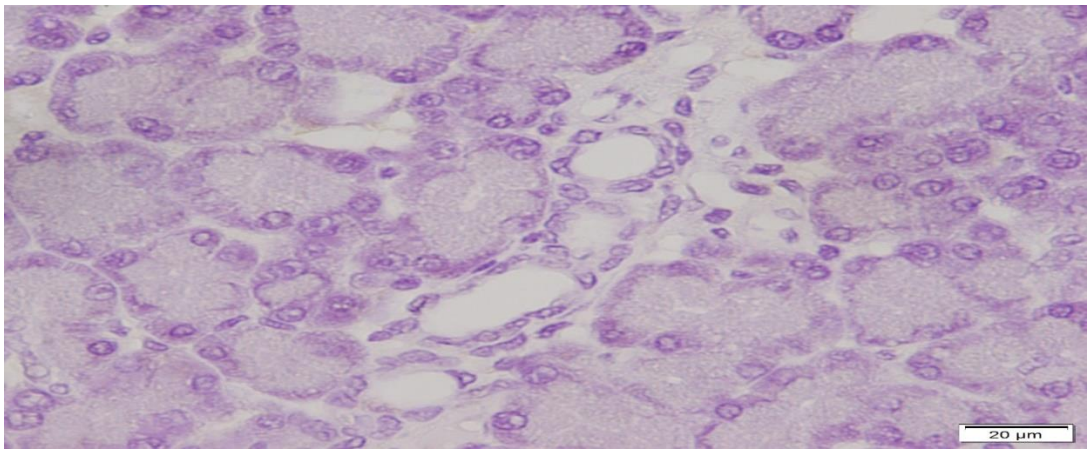

Figure 4: COX2 immunostained sections in pancreas x 400 in control group showing: –ve IE.

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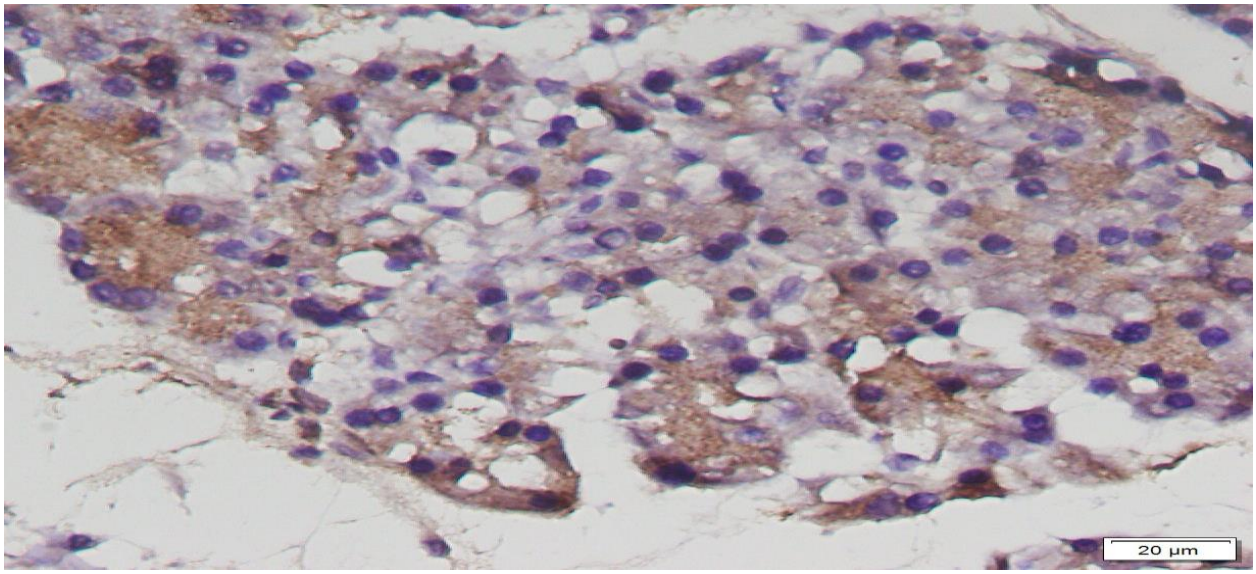

Figure 5: COX2 immunostained sections in pancreas x 400 in AP group showing strong positive (+ve) IE was seen among multiple acini in most fields, among fewer acini in other fields and dense + ve IR was evident among few acini.

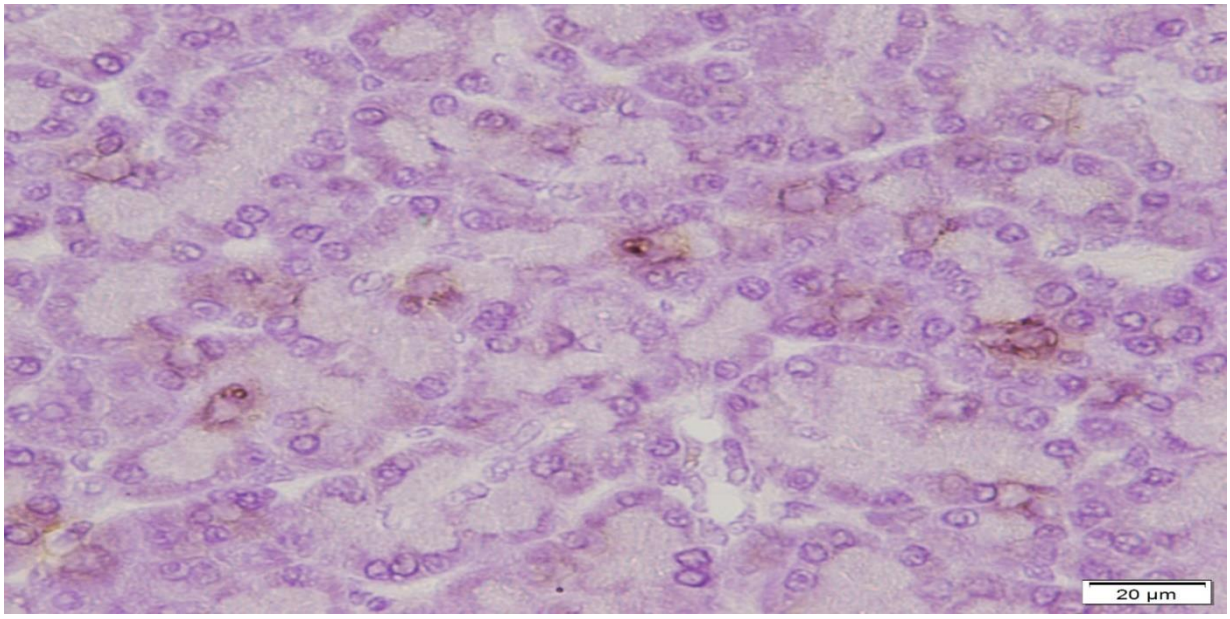

Figure 6: COX2 immunostained sections in pancreas x 400 in AP treated with verapamil group showing weak + ve IE among few acinar cells.

Table 1: Mean values (± SD) of serum amylase and lipase

Groups	Serum Amylase (U/ml)	Serum Lipase (U/ml)
Group I (Control)	2008.20±13.21	42.82±2.73
Group II (AP group)	10786.24±9.50*	1190.07±3.47*
Group III (Verapamil group)	3815.65±13.38^●	476.21±8.21^●

sig ≤ 0.05

* Increase versus control and verapamil group

^ Increase versus control

● Decrease versus AP group

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Histogram 1: Mean values (± SD) of serum amylase and lipase

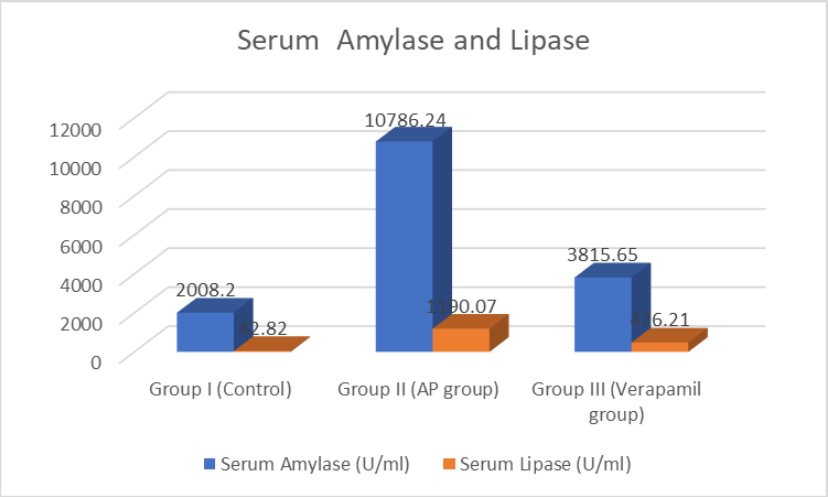

Table 2: Mean values of MDA and Catalase

Groups	MDA (nM/gm)	Catalase (U/gm)
Group I (control)	24.04±3.20	33.65±2.93
Group II (AP group)	87.08±5.32*	8.97±0.98**
Group III (verapamil group)	41.12±5.18^	28.51±5.11^^

sig ≤ 0.05

* Increase and ** decrease versus control and treated group.

^ Decrease and ^^ increase versus group II.

Histogram 2: Mean values of MDA and Catalase

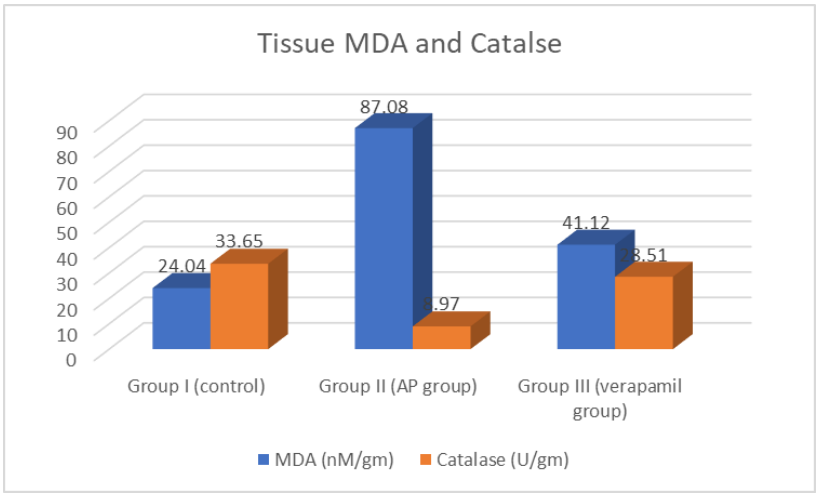

Table 3: Table 3: Mean area (µ2) (± SD) of dark nuclei and interstitial ZGs.

Groups	Area of dark nuclei	Area of interstitial ZGs
Group I (Control)	0	0
Group II (AP group)	20.19±3.21*	10.84±1.24*
Group III (Verapamil group)	4.15±0.31^	2.05±0.1^

sig ≤ 0.05

* Increase versus control and treated group.

^ decrease versus group II.

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Histogram 3: Mean area (µ2) (± SD) of dark nuclei and interstitial ZGs.

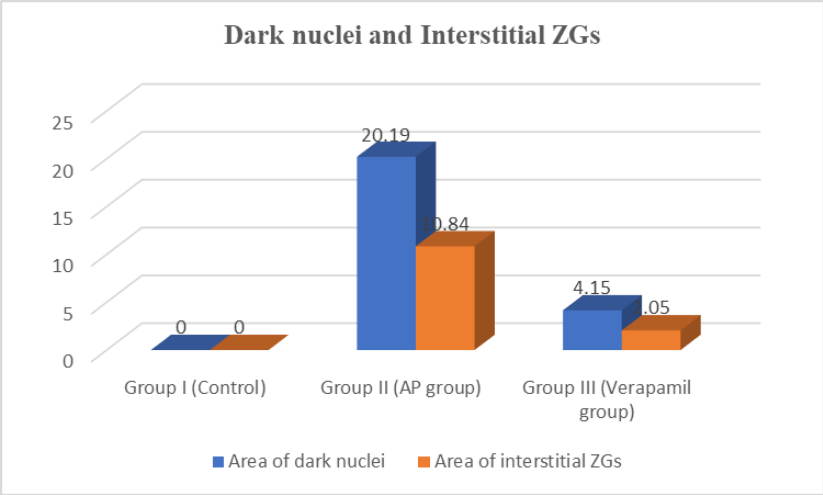

Table 4: Mean area% (± SD) of COX2 IE and mean optical density of COX2 IR.

Groups	Area% of COX2 IE	Optical density of COX2 IR
Group I (Control)	0	0
Group II (AP group)	25.71±4.29*	0.83±0.2*
Group III (Verapamil group)	3.67±0.89^	0.09±0.09^

* P≤ 0.05 increase versus control and treated group.

^ P≤ 0.05 decrease versus group II.

Histogram 4: Mean area% (± SD) of COX2 IE and mean optical density of COX2 IR.