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RESEARCH ARTICLE

ANTI-INFLAMMATORY AND ANTI-APOPTOTIC THERAPEUTIC EFFECTS OF MESENCHYMAL STEM CELLS-DERIVED EXOSOMES ON THE PANCREAS OF STREPTOZOTOCIN-DIABETIC RATS

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Abstract

The rising prevalence and serious complications of type 1 diabetes mellitus (T1DM)have made it a huge challenge that significantly jeopardizes public health, with high morbidity resulting in poor quality of life.The safety and therapeutic excellence of cell-free exosomes(EXs) derived from mesenchymal stem cells (MSCs) over MSCs transplantation suggested a novel perspective for DM and the treatment of its complications. The current design wasconducted toinvestigate the therapeutic potentialities of management in rats. For 4 consecutive weeks, 4rat groups were conducted as control, EXs, STZ-diabetic (D), and D+EXs groups. The results showed that the untreated diabetic rats exhibited marked hyperglycemia evidenced by elevated serum glucose and HbA1c levels and declined insulin and C-peptide levels compared to control rats. In addition, pancreatic tissues of the untreated diabetic rats revealed significant progression in oxidative stress (↑ MDA, H_2O_2, and NO), inflammation (↑ IL-6, TGF-β, and TNF-α), and apoptotic statuses (↑annexin V, P53, caspase-3, and ↓Bcl-2) regarding control. However, all results reverted near to normal levels following intravenous injection of diabetic rats with a single dose of MSCs-EXs (500 µg/mL) compared to the untreated diabetics. These results were confirmed by the enhanced histological picture of diabetic rats' pancreas, concerning the diabetic-untreated rats.The biochemical and flow cytometric assessments evidenced that diabetic rats treated with marked pancreatic regenerative anti-inflammatory, immunomodulation, antioxidant, and anti-apoptotic potentialities in counteracting the induced hyperglycemia.

Introduction:-

Diabetes mellitus (DM) is a chronic condition of carbohydrate metabolism characterized by hyperglycemia, and long-term high blood glucose levels, due to either insulin insufficiency (type 1, T1DM) or poor response of the recipient cell to insulin (type 2, T2DM) **(1).** Driven by lifestyle changes in modern society caused by an increase in caloric foods and a decrease in physical activity, diabetes is considered a modern pandemic and a huge challenge posing a great health and economic burden that significantly jeopardizes public health globally **(**2,3**).** Diabetes

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incidence and prevalence are on the rise becoming the ninth leading cause of death worldwide, with 4 million annual deaths worldwide. According to the 2023 International Diabetes Federation (IDF) estimation, more than 537 million people are currently affected by the diabetes epidemic, with approximately 1 in 11 adults having diabetes, which is expected to reach 700 million diabetic patients by 2045 **(**4**).**Also, according to the IDF, Egypt ranks ninth in the prevalence of DM worldwide, and the number of adult diabetic patients was 8,850,400 in early 2020, with a prevalence of 15.2%, which may be an underestimation **(**2).

Notably, T1DM is an autoimmune metabolic disease characterized by hyperglycemia, which occurs due to absolute deficiency of insulin secretion caused by the autoimmune attack of autoreactive $CD4^+$ and $CD8^+$ T lymphocytes to pancreatic β islets, resulting in lysosome dysfunction with subsequent autophagy and apoptosis **(1,3).** It often presents with symptoms such as thirst, weight loss, and polyuria closely associated with various microvascular and macrovascular complications**(5,6).** On account of insulin deficiency, reduced glucose uptake in target tissues prompts the body to break down fat adaptively as a response, thus producing many acetyl coenzyme A, which is the building block of cholesterol, contributing to thickened blood and stiffened arteries **(1,**7**).**

Although suggested to account for only 10% of DM cases, TIDM is considered more dangerous than T2DM, since it is commonly complicated by ketosis, and is generally lethal unless being treated with daily exogenous insulin injections, which was and still is the gold primary standard treatment **(**2,7**).**However,traditional therapies including exogenous insulin injection cannot slow the pancreatic β-cells' emergence failure or at least prevent diabetic complications progression, since they cannot mimic the endogenous insulin released by a healthy pancreas in T1DM patients **(1,8)**.Addressing auto-immunity and inflammatory responses has been suggested recently to provide an opportunity for T1DM therapy **(9,10).**

In this context, mesenchymal stem cells (MSCs) were suggested as a potential therapeutic protocol for diabetic patients because of their confirmed immunoregulatory and regenerativepotentialities **(1,11).** MSCs are a class of mesodermal heterogeneous pluripotent subset of stromal stem cells, that can be isolated from various tissues (bone marrow, adipose tissue, perinatal tissues (umbilical cord blood, amniotic fluid, placenta), exhibiting self-renewal and multidirectional differentiation potential **(1).** Unfortunately, the application of MSCs in regenerative medicine showed many limitations; involving cell senescence, low survival rate in vivo, organ residence, tumorigenicity potential,and thrombogenesis **(11,12).**

The paracrine therapeutic mechanisms of MSCs were attributed to the release of many cytokines, antioxidants, immunomodulatory, and growth factors contained in various types of extracellular vehicles (EVs), including exosomes **(13).**Exosomes (EXs) are nanoparticles (40-150 nm) of endosomal origin involved in cell-to-cell communication, to repair damaged tissues and regulate metabolism, via transporting their cargo (various proteins and functional RNA) to neighbor cells**(6,12,14).**EXs possess various surface proteins used as biomarkers for their phenotypic identification,such as thetetraspanin protein family (CD9, CD63, CD81)**(15,16,17).**

Because they naturally exhibit their original parental cell characteristics, they were represented as mini versions of the originator cells**(17,18).**Likely, mesenchymal stem cell-derived exosomes (MSCs-EXs) not only inherit the ability of MSCs to repair damaged tissues but also,were able to avoid the limitations as mentioned above, besides its easier quantification, maintenance of bioactivities during transportation or storage, and direct tumorigenicity lack, suggesting being a safer therapeutic option**(9,11,15,18,19).**In this line, MSCs-EXs have demonstrated equal **(20,21)** or even superior efficiency to MSCsthemselves**(1,6,12).**Like the parent cells, MSCs-EXs offer cell protection against apoptosis via modifying the recipient cells' proteome and transcriptome, thereby regulating their proliferation and differentiation and modulating apoptosis pathways **(6,12,17).**

Of note, the therapeutic potential of MSC-EXs was confirmed to rely on the known immunomodulatory efficacy of their bioactive contents (growth factors, lipids, immunoregulatory proteins, cytokines, and microRNA) to promote both tissue regeneration and repair **(15,18,22,23).**Since they have shown great promise in tissue regeneration, MSCs-EXs could be represented as a novel cell-free treatment option for T1DM patients, providing further pancreatic β-islets protection from apoptosis and autoimmune targeting, to cure disease or at least slow its progression **(9,17).** Therefore, we conducted this study to investigate the possibility of restoring normal insulin secretion in type 1 diabetic rats, via exploring MSCs-EX potentiality as an anti-inflammatory and anti-apoptotic agent for modulating the pathological pancreatic status.

Methods:-

Chemicals

MSCs-EXs (500 ug/ml) were purchased from NAWAH Co. (EGYPT), while Streptozotocin (STZ)was purchased from Sigma Aldrich Co. (USA).

MSCs- EXs preparation & characterization

MSCs-EXs stock (500 ug/ml) were sonicated,for 10 minutes, in phosphate buffer saline (PBS, pH 7.4) to ensure complete solubilization. Before being used as an EXs-control group or applied for T1DM treatment, EXs undergo both morphological and phenotypic characterization to confirm their identity and availability.

To evaluate their integrity and morphology, 2.5 µl of a diluted EXs sample was loaded onto freshly 'glow discharged' 300 mesh formvar/carbon-coated TEM grids (Ted Pella, Redding, CA), negatively stained with 2% aqueous uracyl acetate and observed under a Hitachi H7600 transmission electron microscope (TEM, Zeiss, LEO 906E, Germany) operated at 80kV. Images were captured with a side-mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA) **(24).**In addition, FACS Caliber flow cytometer (Becton Dickinson, USA) and Sigma Aldrich (USA)Antibodies kits were used to detect the presence of specific surface markers (CD9, CD63, and CD81), to confirm EXs phenotype identity **(12,22)**.

Induction of diabetes

Following the dissolution in cold sodium citrate buffer (0.1 M, pH 4.6), a single STZ dose (60 mg/kg)was injected intraperitoneally (IP) in overnight fasted rats. The fasting blood glucose (FBG) level from the tail vein was assessed, after three days of diabetes induction, using ACCU–CHEK Go glucometer (Roche Co, Germany), where animals with FBG over 200 mg/dl, only were considered as diabetic, and others were excluded **(25).**

Experimental design

Thirty-two male albino Wistar rats (180-210g) were housed in plastic cages and were maintained under conventional laboratory conditions throughout the study (air-conditioned, 12/12 h darkness/daylight and pathogen-free), where water and chow were ad libtum. Rats were allowed to acclimate for 2 weeks, divided into 4 groups each of 8 rats for 4 weeks, as follows:

1. Control group: injected with citrate buffer (2 ml/kg, pH 4.6, IP, once).
2. EXs group: injected with 0.5 ml EXs (IV, once).
3. Diabetic (D) untreated group:injected with STZ (60 mg/kg, IP, once).
4. Diabetic EXs-treated group:diabetic rats injected with 0.5 ml EXs (IV, once).

Samples collection

Diethyl ether was used to anesthetize overnight fasted rats before being dissected.A cardiac puncture was performed to collect blood samples. A few blood droplets were placed in heparinized tubes to assess glycosylated hemoglobin (HbA1c). The collected blood samples were then centrifuged for 15 minutes at 1000 x g to obtain plasma, separate sera, and stored at -20 ℃. On the other hand, the pancreas was separated,where(1) one part was used to obtain a 10% (w/v) cold distilled water homogenate, for later biochemical estimations,(2) another part for flow cytometric analysis was kept at -80 ℃, and (3)remaining part for histopathological examination was stored in 10% formalin solution.

Histochemical examination

A routine Hematoxylin and Eosin staining was applied following the method of **Suvarna et al. (26).**Briefly, the pancreas was cut (1 cm thickness), fixed in 10% neutral buffer formalin, and embedded in paraffin wax to form sample blocks. A microtome was used to cut the wax blocks into 3-5 µm thickness sections, which were mounted later on labeled glass slides. A Leica Auto Stainer was then used to stain the slides with Hematoxylin and Eosin. Afterward, a light microscope was used to examine the pancreatic samples.

Biochemical assays

Levels of both C-peptide and insulin in serum were detected using Bio Vision Company, USA, ELISA kits. Meanwhile,SPINREACT diagnostics kits (Spain) were applied for the serum glucose, HbA1c, and amylase levels estimation. However, the pancreatic oxidative stress status was explored via the detection of hydrogen peroxide (H_2O_2), nitric oxide (NO), and malondialdehyde (MDA) contents.On the other hand, pancreatic antioxidant capacity was explored via assessment of glutathione peroxidase (GPX), catalase (CAT), glutathione (GSH), and superoxide

dismutase (SOD)contents; using diagnostic kits obtained from Bio Diagnostic Co, Egypt. The supplier's instructions were followed precisely to perform all the measurements mentioned above.In addition,pancreatic cell cycle phases,inflammatory (IL-6, TGF-β, and TNF-α), and apoptotic markers (Bcl-2, annexin V, P53, and caspase-3)were assessed by flow cytometry following **Tribukaitet al. (27**) method**.**

Statistical analysis

The obtained data were analyzed using ANOVA followed by post-hoc Tukey multiple range tests, where results were expressed as the Mean±standard error (SEM), where n=8. The level of significance was set at $p \leq 0.05$ for all statistical tests.

Results:-

TEM for MSCs-EXs morphology observations showed circular and intact vesicles with a size range of < 100 nm **(Figure 1A).** Flow cytometric analysis showed very high CD9 (96.2 %), CD63 (99.3 %), and CD81 (97.0 %) expression %, confirming the MSCs-EXs phenotype identity **(Figure 1B).**

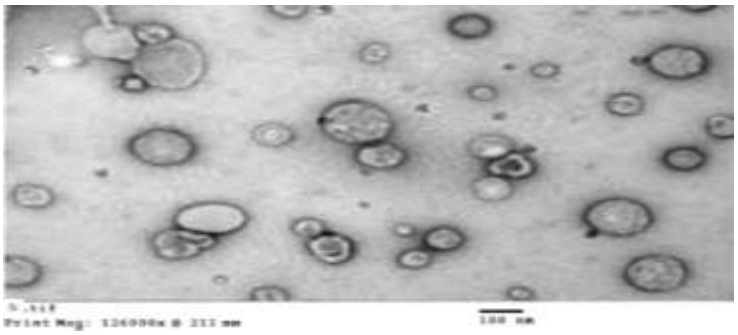
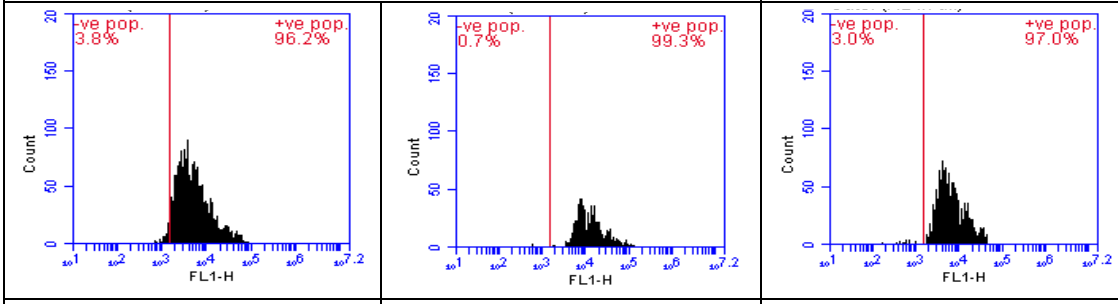


Figure 1A:- EXs morphology.



CD9 **CD63** **CD81**

Figure 1B:- MSCs-EXs surface markers flow cytometric analysis.

Diabetic rats' results in **Table 1** exhibited a marked decline in insulin, C-peptide, and amylase levels, with a marked elevation in both serum glucose and HbA1c levels, concerning the control rats. On the contrary, great hypoglycemic activity was recorded in the diabetic rats injected with EXs, evidenced by the obvious improvement in the previously mentioned parameters,compared to the untreated diabetics.

Table (1):-Serum glucose, HbA1c, insulin, C-peptide, & amylase levels.

		Control	EXs	Diabetic(D)	D + EXs
Glucose (mg/ 100 ml)	**Mean ±SEM**	96.00 ± 4.06	83.00 ± 3.83	348.00 [a] ± 27.87	175.00[ab] ± 16.54
HbA1c (%)	**Mean ±SEM**	4.43 ± 0.12	4.39 ± 0.32	10.20 [a] ± 2.02	7.08[ab] ± 1.33
Insulin (µ I U/ml)	**Mean ±SEM**	17.48 ± 0.54	16.06 ± 0.03	8.20 [a] ± 1.43	12.31[ab] ± 1.32
C-peptide (ng/ml)	**Mean ±SEM**	0.82 ± 0.03	0.79 ± 0.02	0.31 [a] ± 0.01	0.55[ab] ± 0.01
Amylase (U/L)	**Mean ±SEM**	964 ± 49.33	1015 ±60.57	478 [a] ± 20.53	760[ab] ± 40.12

[a&b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

Regarding the control, a marked elevation in the pancreatic oxidative stress (MDA, H_2O_2, and NO) content was highlighted in the diabetic group, as shown in **Table 2**. Interestingly, the EXs-treated diabetic rats reflected significant oxidative stress status amelioration, relative to the diabetic-untreated rats.

Table (2):- Pancreatic MDA, NO & H_2O_2 contents.

		Control	EXs	Diabetic(D)	D + EXs
MDA (n mol/g)	**Mean ±SEM**	5.12 ± 1.35	5.32 ± 1.44	20.32 [a] ± 4.56	10.01[ab] ± 3.24
H_2O_2 (mM/g)	**Mean ±SEM**	0.23 ± 0.03	0.21 ± 0.02	0.82 [a] ± 0.03	0.54[ab] ± 0.02
NO (µM/g)	**Mean ±SEM**	63.40 ± 7.25	61.20 ± 5.87	147.00 [a] ± 18.43	91.23[ab] ± 11.62

[a & b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

The pancreatic antioxidant content of diabetic rats (**Table 3)** revealed a marked decline, regarding the control. Meanwhile, EX injection in diabetic rats showed tremendous antioxidant up-regulation, compared to the untreated diabetic rats.

Table (3):- Pancreatic GSH, SOD, CAT & GPX contents.

		Control	EXs	Diabetic(D)	D + EXs
GSH (mg/g)	**Mean ±SEM**	61.83 ± 4.12	61.73 ± 5.65	20.38 [a] ± 3.45	44.14 [ab] ± 3.23
SOD (U/g)	**Mean ±SEM**	8.44 ± 1.15	7.97 ± 0.97	3.51 [a] ± 0.23	5.18 [ab] ± 0.36
CAT (U/g)	**Mean ±SEM**	3.04 ± 0.36	2.95 ± 0.44	1.32 [a] ± 0.03	2.12 [ab] ± 0.04
GPX (U/mg)	**Mean ±SEM**	6.14 ± 0.76	6.11 ± 0.48	2.23 [a] ± 0.12	4.86 [ab] ± 0.47

[a & b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

Relative to the control,**Table (4)** results cleared marked pancreatic inflammation induction in diabetic rats, which was markedly downregulated following injection with MSCs-EXs.

Table (4): Pancreatic TNF-α, TGF-β, and IL-6 contents.

		Control	EXs	Diabetic(D)	D + EXs
TNF-α (%)	**Mean ±SEM**	26.60 ± 3.24	26.80 ± 4.73	50.30 ± 5.17 [a]	38.30 ± 4.22 [ab]
TGF-β (%)	**Mean ±SEM**	17.50 ± 2.13	17.00 ± 1.65	45.40 ± 4.13 [a]	30.50 ± 3.03 [ab]
IL-6 (%)	**Mean ±SEM**	21.16 ± 2.43	21.45 ± 2.16	49.00 ± 4.14 [a]	34.80 ± 3.72 [ab]

[a & b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

Table 5 revealed an obvious up-regulation in P53, Bax, and caspase-3 % (apoptotic indicators)associated with an anti-apoptotic Bcl-2 % remarkable diminishing, in comparisonwith control. However, EXs-diabetic treated rats reported a notable anti-apoptotic capacity.

Table (5):- Pancreatic P53, Caspase-3 (apoptotic) & BCl-2 (anti-apoptotic)%.

		Control	EXs	Diabetic(D)	D + EXs
P53 (%)	**Mean ±SEM**	17.30 ± 3.65	18.50 ± 3.44	53.00 [a] ±5.21	38.50 [ab] ±3.06
Caspase -3 (%)	**Mean ±SEM**	17.00 ± 3.04	16.20 ± 2.63	55.00 [a] ±4.33	36.00 [ab] ±3.93
Bax (%)	**Mean ±SEM**	29.00 ± 2.65	30.20 ± 3.08	50.00 [a] ±3.23	40.00 [ab] ±2.43
Bcl-2 (%)	**Mean ±SEM**	44.00 ± 4.52	45.50 ± 5.49	17.30 [a] ±1.16	33.55 [ab] ±3.94

[a & b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

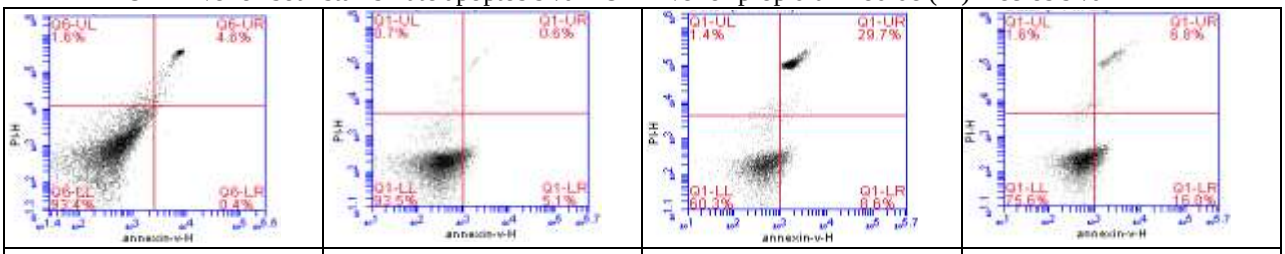
Table 6 & figure 2 showed various pancreatic annexin V % indications in different rat groups. Diabetic untreated rats exhibited a marked decline in viable cells % coupled with significant elevations in apoptotic and necrotic cells %, compared to control. Following EX protocol treatment, diabetic rats showed a notable increase in viable cells %, with a marked decrease in both apoptotic and necrotic cells %.

Table (6):- Pancreatic annexin-V (necrotic & apoptotic) content.

		Control	EXs	Diabetic(D)	D + EXs
Viable cells (%)	**Mean ±SEM**	93.40 ± 3.37	93.50 ±3.16	60.30 [a] ±1.22	63.30 [ab] ±2.43
Early Apoptosis (%)	**Mean ±SEM**	0.40 ± 0.45	5.10 ±0.21	8.60 [a] ±0.63	11.10 [ab] ±0.26
Late apoptosis (%)	**Mean ±SEM**	4.60 ± 1.22	0.60 ± **1**.42	29.70[a] ±2.71	24.20 [ab] ±2.93
Necrosis (%)	**Mean ±SEM**	1.60 ± 0.16	0.70 ± 0.12	1.40[a] ±2.21	1.30 [ab] ± 0.02

Values expressed as mean ± SEM (n = 8). [a & b] are significant differences ($P \leq 0.05$) compared to control and diabetic untreated groups respectively.

Where: **LL**= -ve for both stains=viable cells % **LR**=+ve for annexin V=early apoptosis %
UR= +ve for both stains=late apoptosis % **UL**=+ve for propidium iodide (PI)=necrosis %



Control	*sXE*	D	D + EXs

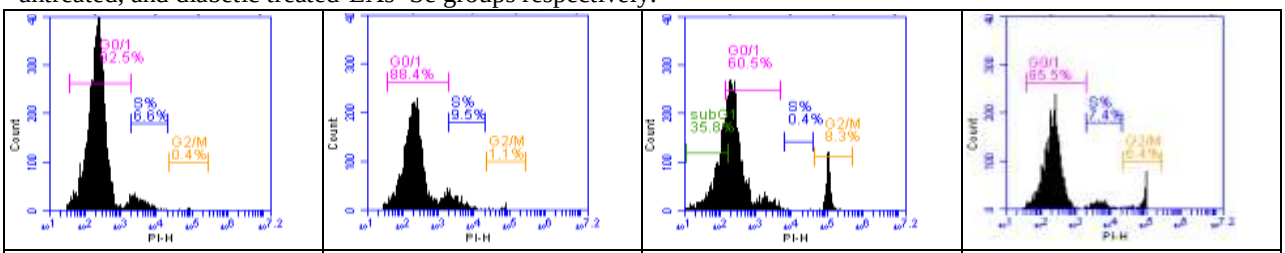
Fig. 2:-Pancreatic Annexin V %.

Table 7 & figure 3 data summarize different pancreatic cell cycle phases in different rat groups.Diabetic untreated rats revealed a marked down-regulation in the G0/G1 cells %, which reflects the pancreatic viable cells %, regarding control rats. In contrast to untreated diabetic rats, EXs injection in diabetic rats markedly resulted inapancreatic viable cell % up-regulation.

Table (7):- Pancreatic cell cycle (G1/G0)) content.

		Control	EXs	Diabetic(D)	D + EXs
G0/G1 phase (%)	**Mean ±SEM**	92.50 ± 3.37	88.40 ±3.16	60.50 [a] ±1.22	85.50 [ab] ±2.43
S Phase (%)	**Mean ±SEM**	6.70 ± 0.02	9.50 ±0.01	0.40 [a] ±0.03	7.40 ±0.02
G2/M phase (%)	**Mean ±SEM**	0.40 ± 0.02	1.10 ±0.02	8.30 [a] ±0.01	6.40 [ab] ±0.03

Values expressed as mean ± SEM (n = 8). [a, b & c] are significant differences ($P \leq 0.05$) compared to control, diabetic untreated, and diabetic treated-EXs+Se groups respectively.



Control	*sXE*	D	D + EXs

Fig. 3:-Pancreatic Cell cycle (Viable cells = G0/G1 %).

Figures 4a&b demonstrated the pancreatic tissues histological examination of control and EXs groups **(C & EXs)**respectively.Both groups showed no pathological anomalies in the pancreatic standard architect (islets intact construction with prominent nuclei, clear borders, and connective tissue septa in between. In contrast to the previous groups, pancreatic tissues of diabetic-untreated rats **(D)** represented in **Figure** clarifies that STZ administration results in a moderate destructive injury represented in the notable islets' mass size reduction with marked vacuolation besides numerous pyknotic nuclei and congested blood vessels occurrence. Nevertheless, the **figure revealed** notable amelioration in the pancreas' morphological structure of diabetic rats treated with EXs,reflected by the obvious restoration of the normal islets' mass size and the limited presence of both congested blood vessels and apoptotic nuclei, relative to the untreated-diabetic rats.

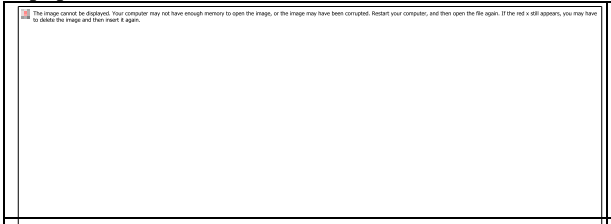


Fig. 4a: C

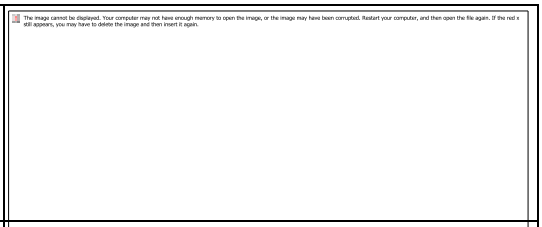


Fig. 4b: EXs

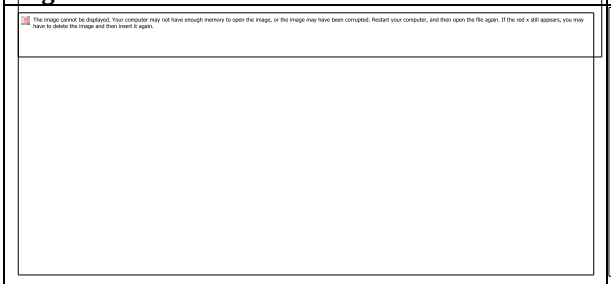


Fig. 4c: D

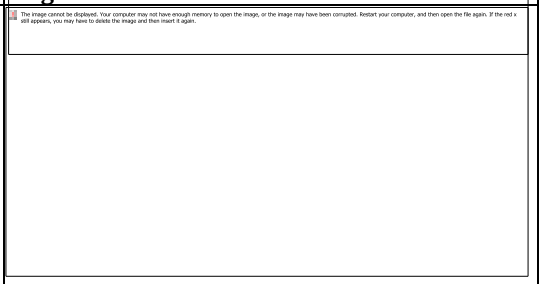


Fig. 4d: D+EXs

Figure (4): Photomicrograph of HE-stained pancreatic sections
(Low magnification → 100 X: bar 100 & High magnification → 400 X: bar 50).
a &b → Control &EXsnormal groups respectively
showed no histopathological alteration with normal structure of either the exocrine portion; represented by acinar cells **(AC)**; or the endocrine portion; represented by islands of Langerhans **(IL)**; containing α-cells**(black arrows)**&β-cells**(red arrows)**.
c → D (STZ-treated) group displayed evident **IL** degeneration illustrated in
severe hydropic degeneration in most α-cells &β-cells, with few apoptotic β-cells**(blue arrow),** and pyknotic nuclei of epithelial lining acini**(arrowheads).**
d → EXs-treated diabetic groupdemonstrated much less hydropic degeneration and markedly improved AC and IL histological picture **(α & β-cells).**

Discussion:-

Because of their nano-sized dimension, MSCs–EXs can distributed easily via biological fluids, penetrating even distant tissues and reaching the target cells, performing both paracrine and endocrine effects **(22,23).** Due to such benefit advantages, besides their known immunomodulatory and regenerative properties, they have been highlighted as a novel promising candidate for various auto-immune and inflammatory disorders therapy, including T1DM **(1,3).**

1. Pancreatic hazards of STZ-induced T1DM

STZ is well known for T1DM induction through pancreatic β-islets' selective destruction, causing irreversible necrosis progression, with loss of pancreatic cellular architecture, compared to the control rats. These harmful effects could be attributed to the marked alkylation of DNA and elevated ROS and NO production, resulting in significant hyperglycemia and insulin insufficiency **(28).** Our results reported a marked FBG and HbA1c levels elevation coupled with marked serum insulin and C-peptide decline, following a single STZ (60 mg/kg) injection, relative to control rats. According to **Sabry et al. (29),**in addition to provoking hyperglycemia, STZ administration

decreased insulin-producing β-cells count andinducedβ-cell size shrinkage, which may be due to increased inflammation, fibrosis, vacuolar congestion, and degeneration.

On the other hand, in the ongoing study, STZ injection resulted in marked β-cells' oxidative stress development, evidenced by the significant MDA, H_2O_2, and NO elevation, accompanied by various antioxidants (GSH, SOD, CAT, and GPX) content decline. A similar assumption was made by **Jamshidiet al. (30) and** Furman (31**)** who found that increased intracellular glucose levels resulted in an overproduction of ROS, accompanied by the decreased activity of antioxidant enzymes, such as SOD, GSH, CAT, and GPX, suggesting the exhaustion of these antioxidants to overcome the increased oxidative stress' deleterious effects.

In addition to provoking hyperglycemia, data in this study showed that STZ could significantly induce a marked pancreatic inflammatory progress in diabetic rats, demonstrated by the increased IL-6, TGF-β, and TNF-α. Accumulating evidence declared a marked proinflammatory cytokines MCP-1, IL1-β, IFN-γ, IL-8, TNF-α, and TGF-β, up-regulation in both serum and tissue of STZ-diabetic rats, concerning the control group; attributed to the hyperglycemia-increased inflammation and oxidative stress **(**31**).**

Regarding apoptosis,progressive pancreatic β cell loss can be caused by STZ injection, which induces pancreatic β-cell apoptosis and decreases insulin secretion, thereby accelerating the hyperglycemic state **(12)**. These findings are consistence with our results which reported significant pancreatic caspase 3, annexin V, Bax, and P53 % elevations, in contrast to a marked Bcl-2 % decline in diabetic rats, relative to the control. Conceivably, near total loss (>80% reduction) of β-cell mass was associated with the enhanced glycemic overload and ROS synthesis, resulting in the amplification of endoplasmic reticulum (ER) stress and consequent cellular damage, inhibiting cell proliferation and initiating either cell necrosis or apoptosis **(32).** In this line, STZ injection was found to upregulate many ER stress-dependent apoptosis signaling molecules, including cytochrome c, BAX, P53, and caspase-3, with marked anti-apoptotic Bcl-xL and Bcl-2 decline; collectively resulting in obvious apoptosis in β-cells of diabetic rats **(**31**).**

2. MSCs-EXs characterization

Following the minimum requirements for the reproducible and accurate investigation of EXs delineated by the International Society for Extracellular Vesicles (ISEV) IN 2018 **(33,34,35)** and before being applied in our study for T1DM treatment, both morphological and phenotypic identity of MSCs-EXs were confirmed using TEM and flow cytometry, respectively. Herein, MSCs-EXs appeared as circular and intact vesicles with a size range of < 100 nm **(Figure 1A).** Such observation was in line with a previous study by**Hosseini-Beheshti et al. (24)** who reported a very homogenous EXs mixture with a typical cup-shaped and round morphology with a diameter range of 30-100 nm when examined by TEM before being applied for prostate cancer treatment.which highly expressed many surface protein markers, including CD9 (96.2 %), CD63 (99.3 %), and CD81 (97.0 %). Such results**.**In agreement with our findings,Kooget al. (16) and **Roszkowski (17)** reported that MSCs-EXs possess various surface proteins that are used as biomarkers for their phenotypic identification,such as the tetraspanin protein family (CD9, CD63, CD81).

3. MSCs-EXs for T1DM treatment

MSCs-EXs have been presented as a novel DM protocol therapy since they have shown marked therapeutic superiority to their parent MSCs **(1).** Herein, diabetic rats injected with MSCs-EXs reported a marked pancreatic antioxidant elevation, which suggested the main direct cause of the significant pancreatic oxidative stress suppression in diabetic rats. Such events consequently minimized the pancreatic oxidative stress and inflammatory progression, leading to a marked pancreatic apoptosis suppression and elevation in the number of viable β-cells. Together, these events were suggested to alleviate STZ-induced hyperglycemia by restoring relatively normal insulin secretion and glycemic control.

To our findings, MSCs-EXsinjectioninto STZ-diabetic animals were found to greatly enhance glycemic control and reverse hyperglycemia, besides restoring the normal insulin secretion and pancreatic β-islets mass, by suppressing apoptosis and enhancing their survival and viability,as a consequence of the MSCs-EXs capacity to minimize oxidative stress and inflammation in diabetic pancreas(**36,37).**Another study by Suet **al. (38)** reported that MSCs-EX injection significantly ameliorated insulin resistance in aged mice and helped regulate the blood glucose level.Recently, **He et al. (9)** found that MSCs-EXs effectively alleviated hyperglycemia, decreased FBG, improved insulin resistance, and increased insulin sensitivity in diabetic rats, via improving islet function, promoting glycolysis/glycogen synthesis, and inhibiting gluconeogenesis.

Such notable hypoglycemic effect was suggested as a result of the therapeutic potency of MSCs-EXs cargoofvarious cytokines (IL-6, IL-10), immunomodulatory agents (miRNA-146a, miRNA-155), and growth factor; to (1) recover β-cell function and mass and promoting their repair, (2) mitigate the undesired pancreatic inflammatory responses and auto-reactivity, and(3) regulating the immune microenvironment; by efficiently minimizing the leucocyte influx into the inflamed tissues,suppressing the inflammatory $CD4^+$ (Th1, Th2, and Th17) lymphocytes as well as B-lymphocytes and M1 macrophages (mainly secrete pro-inflammatory mediators), while inducing immunosuppressive T-regs and M2 macrophage (possess anti-inflammatory functions) expansion, contributing to the attenuation of on-going inflammation in diabetic rats which may participate in alleviating diabetic complications **(12,15,18,28)**.

Similar to the parent MSCs, MSCs-EXs offer cell protection against apoptosis, via repairing cells and protecting them from death **(12),** which could be attributed to the MSCs-EXs' expression of many adhesion protein molecules (CD29, CD44, and CD73), that enable their homing to the inflamed tissues **(39).** Co-culturing diabetic pancreatic β-islets with Wharton's jelly-MSCs-EXs in Vitro was found to downregulate BAD, BAX, Annexin-V, P53, and caspase-3 (apoptotic genes), in contrast to the anti-apoptotic Bcl-2 gene expression upregulation, which suppressedβ-islets' apoptosis and greatly improved their viability, survival, and function**(40).**

Interestingly, **Mahdipour et al.(41); He et al. (9)** and **Yap et al.(42)** confirmed the accumulation of MSCs-EXs in the pancreas of diabetic rats after being intravenously injected, promoting the β-cells' regeneration**.** In a similar design conducted by **Sabry et al. (29)**, the pancreatic histopathology examination of diabetic rats treated with BM-MSCs-EXs revealed marked regeneration and elevation of both the β-islets count and size, accompanied by a marked inflammation and fibrosis suppression. Nevertheless, co-culturing islets with MSCs-EXs greatly improved islets' vascularization and reduced apoptosis, resulting in increased survival and function of islet β-cells both **in vitro** and after engraftment in animals **(Luo** et al**., 43).** Moreover, **Sharma et al. (28)** reported that after 7 days of MSCs-EXs injection, hyperglycemia was attenuated in STZ-diabetic mice, with a concomitant insulin production elevation, which could be attributed to the obvious histological β-cell regeneration and improved pancreas architecture, compared to control mice. Also, **Mahdipouret al. (41)** have shown that EXs derived from menstrual blood MSCs can regulate β cell regeneration through a PDX1-dependent mechanism in the T1DM rat model. According to **Atkinson** et al**. (44),** there is a possibility that the reparative properties of MSCs-EXs could cause a functional recovery of the already-existing islets, making its injection potentially equally effective to MSCs themselves, while not requiring immunosuppressants to prevent graft rejection.

Conclusion:-

Growing evidence suggests MSCs-EXs represent an emerging safer strategy for cell-free therapy, as an MSCs alternative, for T1DM complications alleviation. Our study has demonstrated that MSCs-EVs show effective regenerative potential in the attenuation of pancreas injury leading to alleviating hyperglycemia in diabetic rats, by improving β-cell mass, promoting insulin secretion, and increasing antioxidant contents and counteracting the oxidative stress status, besides minimizing the inflammation and apoptosis of pancreatic islets. However, we suggest that proper modification can enhance the contents, biodistribution, and bioactivity of MSCs-EVs, which may maximize their therapeutic potential.

List Of Abbreviations

AD-MSCs: Adipose-derived mesenchymal stem cells, **CAT:** Catalase, **CD:** Cluster of differentiation, **D:** Diabetic, **DM:** Diabetes mellitus, **DMEM:** Dulbecco's modified Eagle's medium, **ER:** endoplasmic reticulum,**EXs:** Exosomes, **FBG:** Fasting blood glucose, **FBS:** Fetal bovine serum, **GPX:** Glutathione peroxidase, **GSH:** Glutathione**,HbA1c:** glycosylated hemoglobin, **H_2O_2:** Hydrogen peroxide, **IDF:** International Diabetes Federation, **ISEV:** International Society for Extracellular Vesicles, **MDA:** Malondialdehyde, **MSCs:** Mesenchymal stem cells, **PBS:** Phosphate-buffered saline, **ROS:** Reactive oxygen species, **SEM:** Standard error of the mean**, SOD:** Superoxide dismutase, **SPSS:** Statistical Package for Social Scientists, **STZ:** Streptozotocin, **T1DM:** Type 1 diabetes mellitus, **T2DM:** Type 2 diabetes mellitus, **T-reg:**regulatory T-cells

Declarations

Ethics approval and consent to participate:

This design was approved by the Animal Ethics Committee of the Faculty of Science, Mansoura University, Mansoura, Egypt [MU-ACUC (SC.PhD.23.03.6)].

Consent for publication:
Not applicable.

Availability of data and material:
All data generated or analyzed during this study are included in this published article.

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The authors declare that they have no competing interests.

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DYK, RHH, and WME conceived and designed the study. Experiments and lab work, data tabulating and acquisition, searching for literature, and preparing the first draft of the manuscript were performed by DYK. All authors have read and agreed to the published version of the manuscript.

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