Original Article

Comparing the Therapeutic Potential of Mesenchymal Stem Cells-Derived Microvesicles and Infliximab in Chronic Pancreatitis Induced in Rats (Histological and Biochemical Study)

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ABSTRACT

Introduction: Chronic pancreatitis (CP) is a progressive fibro-inflammatory pancreatic disorder that eventually leads to damage of the gland. To date, appropriate therapy is still limited. This work investigated the effects of MSC-derived MVs and infliximab on CP induced by the repeated injection of L-arginine, and explored the possible underlying mechanisms.

Materials and Methods: Forty adult male Wistar rats were randomized into control, arginine-treated, microvesicles-treated and infliximab-treated groups. Serum amylase, lipase and glucose levels were estimated. Rats were sacrificed and pancreatic sections from all groups were subjected to hematoxylin & eosin, Masson's trichrome, Sirius red and immunohistochemical stains (insulin receptors and Bax). RNA was extracted for gene expression of TGF- β1, Pdx1, Fn-1, Collagen 1, mTOR, LC3-II, Beclin-1 and GAPDH.

Results: Our results provided evidence for inflammation, fatty replacement and progressive fibrosis in arginine treated group. There was downregulation of insulin receptor expression and upregulation of Bax immune localization. Administration of infliximab and microvesicles ameliorated the arginine-induced effects with superior and more rapid effect regarding microvesicles.

Conclusion: Microvesicles derived from mesenchymal stem cells provide a novel opportunity in comparison to infliximab as a promising theraputic for experimentally-induced chronic pancreatitis.

Key Words: Bax, infliximab, L arginine, microvesicles, Pancreas, Sirius red.

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INTRODUCTION

Chronic pancreatitis (CP) is an irreversible disease that is characterized by chronic inflammation and progressive fibrosis. Destruction of the gland causes eventual loss of exocrine and endocrine functions. CP may develop complications such as chronic abdominal pain syndrome, metabolic bone disease and pancreatic cancer[1].

Chronic pancreatitis is an utmost complex disease that is pathogenetically linked to pancreas-intrinsic as duct obstruction, toxic and genetic factors. One of the accepted theories is that CP results from repeated episodes of acute pancreatitis (AP)[2]. However, the pathophysiology of CP remains poorly defined. Therefore, appropriate therapies are still limited, and prognosis has not improved[3].

Studying such a complex disease naturally requires validated experimental models. Classic models as caerulein, duct-ligation or L-arginine models reflect the first category of CP etiologic factors. These models were the first that help to gain insight into the CP pathogenesis by imitating human CP in laboratory animals[4, 5].

Despite the large interest on AP, only a few studies have explored CP. The conventional treatments for pancreatitis; both acute and chronic, have mainly been anti-inflammatory to prevent the progression or improve the outcome. However, their effects were not as satisfactory as expected[6].

In AP, tumor necrosis factor alpha (TNF-α); a multifunctional cytokine, was found to contribute to pancreatic acinar cell death and expression of other pro-inflammatory factors[7]. In animal studies, blockade of TNF-α with anti-TNF-α antibodies or receptors decreased the severity of AP and the associated mortality rate[8]. In agreement, infliximab (monoclonal anti-TNF-α antibody) was found to be effective in AP-complicated acute Kawasaki or active Crohn's disease. Also, it had a preventive effect on AP in a patients with recurrent acute exacerbation of CP[9].

Based on animal studies, mesenchymal stem cells (MSCs) are promising for treatment of pancreatitis as

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in many intractable diseases. However, its mechanism remains unclear. The available data determining the best type of MSCs to treat AP and CP were insufficient; therefore, clinical trials investigating the use of MSCs as therapy are not warranted[10].

Recently, not only the MSC live-cell experiments are highlighted, but also the opportunities afforded by the emergence of the newly identified field of MSC necrobiology or derivatives (MSC-derived microvesicles or MSC-MVs). The putative employment of MSC derivatives provides a newer and simpler therapeutic approach that may have significant advantages over the use of cells themselves[11].

It is possible for this work to participate or support other studies in the same field to determine the efficiency and accuracy of this new line treatment. From here, this study aimed to analyze the effects of MSC-derived MVs and infliximab on CP induced in rats by repeated injection of L-arginine, and explore the possible underlying mechanisms.

MATERIALS AND METHODS

Chemicals & materials:

- L- arginine monohydrochloride (Cat. No. A5131); in a powder form, was purchased from Sigma-Aldrich chemicals (India).

- Infliximab (Remicade®); TNF-α blocker, in a powder form (Sigma-Aldrich, Steinheim, Germany).

- PKH26 labeled mesenchymal stem cells-derived microvesicles (MSCs-MV) were purchased from Biochemistry Department, Kasr Al-Ainy Medical School

Animals

Forty adult male Wister rats (200±50 g) were acclimatized for one week. They were kept in polypropylene cages under standard laboratory conditions of temperature and 12 hour light/ dark cycle. Rats were fed standard laboratory diet and water ad libitum throughout the period of experimental protocol. The study had been conducted in the Animal House of the Faculty of Medicine, Zagazig University, Egypt, following the guidance of Ethical Committee for Animal Handling at Zagazig University.

Experimental Protocol

Rats were equally divided into four groups;

Group I (Control): Rats were subdivided into: subgroup A (5 rats) that were given intraperitoneal (I.P) injections of normal saline (vehicle for arginine) on days 1, 4, 7, 10, 13, 16 and 19; and subgroup B (5 rats) that received single IV injection of 200µg PKH26 labeled MSCs-MVs diluted with 1ml phosphate buffered saline (PBS) via tail vein. All rats were sacrificed on day 21.

Group II (Arginine-treated): The L-arginine solution was prepared freshly before experiment[12]. Rats were given two I.P injections of arginine hydrochloride dissolved in normal saline, and its pH was set to 7.4 with NaOH, at a dose of 250 mg/100gm /day, at 1 hour interval on day 1. The same dose was repeated as a single dose on days 4, 7, 10, 13, 16 and 19, and the rats were sacrificed on day 21[13].

Group III (Microvesicles-treated): After induction of chronic pancreatitis, rats received single IV injection of 200µg PKH26 labeled MSCs-MVs diluted with 1ml phosphate buffered saline (PBS) via tail vein, then they were sacrificed after 3 weeks[14].

Group IV (Infliximab-treated): After induction of chronic pancreatitis, rats received a single weekly I.P injection of infliximab (5mg/kg/week) dissolved in 1ml saline for 3 weeks , then they were sacrificed[15].

Isolation of microvesicles

Mesenchymal stem cells (MSCs) were isolated from the expanding medium, and centrifuged. The supernatant was filtered several times to obtain the microvesicles. MSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM) for 24-48h. The medium was collected and centrifuged at 1000 rpm for 5 minutes, then at 2500 rpm for 15 minutes. The medium was filtered through 0.45µm filter to remove the cellular debris, then ultra-centrifuged at 100,000 g for one hour at 4°C (thermo scientific ultracentrifuge) to prepare the MVs. The pellets were suspended in PBS and stored at -80°C until further use. The procedures were done in the Medical Biochemistry Department, Kasr Al-Ainy Faculty of Medicine[16].

Characterization of microvesicles

The microvesicles were characterized using transmission electron microscopy (TEM)[17].

Homing of microvesicles

Using PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (PKH26GL, Sigma), unstained sections of paraffin blocks of group III were examined by fluorescent microscope (Olympus BX50F4, No. 7M03285, Tokyo, Japan) to detect PKH26 labelled MSCs- MVs to confirm homing of MSC-MVs into the pancreas[18].

Sample collection

Blood samples from all rats were collected from the heart under ketamine/ xylazine anesthesia (0.1ml/100g I.P) into non-heparinized tubes after overnight fasting for 12 hours. Sera were separated after centrifugation at 3000 rpm for 15min and stored at -20°C for biochemical determination. Then, rats were sacrificed and the pancreas was obtained and cleaned. For biochemical analysis, portions of the pancreas were homogenized and centrifuged. The supernatant was obtained and stored at -20°C for biochemical assay in tissue. For histological and immunohistochemical studies, pancreatic tissue was fixed in 10% formalin until processing.

Estimation of serum amylase and serum lipase

Serum amylase level was measured by amyloclastic method[19] where iodine gives blue colored complex when it comes in contact with starch. Thus, amylase activity of samples was evaluated by recording time in which known amount of starch was hydrolyzed by amylase. Thus, the end point of the reaction is absence of any substrate capable of forming the starch-iodine blue colored complex. The serum amylase activity was expressed as S.U/100 mL (Somogyi unit/100 mL).

Serum lipase level was measured using MacDonald and LeFave method[20] which involves incubation of the sample containing lipase with olive oil emulsion. The fatty acids released during this reaction is then neutralized or titrated with 0.05 N sodium hydroxide. Amount of lipase present in sample is expressed as ml of 0.05 N sodium hydroxide required to neutralize the fatty acids produced by hydrolysis under the conditions of the test.

Estimation of serum glucose level

Fasting serum glucose level was measured by glucose Liquizyme GOD-PAP (Single Reagent) kit (spectrum Cat no # 250 001).

Estimation of serum insulin level

Serum insulin level was assayed using the ELISA kit. This kit was supplied by Chongqing biospes co., Ltd., for quantitative detection of insulin in rat serum, plasma, body fluids and tissue lysates. Catalog No.: BEK1243.

Gene expression analysis:

Total RNA was extracted from pancreatic tissue homogenate using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. For the RNA quality, the A260/A280 ratio was analyzed by the NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States). For cDNA synthesis, a High-Capacity cDNA Reverse Transcription Kit cDNA Kit; (Applied Biosystems™, USA) was used. The gene expression analysis was evaluated by qRT-PCR using 5 uL of the cDNA, 10 pmol/uL of each primer (1 uL each), 10 uL of SYBR Green 2x Master Mix Green (QuantiTect SYBR Green PCR Kits,Qiagen). The real-time RT-PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA). The PCR cycling conditions included, enzyme activation at 95°C for 8 min as one cycle, denaturation occurs at 95°C for 10s and annealing and extension at 60°C for 60 s for forty cycles. The data were normalized against GAPDH and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression. The sequences of the primers are listed in (Table-1).

Table 1: Primer sequences of TGF- β1, Pdx1, Fn-1, collagen 1, mTOR, LC3-II, Beclin-1 and GAPDH (Invitrogen, USA).

Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)
TGF- β1	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC
Pdx1	GGATGAAATCCACCAAAGCTC	TTCCACTTCATGCGACGGT
Fn-1	ATCACCTGGACCCCCGCTCC	CGGTTCCCTGCTGCCCGTTT
Collagen 1	ACGTCCTGGTGAAGTTGGTC	ACCAGGGAAGCCTCTCTCTC
mTOR	TTGAGGTTGCTATGACCAGAGAGAA	TTACCAGAAAGGACACCAGCCAATG
LC3-II	ACTGCCGCCCTAAAGGTTAC	CGAGGTCCAACCCACAAAGA
Beclin-1	CGGCTCCTATTCCATCAAAA	AACTGTGAGGACACCCAAGC
GAPDH	GCATCTTCTTGTGCAGTGCC	GGTAACCAGGCGTCCGATAC

Histological analysis

Specimens of the pancreas were fixed in a 10% formalin solution and processed to prepare 5µm sections that were stained with hematoxylin and eosin (H&E), Mallory trichrome and Sirius red[21]. Paraffin sections were also processed for immunohistochemical staining using the labeled streptavidin-biotin immunoperoxidase technique[22]. The antibodies used were guinea primary polyclonal antibody for insulin (1:100) (N1542, Dako,

Carpinteria, CA, USA), and rabbit polyclonal primary antibody for Bax (1:50) (ab53154, Abcam, Cambridge, MA, USA). The sections were de-paraffinized in xylene, rehydrated in descending grades of alcohol, and washed in phosphate buffer solution (PBS). Sections were treated with 3% hydrogen peroxide and washed with PBS. The primary antibody was applied and washed with PBS. The biotinylated secondary antibody was applied, washed with PBS and incubated with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride (DAB Substrate Kit, Thermo Fischer Scientific, Rockford, IL, USA). Sections were counterstained with Mayer's hematoxylin. Negative controls were prepared by replacing the primary antiserum with PBS.

Insulin and Bax antibodies expression was evaluated with the scoring system reported by Ramos-Vara *et al*.[23].

Morphometric analysis

The following parameters were measured:

I. Area percentage of:

a. Collagen distribution in Mallory trichrome-stained sections.

b. Collagen distribution in Sirius red-stained sections.

c. Anti Bax antibodies immunoreactivity.

II. Optical density of antiinsulin antibodies immunoreactivity.

Leica Qwin 500 software image analyzer computer system (Leica image system Ltd; Cambridge, England) was used. Ten non-overlapping fields were randomly chosen for each section at x400 magnification. The measurement was done at the Pathology Department, Faculty of Dental Medicine, Cairo University.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows[24]. Data were analyzed and presented as means ± SD. Differences between continuous data were analyzed using one-way analysis of variance (ANOVA) to determine the difference between the test groups compared to control. Values were regarded as statistically significant at $p < 0.05$.

RESULTS

Characterization of MVs:

Transmission electron microscopic (TEM) examination of MVs revealed their heterogeneous spherical appearance and the difference in sizes (Fig. 1).

Homing of MVs:

Examination of pancreatic tissue by fluorescent microscope revealed PKH26 positive MVs. MSC-MVs exhibited strong red autofluorescence confirming that these vesicles migrated to the pancreatic tissue (Fig. 2).

Serological results:

Non significant difference was detected between the results of subgroups IA and IB ($p > 0.05$). Thus, the average of group I was considered as control to be compared with the other study groups

- Serum amylase and serum Lipase (Table 2)

There were significant increase in the levels of serum amylase and lipase in the arginine-treated rats when compared with the control rats, indicating deterioration in the pancreatic functions. However, significant decrease in serum amylase and lipase levels was detected in the MVs-treated rats when compared with arginine treated rats (p value \0.001). No significant difference was observed in infliximab group compared to arginine-treated group regarding both amylase and lipase serum levels.

- Serum glucose levels (Table 3)

There was a significant increase in serum glucose levels in the arginine-treated group, compared to the control (p value \ 0.001). However, a significant decrease was observed in infliximab-treated group when compared to arginine-treated group (p value \0.001). Also, there was a significant decrease in MVs-treated group when compared to arginine and infliximab-treated groups (p value \0.001).

- Serum insulin levels (Table 3)

There was a significant decrease in serum insulin levels in the arginine-treated group when compared to the control (*p value \0.001*). However, a significant increase was observed in the MVs-treated group in comparison to the arginine treated group (*p value \0.001*) but no significant difference was detected when compared to the control group. Infliximab treated group revealed a significant increase when compared to the arginine treated group but less than the MVs treated group.

- Gene expression results (Table. 4)

In our study, there was significant up regulation in the expression levels of TGF- β1, Fn-1 and collagen 1 m RNA in the arginine treated group in comparison to the control group while there was significant decrease in their expression levels in the infliximab treated group and more decrease in the MVs-treated group in comparison to

the arginine treated group. On the other hand, there was significant downregulation in Pdx1 mrna expression in the arginine treated group in comparison to control group while there was significant increase in its expression level in the infliximab treated group and more in MVs-treated group in comparison to arginine-treated group.

For autophagy, there was significant upregulation in m TOR gene expression with significant down regulation of LCII and Beclin 1 gene expression in the arginine treated group in comparison to the control. However, there was significant decrease in the m TOR gene expression and significant increase in LCII and Beclin 1 gene expression in the infliximab treated group. Moreover, there was a significant decrease in the m TOR gene expression and significant increase in LCII and Beclin 1 gene expression in MVs treated group.

Histological results

Examination of hematoxlin and eosin-stained sections in the pancreas of the control group revealed pancreatic lobules with acini and islets of Langerhans. The pancreatic acinar cells appeared pyramidal in shape with apical acidophilia, basal basophilia, and vesicular nuclei in the basal parts. The islets of Langerhans appeared pale and surrounded by connective tissue (fig. 3a). In arginine-treated group, there was a thickened capsule with subcapsular fatty and cellular infiltration. Some pancreatic acini were distorted with vacuolated cytoplasm (fig. 3b). There was precipitation of fibrin threads and proteinaceous material and some pancreatic acini appeared distorted with vacuolated cytoplasm (fig. 3c). Also, many intraepithelial lymphocytes were seen, and most of the cells had small dark nuclei (fig. 3d). Moreover, cells of the islets of Langerhans showed degenerative changes in the form of vacuolation and small dark nuclei. Some acinar cells were exfoliated in the lumen while others showed apoptosis with apoptotic bodies formation (fig. 3e). The acinar architecture was markedly disrupted with most acinar cells showing well-circumscribed cytoplasmic vacuoles (picture of fatty degeneration), with proteinaceous material in the interstitium (fig. 3f). Some pancreatic acinar cells were swollen with fragmented nuclear contents while others showed diminished areas of basal basophilia. The interstitium showed congested blood vessels, abundant collagen fibers, cellular infiltration and hyaline casts in a duct lumen (fig. 3g). In microvesicles-treated group, there was restoration of the normal architecture of the majority of the acini with central acidophilia and peripheral basophilia. However, few acini have dark stained nuclei. The islets of Langerhans appeared normal (fig. 3h). The infliximab-treated group, pancreatic acini and islets of Langerhans appeared normal but some cells contained dark nuclei. Bundles of collagen fibers were seen between the lobules (fig. 3i).

Mallory trichrome-stained pancreatic sections showed few collagen fibers around acini, and inbetween cells of the islets of langerhans in the control group (fig. 4a). In arginine-treated group, collagen deposition was increased intralobularly with bundles of collagen fibers surrounding the islets and infiltrating between its cell cords (fig. 4b,c). In microvesicles-treated group, thin interlobular connective tissue septae and minimal amount of collagen fibers were seen around the islets of Langerhans and inbetween their cell cords (fig. 4d). The infliximab-treated group showed increased collagen deposition around ducts and acini with few collagen fibers around the islets (fig. 4e).

Sirius red-stained sections in the control group showed few collagen fibers around lobules and islets (fig. 5a). In the arginine-treated group, abundant fibers were seen in the interstitium (fig. 5b). In the microvesicles-treated group, some collagen fibers were seen around acini and islets and inbetween the islets cell cords (fig. 5c). In the infliximab-treated group, collagen fibers deposited mainly in the interstitium around blood vessels, and fine few ones around the islets (fig. 5d). Sirius red combined with polarizing microscope enabled visualization of highly birefringent thick and thin collagen fibers in all the study groups (fig. 5e-g).

Immune localization of anti-insulin antibodies in the pancreas of the control group showed strong insulin immune reactivity in β-cells which occupied most of the islets of Langerhans (fig. 6a). In arginine-treated group, marked reduction in the β- cells immune reactivity to insulin was detected (fig. 6b). However, the microvesicles-treated group revealed β-cells with strong insulin immune reaction (fig. 6c). The infliximab-treated group showed strong positive β- cells (fig. 6d).

Immune localization of Bax in the pancreas of the control group showed mild expression of Bax in the islet of Langerhans (fig. 7a). In arginine-treated group, strong Bax immune staining was seen in the islet of Langerhans (fig. 7b). On the other hand, the microvesicle-treated group showed weak immune reaction in few cells of the islet of Langerhans (fig. 7c). The infliximab-treated group showed mild to moderate Bax immune expression both in the islets and acinar cells (fig. 7d).

Morphometric results

There was a significant increase in the area percentage of collagen distribution in Mallory trichrome-stained sections in the arginine treated group as compared to the control. The microvesicles treated group revealed a significant decrease when compared to the arginine treated group. Also, the infliximab showed a significant decrease when compared to the arginine treated group but less than the microvesicles group (fig. 4 f).

Concerning the area percentage of collagen distribution in Sirius red-stained sections, a significant increase was noticed in the arginine treated group as compared to the control. The microvesicles treated group revealed a significant decrease when compared to the arginine treated group. Also, the infliximab showed a significant decrease when compared to the arginine treated group but less than the microvesicles group (fig. 5i).

The optical density of antiinsulin antibodies immunoreactivity in the arginine treated group showed a significant decrease when compared to the control. The microvesicles treated group revealed a significant increase when compared to the arginine treated group. Also, the infliximab showed a less significant increase when compared to the arginine treated group (fig. 6e)

As regard the area percentage of Bax immunoreactivity, a highly significant increase was noticed in the arginine treated group as compared to the control. The microvesicles treated group revealed a significant decrease when compared to the arginine treated group. Also the infliximab showed a significant decrease when compared to the arginine treated group but less than the microvesicles group (fig, 7e).

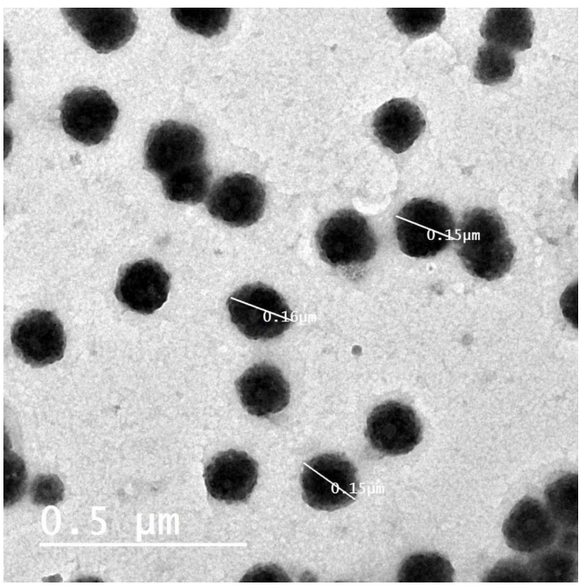


Fig. 1: MVs appear heterogeneous, spherical and different in sizes [TEM]

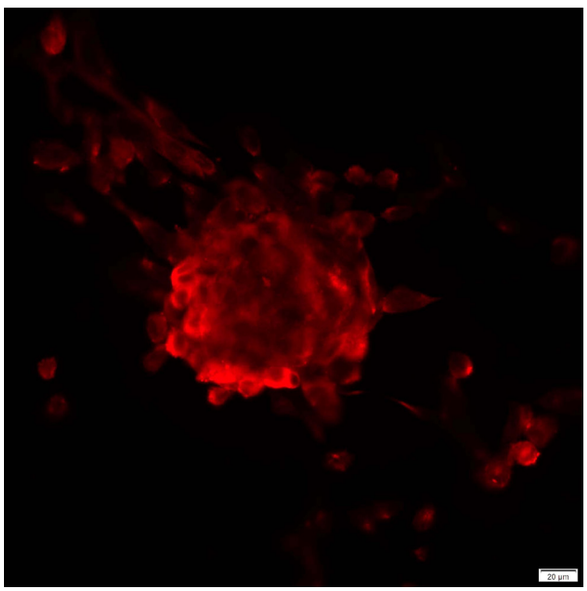


Fig. 2: MVs treated group illustrating the presence of PKH26 labeled MVs in the pancreatic tissue (red fluorescence). [flourescenct microscope]

Table 2: Serum amylase & serum lipase in the study groups

Groups	Control (I)	Arginine-treated (II)	MVs-treated (III)	Infliximab-treated (IV)
Serum amylase	1625.4± 79.1	2809± 85.4	1845±110.9	2701±110.9
Serum lipase	.98± 0.29	3.58± 0.58	1.5±0.25	4.02±0.35

Table 3: Serum glucose & insulin in the study groups

Groups	Control (I)	Arginine-treated (II)	MVs-treated (III)	Infliximab-treated (IV)
Serum amylase	185±18.3	380± 20.6	195± 20.6	210±19.2
Serum lipase	14.3±0.5	6.4±0.4	12.8±0.6	10.8±0.6

Table 4: Gene expression analysis

Groups	Control (I)	Arginine-treated (II)	MVs-treated (III)	Infliximab-treated (IV)	F	*P VALUE*
TGF- β1	1.12±0.12	3.11±0.31	1.39±0.14	2.15±0.21	219.23	*<0.001*
Pdx 1	1.09±0.13	0.54±0.12	1.28±0.15	0.76±0.18	60.96	*<0.001*
Fn-1	1.15±0.11	4.23±0.52	1.52±0.18	1.93±0.30	227.55	*<0.001*
Collagen 1	0.98±0.13	2.50±0.27	1.31±0.17	1.62±0.36	85.92	*<0.001*
mTOR	1.09±0.23	2.43±0.32	1.34±0.13	1.74±0.12	90.31	*<0.001*
LC3-II	1.11±0.07	0.45±0.09	0.92±0.18	0.79±0.10	66.96	*<0.001*

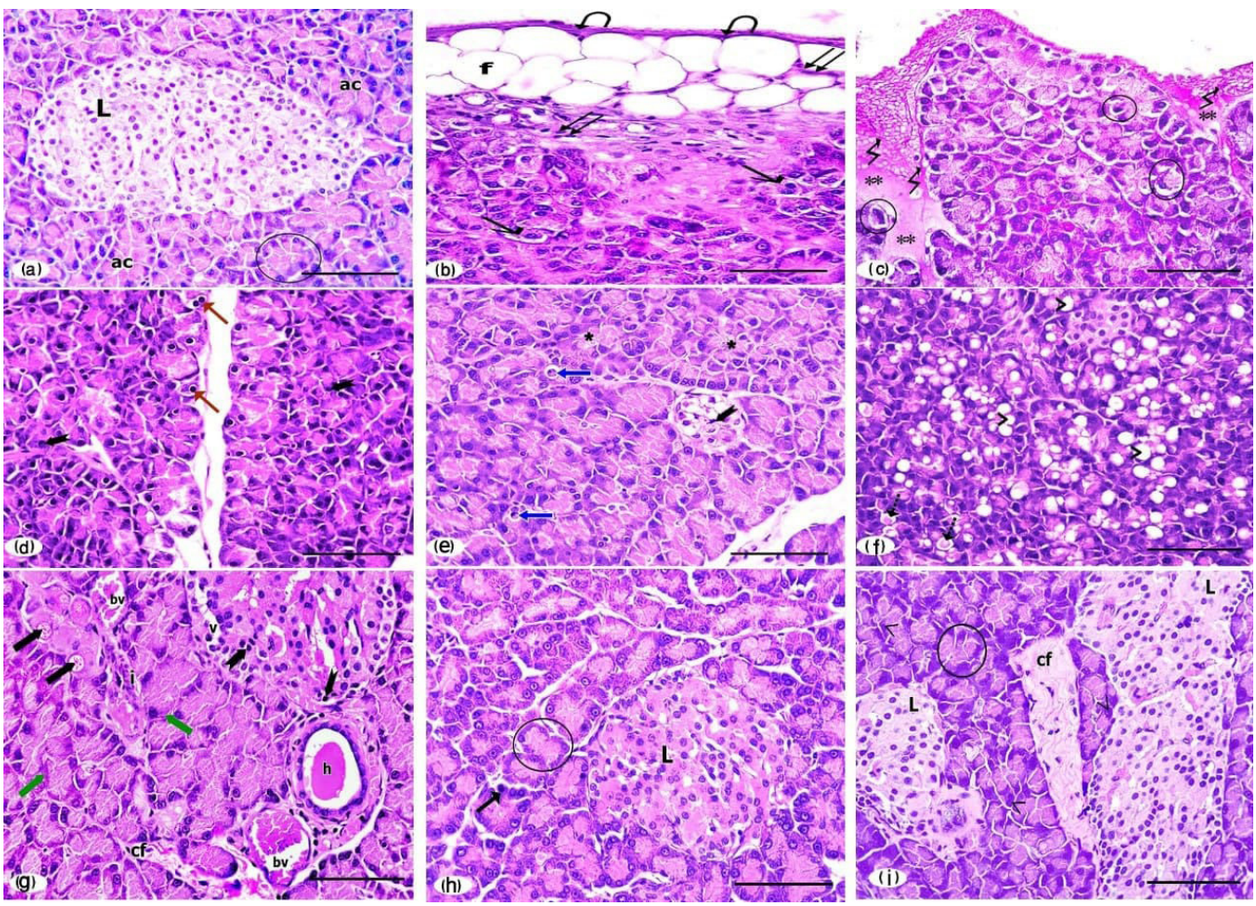


Fig 3: Hematoxlin and eosin-stained sections in the pancreas showing: a) Control group: pancreatic lobules with acini (ac) and an islet of Langerhans (L). The pancreatic acinar cells (circle) appear pyramidal in shape with apical acidophilia, basal basophilia, and vesicular nuclei in the basal parts. The islet of Langerhans appears pale and is surrounded by connective tissue. In arginine-treated group, the capsule is thickened (curved arrow) with subcapsular fatty (f) and cellular (double arrow) infiltration. Some pancreatic acini appear distorted with vacuolated cytoplasm (arrow) (b). In (c), thickened capsule and septa are seen with precipitation of fibrin threads (zigzag arrow) and proteinaceous material (exudate) (**). Some pancreatic acini appear distorted with vacuolated cytoplasm (circle). In (d), distorted pancreatic acini with many intraepithelial lymphocytes (red arrow) are seen. Most of the cells show small dark nuclei (notched arrow). e): Cells of the islet of Langerhans show degenerative changes in the form of vacuolation and small dark nuclei (notched arrow). Some acini appear distorted with exfoliated cells in their lumen (*). Apoptosis of some exocrine pancreatic cells with apoptotic bodies formation (blue arrow) are seen). (f): The acinar architecture is markedly disrupted with most acinar cells showing well-circumscribed cytoplasmic vacuoles (picture of fatty degeneration) (arrow head). There is condensation of proteinaceous material within the interstitium (dashed arrow). g): Some pancreatic acinar cells are swollen with fragmented nuclear contents (thick arrow). Others show diminished areas of basal basophilia (green arrow). The interstitium shows congested blood vessels (bv), abundant collagen fibers (cf), cellular infiltration (i) and an interlobular duct with hyaline cast in the lumen (h). The pancreatic islet reveals degenerative changes as vacuolation (v) and pyknotic nuclei (notched arrow). In microvesicles-treated group (h), There is restoration of the normal architecture of the majority of the acini with central acidophilia and peripheral basophilia (circle). Few acini have dark stained nuclei (arrow). The islet of Langerhans appears normal (L). The infliximab-treated group (i), pancreatic acinar cells (circle) appear with their characteristic basal basophilia and apical acidophilia. Dark nuclei are seen in the basal parts of some cells (arrow head). Pale areas of islets of Langerhans (L) are also seen. Bundles of collagen fibers (cf) are running between the lobules. [H&E, Scale bar = 50μm]

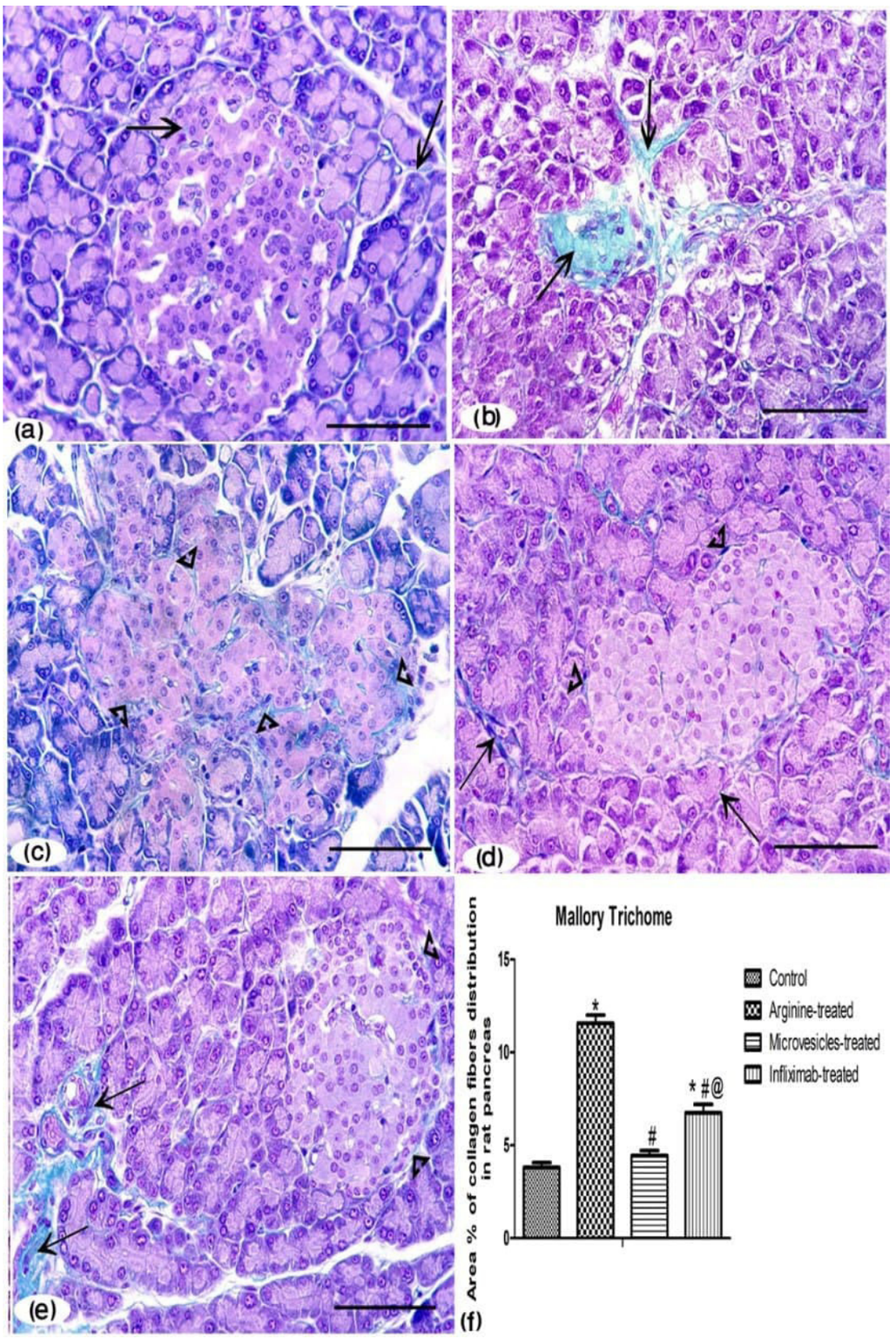


Fig 4: Mallory trichrome-stained pancreatic sections showing: in the control group (a), few collagen fibers (arrow) around acini, and inbetween cells of the Islet of langerhans. In arginine-treated group, collagen deposition (arrow) is increased intralobularly (b) with bundles of collagen fibers surrounding the islet and infiltrating between the cell cords (arrow head). (c). In microvesicle-treated group (d), thin interlobular connective tissue septae (arrow) and deposition of minimal amount of collagen fibers (arrow head) around the islet of Langerhans and inbetween its cell cords. In the infliximab-treated group (e), collagen deposition is increased around ducts and acini (arrow), with few collagen fibers around the islet (arrow head). [Mallory trichrome, Scale bar = 50µm]. (f): Area percentage of Collagen distribution in Mallory trichrome-stained sections in all the study groups.

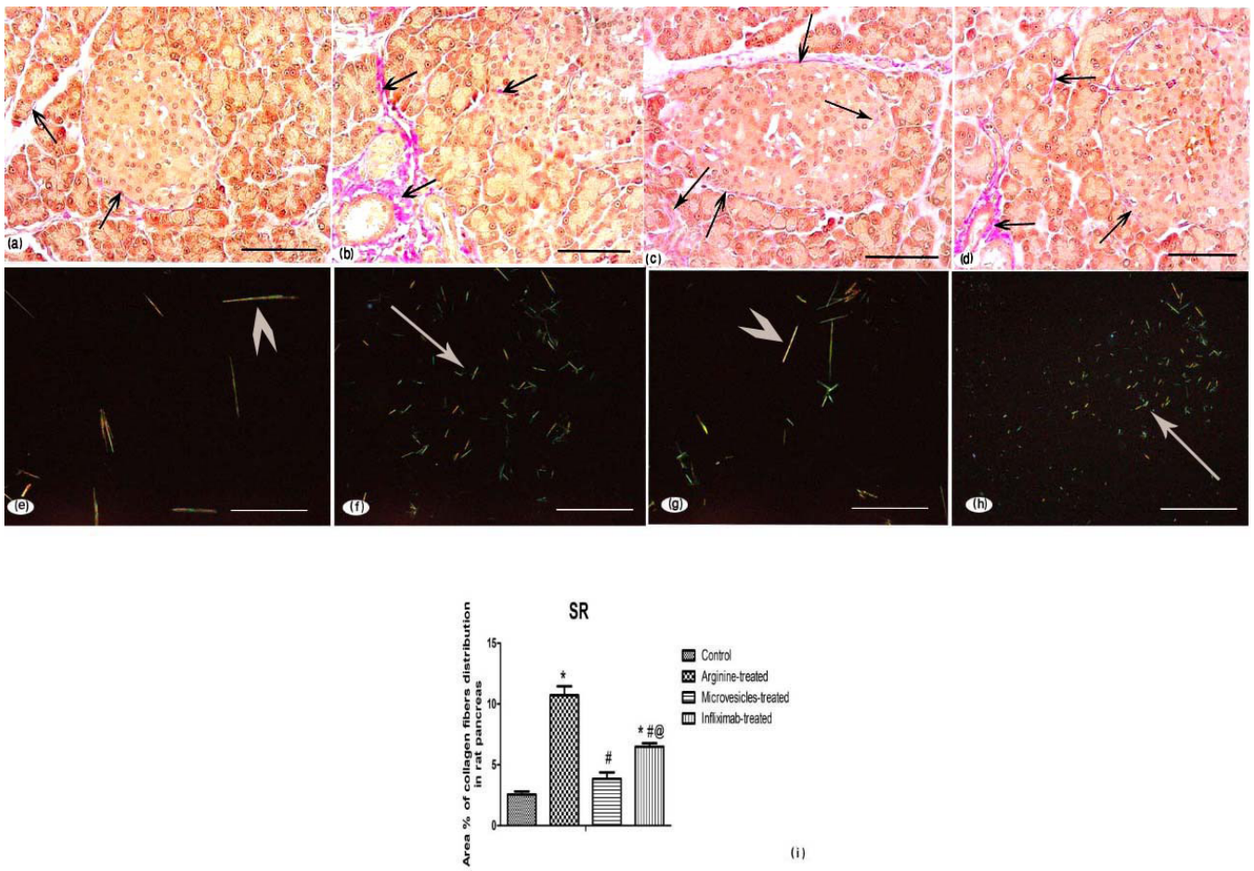
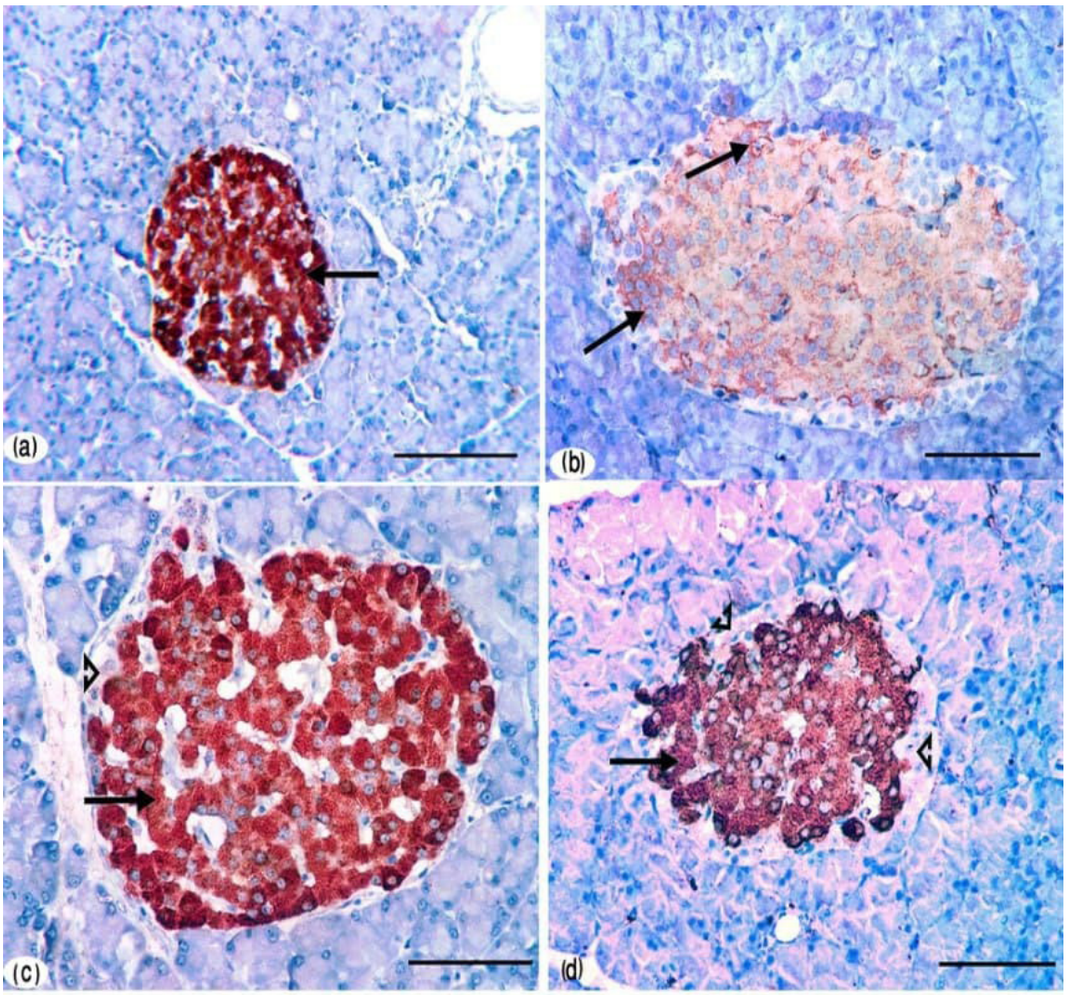


Fig 5: Sirius red-stained pancreatic sections showing: in the control group (a), few collagen fibers (arrow) surround the lobules and the islets. In the arginine-treated group (b), abundant collagen fibers (arrow) are seen in the interstitium. In the microvesicles-treated group (c), some collagen fibers (arrow) are seen intralobularly, around the islet and inbetween the islets cell cords. In the infliximab-treated group (d), collagen fibers (arrow) deposit mainly in the interstitium around blood vessels, and fine few ones around the islet. [Sirius red, Scale bar = 50µm]. (e-g): Sirius red combined with polarizing microscope enables visualization of highly birefringent thick (arrow head) and thin (arrow) collagen fibers. e): Control group, f): arginine-treated, g): Microvesicles treated and h): Infliximab treated [Scale bar = 50 µm]. (i): Area percentage of collagen distribution in Sirius red-stained sections in all the study groups.



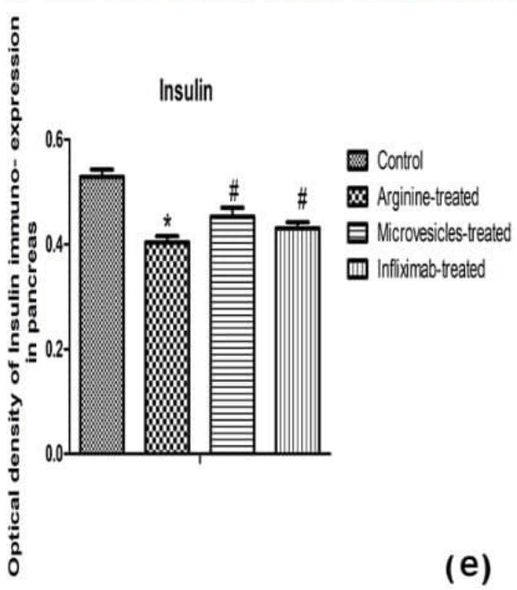


Fig. 6: Immune localization of anti-insulin antibodies in the pancreas of all the study groups showing: in the control group (a), strong insulin immune reactivity (arrow) in β-cells which occupy most of the islet of Langerhans. In the arginine-treated group (b), marked reduction in the β- cells immune reactivity to insulin (arrow) is detected. However, the microvesicles-treated group (c) reveals β-cells with strong insulin immune reaction (arrow). Note. Negative non- β-cells at the periphery (arrow head). The infliximab-treated group (d) shows strong positive β- cells. [Immuneperoxidase, Scale bar = 50μm]. (e): Optical density of antiinsulin antibodies immunoreactivity in all the study groups.

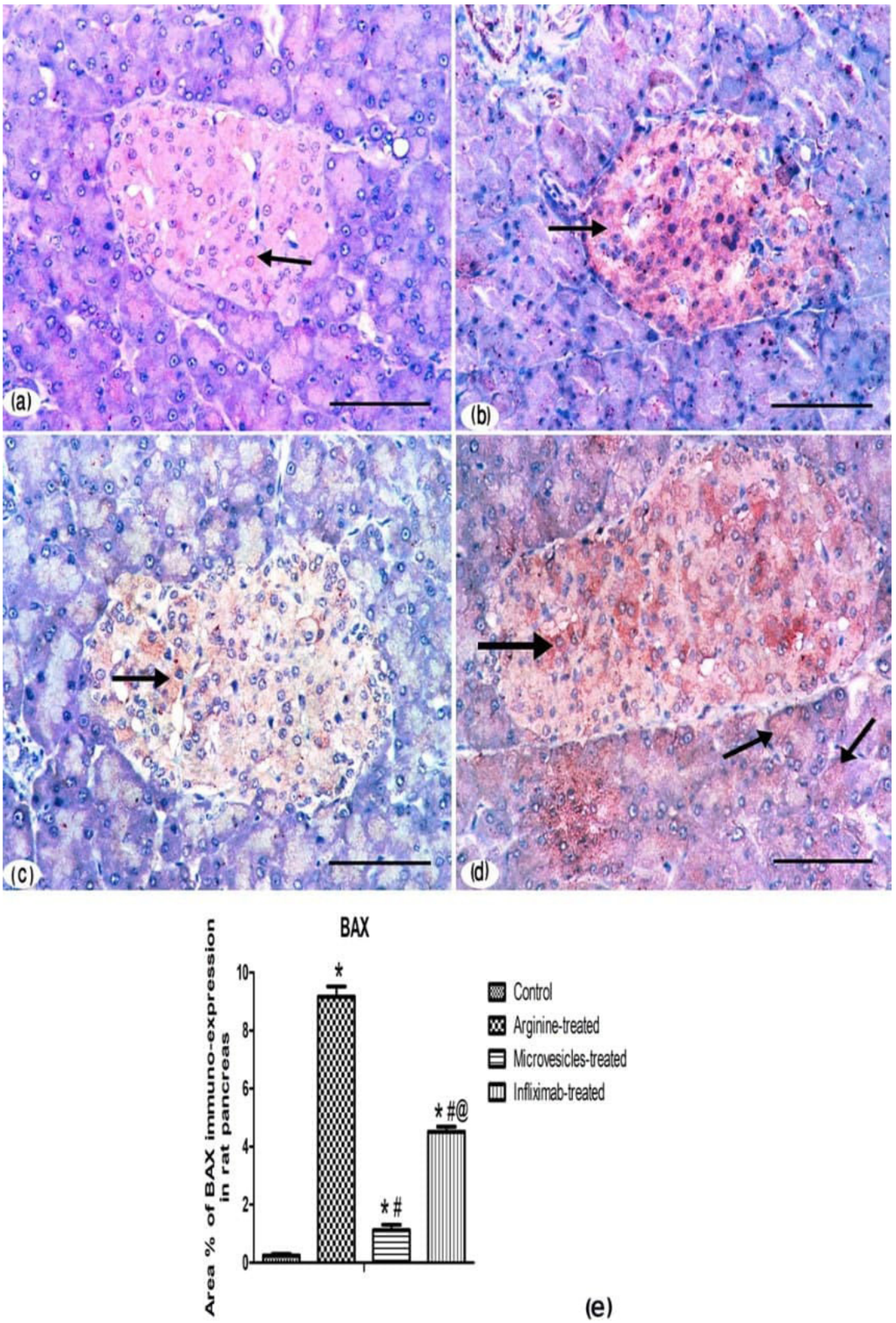


Fig 7: Immune localization of Bax in the pancreas of all the study groups showing: in the control group (a), mild expression of Bax in the islet of Langerhans (arrow). In the arginine-treated group (b), marked Bax immune expression in the islet of Langerhans. In the microvesicles -treated group (c), weak immune reaction (arrow) is detected in few cells of the islet of Langerhans. The infliximab-treated group (d) shows mild to moderate Bax immune expression (arrow) both in the islet and acinar cells. [Immuneperoxidase, Scale bar = 50μm]. (e): Area percentage of Bax immunoreactivity in all the study groups.

DISCUSSION

Chronic pancreatitis (CP) describes a wide spectrum fibro-inflammatory disease characterized by severe pain, development of fibrosis, and progression of endocrine and exocrine insufficiency. No treatment has been shown to be effective in halting the fibrogenic process in CP[25].

Elucidation of the molecular mechanisms for CP may develop novel therapeutic approaches to minimize or reverse the pancreatic fibrosis. Therefore, the current work used a non-surgical chronic pancreatitis rat model by repeated I.P injections of L-arginine. Serum enzymes; amylase and lipase, were the diagnostic biochemical tests as they are technically simple and readily available. Thus, they are continued to be used routinely in clinical settings[26].

Excessive dose of arginine was found to injure the pancreas through oxidative stress (OS) which contributes to elevated pancreatic lipid peroxidation (LPO) that alter the membrane integrity, and thus could account for the onset of AP and CP[27]. The increased LPO is associated with a decrease in GSH level as glutathione is consumed to detoxify the peroxides[28], and enhanced expression of MDA that may indirectly reflect oxygen free radicals (OFR) activity[29]. Our results supported the previous theory as the arginine-treated group revealed significant increase in MDA level and significant decrease in GSH level, as compared to the control.

The role of ROS in the development of pancreatitis was attributed to their cytotoxic capacity and their ability to activate neutrophils and macrophages which enhance the initial inflammatory process[30]. Also, generation of OFR activates pancreatic stellate cells (PSCs); a critical participant in the initiation of CP[25]. In agreement, our results showed cellular infiltration, abundant collagen fibers and congested blood vessels in the arginine-treated group.

Exposure of β-cells to cytokines is expected to rapidly generate proapoptotic signals, as indicated by the activation of nuclear factor-kB and MAPKs and up-regulation of iNOS. Although potent proapoptotic signals are generated early in cytokine-exposed β-cells, their death by apoptosis is delayed suggesting a protective mechanism that was maintained by glucose-induced synthesis of antiapoptotic proteins. Apoptosis induction in primary β-cells requires sustained exposure to the causative conditions[31]. In agreement, our results revealed significant increase in the area percent of bax immune expression in the chronic pancreatitis group as compared to the control.

Pancreas/duodenum homeobox protein 1(Pdx1) is one of the transcription factors implicated in pancreatic organogenesis. It is necessary for beta cell differentiation, maturation and insulin hormone production[32]. Preadipocyte factor 1 activates a signaling pathway that induces Pdx1 gene transcription[33]. The present study supported the previous theory as the arginine-treated group showed significant down regulation in Pdx1 mrna expression and insulin receptor immune expression, in comparison to the control.

In the present work, some pancreatic acinar cells in the arginine-treated group were swollen with rarified nucleoplasm, indicating what has been termed ballooning degeneration. Cell swelling occurs when the cell loses its ability to precisely control Na^+ and H2O influx, and K^+ efflux. The swelling reflects insufficient capacity of the Na^+/K^+ ATPases to exchange Na^+ for K^+ at a rate needed to maintain ionic gradients and water balance. Direct damage to the pumps, inadequate supplies of their essential ATP substrate, or inability to keep up with the Na+ influx due to plasma membrane damage may all contribute to cell swelling that may indicate relatively mild injury; however, cells that are severely injured also go through a phase of swelling[34].

Gukovskaya and Gukovsky[35] suggested impairment of autophagy through lysosomal dysfunction as a key initiating event in pancreatitis. Zhang *et al*.[36] reported morphological changes in acinar cells as autophagic vacuoles, autophagosomes, and autolysosomes. In agreement, the current study showed significant upregulation in m TOR gene expression and down regulation of LCII and Beclin 1 gene expression in arginine-treated rats.

In the present work, many intraepithelial lymphocytes were detected in the pancreas of the arginine-treated group. Lymphocytes, especially of the $CD4^+$ subset, were found mainly in the fibrous stroma, while T cells were also observed periductally. A T-cell subset bearing the phenotype CD8+CD103+ was found intercalating between the ductal epithelial cells. Phenotyping of the T lymphocytes in chronic pancreatitis supports the role of this T-cell subset as a first-line defense against deleterious epithelial events[37]. The spontaneous cytotoxicity of intraepithelial lymphocytes differs from that of peripheral blood lymphocytes in their target cell restriction, lack of response to interferon gamma, and effector cell phenotype[38].

The current work showed fibrosis in the chronic pancreatitis group evidenced by significant increase in the area percent of collagen deposition in Mallory trichrome and Sirius red-stained sections. Similar results were reported by Sharma *et al*.[13]. Development of fibrosis rather than glandular parenchyma restricts the pancreatic functions[39].

In response to pancreatic injury, quiescent PSCs are transformed into myofibroblast-like cells. Activated

PSCs proliferate, migrate, produce ECM, and stimulate cytokines and chemokines production[40]. Activation of PSCs could occur by both autocrine and paracrine mechanisms, indicating that its effects, primarily inflammation, and resultant fibrosis could further progress even after removing the primary source[41]. A large variety of growth factors, cytokines and chemokines activate PSCs in vitro. Other known activators include oxidant stress, hyperglycemia, pressure, protease, alcohol, endotoxin, and factors pertinent to pancreatic injury[42].

Fibronectin (FN1); a major constituent of the extracellular matrix, is produced mainly by fibroblasts and also tumor cells. Normally, it supports cell-ECM interactions and is essential for wound healing, development, and tissue homeostasis[43]. On the other hand, Hu *et al*.[44] reported that expression of FN1 is abundant in the pancreatic ductal adenocarcinoma, while there is minimal expression in normal pancreatic tissue. Stromal FN1 expression was associated with aggressive tumor properties. Our results revealed significant up regulation in the expression of TGF- β1, Fn-1 and collagen 1 m RNA in arginine-treated group in comparison to the control group.

Sirius red staining is one of the best understood histochemical methods that can highly and specifically underline fibers when combined with polarized light microscopy (PLM). This technique is relatively inexpensive and relies on the birefringent properties of collagen molecules. It provides complementary information about collagen fibers, such as orientation, type and spatial distribution[45]. In our work, Sirius red combined with polarizing microscope enabled visualization of highly birefringent thick and thin collagen fibers. According to Rittié[46], highlighting the organization of a collagen-containing matrix can be very useful for understanding or grading pathophysiological conditions.

In our work, the pancreas of arginine-treated rats showed subcapsular fatty infiltration with most acinar cells showing fatty degeneration. These results were in accordance with Yamaguchi *et al*.[47] who reported acinar destruction with adipose tissue replacement in L-arginine treated group. However, ductal, vascular, and islet cells were spared. This method of inducing chronic pancreatitis in rats is simple and reproducible but the histological features completely differ from those in humans as fibrosis is not found and adipose tissue completely replace the destroyed acinar cells.

Infliximab was found to have promising results in the treatment of experimental pancreatitis. Infiltrating macrophages release TNF-α; a key mediator of inflammation. The concentration of TNF-α correlates with the severity of pancreatitis[48]. In a study of different types of experimental pancreatitis, rats with acute edematous pancreatitis and severe necrotizing pancreatitis had significant decreases in serum amylase value and improvement in their histopathologic score following treatment with infliximab[49].

After administration of infliximab to the chronic pancreatitis rats, our work revealed no significant difference regarding both serum amylase and lipase levels, compared to diseased group. However, the treated groups; infliximab and microvesicles, showed decrease in blood glucose levels together with increase in plasma insulin levels indicating regeneration of pancreatic beta cells with re-production of insulin hormone.

Moreover, non significant difference in optical density of insulin receptor immune expression between infliximab-treated group and microvesicles-treated group in comparison to the control. However, there was significant increase in Pdx1 mrna expression level in the infliximab-treated group and more in microvesicles-treated group in comparison to arginine-treated group.

On the other hand, there was significant decrease in the expression levels of TGF- β1, Fn-1 and collagen 1 m RNA in the infliximab treated group and more decrease in the microvesicles treated group in comparison to the arginine treated group.

Our results also showed significant decrease in the m TOR gene expression and significant increase in LCII and Beclin 1 gene expression in the microvesicles treated group and infliximab treated group.

Rat studies proved that infliximab reduced acute pancreatitis-related complications. Treatment with a soluble TNF-α receptor antagonist also showed a decrease in the mortality and histologic severity of experimental pancreatitis[50]. A single case report showed improvement after infliximab infusion in an adult patient with Crohn's disease complicated with pancreatitis[51]. However, a subsequent case report described recurrent pancreatitis in a patient with Crohn's disease receiving chronic infliximab treatment, thus, pancreatitis was added as a potential adverse event of infliximab treatment by the FDA. A causal role for infliximab in pancreatitis is unclear due to concomitant administration of other anti-inflammatory and immunosuppressive drugs in these patients and the fact that pancreatitis has been reported as a complication of Crohn's disease in the absence of infliximab therapy[52].

MSCs have been thought hopeful for the cure of type 1 DM in the last few years, because they have the capability to differentiate into glucose responsive insulin producing cells. The MSCs regenerative ability can be achieved by the release of certain paracrine factors which includes extracellular vesicles (EVs) as microvesicles and exosomes[53].

Therapy with live MSCs has a singular advantage over the use of their derivatives: The cells can differentiate to provide the connective tissue stroma of the recipient organ. However, the cell implantation time is too short for differentiation to occur[54]. Recently published studies found many superiorities of the therapeutic effects of MSCs derived microvesicles over the direct use of MSCs itself. Weiss *et al*.[55] succeeded to address the underlying cell biology responsible for this profound efficacy. They noted that the basic MSCs biology and their mode of action greatly depend on the surrounding tissue environment and the released cellular signals that might be activators to MSCs or stop their action and being pro- apoptotic. MSCs response to such cellular stresses depends on the degree of inflammation and the exposure time.

The term "necrobiology" describes the cellular processes associated with morphological, biochemical, and molecular changes which predispose, precede, and accompany cell death, as well as the consequences and tissue response to cell death[56]. MSC viability and efficacy are not necessarily correlated suggesting that the efficacy of MSC can be attributed to either viable or dead MSCs, and their benefits are evoked by the biological activity of the intended therapeutic component (the viable MSC itself or its derivatives) and/or the recipient's response to MSCs that are in the process of dying[57].

Healthy, viable MSCs are well-characterized producers of a wide range of EVs with different cargos that are now recognized as powerful mediators of intercellular communication locally and systemically. On the other hand, MSC derived vesicles (even in the absence of their viable MSC producer) have detectable therapeutic influences in human systems[55]. Microvesicles are capable of cytokine release, immune differentiation, T cell activation as well as angiogenesis promotion[58].

Necrobiology provides four mechanisms that enable mesenchymal stromal cell derivatives to maintain significant clinical efficacy: apoptosis, autophagy, mitochondrial transfer, and extracellular vesicles. MSC derivatives from these pathways have been proven to ameliorate a plethora of pathologies and to be as therapeutically beneficial as living MSCs, but have the advantage of passing easily through the circulation[59]. In the same context, pre-apoptotic MSCs used as therapies in inflammatory microenvironments could be responsible for a switch toward an anti-inflammatory response through subcellular particles through their intra-vesicular or surface cargo. Moreover, apoptotic cells can produce a range of EVs and apoptotic bodies that can influence their microenvironment[60]. EVs were found to promote mitochondrial transfer to the macrophages increasing their phagocytic capacity and inducing an anti-inflammatory response[55].

Therefore, the recent emergence of a variety of methods to produce MSC derivatives provides a newer and simpler approach that could have significant advantages over the use of cells themselves including simpler production, lower regulatory barriers, and easier systemic transport upon intravenous delivery[11].

CONCLUSION

In conclusion, repeated I.P injections of arginine hydrochloride induced chronic pancreatitis in rats, manifested by degenerative changes in both acini and islets of Langerhans, fibrosis and fatty degeneration, that were proved by histological and biochemical methods. Both infliximab and MSCs-derived microvesicles had therapeutic effects when injected in the chronic pancreatitis rats possibly through antioxidant, anti-inflammatory and antifibrotic effects as well as enhancement of autophagy. The therapeutic potential of the microvesicles was found to be superior and more rapid than that of infliximab. Future human studies are needed to adjust the microvesicles dose and duration of treatment. Also, the necrobiology of MSC will be a fruitful area for future study for improving the efficacy or removing confounding influences on cell therapy.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

مقارنة الامكانات العلاجية للحويصلات الدقيقة المشتقة من الخلايا الجذعية الوسيطة والانفليكسيماب في التهاب البنكرياس المزمن المحدث في الفئران

(دراسةهستولوجية وبيوكيميائية)

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التهاب البنكرياس المزمن هو اضطراب التهابي تدريجي في البنكرياس يؤدي في النهاية إلى تلف الغدة. وحتى الآن لا يزال العلاج المناسب محدودًا. وقد فحص هذا العمل تأثير الحويصلات الدقيقة المشتقة من الخلايا الجذعية الوسيطة والانفليكسيماب على التهاب البنكرياس المزمن الناجم عن الحقن المتكرر لـلأرجينين كما استكشف الآليات الكامنة المحتملة.

الطرق: تم اختيار أربعين ذكرًا بالغًا من فئران Wistar, قسمت بشكل عشوائي في مجموعات الضابطة والمعالجة بالأرجينين والمعالجة بالحويصلات الدقيقة والمعالجة بالإنفليكسيماب. وتم تقدير مستويات الأميليز والليباز والجلوكوز في الدم. وأخذت عينات البنكرياس من الفئران في كل المجموعات وصبغت بالهيماتوكسيلين والأيوسين ، ثلاثي كروم ماسون ، أحمر سيريوس وصبغات كيميائية مناعية (لمستقبلات الأنسولين والباكس). وتم استخراج الحمض النووي الريبوزي للتعبير الجيني عن TGF-β1 و Pdx1 و Fn-1 و Collagen 1 و mTOR و LC3-II و Beclin-1 و GAPDH. النتائج: قدمت نتائجنا دليلاً على وجود التهاب واحلال دهني وتليف تدريجي في المجموعة المعالجة بالأرجينين. وكان هناك انخفاض في التعبير عن مستقبلات الأنسولين وزيادة في التعبير عن الباكس. وأدي استخدام الإنفليكسيماب والحويصلات الدقيقة إلى تحسين التأثيرات التي سببها الأرجينين مع تأثير أعلى وأسرع فيما يتعلق بالحويصلات الدقيقة.

الاستنتاج: الحويصلات الدقيقة المشتقة من الخلايا الجذعية الوسيطة توفر فرصًا غيرتقليدية بالمقارنة مع الإنفليكسيماب كعلاج واعد لالتهاب البنكرياس المزمن المحدث.