Egyptian Journal of Cell and Tissue Research
Print ISSN: 2812-5436 / Online ISSN: 2812-5444

The Possible Protective Effect of Nifedipine Against L-Arginine Induced Acute Pancreatitis in Adult Male Albino Rats: Histological and Immunohistochemical Study

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Abstract:

Background: Acute pancreatitis (AP) is a global inflammatory disease with high morbidity and mortality rates. Nifedipine, an antioxidant and anti-inflammatory, can prevent oxidative stress-induced acinar cell damage. **Aim of the work:** The study aimed to investigate the possible protective effect of Nifedipine against L-arginine induced AP in adult male albino rats. **Materials and methods:** The study involved 40 adult male albino rats in five groups (n=8, each group): normal control, Nifedipine (5mg/Kg/day/orally for one week), AP induced in the other groups by 2 doses of L-arginine Hcl injections (250mg/100gm) with 1 hour interval between the two doses, then divided in to L-arginine group (AP), L-arginine + Nifedipine for 1 week, and recovery group which is L-arginine group left for 2weeks. After sacrifice, rats' pancreas were examined for biochemical, histological, immuno-histochemical, and morphometric studies. **Results:** The study revealed distortion & irregular acinar architecture, dilated and congested blood capillaries, inflammatory cell infiltration, cytoplasmic vacuolations and widened spaces CT stroma between the pancreatic lobules and around the acini. The islets appeared atrophied. The L-arginine + Nifedipine group showed improvement in histological architecture. Nifedipine attenuated AP by decreasing serum amylase and lipase levels, oxidative stress stimulation,

and pancreatic tissue levels of MDA, MPO, and TNF-α. It also increased GSH and Nrf2 content in addition to a significant decrease in immunoreactivity of Caspase-3 & NF-kB. The recovery group showed mild improvement in some histological and biochemical findings. **Conclusion:** Nifedipine is recommended as an adjuvant treatment due to its strong therapeutic effects during acute pancreatitis course treatment**.**

Keywords:

Acute Pancreatitis, Nifedipine, L-Arginine, oxidative stress, NF-kB, Caspase-3.

1. Introduction:

Acute pancreatitis (AP) is a mild to severe inflammatory condition of the exocrine pancreatic parenchyma with substantial morbidity and mortality **(1)**. Life-threatening AP, a reversible process that generates interstitial edema, inflammatory cell infiltration, apoptosis, necrosis, and bleeding, mostly causes exocrine dysfunction, but recurrent inflammation and fibrosis can cause endocrine dysfunction **(2)**.

AP is primarily caused by gallstones (40-70%), alcohol, (smoking25-35%), hypertriglyceridemia, post-endoscopic retrograde cholangiopancreatography (ERCP), genetic risk, medications, and pancreatic duct injury **(3-5)**. Drug-induced pancreatitis is rare, with generally excellent prognosis and low mortality **(6)**. Pancreatic duct injury, biliary sludge, microlithiasis, biliary obstruction, autoimmune pancreatitis, hypercalcemia, and infections are rare causes of acute pancreatitis. These injuries can cause acute duct rupture, pancreatic ascites, scarring, and stricture, while infections and toxins can also contribute **(7-9)**.

AP is caused by intra-acinar zymogen activation, autophagy, oxidative stress, mitochondrial dysfunction, endoplasmic stress, inflammatory responses, and multi-organ dysfunction syndrome. Premature trypsinogen activation is a key early illness stage regulated by calcium homeostasis, lysosome-zymogen co-localization, and pH change **(10-12)**.

Pancreatic damage crucially involves oxidative stress. Increased reactive oxygen species (ROS) in acinar cells promotes apoptosis and inflammation. After acinar damage, active neutrophils may induce local and

systemic inflammation **(13)**. An imbalanced cellular redox condition causes oxidative damage and signals NFκB (Nuclear factor kappa B) to upregulate pro-inflammatory genes **(14)**. In AP, excessive ROS and NO (Nitric oxide) production damages mitochondria due to mitochondrial malfunction. The mitochondrial permeability transition pore (PTP) opens, membrane potential drops, and cells are damaged **(15)**.

L-arginine, an essential amino acid found in all living proteins, has been intensively used as a NO donor in cardiovascular studies. It treats angina, congestive heart failure, hypertension, and peripheral artery disease **(16)**. Athletes use L-arginine to boost growth hormone and exercise metabolism **(17, 18)**. In rodents, excessive L-arginine supplementation can promote AP by creating oxygen free radicals and inflammatory mediators needed for disease progression **(19)**.

L-arginine can cause AP in rats through various mechanisms, including the generation of oxygen and nitrogen free radicals, which distort zymogen granules' cellular membranes and release digestive enzymes, cellular proteins, and increased inflammatory mediators. Nitric oxide synthase (NOS) converts L-arginine into L-citrulline and nitric oxide, while arginase degrades it into L-ornithine and urea. Inflammatory oxidative stress can lead to cell damage **(20)**.

NO is a key stimulator of inflammatory NF-kB signaling in endothelial cells, inducing release of pro inflammatory Tumor necrosis factor alpha (TNFα) and other cytokines **(21)**. ROS and lipid peroxidation products activate Caspase-3 , causing apoptosis, cell death, and tissue damage **(22)**. Cellular calcium overload can also cause L-arginine-induced-AP, as seen in caerulein hyperstimulation, bile acid infusion, and fatty acid plus ethanol **(23, 24)**.

Nifedipine, a dihydropyridine calcium channel blocker, is often used for treatment of hypertension and angina. In experimental research on various disorders, Nifedipine exhibited antioxidant, anti-inflammatory, and anti-apoptotic properties. These qualities have been shown to inhibit cell death. It reduced ROS, NF-kB, Interleukin-1(IL-1a), Interleukin-6 (IL-6), TNF- α, Inducible nitric oxide synthase (Inos), and Cyclooxygenase-2 (COX-2), and

other inflammatory and oxidative stress markers **(25-29)**. This study used Nifidepine to prevent and treat AP due to its anti-inflammatory, antioxidant, and Ca overload-lowering properties.

2. Materials and Methods:

2.1. Drugs

Nifidepine tablets (Adalat, Bayer Co., Egypt) were purchased and dissolved in 0.9% normal saline for oral use. L-arginine Hcl (DOP, Organic Kimya, Turkey) was prepared from L-arginine powder in a 20% concentration solution, adjusted to 7 PH and volume to 10 ml.

2.2. Animals

Forty adult male albino rats weighing 180-200g, aged 5-6 months, purchased from Nile Pharma Company, and kept in a hygienic laboratory at Beni-Suef University. The rats were kept under a standard chow diet and tap water, and acclimatized for a week before the experiment. The current experiment was approved by the Research Ethics Committee, Faculty of science, Beni-Suef University with approval reference number: (022-266).

2.3. Experimental design

The rats were randomly divided into 5 groups, eight rats each:

Group I (Control):

Received 2 ml 0.9% normal saline orally for one week and received two intraperitoneal (IP) injections of 1ml saline with 1hour interval between the two doses.

Group II (Nifedipine):

Received Nifedipine orally (5mg/Kg) for 1week **(29)**.

Group III (L-Arginine):

Received two IP injections of L-arginine Hcl (250mg/100gm) with 1 hour interval between the two doses and rats were kept for 1week **(30)**.

Group IV (L-arginine + Nifedipine):

Received Nifedipine orally (5mg/Kg) after 1hour of L-arginine dose for 1week **(29)**.

Group V (Recovery):

Received two IP injections of L-arginine Hcl (250mg/100gm) with 1hour interval between the two doses and rats were kept for 2weeks.

2.4. Experimental procedure:

Experimental procedures were carried out at the animal laboratory of the Faculty of Veterinary medicine, Beni-Suef University. After the last day of the experiment, body weight of rats was recorded, and then anesthetized with ketamine, followed by collecting blood

samples. Animals from control and experimental groups were sacrificed via cervical dislocation, and pancreas was separated through an incision in the abdominal cavity, washed with ice-cold saline. Pancreatic weight was recorded for each rat. Sections were homogenized in phosphate-buffered saline. The tissue specimens were cut longitudinally and fixed in a neutral buffered formalin solution for 24 hours **(31)**. Post-fixation, the specimens were processed for the paraffin technique, and 5-6 µm thick serial sections were cut and mounted on charged slides for immunohistochemical studies.

2.5. Pancreatic weight index (PWI)

The mean pancreatic weight as a ratio of body weight was calculated (PW/BW ratio) **(32)**.

2.6. Biochemical assay:

Serum lipase and α amylase were measured in blood samples. Malondialdehyde (MDA), Myeloperoxidase (MPO), Glutathione (GSH), Tumor necrosis factor alpha (TNFα), and Nuclear factor erythroid 2-related factor 2 (Nrf2) were measured in pancreatic tissue homogenate using suitable colorimetric and ELISA kits, following manufacturer's instructions.

2.7. Histological study:

Prepared paraffin sections were subjected to:

1. Hematoxylin and Eosin (H &E) to demonstrate the histological changes **(33)**.

2. Masson's Trichrome stain to demonstrate stromal changes in collagen deposition **(33)**.

3.Toluidine blue stain to detect zymogen granules in the apical part of pancreatic acinar cells and evaluate secretory function of pancreatic acinar cells **(34)**.

2.8. Immunohistochemical study:

Caspase-3 (predominantly cytoplasmic with some nuclear staining) was used as an indicator of apoptosis **(35)**, Nuclear Factor kappa B (NF-kB) (mainly cytoplasmic) was used as an indicator of inflammation **(35).**

Paraffin sections were mounted on ready-to-use slides, deparaffinized in xylene, rehydrated in descending grades of ethanol, and placed in a humidity chamber. Boiling tissue sections in 1mM EDTA and chilling at room temperature brought out antigens. Sections were incubated in H_2O_2 for 15 minutes and rinsed in PBS to suppress endogenous peroxidase activity. Covering portions with serum blocking solution and

incubating at room temperature for 10 minutes eradicated non-specific background. Secondary antibodies were added to sections after primary antibodies (Caspase-3: Labvision, Cat. RB-1197-R7, NF-kB P65: GeneTex, Cat. GTX107678). After applying "Streptavidin peroxidase conjugate" to slides, PBS rinsed and dried them. The sections were counterstained with Mayer's hematoxylin after applying a substrate-chromogen combination. Before Histomount and coverslip mounting, slides were dehydrated in ethanol and cleaned in xylene.

2.9. Morphometric study:

Mean area percent of collagen stained with Masson's trichrome, in addition to Caspase-3 , and NF-kB Immunopositive reaction were measured in all five groups using an image analyzer computer system with Leica Qwin 500 software at Beni-Suef University's Faculty of Veterinary Medicine (Cambridge, England). Ten randomly selected, non-overlapping fields were evaluated at a magnification of 400 for each region.

2.10. Statistical analysis:

Data were statistically analyzed using the Statistical Package of Social Science (SPSS version 25) software. The mean of each group was compared with the others using the one-way analysis of variance (one way ANOVA) followed by the post Hoc "Tukey" test. The significant difference was defined as P-values < 0.05. Graphs were done using graphpad prism software version version 8 (California, U.S.) **(36)**.

3. Results:

3.1. Mean Pancreatic Weight Index in control and experimental groups

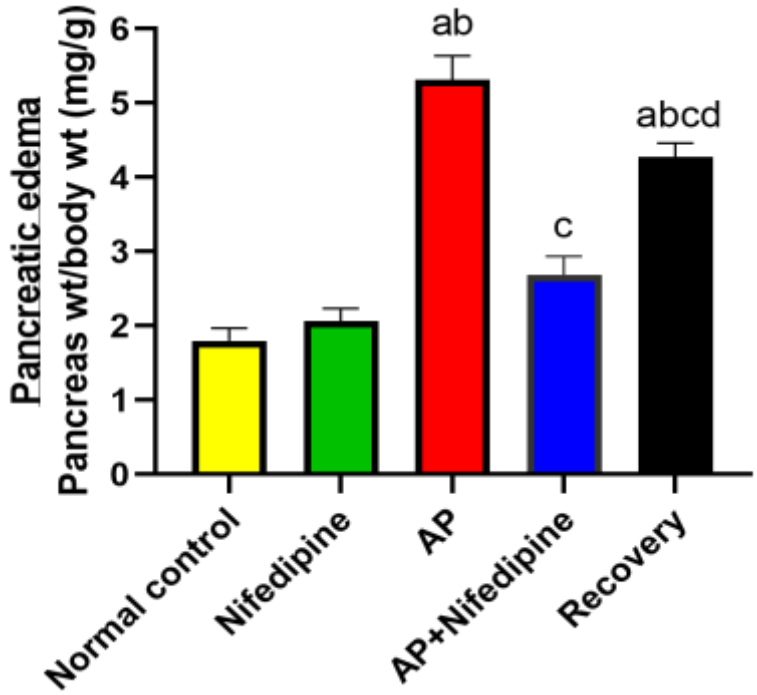
Results are presented in Table (1) and Histogram (1).

Pancreatic edema did not significantly differ between Nifedipine and control groups. In addition, L-arginine-induced-AP caused significant increased pancreatic edema than control and Nifedipine. AP rats treated with Nifedipine showed significant decreased pancreatic edema than AP and recovery groups, restoring it to normal levels. Recovery group had significant more pancreatic edema than control and Nifedipine groups and less than AP group.

Table (1): Mean Pancreatic Weight Index in all experimental groups.

Groups Mean±SEM	**Pancreatic weight index (mg/g)**
Control	1.80±0.169
Nifedipine	2.06±0.170
AP	5.31±0.327[ab]
AP+Nifedipine	2.69±0.25[c]
Recovery	4.28±0.190[abcd]
Sample size	8 rats in each group
F.value (df: 4,35)	43.21
P.value	<0.0001[*]

[a]Significant difference with normal control group, [b]Significant difference with Nifedipine group, [c]Significant difference with AP group, [d]Significant difference with AP+Nifedipine group. [*]Significantly different at P<0.05



Histogram (1): Mean Pancreatic Weight Index in all experimental groups.

3.2. Serological biochemical assay

Mean serum α amylase and lipase in control and experimental groups

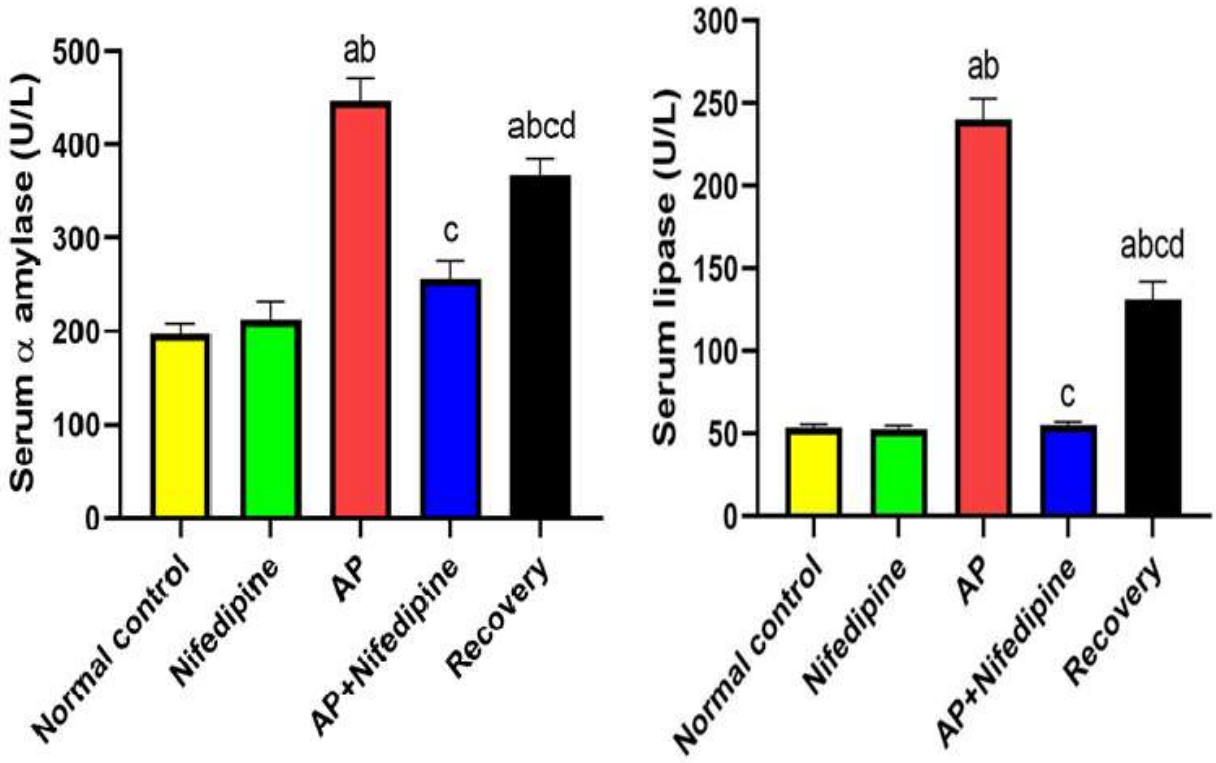
Results are presented in Table (2) and Histogram (2).

Serum α amylase and lipase did not significantly differ between Nifedipine and control groups. In addition, L-arginine-induced-AP caused significant increased α amylase and lipase than control and Nifedipine groups. AP rats treated with Nifedipine showed significant decreased α amylase and lipase than AP and recovery groups, restoring them to normal levels. Recovery group had significant higher α amylase and lipase than control and Nifedipine groups and lower than AP group.

Table (2): Mean serum α amylase and lipase in control and experimental groups.

Groups Mean±SEM	**Serum α amylase (U/L)**	**Serum lipase (U/L)**
Control	197.1±11.78	53.53±2.15
Nifedipine	212.7±18.80	52.94±1.83
AP	446.5±24.65[ab]	240.1±12.65[ab]
AP+Nifedipine	255.9±20.74[c]	55.34±1.70[c]
Recovery	367.4±18.40[abcd]	131.4±10.84[abcd]
Sample size	8 rats in each group	
F.value (df: 4,35)	30.81	116.00
P.value	<0.0001[*]	<0.0001[*]

[a]Significant difference with normal control group, [b]Significant difference with Nifedipine group, [c]Significant difference with AP group, [d]Significant difference with AP+Nifedipine group. [*]Significantly different at P<0.05



Histogram (2): Mean serum α amylase and lipase in all experimental groups.

3.3. Pancreatic tissue biochemical assay

Mean pancreatic oxidative stress and inflammatory markers in control and experimental groups

Results of oxidative stress and inflammatory markers; MDA, MPO, and TNFα in addition to antioxidant markers; GSH, Nrf2 are presented in Table (3) and Histogram (3).

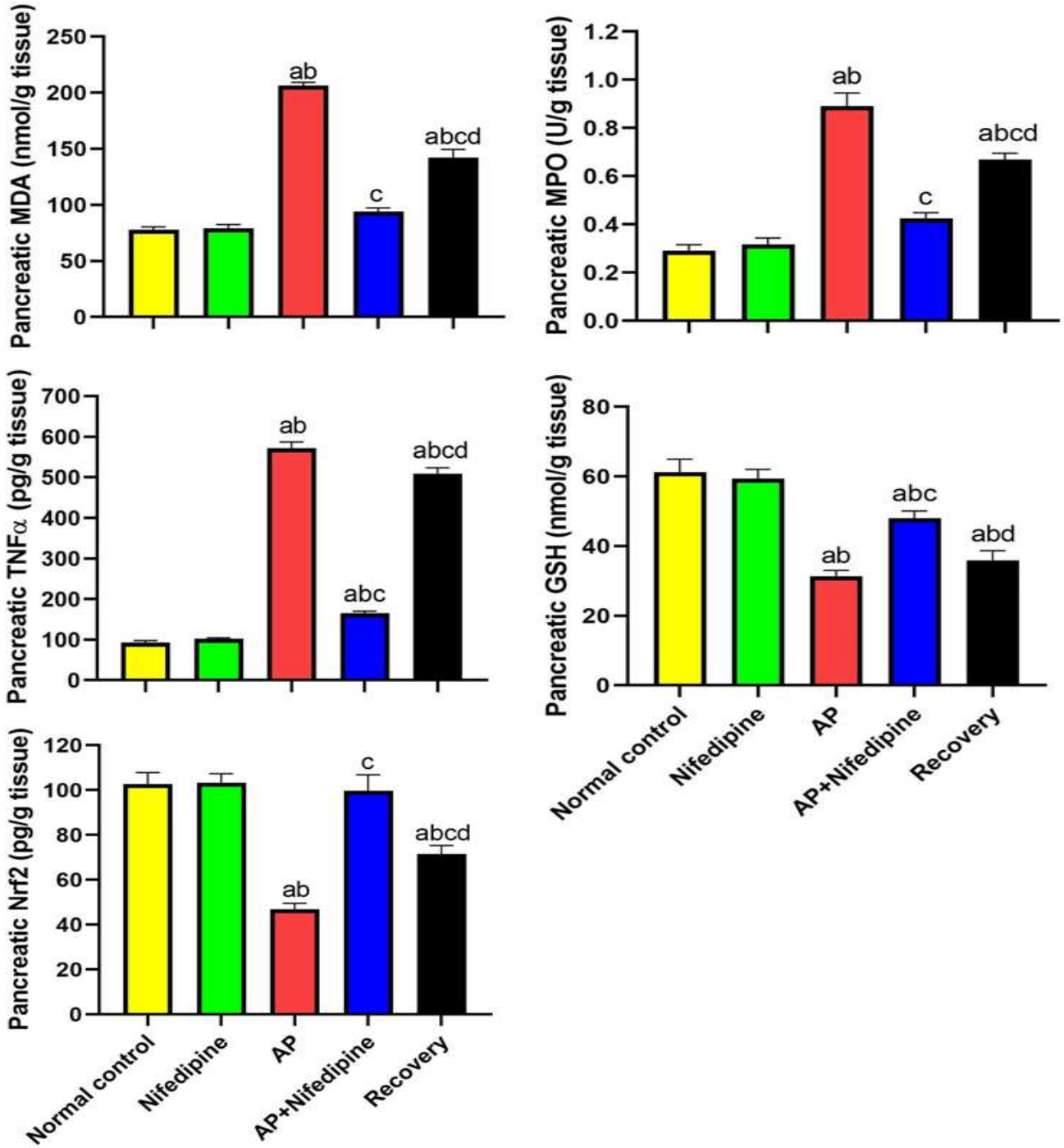
Pancreatic MDA, MPO, TNFα, GSH, and Nrf2 did not significantly differ between Nifedipine and control groups. In addition, L-arginine-induced-AP caused significant higher MDA, MPO, TNFα and significant lower GSH, Nrf2 than control and Nifedipine. AP rats treated with Nifedipine showed significant lower MDA, MPO, TNFα and significant higher GSH, Nrf2 than AP rats and recovery rats, restoring MDA, MPO, Nrf2 to normal levels. In addition, AP rats treated with Nifedipine showed significant higher TNFα and lower GSH as compared to control and Nifedipine treated rats. Recovery group had significant higher MDA, MPO, TNFα and significant lower GSH, Nrf2 than control and Nifedipine groups. Recovery group had significant lower MDA, MPO, TNFα and

significant higher Nrf2 than AP group. Moreover, Recovery group did not significantly change as compared to AP group.

Table (3): Mean pancreatic oxidative stress and inflammatory markers in control and experimental groups.

Groups Mean±SEM	**Pancreatic MDA (nmol/g tissue)**	**Pancreatic MPO (U/g tissue)**	**Pancreatic TNFα (pg/g tissue)**	**Pancreatic GSH (nmol/g tissue)**	**Pancreatic Nrf2 (pg/g tissue)**
Control	77.78±2.66	0.291±0.024	93.10±4.85	61.23±3.73	102.6±5.18
Nifedipine	79.26±3.33	0.316±0.028	101.6±2.91	59.34±2.69	103.3±4.10
AP	206.4±2.94[ab]	0.891±0.054[ab]	571.4±15.61[ab]	31.31±1.69[ab]	46.86±2.73[ab]
AP+Nifedipine	94.24±3.08[c]	0.425±0.023[c]	165.5±4.78[abc]	48.01±2.09[abc]	99.78±7.04[c]
Recovery	142.1±7.31[abcd]	0.669±0.027[abcd]	509.2±14.43[abcd]	35.91±2.79[abd]	71.43±3.94[abcd]
Sample size	8 rats in each group				
F.value (df: 4,35)	168.00	59.72	535.40	25.06	26.91
P.value	<0.0001[*]	<0.0001[*]	<0.0001[*]	<0.0001[*]	<0.0001[*]

[a]Significant difference with normal control group, [b]Significant difference with Nifedipine group, [c]Significant difference with AP group, [d]Significant difference with AP+Nifedipine group. [*]Significantly different at P<0.05



Histogram (3): Mean pancreatic oxidative stress and inflammatory markers in all experimental groups.

3.4. Histological results

3.4.1. Hematoxylin and Eosin-stained pancreatic sections

Group I showed normal exocrine and endocrine pancreatic tissue. Normal pale stained islets of Langerhans are regular with normal size. Interlobar ducts were noticed. Centroacinar cells were detected. Normal blood capillaries were observed (Figs.1A). **Group II (Nifedipine)** showed similar findings as control (Figs.1B). **Group III (L-arginine)** showed significant structural changes in pancreatic tissue compared to the control and Nifedipine groups, including loss of normal histological architecture of exocrine and endocrine parts, widened spaces between lobules, distortion of pancreatic acini, cytoplasmic vacuolations in acinar and islet cells, dilated blood vessels, massive inflammatory cell infiltration, and atrophied pancreatic islets. Widened C.T. stroma between the pancreatic lobes and lobules were noted (Figs.1C), compared to group I and II.

Group IV (L-arginine+Nifedipine) revealed improved pancreatic structure, normal pancreatic acini with basal basophilia, apical acidophilia, and rounded nuclei, with minimal inflammatory cell infiltration and delicate separation of pancreatic lobules, compared to group III. The ducts and islets of Langerhans showed normal appearance, with normal architecture and size, and blood capillaries were observed (Figs.2D). **Group V (Recovery)** revealed widened spaces between pancreatic lobules, irregular architecture of pancreatic acini, dilated blood vessels, inflammatory cell infiltration, normal islet, irregular interlobular duct architecture, and some acini showing cytoplasmic vacuolation (Figs.2E).

3.4.2. Masson's trichrome stained sections

Group I (Control) showed delicate collagen fibers in thin septa and around the pancreatic acini (Figs.3A). **Group II (Nifedipine)** showed similar findings as group I (Figs.3B). **Group III (L-arginine)** showed remarkable increased collagen C.T. fibers deposition in the exocrine pancreas around blood vessel, pancreatic duct, and islets of Langerhans (Figs.3C), compared to group I and II. **Group IV (L-arginine+Nifedipine)** showed minimal collagen C.T. fibers deposition in the exocrine pancreas around blood vessel, pancreatic duct, and islets of Langerhans (Figs.4D), compared to group III. **Group V (Recovery)** showed increased collagen C.T when compared to control and Nifedipine groups. Fibers deposition in the exocrine pancreas between acinar cells and around the blood vessels was detected. Moderate amount of collagen fibers associated with the pancreatic ducts was also observed (Figs.4E).

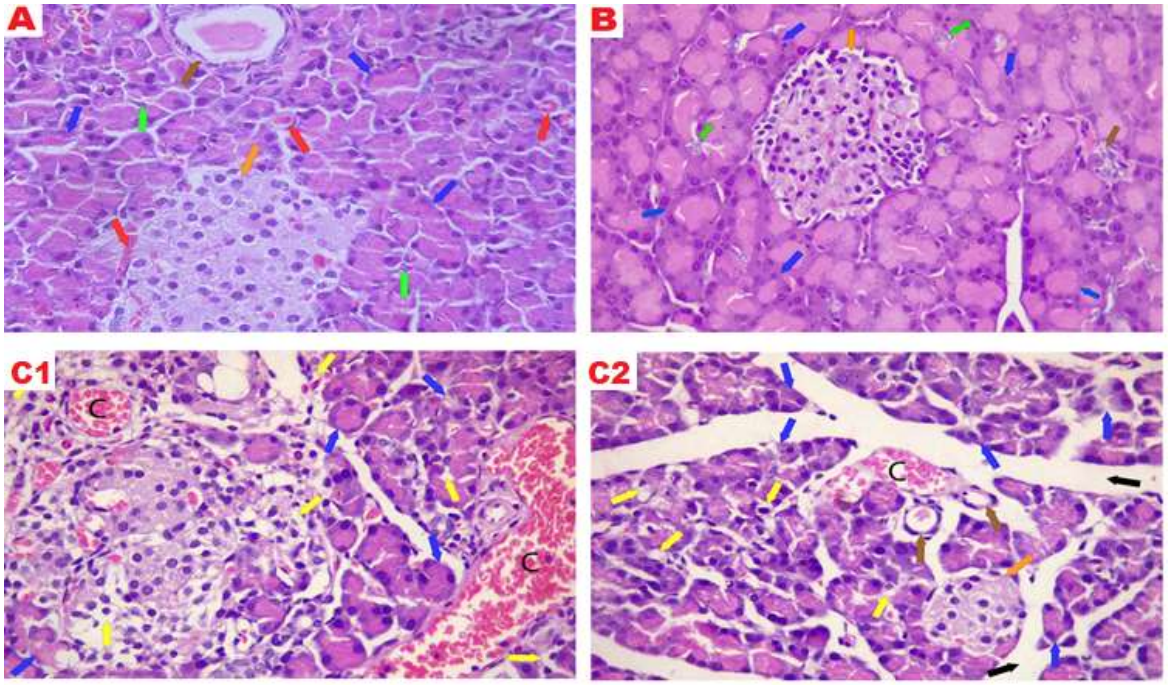


Fig.1: Hematoxylin and Eosin-stained pancreatic sections. A: Control, B: Nifedipine, C: L-arginine.

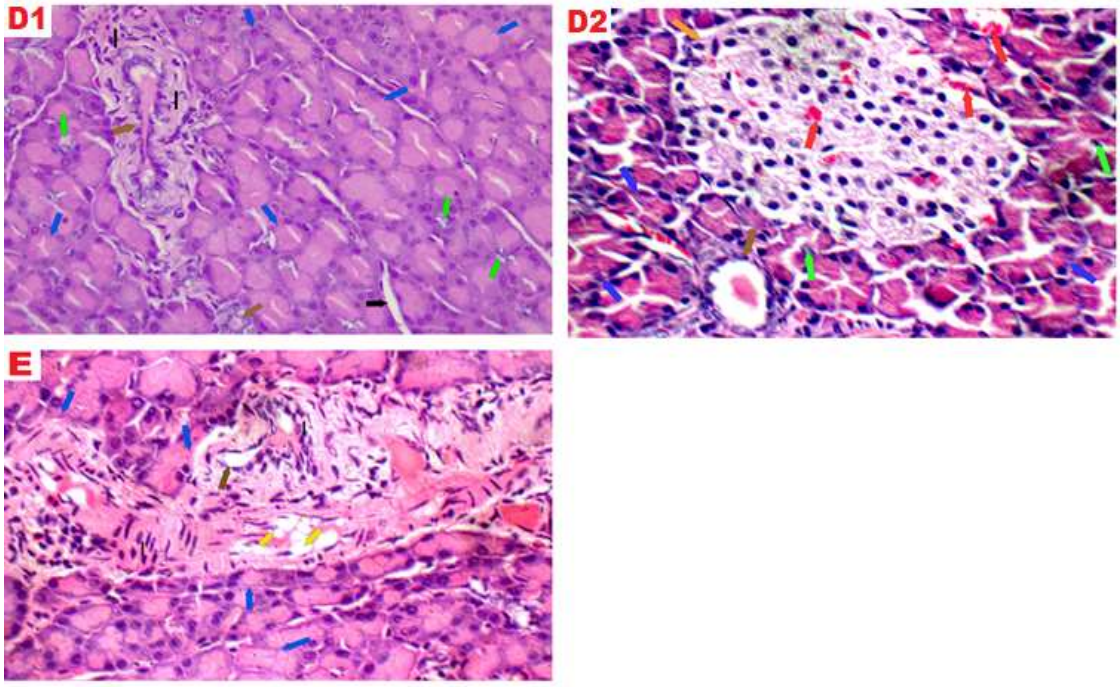


Fig.2: Hematoxylin and Eosin-stained pancreatic sections. D: L-arginine+Nifedipine, E: Recovery.

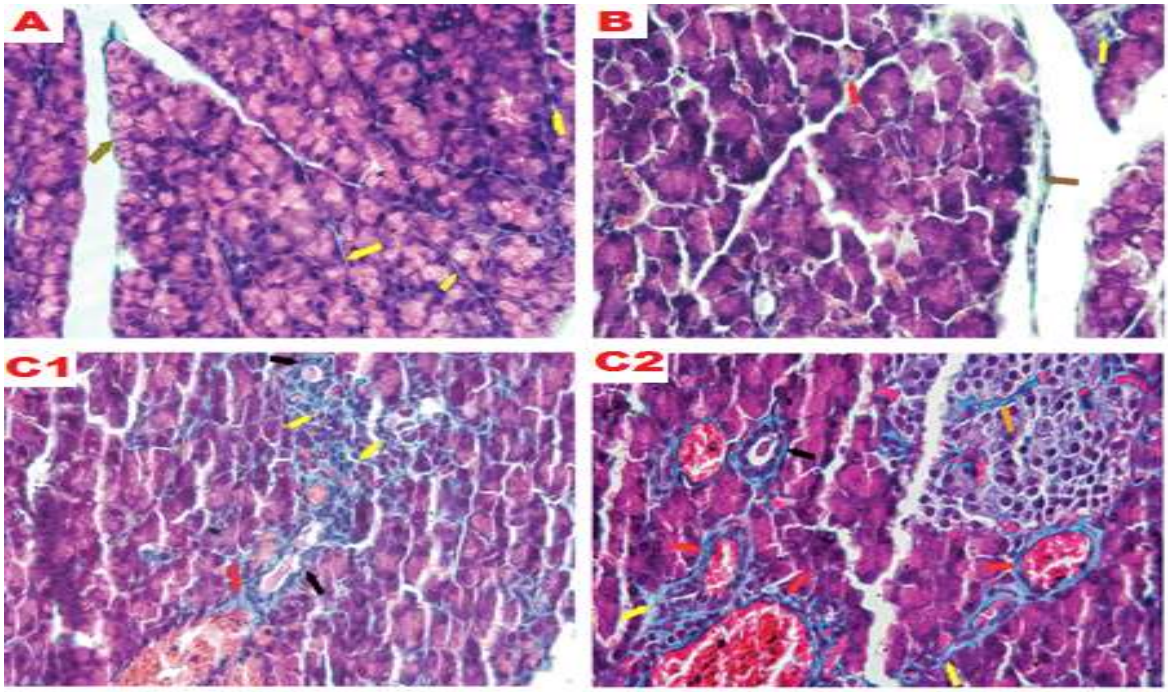


Fig. 3: Masson's trichrome stained sections. A: Control, B: Nifedipine, C: L-arginine.

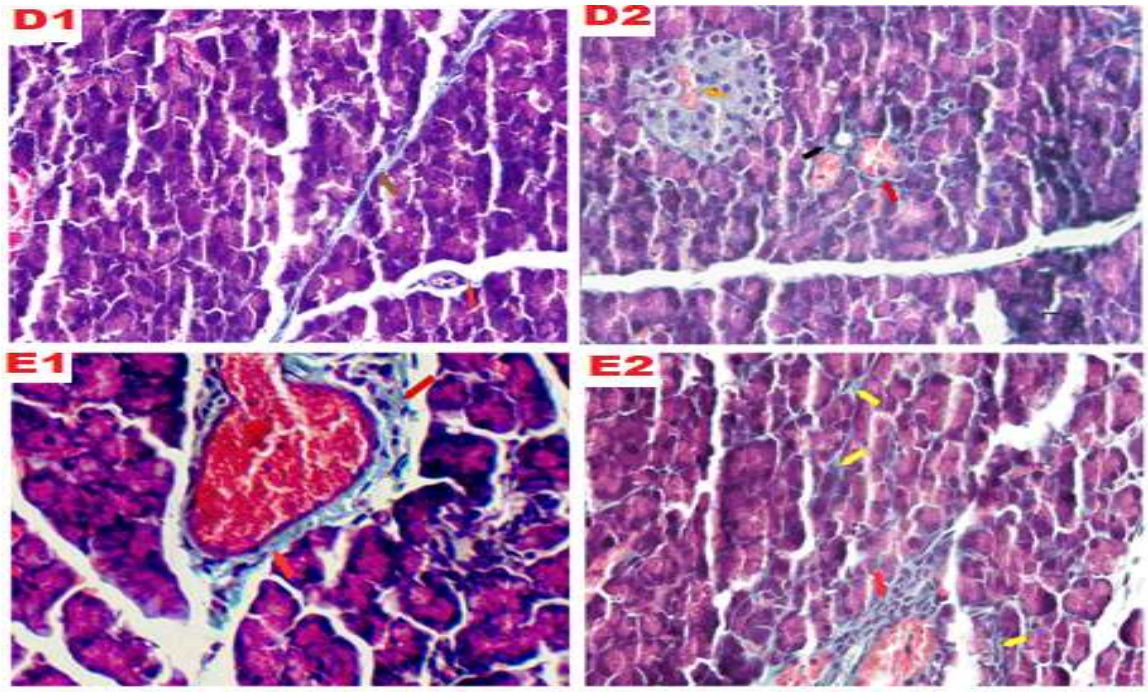


Fig. 4: Masson's trichrome stained sections. D: L-arginine+Nifedipine, E: Recovery.

3.4.2. Toluidine blue stained sections

Group I (Control) showed preserved zymogen granules with average amount in the apical part of pancreatic acinar cells (Figs. 5A1). The islets were distinct and had beta cells that exhibited normal nuclei and average amount of cytoplasmic granules (Figs.5A2). **Group II (Nifedipine)** showed similar findings as group I (Figs.5B1, B2). **Group III (L-arginine)** showed decreased amount of the zymogen granules in the apical part of pancreatic acinar cells (Figs 5C1) and decreased amount of cytoplasmic granules in the beta cells (Figs.5C2). **Group IV (L-arginine+Nifedipine)** showed restoration of the zymogen granules in the apical part of pancreatic acinar cells (Figs.6D1). Average amount of cytoplasmic granules were observed in the beta cells (Figs.6D2), compared to group III. **Group V (Recovery)** showed reduction of zymogen granules in the apical part of pancreatic acinar cells (Figs.6E2), and decreased amount of cytoplasmic granules in the beta cells (Figs.6E1).

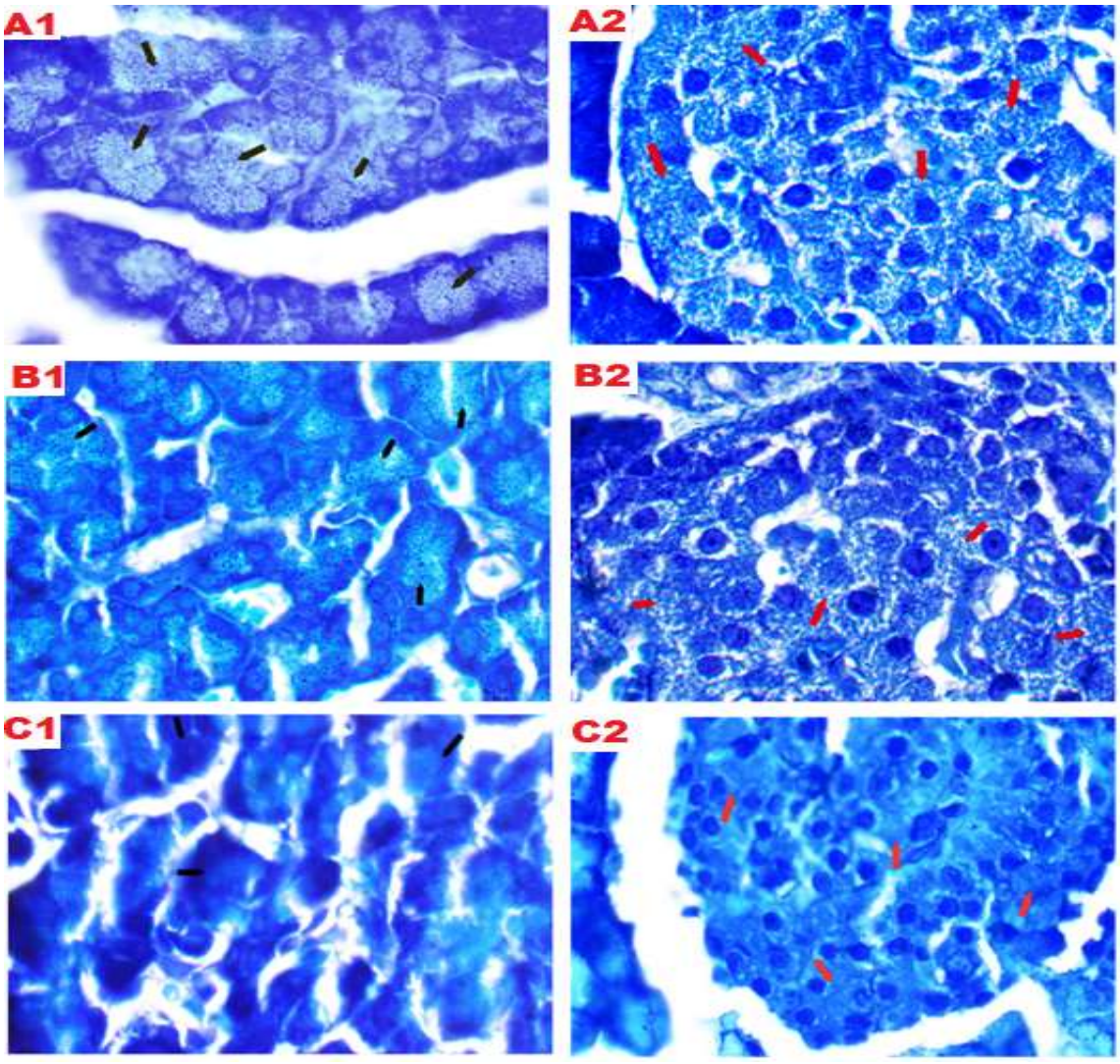


Fig. 5: Toluidine blue stained sections**.** A: Control, B: Nifedipine, C: L-arginine.

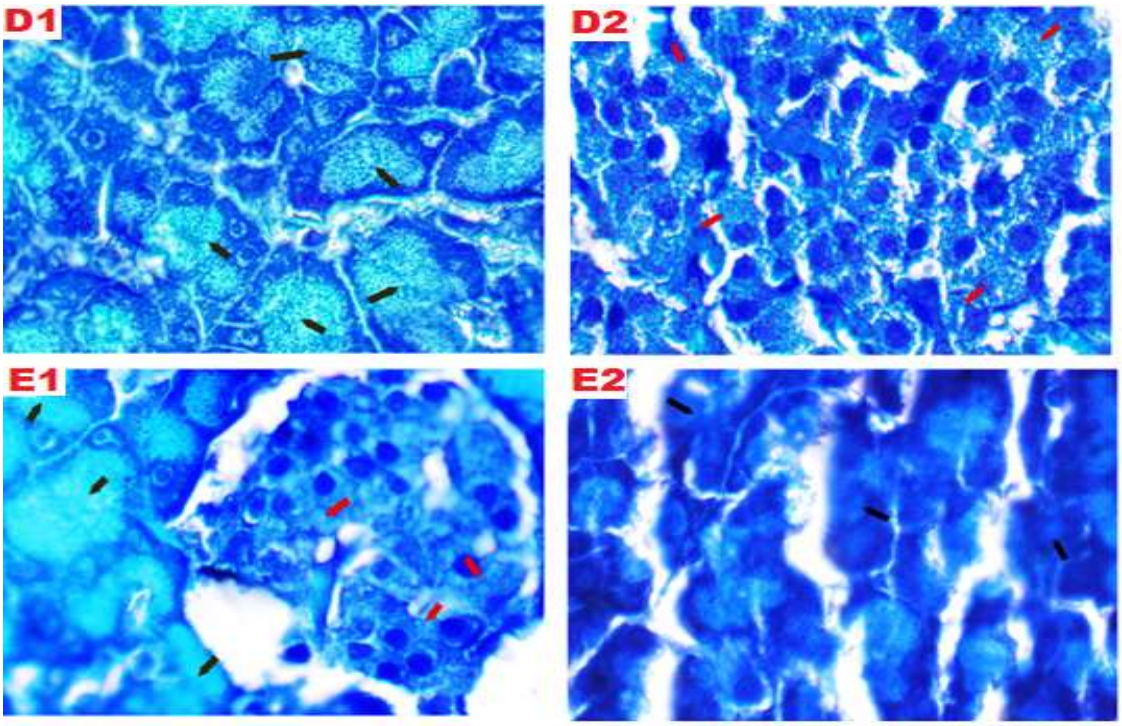


Fig. 6: Toluidine blue stained sections**.** D: L-arginine+Nifedipine, E: Recovery.

3.5. Immunohistochemical results

3.5.1. Anti-Caspase-3 Immunostained Sections

Group I (Control) showed negative cytoplasmic immune-expression of Caspase-3 in the pancreatic acinar cells (Figs.7A) and islets of Langerhans (Figs.7A2). **Group II (Nifedipine)** showed similar findings as group I (Figs.7B1, B2). **Group III (L-arginine)** showed strong positive cytoplasmic immune-expression of Caspase-3 as a brown cytoplasmic reaction in the acinar cells (Figs.7C1) and islets of Langerhans (Figs.7C2), compared to group I and II. **Group IV (L-arginine+Nifedipine)** showed weak cytoplasmic immune-expression of Caspase-3 in the acinar cells (Figs.8D1) and islets of Langerhans (Figs.8D2), compared to group III. **Group V (Recovery)** showed moderate cytoplasmic immune-expression of Caspase-3 in the acinar cells (Figs.8E1) and islets of Langerhans (Figs.8E2).

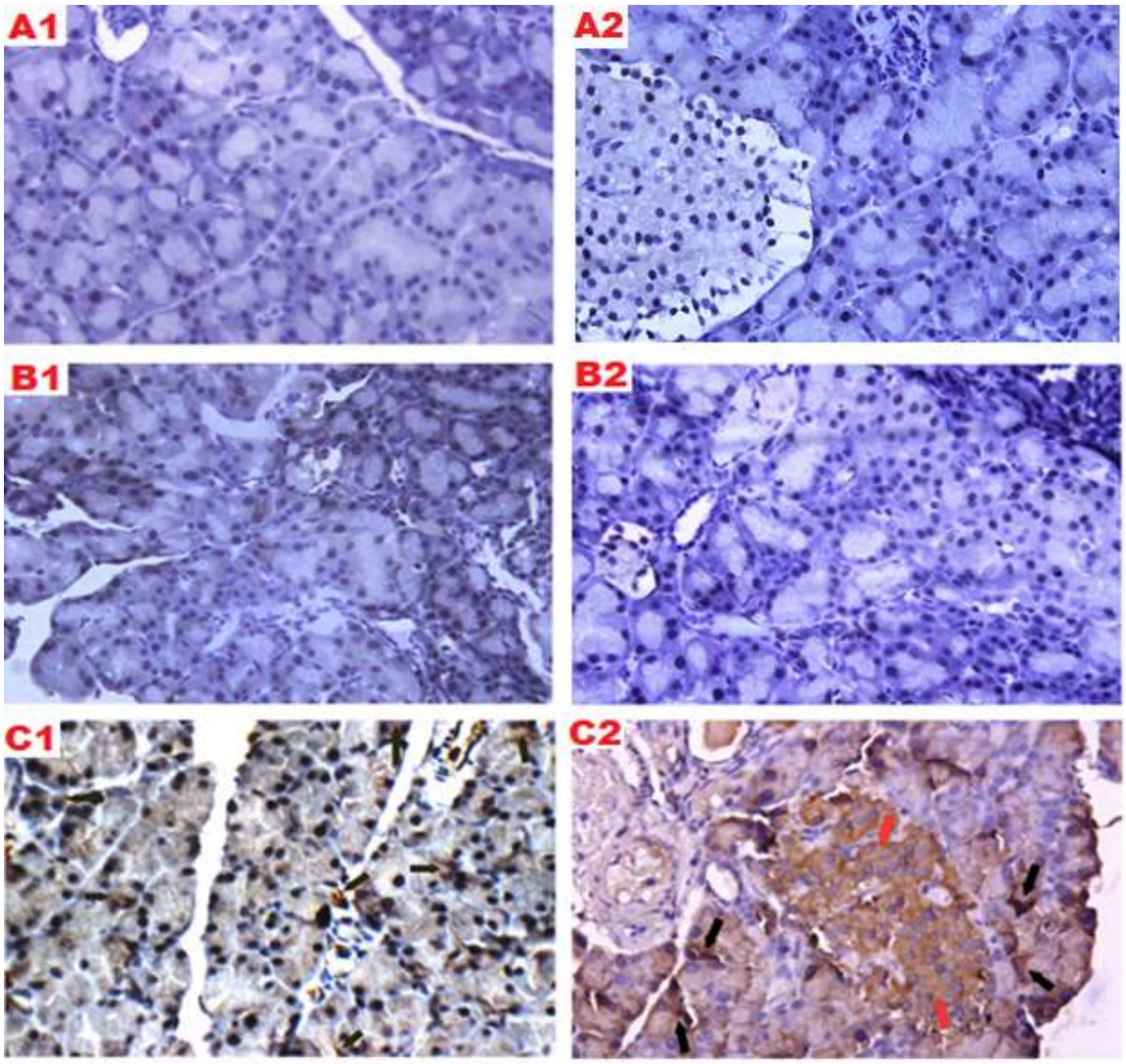


Fig. 7: Anti-Caspase-3 Immunostained Sections. A: Control, B: Nifedipine, C: L-arginine.

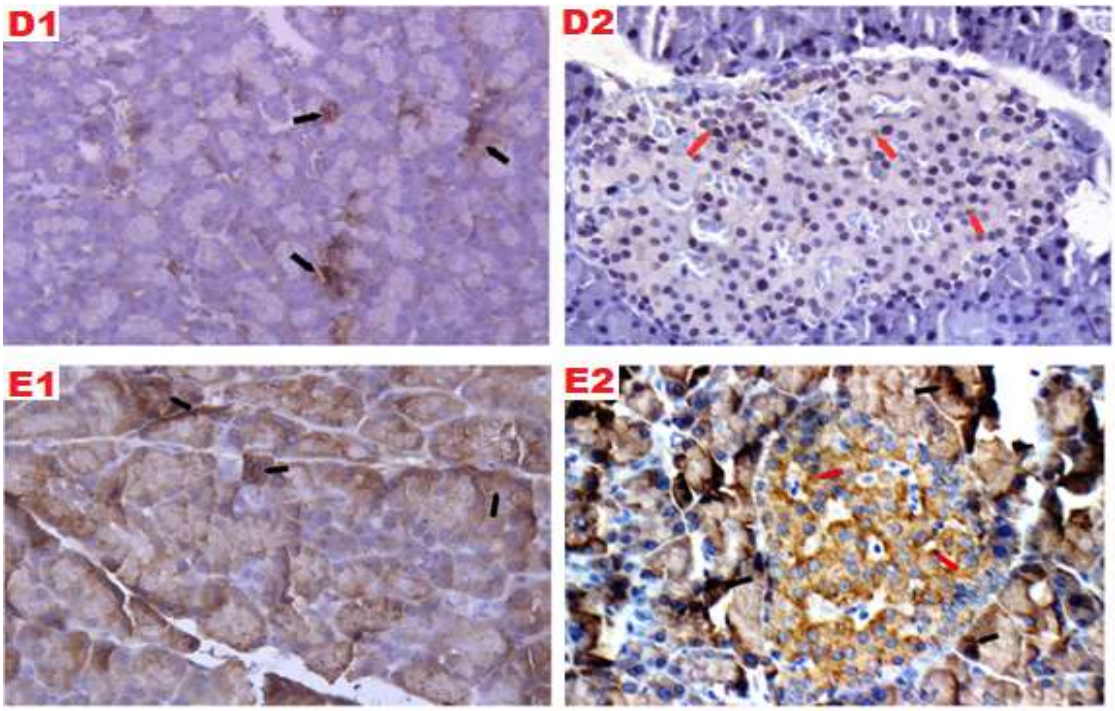


Fig. 8: Anti-Caspase-3 Immunostained Sections. D: L-arginine+Nifedipine, E: Recovery.

3.5.2. Anti-NF-kB Immunostained Sections

Group I (Control) showed negative cytoplasmic immune-expression of NF-kB in the pancreatic acinar cells and islets of Langerhans (Figs.9A). **Group II (Nifedipine)** showed similar findings as group I (Figs.9B). **Group III (L-arginine)** showed strong positive cytoplasmic immune-expression of NF-kB as a brown cytoplasmic reaction in the acinar cells (Figs.9C1) and islets of Langerhans (Figs.9C2), compared to group I and II. **Group IV (L-arginine+Nifedipine)** showed weak cytoplasmic immune-expression of NF-kB in the acinar cells and islets of Langerhans, compared to group III (Figs.9D). **Group V (Recovery)** showed moderate cytoplasmic immune-expression of NF-kB in the acinar cells and islets of Langerhans (Figs.9E).

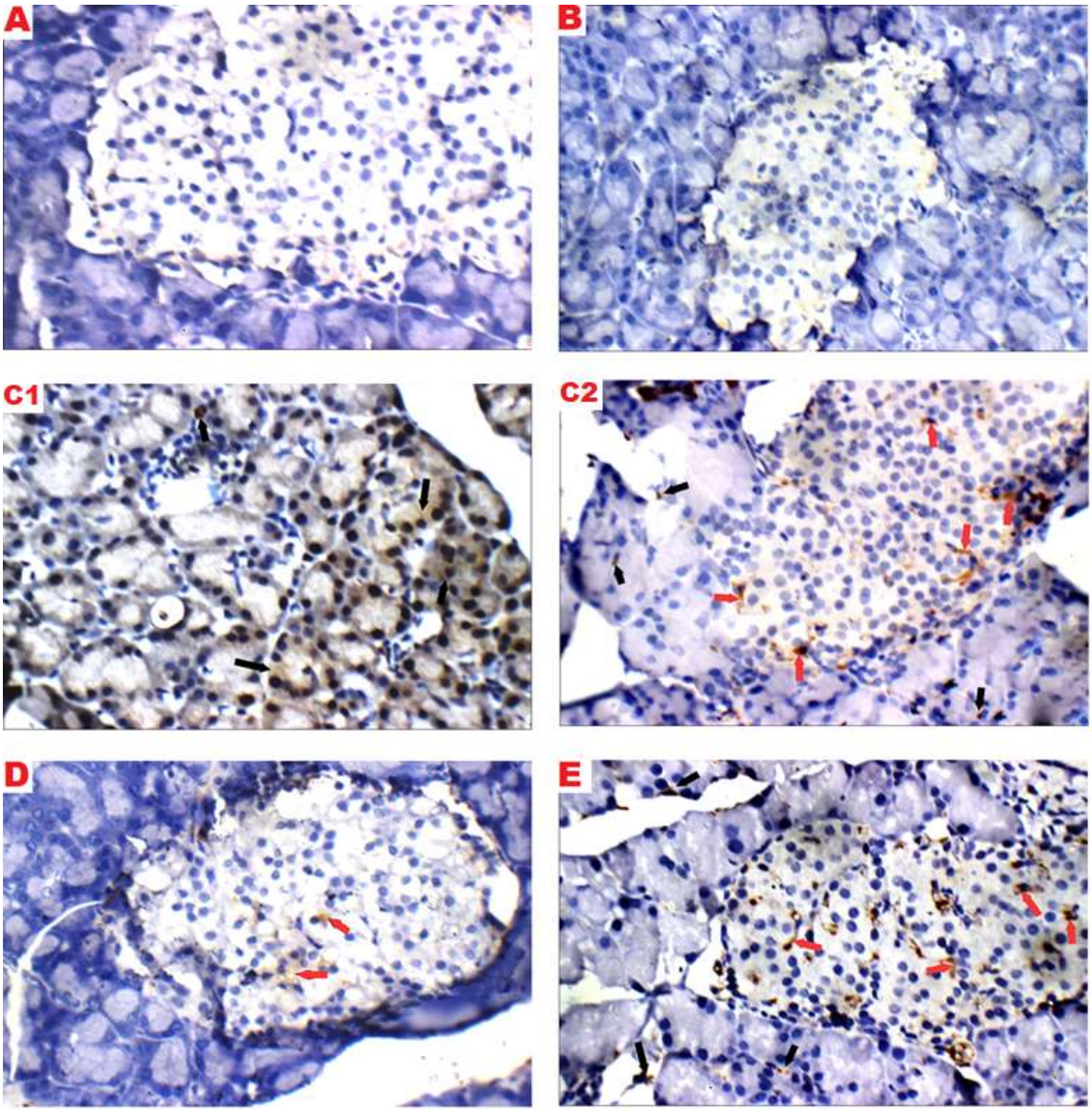


Fig. 9: Anti- NF-kB Immunostained Sections. A: Control, B: Nifedipine, C: L-arginine, D: L-arginine+Nifedipine, E: Recovery.

3.5. Morphometric results

Mean Area Percent of Collagen deposition, Caspase-3, and NF-kB immunoexpression in all experimental groups

Results of Collagen deposition, Caspase-3, and NF-kB immunoexpression are presented in Table (4) and Histogram (4).

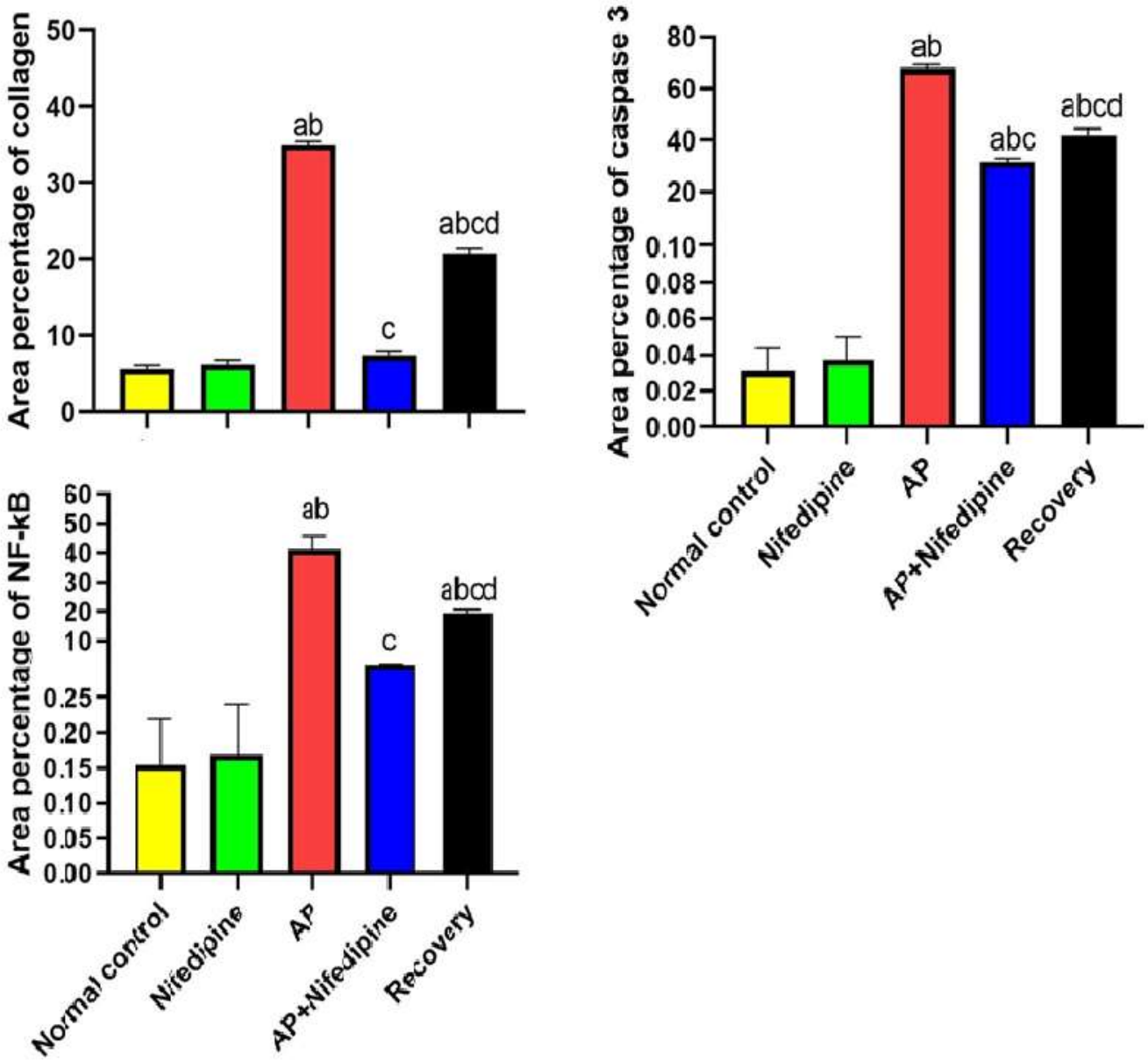
Mean area percent of collagen deposition, Caspase-3, and NF-kB immunoexpression did not significantly differ between Nifedipine and control groups. In addition, L-arginine-

induced-AP caused significant increased area percent of collagen deposition, Caspase-3, and NF-kB immunoexpression than normal control and Nifedipine. AP rats treated with Nifedipine showed significant decreased area percent of collagen deposition, Caspase-3 and NF-kB immunoexpression than AP rats and recovery rats, restoring collagen and NF-kB to normal levels. In addition, AP rats treated with Nifedipine showed significant higher Caspase3 expression as compared to normal and Nifedipine treated rats. Recovery group had significant higher area percent of collagen deposition, Caspase-3, and NF-kB immunoexpression than normal control and Nifedipine groups and lower than AP group.

Table (4): Mean Area Percent of Collagen deposition, Caspase-3 , and NF-kB immunoexpression in all experimental groups.

Groups Mean±SEM	**Mean Area Percent of collagen**	**Mean Area Percent of Caspase-3**	**Mean Area Percent of NF-kB**
Normal control	5.65±0.457	0.03±0.012	0.15±0.066
Nifedipine	6.17±0.584	0.04±0.013	0.17±0.072
AP	34.92±0.549^{ab}	67.86±1.24^{ab}	41.14±4.65^{ab}
AP+Nifedipine	7.39±0.558^{c}	31.40±1.44^{abc}	1.60±0.268^{c}
Recovery	20.73±0.714^{abcd}	41.85±2.50^{abcd}	19.54±1.20^{abcd}
Sample size	10 fields in each group		
F.value (df: 4,35)	487.90	425.30	69.98
P.value	<0.0001*	<0.0001*	<0.0001*

^{a}Significant difference with normal control group, ^{b}Significant difference with Nifedipine group, ^{c}Significant difference with AP group, ^{d}Significant difference with AP+Nifedipine group. *Significantly different at P<0.05



Histogram (4): Mean area percent of collagen deposition, Caspase-3 and NF-kB immunoexpression in all experimental groups.

4. Discussion:

Acute pancreatitis is a prevalent gastrointestinal disease, but its overall mortality rate has not significantly improved. Early management, diagnosis, and severity assessment are crucial for reducing morbidity and mortality. Oxidative stress and inflammation are central to the pathogenesis of AP, according to experimental and clinical studies **(14)**.

This study used L-arginine induced AP as a reliable, reproducible, and non-invasive model to induce pancreatitis with a similar presentation to human pancreatitis **(37)**.

Nifedipine, a calcium channel blocker, is used to treat hypertension and cardiovascular disorders. It has anti-inflammatory and antioxidant properties in various pathological disorders **(29)**. This study investigates Nifedipine's potential protective effect against inflammatory and oxidative stress pathways of AP in rats. The study involved biochemical, histological, immunohistochemical, and morphometric assessments to evaluate its effect on L-arginine-induced AP.

IP injections of L-arginine caused AP, as shown by a rise in PW/BW ratio, a crude test for pancreatic edema. Compared to group I&II, L-arginine administration in group III&V increased PWI significantly. AP produced with L-arginine increased pancreatic edema, as reported by **El-Ashmawy et al. (2018) (38)**. The vasodilator effect of NO and pro-inflammatory cytokines from injured pancreatic acini due to L-arginine toxicity may cause pancreatic edema and acinar cell damage **(32)**. **Tsuburaya et al. (2016)** found that Nifedipine's anti-inflammatory and vasculoprotective properties lowered edema and pathology score as seen in group IV **(39)**.

AP produces hydrolytic enzymes that hydrolyze phospholipids, leading to cytotoxic properties. Excessive ROS production and decreased anti-oxidative defense system cause premature activation of lysosomal and digestive enzymes, leading to autodigestion, necrosis, and cell death **(30)**.

The current study found that L-arginine increased serum amylase and lipase levels, agreeing with previous studies **(30, 40)**. Nifedipine reduced these levels, demonstrating its antioxidant effect by inhibiting oxidative stress by activating the Nrf2 pathway **(25)**.

The study examined H&E stained sections of the pancreas of control and Nifedipine groups, revealing normal histological architecture. It showed normal exocrine and endocrine pancreatic tissue, with densely packed serous acini and columnar to pyramidal epithelial cells. The endocrine component consisted of pale stained pancreatic islets dispersed throughout the exocrine part.

The AP group's pancreatic tissue showed broad interacinar and interlobular gaps between irregular, disordered pancreatic acini, edema and vascular congestion. The distortion of pancreatic acini widened these gaps, causing interstitial pancreatic edema and congestion **(41)**. The widening in the interacinar and interlobular spaces was previously observed by **Ibrahim et al. (2019)** and attributed this change to pancreatic edema which occurs due to increased production of hyaluronic acid with its hydrophilic property resulting in attraction and accumulation of water in the interstitial tissues.

Sections of group III showed areas of acinar cell degenration, which may be due to ROS. ROS damage acinar cells by denaturing their membrane proteins, causing structural loss and extracellular leakage of pancreatic protease enzymes, which degenerates the gland, agreeing with **El-Ashmawy et al. (2018) (38)**. ROS was reported to denaturize cytoplasmic proteins, organelles, and the cytoskeleton.

L-arginine group interlobular ducts were dilated with irregularity and rupture of their walls, and some had flattened epithelial cells.**Abdel Wahab et al. (2017)** and **Rizk et al. (2017)** found that pancreatic duct inflammation is caused by digestive and lysosomal enzymes released from acinar cells. This leads to inflammation, limited pancreatic regeneration, obstruction, and duct dilatation **(42, 43)**. **Nolte et al. (2016)** and **Aziz et al. (2017)** suggested that flattened epithelial cells in interlobular ducts may result from duct dilation and cystic formation **(44, 45)**.

The present work found perivascular inflammatory cellular infiltration in pancreatic tissue due to acute inflammation, where leucocytes, particularly neutrophils, are recruited and activated by cytokines released from both injured pancreatic tissue and the resident pancreatic macrophages **(46)**.

Multiple cytoplasmic vacuoles in pancreatic acinar cells of the L-arginine-

treated group may be due to vacuole membrane protein 1, which causes cytoplasmic vacuolation and cell death **(47)**.

The pancreas' exocrine and endocrine parts are linked **(48)**. The L-arginine group had worse endocrine function than the control and Nifedipine groups, with islets of Langerhans atrophy, diminished cellularity, cytoplasmic granules, vaculation, and pyknotic nuclei. In AP, pancreatic enzymes may degrade insulin-producing β-islet cells, as suggested by **Butler et al. (2017) (49)**. In the more severe form of acute pancretitis, necrosis of pancreatic tissue affects acinar and ductal tissues as well as islets of langerhans**(50)**. **Mastracci et al. (2022)** reported that the onset and severity of the endocrine dysfunction depend on degree of parenchymal destruction of the pancreas **(51)**, which results in islet β-cell insufficiency and finally occurrence of diabetes mellitus **(52).**

Light microscopic examination of L-arginine +Nifedipine group exhibited recovered acinar cells. However, few inflammatory cells remained. This study found that Nifedipine improved pancreatic islet damage and endocrine function. Nifedipine safeguards β-cells, reduces oxidative stress, enhances antioxidants, and lowers pancreatic inflammation **(25)**. It reduces ROS, which can disrupt insulin signaling and regulate glucose levels **(53)**. Nifedipine's antioxidant/anti-inflammatory mechanism has been shown to treat diabetic rats' testicular toxicity, mice's demyelination and behavioral dysfunction, cell death in hypoxic A549 cells, and chilblain **(26-29)**.

Compared to control and Nifedipine groups, Group V had disordered acini and ducts, and mild edema, dilated congested blood vessels with moderate cellular inflammation. AP affects exocrine and endocrine pancreatic activities depending on morphological damage, according to **Abdelwahab et al. (2017)**. Interstitial pancreatitis recovers in 4-12 weeks, although necrotizing pancreatitis may cause pancreatic insufficiency in half of patients **(42)**.

Group I&II rats had limited collagen deposition in Masson's Trichrome sections between pancreatic acini and blood capillaries, while group III rats had abundant collagen fiber deposition between pancreatic ducts, acini, and congested blood vessels. This

matches **Ammar et al.'s 2021** results of extensive collagen fiber deposition in deteriorated pancreatic acini and inflammatory infiltration after L-arginine-induced-AP in adult rats **(54)**. **Apte et al. (2023)** found that xanthine oxidase-generated free radicals directly activate pancreatic stellate cells, inducing fibrosis. Cytokines like TGF-β secreted by acinar cells after injury may cause pancreatic stellate cell fibrosis by encouraging myofibroblast-like cell proliferation and differentiation, leading to extracellular matrix production and collagen fiber deposits **(55)**.

In group IV, collagen fiber deposition decreased and appeared normal in pancreatic tissue and blood capillaries. Nifedipine reduces ROS generation, lipid peroxidation, and protein oxidation and scavenges free radicals before they harm cells and produce pancreatic fibrosis. Nifedipine reduced oxidative stress and collagen deposition in hepatic fibrosis in a non-alcoholic steatohepatitis model in ~~a~~ previous study **(56)**.

Group V rat sections exhibited moderate collagen fiber accumulation in pancreatic tissue and blood capillaries agreeing with **Alves et al. (2022) (57)**.

In the morphometric study, L-arginine significantly altered collagen fiber deposition compared to the control and Nifedipine groups. L-Arginine+Nifedipine significantly decreased mean area percent of collagen compared to the L-arginine and Recovery groups and increased compared to the control and Nifedipine-treated groups.

Group I and II had average cytoplasmic granules in pancreatic acinar cells in toluidine blue stained sections, but group III had substantial depletion. Calcium disturbance, a critical intracellular messenger in pancreatic acinar cell secretion, is the leading factor. Cytosolic calcium overload impair zymogen granule apical exocytosis, a critical molecular step in AP etiology **(58)**. The increase in cytosolic calcium results in a sustained increase in nuclear calcium, disrupting calcium-induced gene transcription and inhibiting pancreatic cell secretory function **(59)**. Cytoplasmic granules were retained in group IV rats but reduced in group V rats. **Mukherjee et al. (2015)** found that Nifedipine may improve calcium signaling and intracellular Ca^{2+} overload in AP, restoring pancreatic cell secretory function **(60)**.

In the current work, L-arginine-group islets of Langerhans and pancreatic acini cells tested positive for cytoplasmic Caspase-3, an apoptotic marker. Other studies showed that L-arginine upregulated pancreatitis-associated Caspase-3 expression, destroying acinar cells via the apoptotic caspase3 pathway **(30, 32, 61)**. They found that lipid peroxidation activates Caspase-3 and acinar cell apoptosis. Nifedipine therapy in group IV improved apoptosis-dependent Caspase-3 pathway, with modest positive cytoplasmic Caspase-3 immunostaining. **Bolnick et al. (2018)** reported that the Nifedipine interrupts intracellular Ca^{2+} signaling, playing a central role in the apoptotic pathway **(62)**. Contrary to present finding, other studies had 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 for overcoming AP injury in those studies **(Chen et al. 2019; Marwan 2022).**

The morphometric study found significant increase in mean area percent Caspase-3 immunostaining in the L-arginine group compared to the control and Nifedipine-treated groups, while the L-Arginine+ Nifedipine group significantly decreased Caspase-3 mean area percent.

As a transcription factor, NFκBcontrols pancreatic inflammatory mediator expression**(63)**. Like current results, **Jakkampudi et al. (2016)** observed that L-arginine boosted cytoplasmic NFκB immunostaining, demonstrating that calcium signaling and ROS production activate NF-kB **(64)**. However, L-arginine+ Nifedipine group showed a significant decrease in mean area percent of NFκB immunostaining compared to L-arginine and recovery groups. Blocking NFκB activation is beneficial in acute experimental pancreatitis, but it also limits tissue injury and inflammatory response spread. In rat model of L-Arginine-induced pancreatitis, Nifedipine treatment can reduce NFκB activation and protect against pancreatic damage as reported by **Choe et al. (2021) (65)**.

This study aimed to assess the pancreatic oxidative stress and its effects on lipid peroxidation and cell membrane damage in rats with AP. The pancreatic oxidant MDA and antioxidant GSH levels were assessed, and it was found that redox imbalance and disturbance of oxidative stress/antioxidant balance were

significant in the AP group, agreeing with **Pérez et al. (2015) and Mirmalek et al. (2016) (66, 67)**. Nifedipine treatment significantly increased pancreatic GSH, and decreased pancreatic MDA concentration relative to the AP group, demonstrating its antioxidant effect.

The perivascular infiltration of inflammatory cells in the pancreatic tissue was confirmed biochemically in L-arginine group by the elevated pancreatic tissue level of myeloperoxidase (MPO), an indirect biomarker of neutrophil infiltration and an index of acute inflammation in various tissues **(68)**. Nifedipine treatment significantly decreased the pancreatic tissue level of MPO, demonstrating its anti-inflammatory effect. It also decreased other inflammatory markers such as IL-1B and IL-6 as reported by **Aleksiuk et al. (2023)** who declared that decreasing of these inflammatory markers by Nifedipine in a murine model of osteoarthritis.

TNF-α, a pro-inflammatory cytokine, is an important mediator in the pathogenesis of AP, promotes pancreatic local inflammation followed by systemic inflammatory response **(69)**. Following L-arginine administration, the amount of TNF-α in pancreatic tissue was noticeably increased due to the excessive production of ROS that activate NF-kB, resulting in the upregulation of various inflammatory cytokines, particularly IL-1β and TNF-α **(70)**. Treatment with Nifedipine notably decreased TNF-α compared with animals received L-arginine alone, possibly due to Nifedipine's correction of redox unbalance that causes not only oxidative damage but also acts as an intracellular signal in inflammatory processes, particularly up-regulating pro-inflammatory genes. This result agreed with a previous study, which reported that Nifedipine reduced TNF-α and IL-1β in cuprizone-induced demyelination and behavioral dysfunction in mice **(29)**.

The present study measured pancreatic antioxidant Nrf2 which was significantly reduced in the L-arginine group. This result in agreement with a previous work that showed that Nifedipine raises the Nrf2 level and stops its deterioration through restoring detoxification and antioxidant defense mechanisms **(30)**.

The current results agreed with **Petersen et al. (2021)**, they reported that Nifedipine signifcantly reduced the

amount of free radicals produced by L-arginine-induced-AP. Pretreatment with pharmacological Ca^{2+} blockers was found to prevent premature digestive enzyme activation, vacuolization, and acinar cell necrosis induced by Ca^{2+} overload **(71)**.

The treatment of AP rats with calcium channel blocker Nifedipine for 7 days significantly protected the pancreas from the injurious effect of L-arginine, possibly due to its ability to inhibit inflammation, oxidative stress, apoptosis, and NF-kB activation, as well as promote acinar cell proliferation, thus alleviating pancreatic histopathological changes in L-arginine-induced-AP in adult albino rats.

5. Conclusion:

Based on current results, it can be concluded that L-Arginine administration induced remarkable damage of normal exocrine and endocrine pancreatic architecture. Nifedipine administration decreased biochemical parameters and restored the pancreatic tissue in histological and morphometric results after L-Arginine-induced-AP.

6. Recommendations:

- Better to use Nifedipine due to its anti-inflammatory, anti-oxidant and free radical scavenger effect.
- Limit the usage of high doses of L-Arginine as a supplement due to its serious toxic effect on the pancreas.
- Further studies are required to uncover other therapeutic mechanism of action of Nifidepine in acute pancretitis are highly recommended.

Conflict of interest

No potential conflicts of interests exist.

Legend of Figures

Fig. 1: A photomicrogragh of a section in the pancreas of **group I (A)**, and **group II (B)** showing the organized acinar structure into apical acidophilic region, basal basophilic region and basal rounded nuclei (blue arrows). Normal pale stained islets of Langerhans (orange arrow). Pancreatic lobules are separated by CT septa (black arrows). Normal pale stained islets of Langerhans (orange arrows). Interlobular duct (brown arrow) and centroacinar cells (green arrows) are noticed. Normal Blood capillaries (red arrows) are seen. **Group III (C)** shows loss of the normal organization of the pancreatic acini (blue arrows) and ducts with flattened lining epithelial cells are

noted (brown arrows). Notice the atrophied islet of langerhans (orange arrow). Some acinar and islet cells exhibit vacuolated cytoplasm (yellow arrows). Widened CT stroma between the pancreatic lobes and lobules are noted (black arrows). Dilated congested blood vessels are observed (C). **(H&E X400).**

Fig. 2: A photomicrogragh of a section in the pancreas of **group IV (D)** showing the characteristic appearance of relatively normal pancreatic acini with basal basophilia of its cytoplasm and apical acidophilia (blue arrows). Minimal inflammatory cell infiltration (I). Normal pale stained islets of Langerhans (orange arrow). Interlobular duct & interlobular duct (brown arrows) and centroacinar cells (green arrows) are noticed. Pancreatic lobules are separated by adelicate CT septa (black arrow). Blood capillaries (red arrows) are seen. **Group V (E)** shows inflammatory cell infiltration (I). Irregular distorted architecture of pancreatic acini (blue arrows) and the interlobular ducts (brown arrow) are noticed. Some acini show cytoplasmic vacuolation (yellow arrows). **(H&E X400).**

Fig. 3: A photomicrogragh of a section in the pancreas of **group I (A)**, and **group II (B)** showing delicate scanty collagen fibers in stroma between the acini (yellow arrows), between the pancreatic lobules (brown arrow), and around blood capillaries (red arrow). **Group III (C)** shows remarkable increase in collagen fibers in stroma between the acini (yellow arrows), between the pancreatic lobules (brown arrow), and around blood capillaries (red arrow), with the pancreatic ducts (black arrows), and the islets (orange arrow). **(Masson's Trichrome X400).**

Fig. 4: A photomicrogragh of a section in the pancreas of **group IV (D)** showing minimal collagen fibers in stroma between the acini (yellow arrow), between pancreatic lobules (brown arrow), around blood capillaries (red arrow), and the pancreatic ducts (black arrow). **Group V (E)** shows moderate deposition of collagen fibers in stroma between the acini (yellow arrows), and around the blood vessels (red arrows). **(Masson's Trichrome X400).**

Fig. 5: A photomicrogragh of a section in the pancreas of **group I (A)**, and **group II (B)** showing preserved cytoplasmic granules in most of acinar cells (black arrows), and distinct & normal beta cells exhibiting normal nuclei and average

amount of cytoplasmic granules (red arrows). **Group III (C)** shows marked reduction of cytoplasmic granules in most acinar cells (black arrows), and beta cells (red arrows). **(Toluidine blue X1000).**

Fig. 6: A photomicrogragh of a section in the pancreas of **group IV (D)** showing preserved cytoplasmic granules in most acinar cells (black arrows) in addition to distinct & normal beta cells exhibiting normal nuclei and average amount of cytoplasmic granules (red arrows). **Group V (E)** shows reduction of cytoplasmic granules in most of acinar cells (black arrows), and beta cells (red arrows). **(Toluidine blue X1000).**

Fig. 7: A photomicrogragh of a section in the pancreas of **group I (A)**, and **group II (B)** showing negative Caspase-3 immuno reactivity in the pancreatic acini, and islets of Langerhans (red arrows). **Group III (C)** shows strong positive cytoplasmic immuno reaction for Caspase-3 in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **(Caspase-3 immunostain X400).**

Fig. 8: A photomicrogragh of a section in the pancreas of **group IV (D)** shows weak cytoplasmic immune reactivity for Caspase-3 in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **Group V (E)** shows moderate cytoplasmic immune reactivity for Caspase-3 in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **(Caspase-3 immunostain X400).**

Fig. 9: A photomicrogragh of a section in the pancreas of **group I (A)**, and **group II (B)** showing negative NF-kB immuno reactivity in the pancreatic acini, and islets of Langerhans (red arrows). **Group III (C)** shows strong positive cytoplasmic immuno reaction for NF-kB in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **Group IV (D)** shows weak cytoplasmic immune reactivity for NF-kB in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **Group V (E)** shows moderate cytoplasmic immune reactivity for NF-kB in the pancreatic acinar cells (black arrows), and islets of Langerhans (red arrows). **(NF-kB immunostain X400).**

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