

Article

Efficacy of Mesenchymal Stem Cell and Vitamin D in the Treatment of Diabetes Mellitus Induced in a Rat Model: Pancreatic Tissues

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Citation: Hamza, R.Z.; Al-Eisa, R.A.; El-Shenawy, N.S. Efficacy of Mesenchymal Stem Cell and Vitamin D in the Treatment of Diabetes Mellitus Induced in a Rat Model: Pancreatic Tissues. *Coatings* **2021**, *11*, 317. <https://doi.org/10.3390/coatings11030317>

Academic Editor: El-Sayed Abd El-Aziz

Received: 13 February 2021

Accepted: 3 March 2021

Published: 10 March 2021

Retracted: 16 August 2022

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Abstract: Treatment with mesenchyme stem cells (MSCs) plays a significant role in the therapies of many diseases such as diabetics. Vitamin D plays a significant role in the development of insulin and can increase the insulin action sensitivity of peripheral tissues. Moreover, there is limited research concerning the mechanism of the therapeutic action of MSCs with the combination of vitamin D (vit. D). Therefore, we evaluated the effect of MSC intervention in a diabetic animal model. Diabetes was induced by streptozotocin (STZ) injection at a dose of 50 mg/kg in adult male rats. The diabetic rats were injected with MSCs derived from bone marrow (2×10^6 per rat), either alone or in combination with vit. D through the tail vein for four weeks. Serum insulin, glucose, C-peptide, glycosylated hemoglobin, and lipid profile levels were determined. Pancreatic oxidative stress, histology, and electron microscopy were evaluated, and the gene expression of cytokines was assessed by real-time polymerase chain reaction (PCR). MSC treatment suppressed pancreatic inflammatory cytokine secretion and oxidative stress in diabetic rats, resulting in improved pancreatic histology and cellular structure, and the complication of hyperglycemia was observed. Engrafted MSCs were found inside degraded pancreatic regions and regulated inflammatory cytokines. Our results demonstrated that treatment with MSCs and vit. D in combination prevented pancreatic injury via antioxidant and immune regulation in diabetic rats, contributing to the prevention of pancreatic dysfunction, improvement of lipid metabolism, and regulation of cytokine gene expression compared with each one separately. All these mechanisms also improved the histological structure of the pancreas based on transmission electron microscopy. The combination of MSCs and vit. D appears to have contributed to a greater improvement in the diabetic pancreatic complication of rats than was observed by each one separately. Therefore, this association can be used as antidiabetic therapy.

Keywords: diabetes mellitus; stem cells; vitamin D; pancreatic tissues

1. Introduction

Diabetes mellitus (DM) type II is a metabolic condition arising as a result of the pancreas' deficient development of insulin. Physiological regulation of blood glucose levels can occur in a variety of ways: external insulin administration, insulin-stimulating medicines, medicines for reducing insulin resistance, and/or regeneration of β cells that are precursors of insulin. Progressive and ineluctable failure of beta-cells is characteristic of type 2 diabetes mellitus (T2DM), and reconstruction of beta-cells using stem cell therapy may be a successful tool [1].

Using embryonic stem cells was the best model for pancreatic regeneration studies [2]. A further important technique for producing β cells is the use of adult stem cells. Mesenchymal stem cells (MSCs) have many benefits for clinical use such as their abilities to

move to tissue injury sites, powerful immunosuppressive effects [3], and improving safety followed injection of allogeneic MSCs [4]. MSCs are known as the most desirable sources of regenerative medicine for cells. MSCs have multi-potential, including the ability to self-renew, pluripotent, low antigenicity, declining toxicity, and ease of cultivation, and in vitro expansion is required to obtain sufficient cells used for treatment. These cells are found in the umbilical cord blood amniotic fluids, adipose tissue, and bone marrow [5].

MSCs differentiate into hepatocytes, neurons, cardiomyocytes, adipocytes, epithelial cells, and vascular cells, making them a valuable tool for treating severe human diseases. MSCs' therapeutic effects depend not only on their differentiating ability to repair scar tissue but also on their ability to improve the surrounding environment, stimulate endogenous progenitor cells, and secrete numerous variables [6].

Vitamin D is one of the fat-soluble vitamins and micronutrients of which deficiency is associated with the pathogenesis of insulin-resistance-related diseases, including obesity and diabetes mellitus type 1 and type 2. Both vitamin D (vit. D) genomic and non-genomic action is related to insulin signaling. Vitamin D linked to insulin secretion, insulin resistance, and dysfunction of β -cells in the pancreas was demonstrated [7].

It was found that vitamin D's molecular action is involved in maintaining normal ROS and Ca^{2+} resting levels, not only in pancreatic β -cells but also in insulin-responsive tissues. Therefore, vit. D decreases the severity of insulin resistance-related pathologies such as oxidative stress and inflammation. Vit. D also prevents epigenetic changes associated with insulin resistance and diabetes as well as speeds up insulin production [8]. Liu et al. [9] indicated that vit. D has a therapeutic benefit on diabetes-induced liver complications in diabetic rats.

Diabetes mellitus is a significant problem in the health service systems around the world, as it is correlated with many key risk factors for health. Presently marketed medications are inadequate to maintain long-term blood glucose regulation levels and cause severe side effects. There is still a pressing interest in developing more new treatments for metabolic disorders. Scientific proof from laboratory and field trials is incomplete and insufficient; therefore, evidence of the causal relationship between treatment with vitamin D/MSCs and glycemic regulation is not appropriate.

Therefore, this study was designed to examine the benefits and mechanisms of vit. D and/or MSCs on the pancreatic tissue of diabetic model rats. The levels of insulin and glucose and the lipid profile in serum as well as pancreatic oxidative/antioxidant and cytokine transcript levels (TNF- α , IL-1 β , and IL-6) and the gene expression of experimental diabetic rats were determined. In addition, the relationship between vit. D and MSCs in the treatment of diabetes mellitus was clarified as shown in Figure 1.

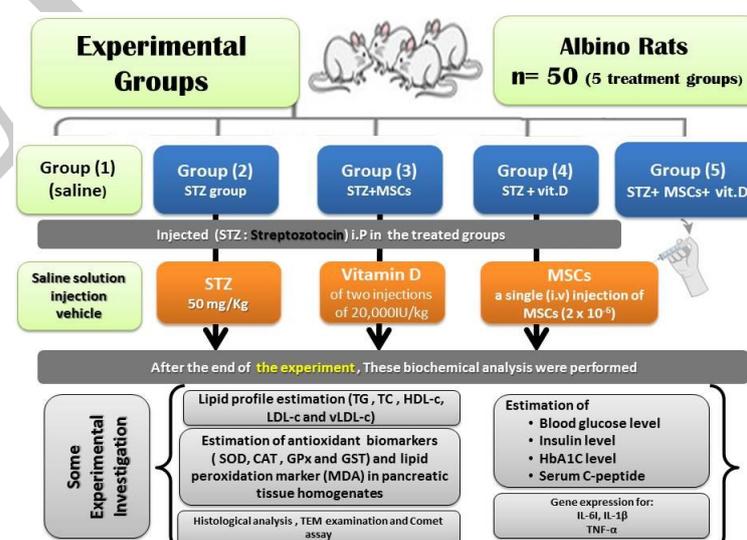


Figure 1. Experimental protocol and conclusion of the study.

2. Materials and Methods

2.1. Chemicals

Streptozotocin (STZ) was obtained from Sigma Chemical Co. (Sigma–Aldrich, St. Louis, MO, USA). Vitamin D (vit. D; Drop injections) was purchased from a local pharmacy, Zagazig, Egypt.

2.2. Experimental Animals

Adult male albino rats ($n = 50$) with a weight of 180–195 g were housed under standardized conditions in metal hygiene cages and allowed access to food and water. The experiment protocol was approved by Ethical committee, Deanship of scientific research, Taif University (approval number: 40-31-0189) and followed the international guidelines of the animal care committee. Animals were housed under controlled temperature (23 ± 1 °C), humidity (45–65 percent), and a 12-h light/dark cycle with free access to food and water.

2.3. STZ-Induced Diabetes in the Rat Model

Experimental diabetes was induced in the fasted animals by freshly prepared STZ dissolved in 0.1 M sodium citrate buffer with pH 4.5 by a single intraperitoneal (i.p) injection of 50 mg/kg STZ within a few minutes after preparation [10].

Diabetes was evaluated by measuring the level of blood glucose from the lateral tail vein by glucose monitoring using Strips of Aqua Check Active after three days of STZ injection. Rats with a blood glucose level higher than 250 mg/dL were considered diabetic and included in the experiment. The treatments began after 72 h of diabetes induction and proceeded every day for 30 consecutive days.

2.4. Isolation and Culture of Bone Marrow MSCs

MSCs were isolated from bone marrow by the method of Wang et al. [11]. Bone marrow was harvested from the tibiae and femoral bone of 10 male albino rats; harvested cells were isolated by separating mononuclear cells (MNCs) with Ficoll-Hypaque solution. Then, cells were grown in modified Eagle medium (DMEM, GIBCO/BRL) with low-glucose Dulbecco. Nucleated cells were isolated with a density gradient (Ficoll/Paque; Pharmacia) and re-suspended in the complete culture medium with 1 percent penicillin-streptomycin (Gibco/BRL). Cells were incubated for 12–14 days at 37 °C in 5 percent humidified CO₂ before large colonies formed (80–90 percent confluence). The crop was washed with PBS and released at 37 °C for 5 min with 0.25 percent trypsin in 1 mM/L EDTA (Gibco/BRL). The cells were re-suspended with medium after centrifugation and incubated in a culture flask. The result was referred to as the cultures of the first passage [12]. They were characterized by their adhesivity and fusiform shape in the media [13].

2.5. Flow Cytometric Analysis

A flow cytometer (Becton Dickinson, Holdrege, NE, USA), was used for the cytometric study of the bone marrow MSCs. CD 29, which is known as the stromal precursor of bone marrow and is expressed highly in MSCs, was observed in the following cell surface antigens. We acquired and analyzed 100,000 labeled cells using Cell Quest tools [14].

2.6. Labeling MSCs with PKH26 and Its Detection in Pancreatic Tissue

PKH26 is a red fluorochrome; MSCs obtained from Sigma Company (Saint Louis, MO, USA) were labeled with PKH26. The experimental procedure was performed in the dye kit at room temperature according to the protocol. After 30 successive days, pancreatic tissues were examined with a fluorescence microscope to determine home-labeled cells (Leica, Germany).

The final concentrations of 2×10^6 M PKH26 dye and 1×10^7 cells/mL were stained in a total volume of 2 mL using the following standard methods as per the Sigma protocol. This solution was incubated in a conical tube for 2 min and was inverted gently and consistently to ensure thorough mixing. An equal volume of FBS was added to stop the

reaction, and the resulting mixture was incubated for 1 min. The solution was diluted with an equal volume of serum-containing DMEM to remove cells from the staining solution and centrifuged for 10 min at 25 °C at 400× g. The supernatant was removed, and the cell pellet was transferred to a new tube for further washing (a minimum of three washes). The cells were examined using fluorescence microscopy. The precipitated cells were then washed three times with an equal volume of serum-containing DMEM, and cells were suspended at a complete cell medium density of 1×10^5 cells/mL, then cultivated to obtain cell line LP1. A fluorescence microscope was used to observe the labeling of the LP1 cells.

2.7. Animal Grouping and MSC Transplantation

After the period of adaptation, male albino rats weighing 180–195 g were divided into five groups (10 rats per group):

Group I: normal control (NC) healthy group treated intraperitoneally (i.P) with physiological saline.

Group II: (Diabetic untreated group) (DC). This group was regarded as diabetic control (STZ-group) untreated animals receiving injections of 0.5 mL 0.9% saline.

Group III: diabetic rats treated with a single i.v. injection of MSCs (2×10^{-6}); this group was the STZ-stem cell group.

Group IV: diabetic rats injected i.P with vit. D (STZ + vit. D) at a dose of two injections of 20,000IU/kg, diluted in sesame oil, on two days every week (intramuscular (IM)) [15].

Group V: diabetic animals administrated both stem cells and vit. D in the same manner as mentioned above (STZ + stem cell + vit. D).

2.8. Biochemical Determination

After one month of treatment, all fasting animals were suddenly decapitated under light ether anesthesia. Levels of blood glucose were evaluated by using commercial kits. The blood samples were taken using capillary tubes from the eye plexus and were centrifuged at 5000× g for 15 min for further biochemical analyses.

Serum total cholesterol (TC) and triglycerides (TG) were determined following Carr et al. [16] and Warnick et al. [17], respectively. Then, the calculation of LDL-c and v-LDL-c was performed.

A rat enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Diagnostics, Kew-aydin, NH, USA) was used to evaluate the fastening serum insulin levels. C-peptide enzyme commercial immune assay (Sigma–Aldrich) and glycosylated hemoglobin level (HbA1c) ELISA (Cusabio Co., Hubei, China) kits were used according to the manufacturers' protocols.

2.9. Preparation of Pancreatic Tissue Homogenates and Oxidative Stress/Antioxidant Determination

The pancreatic tissues were kept after animal decapitation at -80 °C for further investigations. A small portion of the pancreatic tissues was used for the estimation of the antioxidant biomarkers. Pancreatic tissues were homogenized in 5 mL cold buffer/g and centrifuged at 5000× g for 1/2 h to obtain the supernatant that was kept at -20 °C.

Supernatants of the homogenates pancreatic were used for the estimation of myeloperoxidase (MPO) and xanthine oxidase (XO). The MPO Kit provided a rapid and real method of MPO detection. XO was determined by the method of Litwack et al. [18]. Catalase (CAT) and superoxide dismutase (SOD) activities were evaluated according to Xu et al. [19] and Marklund and Marklund [20], respectively. Glutathione peroxidase (GPx) activity was determined as described by Hafeman et al. [21] and expressed in mmol GSH consumed/min/g tissue. Glutathione-S-Transferase (GST) was determined following Alin et al. [22]. The activity of GST was expressed as U/g tissue (1 unit is the amount of enzyme that conjugates one nmol of CDNB with GSH/min). Pancreas lipid peroxidation expressed as malondialdehyde (MDA) levels was evaluated following Ohkawa et al. [23].

2.10. RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction

Gene expressions of pancreatic tissues were examined using PCR. Total RNA was isolated by using the reagent Trizol [24].

2.11. Polymerase Chain Reaction (PCR) for Determination of Cytokine

In male rat pancreatic tissues, cytokine transcript levels of TNF- α , IL-1 β , and IL-6 were measured using the reverse transcriptase (RT)-PCR technique. Total RNA from the pancreas was extracted using iScriptTMRTqPCR [25].

2.12. Histological Analysis of Pancreas

Pancreatic tissues were fixed in 10 percent neutral-buffered formalin. The samples were pressed into paraffin after fastening, and then thin parts were cut and stained with hematoxylin and eosin (H&E). Finally, a light microscope was used to examine the specimens.

2.13. Single-Cell Gel Electrophoresis (SCGE) (Comet Assay)

This is a sensitive technique for analyzing DNA damage in individual cells. Pancreatic tissues were placed into a Petri dish with ice solution (Ca²⁺, Mg²⁺ free with EDTA). The various steps involved in the alkaline comet assay followed the method of NandhaKumar et al. [26].

2.14. Transmission Electron Microscopic Study

Pancreas portions were fixed in 2.5% glutaraldehyde for 48 h; the other processes followed Hayat [27].

2.15. Statistical Analysis

Data are presented as the mean \pm standard error (SE) based on one-way analysis of variance followed by the post-hoc test of the SPSS program (Snedecor and Cochran [28]). The significance of the difference was set at $p \leq 0.05$.

3. Results

MSCs were identified by their spindle shape and fibroblasts (Figure 2A) and were detected by stem cell marker CD29. MSCs were +ve for CD29 (>98%). The area of blue in (Figure 2B) represents isotype control IgG expression, and the marker expression is shown in gray lines. Stem cells were labeled with PKH26 dye by fluorescence microscopy in pancreatic tissues of diabetic groups at the end of the experiment (30 days). After transplantation into rats, MSCs labeled with PKH26 displayed heavy red auto fluorescence, indicating that cells were seeded in the pancreatic tissue. The upper part presents the treatment with MSCs alone, and the lower part, the treatment with MSCs and vit. D (Figure 3).

Blood glucose and Hb A1c increased in the DC group by 4.2- and 1.6-fold, respectively, compared with NC animals (Table 1). However, the insulin and c-peptide levels significantly decreased in diabetic rats compared with the NC group. Treatment of diabetic animals with stem cells in combination with vit. D improved all the above parameters better than each one alone.

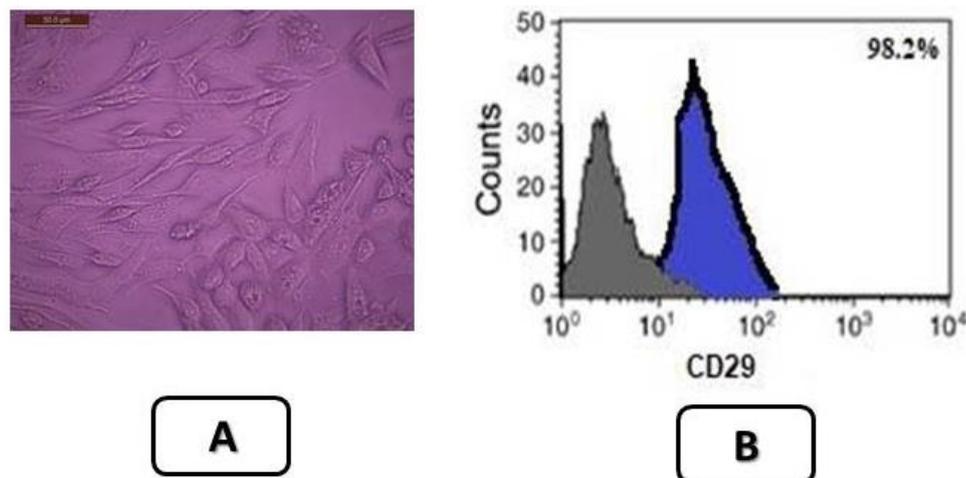


Figure 2. (A) Characterization of isolated bone marrow stem cells of rat that were used after 14 days (Mature stage) X200. (B) Flow cytometry results of the mesenchymal stem cell marker CD29. MSCs were +ve for CD29 (>98%). The blue area represents the isotype control IgG expression, and gray lines depict the marker expression. The results are representative of three experiments. Data results correspond to the mean \pm S.D.

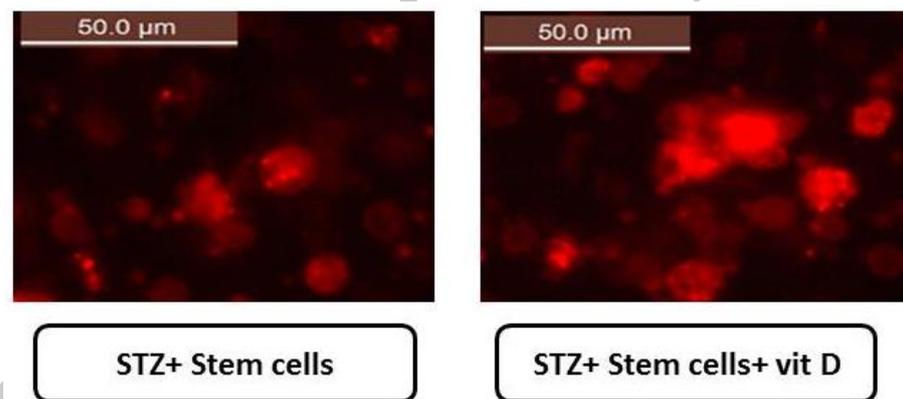


Figure 3. Stem cells labeled with PKH26 dye in pancreatic tissues of diabetic groups at the end of the experiment (50.0 μ m) shown under a fluorescence microscope. MSCs labeled with PKH26 showed strong red autofluorescence after transplantation into rats, confirming that these cells were seeded in the pancreatic tissue. Upper: treatment with MSCs alone, and lower: after treatment with MSCs and vit. D.

Table 1. Blood glucose level, insulin, HbA1c, and fasting serum C-peptide of control and male rats treated with stem cells, vit. D, and their combinations.

Groups	Parameters			
	Blood Glucose (mg/dL)	Insulin (IU/mL)	HbA1c (mmol/mol)	Serum C-Peptide (ng/mL)
Control group	95.61 \pm 4.15 ^e	24.76 \pm 2.25 ^{ab}	6.12 \pm 0.85 ^d	4.22 \pm 0.49 ^{ab}
STZ group	402.27 \pm 7.02 ^a	4.30 \pm 0.25 ^e	9.51 \pm 1.66 ^{ab}	0.72 \pm 0.05 ^d
STZ + stem cell	180.34 \pm 5.03 ^c	18.59 \pm 1.36 ^c	8.98 \pm 1.25 ^b	2.98 \pm 0.77 ^c
STZ + vit. D	200.05 \pm 5.72 ^b	15.38 \pm 2.15 ^d	9.02 \pm 1.02 ^{ab}	2.72 \pm 0.59 ^c
STZ + stem cell + vit. D	132.05 \pm 5.25 ^d	21.65 \pm 2.65 ^b	7.05 \pm 0.59 ^{cd}	3.84 \pm 0.43 ^b

Data are presented as the mean \pm SE. STZ: diabetic group, vit. D: vitamin D. Letters carrying different symbols are significant and assigned alphabetically. The values with the same letters are not significantly different, and the data with different letters are significantly different.

Table 2 shows the lipid profile parameters of the rats treated with MSC stem cells and/or vit. D. TG levels increased by 4.2-fold in DC rats as compared to the NC group. TG levels declined by 60.7%, and 53.2% in diabetic groups treated with stem cells and vit. D, respectively, as compared to DC animals. However, in diabetic rats treated with stem cells and vit. D, the TG level decreased by 70% as compared to the DC group.

Table 2. Lipid profile of control and diabetic male rats treated with stem cells, vitamin D, and their combinations.

Groups	Parameters				
	Triglycerides (mg/dL)	Total Cholesterol (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)	vLDL-c (mg/dL)
Control group	67.32 ± 1.26 ^e	113.82 ± 5.04 ^d	40.12 ± 4.25 ^{ab}	17.03 ± 2.02 ^d	13.46 ± 1.45 ^e
STZ group	280.16 ± 4.14 ^a	320.16 ± 4.27 ^a	20.86 ± 1.02 ^e	51.31 ± 4.36 ^a	27.07 ± 3.02 ^{ab}
STZ + stem cell	110.02 ± 6.25 ^c	141.58 ± 5.25 ^c	35.36 ± 2.14 ^c	28.21 ± 2.56 ^b	20.60 ± 1.36 ^c
STZ + vit. D	131.25 ± 5.25 ^{bc}	175.02 ± 6.36 ^b	33.46 ± 3.58 ^d	29.25 ± 1.36 ^b	24.21 ± 1.36 ^b
STZ + stem cell + vit. D	84.32 ± 5.25 ^d	100.34 ± 4.69 ^e	38.31 ± 3.69 ^b	20.42 ± 1.57 ^{cd}	17.11 ± 1.86 ^{de}

Data are presented as the mean ± SE. STZ: diabetic group, vit. D: vitamin D, HDL-c: high-density lipoprotein cholesterol, vLDL-c: volatile low-density lipoprotein cholesterol. Letters carrying different symbols are significant and assigned alphabetically. The values with the same letters are not significantly different, and the data with different letters are significantly different.

TC levels decreased more in response to STZ + stem cell + vit. D than STZ + stem cell or STZ + vit. D alone as compared to DC animals (Table 2). HDL-c declined by 50% in DC rats as compared to NC animals. The treatment with different protocols enhanced the level of HDL-c in the following order: STZ + stem cell + vit. D > STZ + stem cell > STZ + vit. D. The respective LDL-c and VLDL-c levels were increased by almost 3- and 2.1-fold in DC animals as compared to NC rats. The treatment of the diabetic group with both stem cells and vit. D decreased the LDL-c and VLDL-c levels more than the other treatments with stem cell or vit. D alone (Table 2).

The MPO and XO levels of the rats treated with stem cells alone or in combination with vit. D revealed a significant decrease in four weeks after stem cell transplantation when compared with diabetic rats. In addition, levels were significantly decreased compared to the group treated with vit. D alone (Table 3).

Table 3. Changes in MPO and XO in pancreatic tissues of control and male rats treated with stem cells, vitamin D, and their combinations.

Groups	Parameters	
	MPO (nmol/min/mL)	XO (U/g)
Control group	15.10 ± 1.26 ^d	17.45 ± 1.45 ^e
STZ group	29.56 ± 1.31 ^a	35.25 ± 1.36 ^a
STZ + stem cell	18.49 ± 1.34 ^c	25.17 ± 1.55 ^b
STZ + vit. D	20.44 ± 1.25 ^b	22.31 ± 1.65 ^c
STZ + stem cell + vit. D	18.46 ± 2.08 ^c	19.27 ± 2.52 ^{de}

Data are presented as the mean ± SE. STZ: diabetic group, vit. D: vitamin D, MPO: myeloperoxidase (MPO), and XO: xanthine oxidase. Letters carrying different symbols are significant and assigned alphabetically. The values with the same letters are not significantly different, and the data with different letters are significantly different.

Table 4 shows the changes in the oxidative/antioxidant enzymes in pancreatic tissues of control and male rats treated with stem cells, vit. D, and their combinations. MDA levels in DC animals significantly increased with decreasing antioxidant enzymes (SOD, CAT, GRx, and GST). The combination of stem cells and vit. D decreased the MDA levels and improved all the antioxidants more than treatment with stem cells or vit. D separately.

Table 4. Changes in oxidative/antioxidant enzymes in pancreatic tissues of control and male rats treated with stem cells, vitamin D, and their combinations.

Groups	Parameters (U/g)				
	CAT	SOD	MDA	GRx	GST
Control group	1.48 ± 0.12 ^a	22.45 ± 1.15 ^{ab}	2.35 ± 0.28 ^e	30.05 ± 1.55 ^{ab}	20.35 ± 0.52 ^{ab}
STZ group	0.47 ± 0.11 ^d	5.12 ± 1.15 ^e	39.17 ± 1.96 ^a	7.16 ± 1.15 ^e	4.35 ± 0.48 ^e
STZ + stem cell	1.12 ± 0.32 ^{bc}	16.91 ± 1.48 ^c	20.40 ± 1.47 ^c	24.25 ± 1.17 ^c	12.72 ± 1.35 ^{cd}
STZ + vit. D	1.0 ± 0.67 ^c	12.64 ± 2.18 ^d	25.42 ± 1.02 ^{bc}	20.85 ± 1.12 ^d	10.87 ± 0.49 ^d
STZ + stem cell + vit. D	1.40 ± 0.53 ^a	20.17 ± 1.15 ^b	8.36 ± 1.46 ^d	28.39 ± 1.81 ^b	16.72 ± 0.67 ^b

Data are presented as the mean ± SE. STZ: diabetic group, vit. D: vitamin D, CAT: catalase, SOD: superoxide dismutase, MDA malondialdehyde, GRx: glutathione reductase and GST: glutathione-s-transferase. Letters carrying different symbols are significant and assigned alphabetically. The values with the same letters are not significantly different, and the data with different letters are significantly different.

RT-PCR analysis was used to elucidate whether the inhibitory effects of either stem cell and/or vit. D on inflammation were due to the regulation of mediator genes such as IL-1 β , IL-6, and TNF- α (Tables 5 and 6). The expression of a range of genes was examined (Table 5). The relative mRNA expression of IL-1 β , IL-6, and TNF- α was elevated in STZ-treated rats as compared to those of the control group. In the DC group treated with stem cells + vit. D, the relative levels of mRNA expression of IL-1 β , IL-6, and TNF- α significantly declined as compared to the STZ group (Table 6).

Table 5. Primer sequences for PCR amplification.

Gene	Primer Sequence	Accession No.	Length (bp)
IL-6	Forward: 5'-TCCTACCCCAACTTCCAATGCTC-3'	E02522	79 (Marek et al., 2010)
	Reverse: 5'-TTGGATGGTCTTGGTCCTTAGCC-3'		
TNF- α	Forward: 5AAATGGGCTCCCTCTCATCAGTTC-3'	X66539	111 (Marek et al., 2010)
	Reverse: 5'-TCTGCTTGGTGGTTTGCTACGAC-3'		
Il-1 β	Forward: 5'-CACCTCTCAAGCAGAGCACAG-3'	M98820	79 (Marek et al., 2010)
	Reverse: 5'-GGGTTCCATGGTGAAGTCAAC-3'		

Table 6. Effects of either stem cell and/or vitamin D on inflammation were due to the gene regulation of inflammatory mediators.

Groups	IL-6 mRNA	TNF- α mRNA	Il-1 β mRNA
Control group	0.45 ^a	0.54 ^a	0.35 ^a
STZ group	5.5 ^d	6.54 ^d	8.02 ^d
STZ + stem cell	1.85 ^{b,c}	2.14 ^{b,c}	4.25 ^{b,c}
STZ + Vit. D	1.97 ^c	2.31 ^c	4.54 ^c
STZ + stem cell + Vit. D	0.64 ^a	0.77 ^a	0.62 ^a

Data are presented as the mean ± SE. STZ: diabetic group, vit. D: vitamin D. Letters carrying different symbols are significant and assigned alphabetically. The values with the same letters are not significantly different, and the data with different letters are significantly different.

Normal parenchyma and islets of Langerhans are presented in Figure 4A. The STZ-treated group showed detached pancreatic parenchyma with reduced and disintegrated islets of Langerhans with irregular shapes (Figure 4B). The STZ + stem cell group showed

high restoration of detached pancreatic parenchyma with moderate-sized islets of Langerhans (Figure 4C). Treatment of the diabetic group with vit. D caused improvement in pancreatic parenchyma with moderately irregular islets of Langerhans (Figure 4D). Diabetic rats treated with both stem cells and vit. D for one month showed intact pancreatic parenchyma with an enlarged size of the islets of Langerhans compared with other groups treated separately (Figure 4E) (H & E, $\times 400$).

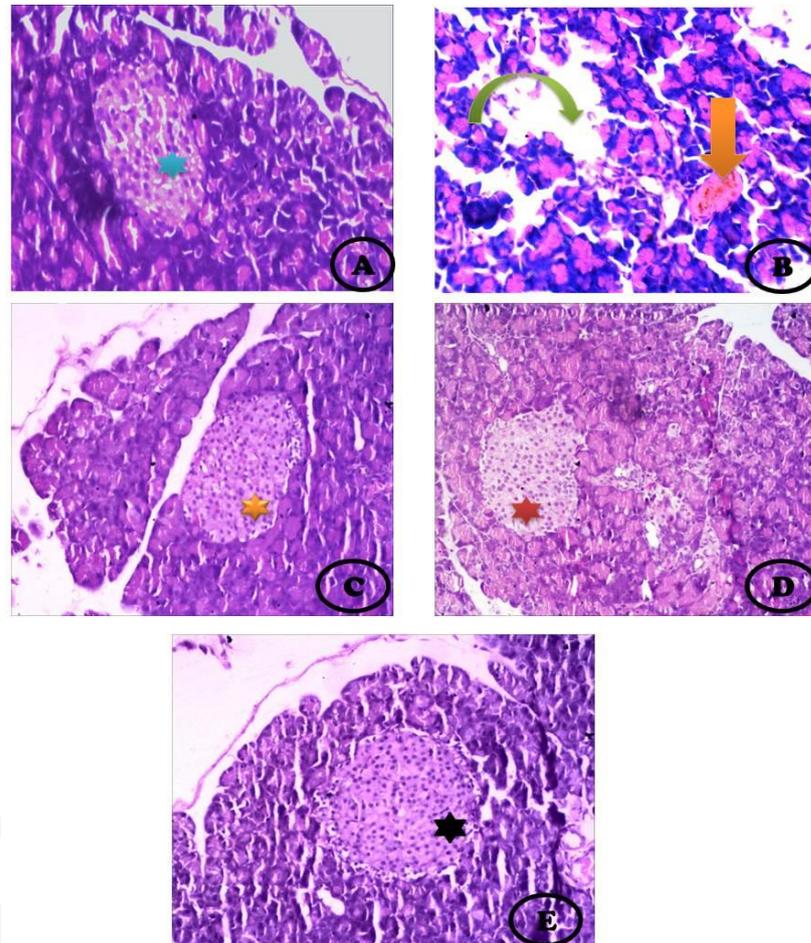


Figure 4. Photomicrograph of the pancreas showing (A) Normal pancreatic parenchyma and normal appearance of islets of Langerhans (blue star), acinar cells stain blue at their base due to the presence of a high content of RNA and nuclei, and their apex (luminal aspect) stains pink where there is a high content of digestive enzymes, (B) STZ-treated group showing large detached pancreatic parenchyma (Inverted green arrow) with very reduced and disintegrated islets of Langerhans (orange arrow), (C) STZ + stem cell group showing high restoration of detached pancreatic parenchyma with moderate-sized islets of Langerhans (orange star), (D) STZ + vit. D showing normal pancreatic parenchyma with moderately irregular islets of Langerhans (red star), (E) STZ + stem cells+ vit. D group showing intact pancreatic parenchyma with enlarged islets of Langerhans (black star) compared with the other groups treated separately (H&E, $\times 400$).

Figure 5 presents the comet images of cells originating from the pancreatic tissues. The control group had intact nuclei and round cells without a hollow tail (Figure 5A). DC showed a high degree of damage with the appearance of more than three apoptotic cells with a large hollow tail, and comet-shaped small head (Figure 5B). When the diabetic rats received a single i.v. injection of stem cells, intact cells appeared but with some apoptotic hollow areas (Figure 5C). The diabetic animals treated with vit. D contained intact cells with the tail appearing as a hollow area (Figure 5D). Combination treatment of the diabetic

rats with stem cell and vit. D showed more intact cells with fewer damaged DNA strands and improved altered nuclei (Figure 5E).

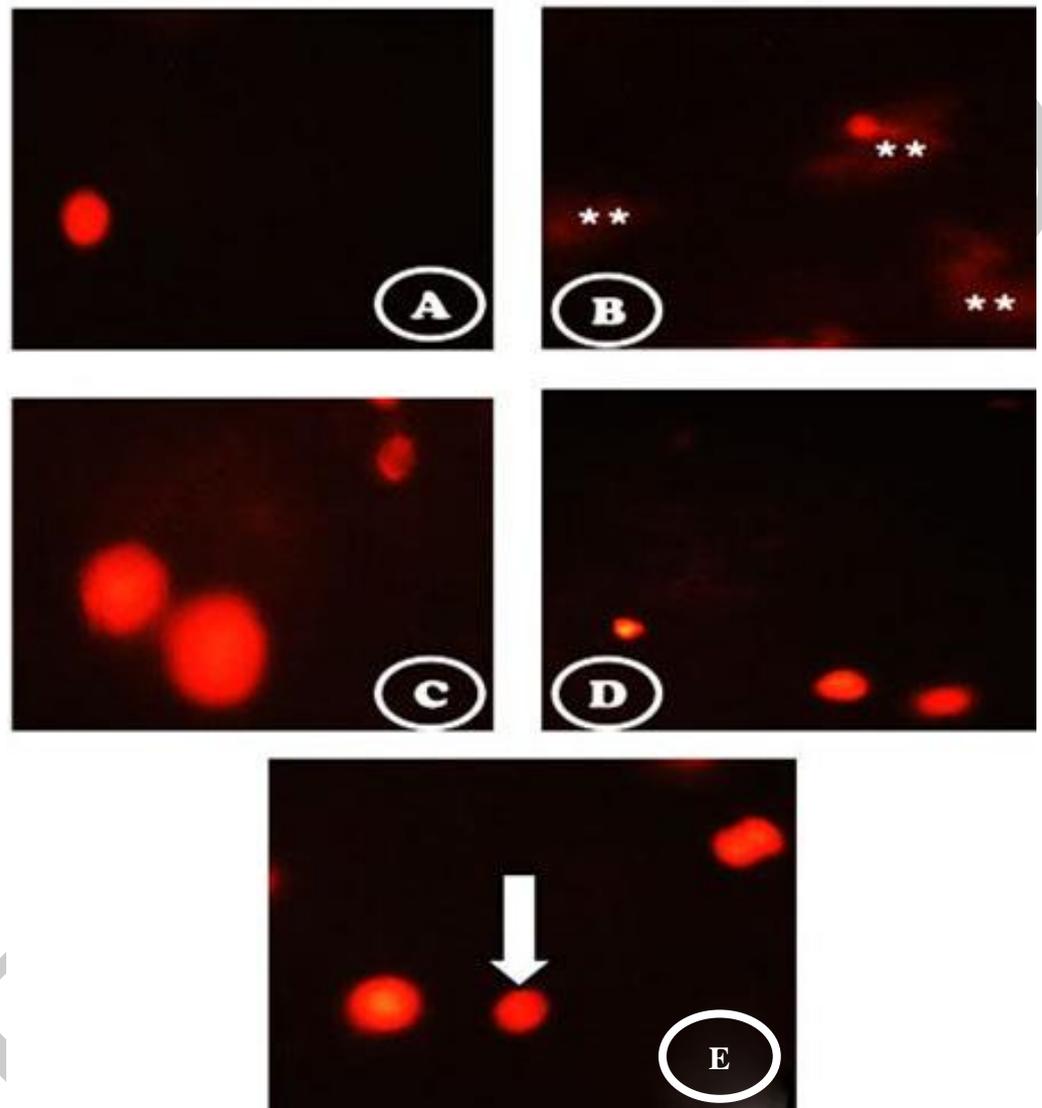


Figure 5. Comet images of cells derived from the pancreatic tissues (A) the control group showed intact nuclei and normal round cells without a hollow tail. (B) the STZ group showed a high degree of damage with the appearance of more than three apoptotic cells (white asterisks) with a large hollow tail and small head in the form of a comet shaped. (C) the STZ + stem cell group showed intact cells but with some apoptotic hollow areas. (D) the STZ + vit. D group contained intact cells with tails appearing as a hollow area. (E) the STZ + stem cell + vit. D group showed more intact cells with fewer damaged DNA strands and less damaged nuclei (white arrow).

Figure 6 presents an electron micrograph of β -cells of the pancreatic tissues. The control group showed normal β -cells with normal multi euchromatic rounded nuclei and normal-sized β -granules (Figure 6A). Fibrotic pancreatic parenchyma with a reduced nucleus was detected in the STZ-treated group. In addition, few beta granules with the appearance of some empty granules in the STZ-treated group (Figure 6B) were observed. The diabetic rats treated with stem cells showed restoration of pancreatic tissue with many moderate, dark, dense β -granules and the appearance of a normal β -granular area as well as a normal euchromatic nucleus with vacuoles (Figure 6C). When the diabetic animals were treated with vit. D, some restoration of pancreatic tissue with many small-sized β -granules was observed in the euchromatic nucleus (Figure 6D). The combined

treatment of Dc rats with stem cells and vit. D showed restoration of almost the entire pancreatic parenchyma with the appearance of many enlarged β -granules. In addition, some moderate-sized β granules, vacuoles, and enlarged Bi-euchromatic nuclei appeared (Figure 6E).

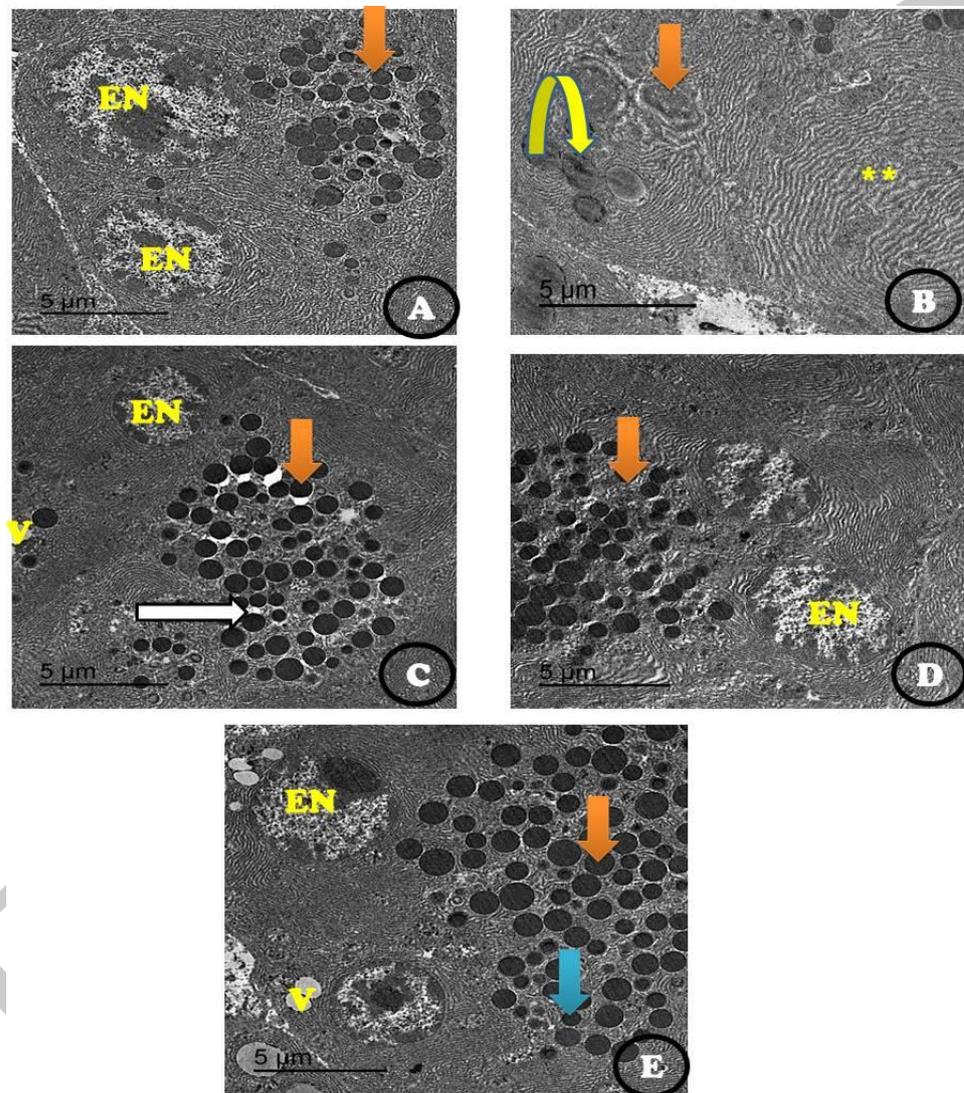


Figure 6. An electron micrograph of β -cells of the pancreatic tissues showing (A) the control group with normal β -cells (orange arrow) and many normal euchromatic rounded nuclei (EN), as well as normal-sized β -granules. (B) STZ treated group showing fibrotic pancreatic parenchyma (yellow star) with a reduced nucleus (orange arrow) with very few beta granules with the appearance of some empty granules (inverted yellow arrow). (C) STZ + stem cell group showing the restoration of almost all pancreatic parenchyma with the appearance of many moderate, dark, dense β -granules (orange arrow) and mild β -granular areas (white arrow) as well as a normal euchromatic nucleus (N) and some vacuoles (V). (D) STZ + vit. D group showing some restoration of pancreatic parenchyma with the appearance of many moderate- to small-sized β -granules (orange arrow) and a euchromatic nucleus (EN). (E) STZ + stem cell + vit. D group showing the restoration of almost all pancreatic parenchyma with many enlarged β -granules (orange arrow) and some moderately sized β granules (blue arrow) as well as the appearance of some vacuoles (V) with an enlarged Bi-euchromatic nucleus (EN) (Scale bar = 5 μ m).

4. Discussion

DM is characterized by increasing the glucose level that results from defects in insulin secretion or action or both [29]. This study used MSCs and vit. D in the treatment of diabetic rats induced by STZ. We tried to identify the pros and cons of each treatment separately and in combination. A novelty of the current study is using both treatments against diabetic pancreatic complications.

The diabetic animals suffered from the elevation of Hb A 1c, glucose levels, and the lipid profile (LDL-C, TC, and TG) except for HDL-c, which decreased. In addition, insulin and c-peptide levels were markedly decreased. Moreover, pancreatic levels of MPO, XO, and MDA were elevated, and all the measured antioxidants (CAT, SOD, GRx, and GST) decreased significantly. Hyperglycemia leads to oxidative stress damage that further accelerates the development of diabetes. The relative expression of mRNA of IL-1 β , IL-6, and TNF- α was elevated in STZ-treated rats as compared to those of the control group. Histopathological and DNA analyses revealed cytoplasmic vacuolation. Decreases in the number and size of the islets of Langerhans as well as apoptotic cells with large hollow tails and a small head in the form a comet shape were observed.

Streptozotocin selectively destroys β -cells in the islets of Langerhans in the pancreas, resulting in inhibition of insulin synthesis and blood glucose elevation. These effects are primarily due to reduced glucose entry into peripheral tissue, adipose tissue, and muscle as well as increased glycogen breakdown, gluconeogenesis, and glucose production by the liver [30]. There were also degenerative morphological alterations and shrinking of pancreatic tissue islets through several mechanisms, including the production of reactive oxygen species, activation of the pancreatic nuclear factor-kB, and induction of immune responses and inflammation. The current data showed that the lipid profile levels were changed by STZ except for the HDL-c, which substantially decreased the C-peptide level. These findings coincide with previously published results [31].

In the current study, treatment with vit. D or MSCs alone or in combination revealed a significant improvement in all the above-mentioned parameters as compared to the diabetic group. Moreover, the combined treatment was better than each one alone after four weeks.

All the lipid profile (TG, TC, LDL-c, and VLDL-c) levels decreased after treatment with MSCs as compared to the diabetic group, by 60.7%, 55.8%, 45.0%, and 23.9%, respectively. This decrease due to the effect of MSCs could be due to increasing insulin that activated lipoprotein lipase [32]. However, vit. D decreased the TG, TC, LDL-c, and VLDL-c levels by 53.2%, 45.3%, 42.9%, and 10.6%, respectively. The combination of MSCs and vit. D in the treatment of hyperglycemic rats decreased the TG, TC, LDL-c, and VLDL-c levels by 69.9%, 68.7%, 60.2%, and 36.8%, respectively; therefore, vit. D acts as a synergistic factor, improving the performance of MSCs to decrease the lipid profile. Decreasing adipogenesis and increasing LDL receptor expression could be one of the mechanisms to decrease the TG that elevated the lipoprotein lipase activity [33]. It was suggested that several mechanisms can explain the effect of calcium on lipids, including its reduction role in fatty acid absorption through the formation of insoluble calcium–fatty complexes in the gut. It is expected that the serum levels of total and LDL cholesterol are reduced by decreased fat absorption, especially saturated fatty acids [34]. In addition, because of its ability to bind with bile acids, calcium can increase the conversion of cholesterol into bile acids [35]. The effect of enteric calcium on lipid absorption is limited and had no significant effect on lipid profiles [36]. Our study confirms the possible ameliorative effect of vit. D on serum lipids levels. In the present investigation, the lipid profile pattern was optimized for the treatment of diabetic rats with MSCs. This result is in agreement with Ahmed et al. [37], who explained how MSCs improved the lipid profile due to improved β cell function and decreased insulin resistance.

In the present experiment, the treatment with vit. D resulted in a significant reduction in glucose levels. The decrease in HbA1c was statistically significant. Furthermore, a significant increase was observed in the level of insulin. These data are in parallel with

previous studies indicating various potential mechanisms to explain vit. D's role in the control of glucose levels. Vit. D deficiency causes glucose impairment by increasing insulin resistance, which in turn reduces the adipose expression of PPAR- γ and decreases the mass of β -cells, impairing their role [38]. Jayanarayanan et al. [39] indicated the importance of vit. D in diabetes treatment by controlling glutamatergic activity.

Some studies suggest oral ingestion for older mice as vit. D increases the metabolism of glucose as an enhancer of GLP-1 [40] and improves the insulin growth factor-1 (IGF-I) level [15].

Another important finding of the present study was the significant decrease in the expression levels of IL-6 mRNA, TNF- α mRNA, and IL-1 β mRNA in diabetic rats following treatment with vit. D as compared to diabetic animals. Therefore, we hypothesized that vit. D has effects on diabetes-induced pancreas complications, possibly by down regulating the expression of IL-6 mRNA, TNF- α mRNA, and IL-1 β mRNA. This could be due to the anti-inflammatory properties of vit. D because some authors suggested an association between a chronic inflammatory state and impaired insulin activity through stimulation of the expression of insulin receptors on β cells [9,41]. The secondary role of vit. D is the regulation of extracellular calcium and the maintenance of body calcium inflow to β cells [42]. In the current study, vit. D could improve the complications of diabetes not only by enhancing sensitivity to insulin and C-peptide, but also by suppressing pancreatic gene expression of IL-6 mRNA, TNF- α mRNA, and IL-1 β mRNA as well as oxidative stress.

MSCs were capable of lowering blood glucose levels and increasing the insulin level compared with the STZ-diabetic non-treated rats. The histopathological and electron transmission results confirmed that MSCs decreased the degenerative alteration in the pancreatic β -cell islets. We explain MSCs' ability along with vit. D, assessing their role in the immune system's modulation.

MSCs can differentiate into islet-like insulin-producing cells (IPCs), endorse the restoration of beta cells in the pancreatic islets, and protect endogenous beta cells against oxidative stress through immunotherapeutic mechanisms. These data are in agreement with those presented by other authors [43–45]. Frequently, MSCs help to regenerate endogenous beta-cells in the pancreatic islets by secreting specific cytokines and growth factors. Si et al. [46] discovered that in a diabetic rat model, the infusion of MSCs resulted in significant endogenous beta-cell regeneration. In addition, MSC infusion significantly enhanced insulin sensitivity as evidenced by phosphorylated insulin receptor substratum 1 (IRS-1), protein kinase B (Akt), and insulin target tissue GLUT4 [46]. Lee et al. [47] also revealed an improvement in regenerated mouse pancreatic islet beta cells that produced murine insulin following the transfer of BM-MSCs into diabetic mice.

The protection of extracellular pancreatic islet cells was improved by the antioxidant and anti-inflammatory activity effects of MSCs. In addition, they have the capability to reduce oxidative stress by decreasing the serum levels of MPO and XO, as well as pancreatic lipid peroxidation. Moreover, they increased the antioxidant effect by increasing SOD, CAT, GPx, and GST as compared to the diabetic hyperglycemic group. However, the elevation of C-peptide after MSCs treatment indicated that the pancreatic beta cell performance was enhanced.

MSCs' immunoregulatory features are of medical importance, showing the effectiveness of stem cell transplants for the treatment of Type 2 diabetes mellitus; see Refat et al. [48]. All patients showed a significant decrease in daily insulin requirements and HbA1c, as well as a significant increase in fasting C-peptide levels.

C-Peptide is formed in pancreatic beta-cells and is secreted into the circulatory system. C-peptide has long been considered important in insulin biosynthesis and is reported to possess relatively low biological activity [49]. In addition, it is an excellent marker for determining the activity of pancreatic β -cells. It has a normal ratio with insulin secretion, longer half-life, and marginal hepatic clearance. Some scientists recommended levels of C-Peptide to levels of insulin when detecting changes in insulin secretion in β -cells.

Vit. D has a protective effect on the skeleton by acting on calcium homeostasis and bone formation. Additionally, vit. D has a direct potent effect on stem cells (MSCs) in stimulating their differentiation [50].

Several studies speculated on the role of vit. D in the differentiation of osteoblasts, and stem cells (MSCs) are known for their abilities in promoting bone repair and regeneration of cells [51].

There is not a lot of data in the literature about vit. D and cell adhesion, but it is well known that interactions between cells and surfaces are involved in the activation of many signals that in turn are responsible for cell commitment and differentiation. The current study indicated that vit. D administration in combination with stem cells (MSCs) increased the insulin hormone level and lowered the blood glucose level.

In the current data, the appearance of edema was one of the main signs of inflammation, as mentioned before [52]. The increase in vascular permeability following an inflammatory stimulus is the primary mechanism for edema formation and depends on inflammatory mediator development and/or release. This inflammatory response can be initiated quickly by mast cells. Once activated, mast cells de-granulate to release histamine and other mediators.

In the present work, these ameliorative effects of vit. D and/or MSCs were confirmed by the improvement in pancreatic architecture, especially their combined effect.

In conclusion, the antioxidative and anti-inflammatory capacity of MSCs could promote pancreatic islet cell survival and thus prevent or decrease the deterioration that results from type II diabetes mellitus. The combination of intramuscular vit. D and i.v. of MSCs appear to have contributed to the improvement in the pancreatic complication of diabetic rats compared with an injection of MSCs or vit. D alone.

Author Contributions: Conceptualization, R.Z.H.; Formal analysis, R.Z.H.; Investigation, R.Z.H.; Methodology, R.Z.H.; Resources, R.Z.H., R.A.A.-E., and N.S.E.-S.; Software, R.A.A.-E. and N.S.E.-S.; Supervision, R.Z.H.; Validation, R.A.A.-E.; Visualization, R.Z.H., R.A.A.-E. and N.S.E.-S.; Writing—original draft, R.Z.H.; Writing—review & editing, R.Z.H. and N.S.E.-S. All authors have read and agreed to the published version of the manuscript.

Funding: Taif University Researcher supporting project number (TURSP-2020/21), Taif University, Taif, Saudi Arabia.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethical committee, Deanship of scientific research, Taif University (approval number: 40-31-0189).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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