Research article

Impact of insulin producing cells derived from adipose tissue mesenchymal stem cells on testicular dysfunction of diabetic rats

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ARTICLE INFO	ABSTRACT
	The present study is to clarify the effect of insulin-producing cells (IPCs) derived from adipose tissue mesenchymal stem cells (AT-MSCs) on diabetic-induced impairments as the abnormalities of testicular tissues, oxidative stress of testes, and defects of spermatogenesis. Diabetes was stimulated by streptozotocin (STZ) injection in male adult Sprague Dawley (SD) rats. Diabetes was confirmed by taking two highly consecutive fasting blood sugar readings; more than 300 mg/dl; within one week. Five million of IPCs derived from AT-MSCs; encased in TheraCyte capsule; were then directly transplanted (one implant for each rat) subcutaneously in diabetic rats. Implants were maintained for 3 months and the fasting blood sugar of the transplanted rats was observed every month. At the end of the experiment; serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were also estimated. The sperm parameters (count, motility, and abnormality) were recorded. In testicular tissue; GPX4, Bcl2, and Bax levels were evaluated, while oxidative stress and antioxidant enzymes activities were measured in the testes homogenate. Also, histopathological alterations were examined in the testes cross-section. In the results, it was found that IPCs treatment enhanced the serum testosterone, FSH, and LH levels. Diabetic-induced impairments in the sperm parameters were noticeably improved post-IPCs transplantation in the diabetic rats. Moreover, the treatment improved the diabetic-associated testicular oxidative stress. Also, it was recognized that the Bax expression decreased, while, GPX4 and Bcl2 expression increased in the treated rats. Meanwhile, the abnormalities showed in the histopathological studies of the hyperglycemic rat's testes were attenuated post-treatment. So, IPCs transplantation improved diabetes and consequently protected against hyperglycemia-induced testicular damages.

1. Introduction

Diabetes mellitus (DM) is a chronic subversive metabolic ailment affecting humans that is caused by problems in insulin secretion and action, or both [1]. This ailment triggers systemic complications including cardiovascular diseases, nephropathies, and neuropathies [2].

Derangement of pituitary testicular axis and serum testosterone impairment have been reported as a result of the hyperglycemic impact of diabetes [3]. The attenuation in the sperm count, augmentation in the sperm mortality, and variation in sexual gonads morphology have also been exhibited in STZ-chemically induced diabetic rats [4].

Recently, the cell population most commonly studied in clinical trials is mesenchymal stem/stromal cells (MSCs) as they showed immunomodulatory properties. Also, studies have validated that MSCs; from various tissues like Wharton's jelly within umbilical cords, bone marrow, and adipose tissue; have the ability to undergo differentiation into insulin-like producing cells [5]. The potential utility of these cells for *in vivo* transplantation therapy of diabetes has been emphasized by the *in vitro* studies [6].

As reported in a previous study [7], Adipose tissue mesenchymal stem cells (AT MSCs) possess advantages as these cells are easily and safely collected with minimal invasiveness and low morbidity. Moreover, large amounts of AT MSCs can be obtained if it compared to bone marrow

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mesenchymal stem cells; 1 g of adipose tissue yields 5000 stem cells/ml, whereas the yield from bone marrow is about 1000 cells/ml.

TheraCyte device might be a suitable option for cell transplantation as their outer membrane allowed the neovascularization whereas their inner membrane; which contained the engrafted cells; was cell-impermeable with 0.4μm pore size. This configuration prevented host immune cells from being in contact with the transplanted cells. Furthermore, its subcutaneous location facilitated the cell transplantation and retrieval if necessary, as well as resulted in a vast surface area for transplantation [8].

The objective of this study was to elucidate that insulin-producing cells (IPCs) could be developed from the differentiation of AT MSCs, clarified the ability of IPCs transplantation to ameliorate DM and subsequently attenuated the testicular dysfunction of diabetic rats chemically induced by STZ.

2. Materials and methods

2.1. Animals

The Urology and Nephrology Center's Animal Research Facility in Mansoura University had provided the experimental animals which were placed in individual cages; kept to a 12/12 h light/dark cycle; offered full diet and water as much as needed. The experiment was achieved in conformity with the guidance of the institutional animal care and use committee (IACUC).

2.2. AT-MSCs isolation and expansion

Adipose tissue mesenchymal stem cells were segregated from the fatty tissue above the testes of adult SD rats; according to the procedures previously described [7]. In brief, adipose tissue was minced by using scissors into very minute parts, digested at 37 °C for 30 min with gentle stirring by using 0.0075g type- I collagenase (Sigma-Aldrich, St. Louis, Missouri, USA); dissolved in 10ml hanks solution (Sigma-Aldrich); followed by inactivation using an equivalent volume of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich); supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT, USA); and centrifuged for 10 min at 1800 rpm. After that, the cells were re-suspended by using 10% complete media (DMEM/10 % FBS) and a 100 μm nylon mesh filter was used for the removal of any debris.

The isolated cells at density 1×10^6 cells/ml were cultured in a flask specific for cell culture (1×25 cm^2) coated with gelatin solution 0.1 % (STEMCELL Technologies, Vancouver, Canada).After the feeding of the cultured cells was performed with DMEM/10% FBS; containing 100 U/mL Penicillin-Streptomycin solution (Sigma-Aldrich) and 1 ml/L 100X Glutamax (Gibco, USA), the cultured cells were placed at 37 °C in an incubator with 5% CO$_2$. Discarding the non-adherent groups was executed after 3 days from the culture of the cells. The adherent MSCs; with spindle-shaped morphology; were fed every 3 days with fresh medium. Detaching of cells was carried out when reaching 80 % confluence by using Trypsin-EDTA, 0.25 % (Sigma-Aldrich); supplied with 1mMol ethylene diamine tetra-acetic acid (EDTA, Sigma-Aldrich). This process was repeated 3 times.

2.3. Rat AT-MSCs phenotyping by flow cytometric analysis

At passage 3, AT-MSCs were collected by trypsin-EDTA solution, centrifuged at 1200 rpm for 10 min at 4 °C, and resuspended in phosphate buffer saline (PBS) at 1×10^6 cells/ml density. The cells were stained with CD14, CD45 fluorescein isothiocyanate (FITC) or CD34, CD29 phycoerythrin (PE, Becton, Dickinson, USA), CD73 PE, and CD90 FITC (BD Biosciences, Tokyo, Japan) specific antibodies for 1 h in dark, then 2ml staining buffer (BD-Pharmingen, USA) was used to wash the cells. Finally, the cells were resuspended in staining buffer (500 μl) to be analyzed. An argon ion laser of FACS Callibur (Becton, Dickinson, USA)

at 488 nm wavelength was used to analyze the stained cells. Events (1×10^4) were analyzed by using Cell Quest software (BD biosciences, USA). The appropriate isotype that matched monoclonal antibodies was used as a control for staining [9].

2.4. AT-MSCs differentiation into IPCs

The 10-day differentiation protocol [10] was executed for the differentiation of the cells. Firstly, serum-free media DMEM low glucose; supplied with 55 nMol Trichostatin-A (TSA); was used in the culture of the cells for 3 days. For extra 7 days, the cells were cultured in a 1:1 DMEM: DMEM F12 medium of high glucose (25 mMol); supplied with FBS (10%), 10 nMol glucagon-like-peptide-1 (GLP-1), and 10 nMol Nicotinamide. The media and growth factors used in the differentiation protocol were purchased from Sigma-Aldrich.

2.5. Differentiated AT-MSC's characterization

2.5.1. Immunofluorescence

The undifferentiated and the fully differentiated rat AT-MSCs were cultured on two chambers-slide (Nunc, Rochester, NY, USA). Initially, fixation of the cells was occurred by using 4% paraformaldehyde for 10 min, followed by permeabilization of the cells that occurred with 100% methanol for 10 min. Blocking of the cells by using 5% goat serum was then performed for 1 h at room temperature (RT). Afterward, the cells were overnight incubated at 4 °C with the primary antibodies [mouse monoclonal anti-rat insulin Antibody 1/200 (Novus Biologicals, Littleton, CO, USA) and rabbit polyclonal anti-rat c-peptide antibody 1/100 (Cell Signaling Technology)]. The cells were kept for 2 h at room temperature with the secondary antibodies [1/200 anti-mouse immunoglobulin G (IgG; HL) Alexa Fluor 488 conjugated antibody and 1/100 anti-rabbit (IgG; HL) Alexa Fluor 555 conjugated antibody (Cell Signaling Technology)] after being washed with PBS. The stain [40, 6-diamidino-2-phenylindole (DAPI, Invitrogen, UK)] was used to counterstain the nuclei. No primary antibodies were utilized for the negative controls. The confocal images were taken finally by the Leica TCS SP8 microscope (Leica Microsystems, Mannheim, Germany).

2.5.2. In vitro insulin and C-peptide release determination in response to exceeding glucose concentrations

Insulin and c-peptide release of the differentiated cells were performed by culturing the differentiated rat AT-MSCs 3 times in a 12-well plate at a cell density of 100, 000 cells per well. Cells firstly were kept in an incubator at 37 °C for 30 min in the buffer of glucose-free Krebs Ringer bicarbonate (KRB) to eliminate any excess insulin. Following that, incubation for 1 h in KRB containing 5.5, 12, and 25 mM glucose concentrations was performed. After the incubation, the supernatant was sampled and assayed by using an ELISA Kit specific for rat insulin and c-peptide (Bioseps, Chongqing, China). Determination of the sample's protein concentration was occurred by using an ELISA plate analyzer instrument (apDia, Belgium, Europe) according to the instructions of the manufacturer. Protein concentrations had been represented as ng/μg protein/hour.

2.6. Experimental design

Thirty adult male SD rats 12 weeks old with weight 250–350 g were randomized into 3 groups. Each group was composed of 10 rats: (i) normal (control), (ii) diabetic (diabetic control) and (iii) diabetic treated by IPCs transplantation (treated group). An intraperitoneal single injection of STZ (Sigma-Aldrich) at a dose of 45 mg/kg body weight; after overnight fasting; was used to induce diabetes [11]. The blood glucose readings above 300 mg/dl were recorded two times consecutively to make sure that the rats were diabetic. Five million of the differentiated cells were equally divided (2.5×10^6 differentiated cells/Capsule) and encapsulated inside 2 capsules (TheraCyte TM capsule, Irvine, CA, USA)

using a Hamilton syringe (2 capsules/rat). After one week from STZ injection; transplantation (one implant for each rat) of 10 rats (treated group) was performed subcutaneously. The transplantation procedures were carried out while the rats were anesthetized.

2.7. Animal sacrifice and sample collection

After 3 months from transplantation; at the end of the observation period; the rats were first weighed, blood samples were collected through the cardiac puncture, and then were sacrificed. The cell-bearing devices were removed and the reproductive organs were revealed by opening the abdominal cavity through a midline abdominal incision. The rat's testes and epididymides were carefully collected and weighed independently. Epididymides were collected for semen analysis. Testes were trimmed free of the adjoining tissues. One of the testes was divided into 2 parts (one part was stored in −120 °C liquid Nitrogen for molecular study and the other part was homogenized for oxidative stress evaluation), while, the other testis was fixed for histological analysis by using 10% formalin. The separated serum was stored at – 20 °C for biochemical assays. Their pancreata were also collected for immunolabeling studies.

2.8. Testes homogenate preparation

The testes were rinsed with 0.9 % normal saline (Sigma- Aldrich, Canada) before the testicular capsules were removed to obtain the seminiferous tubules. The testes were first weighed, then chopped up using a scalpel before being homogenized in a PBS solution with a pH of 7.8. After centrifugation at 3000 rpm, the supernatant was extracted and preserved at -80 °C. The entire procedure was performed in a cold environment. The testes homogenates were used to evaluate the oxidative stress.

2.9. Biochemical analysis

2.9.1. Quantification of serum testosterone, FSH and LH levels
The hormonal assay for rat testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in serum was quantitatively measured by ELISA assay (CUSABIO, Wuhan, China) using a specific kit according to the manufacturer's recommendations [12].

2.9.2. Quantification of TBARS levels in testis
Thiobarbituric acid reactive substances (TBARS) levels were evaluated to measure the lipid peroxidation products; malondialdehyde (MDA) in the tissue of testes according to the method described before by Ohkawa et al. (1979) [13] using a biochemical assay kit (Bio-diagnostic, Giza, Egypt). In Brief, testicular tissue homogenate (0.2 ml) was mixed with 1 ml of chromogen (supplied by the kit) and exposed to heat (95 °C) for 30 min. After being cooled, the absorbance of the supernatant was read against blank and standard against distilled water using Perkin Elmer Lambda UV/VIS spectrophotometer (Beckman, USA) at 534 nm.

2.9.3. GSH levels quantification in testis
Glutathione reduced (GSH) levels in the testes were evaluated according to the method described before by Beutler (1963) [14] using a biochemical assay kit (Bio-diagnostic). Briefly, 0.5 ml of testicular tissue homogenate was mingled with 0.5 ml Trichloroacetic acid (TCA) and left for 5 min at room temperature (RT), followed by centrifugation for 15 min at 3000 rpm. 0.5 ml of the supernatant, 1 ml of buffer (provided by the kit), and 0.1 ml of [5, 5′-dithiobis- (2-nitro- benzoic acid)] (DTNB) were mixed with each other. The absorbance of the clear supernatants was measured using a Perkin Elmer Lambda UV/VIS spectrophotometer at 405 nm to determine the activity of GSH. The absorbance of the sample was read against the blank.

2.9.4. CAT activities quantification in testis
The activity of the testicular catalase (CAT) was evaluated according to the method previously explained by Aebi (1984) [15] using a biochemical assay kit (Bio-diagnostic). In Brief, 0.05 ml of the homogenized rat testes was mingled with 0.1 ml H_2O_2 and 0.50 ml PBS at pH 7.0. The mix was kept in the incubator (25 °C) for exactly 1 min. afterward, 0.2 ml of chromogen-inhibitor was added and 0.5 ml Enzyme: peroxidase 4-Aminoantipyrine preservative was also added. After being incubated for 10 min at 37 °C, the sample was read against sample blank, while, standard against standard blank using Perkin Elmer Lambda UV/VIS spectrophotometer at 510 nm.

2.10. Semen analysis

After the euthanasia of rats, an open castration method was used to perform the orchiectomy. The tunica vaginalis was incised to reveal the testicle and the cauda epididymis was collected. Caudal epididymis was minced by using scissors into parts. Afterward, the sheared caudal parts were kept in 2 ml of warm 0.9% saline for a few minutes to obtain sperm suspension and were incubated for 5 min at 37 °C with 5% CO_2. The semen was then collected by using a 1ml pipette, inserted on a clean slide, and covered with coverslips. The improved Neubauer hemocytometer (Deep1/10mm LABART, Germany) counting chamber was used to examine slides under the light microscope at 400x magnification for evaluating the sperm motility by analyzing the percentage of motile sperms from the total sperm count [16]. The spermatozoa were counted under the light microscope at 200x; as described by Pant and Srivastava (2003) [17]. Briefly, the sperm count was carried out in five Thoma chambers and the count was expressed as million/ml of suspension. The caudal sperm was taken from the original dilution for motility and diluted 1:20 with 0.9 % saline for evaluating the sperm morphology using a light microscope at 100 x; as previously reported by Akunna et al. (2013) [18]. The morphology of the sperms was determined by staining sperm smears on microscopic slides with eosin-nigrosin stain (Bio-diagnostic). In this study, a spermatozoon was considered with defective morphology if it had a rudimentary tail, round or detached head and the percentage of morphologically abnormal sperms was expressed as a percentage of the total sperm count [19].

2.11. Gene expression by real-time quantitative PCR

RNeasy Plus Mini Kit (Qiagen, Germany) was utilized to extract total RNA from tissues of testes. The concentration and purity of the extracted total RNA were evaluated by using the Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher, USA). The RT2 First Strand kit was used to form complementary DNA (cDNA) by using 1.5 μg of the extracted RNA as a template along with random primers; according to the manufacturer's recommendations (Qiagen Sciences, USA). The expression of the following genes; glutathione peroxidase 4 (GPX4), B-cell lymphoma 2 (Bcl-2), and Bcl-2 associated X protein (Bax) in testicular tissue of the treated group was compared to that of the diabetic group using real-time PCR. Amplifications were performed in a 20 μl reaction volume, which included 5 μl of cDNA template, 2μL of primers, 10 μl of 2x QuantiTect® SYBR® Green PCR (Qiagen Sciences, USA) master mix, and 3 μl of water nuclease-free. This technique was carried out using a Bio-Rad CFX96 thermal cycler (Bio-Rad, USA) with the following settings: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 20 s, extension at 72 °C for 20 s and final extension at 72 °C for 3 min. For real-time PCR analysis, each cDNA sample was run 3 times. Testicular tissue of the normal group was included to serve as a positive control, as well as, testicular tissue of the diabetic group was evaluated as a negative control. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the housekeeping gene that was used for mathematical calculations [20] (Table1).

2.12. Immunofluorescent and immunolabeling studies

The transplanted capsules were collected in 10 % formalin and treated according to the instructions of the manufacturer. Blocks were cut

Table 1. List of primers specific for Rat genes; Bax, GPX4, Bcl-2 and GAPDH for RT-qPCR.

Gene	Forward primer	Reverse primer	Accession number
BAX	ATCCACCAAGAAGCTGAGCG	TCCACATCAGCAATCATCCTCTG	NM_017059.2
GPX4	TTCCCCAGACCAGCAACAG	ACGCAACCCCTGTACTTATCC	NM_001039849.3
BCL-2	GAC TGA GTA CCT GAA CCG GC	AGT TCC ACA AAG GCA TCC CAG	NM_016993.1
GAPDH	TGCCACTCAGAAGACTGTGG	TGGTACATGACAAGGTGCGG	NM_017008.4

at 3 μm, taken and fixed on specific positively charged slides which were purchased from Citoglas (Citotest Labware Manufacturing Co., Haimen, China). Sections were firstly dewaxed in xylene, rehydrated in descending grades of alcohol (Ethanol anhydrous denatured, histological grade100% and amp; 95%), and finally embedded in paraffin. Microwave antigen retrieval was carried out in a 10mM sodium citrate buffer at pH 6.0 and the slide was kept for 10 min in a sub-boiling temperature. The non-specific reaction was blocked using 5% natural goat serum before being kept overnight at 4 °C with the primary antibodies previously mentioned. After that, the slide was rinsed 3 times by PBS and kept for 2 h at room temperature with the secondary antibody previously mentioned. DAPI was used to counterstain the nuclei.

The IPCs percentage inside the capsule was evaluated by using the Image J program 1.51h (developed by NIH). Ten fields for cell counting were randomly selected and applied by two independent histopathologists blinded for the clinical data. The previously mentioned work was carried out using Leica TCS SP8 microscope to capture the confocal digital images (Leica Microsystems, Mannheim, and German). The immunolabeling of the harvested native pancreata was done by using anti-insulin mouse monoclonal (primary antibody) and Power-Stain Version 1.0 Poly HRP DAB Kit for mouse (secondary antibody). The primary antibody used in immunolabeling was purchased from Novus Biologicals, while, the used secondary antibody was purchased from Genemed Biotechnologies.

2.13. Histopathological studies of the testicular tissue

With 10 % formaldehyde, testicular tissue samples were fixed. Fixed tissues were embedded in paraffin. Microtome sections of 3 μm thickness were taken from these blocks, and stained with Hematoxylin and eosin (H and E) stain. A light microscope was used to examine and photograph these stained sections. The images of all the stained preparations were taken by Olympus DP72 camera connected to Olympus BX-51 at a magnification of 200 x (Olympus, Tokyo, Japan) [21].

The microscopic examination was performed. The following variables were evaluated for the morphometric study of seminiferous tubules: invagination of the basement membrane, cellular necrosis, lost spermatogonia, detached spermatogonia, primary spermatogonial proliferation, giant cells in the lumen, and Sertoli cells vacuolization. The interstitial region was assessed for the following purposes: edema, thick capillary wall, and Leydig cell atrophy. Ten different fields were selected randomly from each testis section.

2.14. Statistical analysis

Data were represented as Mean ± S.E.M (standard error of means) statistically significant differences and analysis of variance were calculated using ANOVA test (SPSS for Windows, 16.0 version). Differences were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.001$.

3. Results

3.1. Distinctive traits of rat AT-MSCs

AT-MSCs; in case of culture; showed spindle-shaped morphology and adherent character to the culture flask's wall. At the expansion end, the cells expressed the rat AT-MSC's surface markers, CD29, CD90, and CD73, while, the cells neglected the hematopoietic stem cell's surface markers, CD34, CD45, and CD14 (Figure 1).

3.2. Practical estimation of the differentiated rat AT-MSCs

Immunofluorescence stain revealed that the undifferentiated rat AT MSCs were negatively stained for both insulin and c-peptide. While the practical quantification for the rat differentiated AT MSCs demonstrated that rat insulin and c-peptide were found in the fully differentiated cells. In addition to that, there was a co-expression for rat insulin and rat c-peptide in the same cells. The mean percentage of cells positively stained for insulin was 2% and cells positive for c-peptide was 2% as well (Figure 2). Furthermore, increasing the concentration of glucose resulted in a gradual exceed in the release of insulin and c-peptide (Figure 3).

3.3. In vivo transplantation results of rats

It was found that the profile of the fasting blood sugar (FBS) levels in the normal group ranged from 78 to 100 mg/dl. After 3 days from chemical induction of diabetes by STZ, the FBS levels of STZ-injected rats were 300 mg/dl or higher. Excessive urination and progressive weight loss occurred as a result of high blood glucose levels. FBS levels in the treated group began to improve within a month from transplantation. Throughout the three months of the observation period, the transplanted rats remained near the normoglycemia (Figure 4).

3.4. Serum levels of rat testosterone, FSH, and LH

Hyperglycemia lowered the serum testosterone levels highly significantly ($p < 0 .001$) compared to normal animals. IPCs transplantation in diabetic rats significantly ($p = 0.011$) augmented the levels of the serum testosterone compared to the diabetic animals but was highly significant ($p < 0.001$) lower than that of the normal group. There was a highly significant ($p < 0.001$) reduction in the FSH mean value of the diabetic, treated group compared to that of the normal group. However, there was a highly significant ($p < 0.001$) exceed in the FSH level of the treated group when compared with the diabetic group. In addition, the LH mean value of diabetic and treated groups was highly significant ($p < 0.001$) lower compared to that of the normal group. However, there was a significant increase ($p = 0.106$) in the mean value of LH levels of the treated group in comparison with that of the diabetic group as shown in Table 2.

3.5. Progression of oxidative impairments

From this study, the MDA level was highly significant greater in the testis homogenate of the diabetic group ($p < 0.001$); showing that there was an exceed in the protein oxidation and lipid peroxidation in the diabetic cases; when compared to that of the treated and normal group. The level of MDA was increased significantly ($p = 0.011$) in the testis of the treated group compared to that of the normal group. Meanwhile, endogenous antioxidants; which are GSH as well as CAT levels; were highly significant ($p < 0.001$) attenuated in the diabetic group when compared to that of the treated and normal groups. The level of GSH and CAT was highly significant ($p < 0.001$) decreased in the testis of the treated group compared to that of the normal group as shown in Table 2.
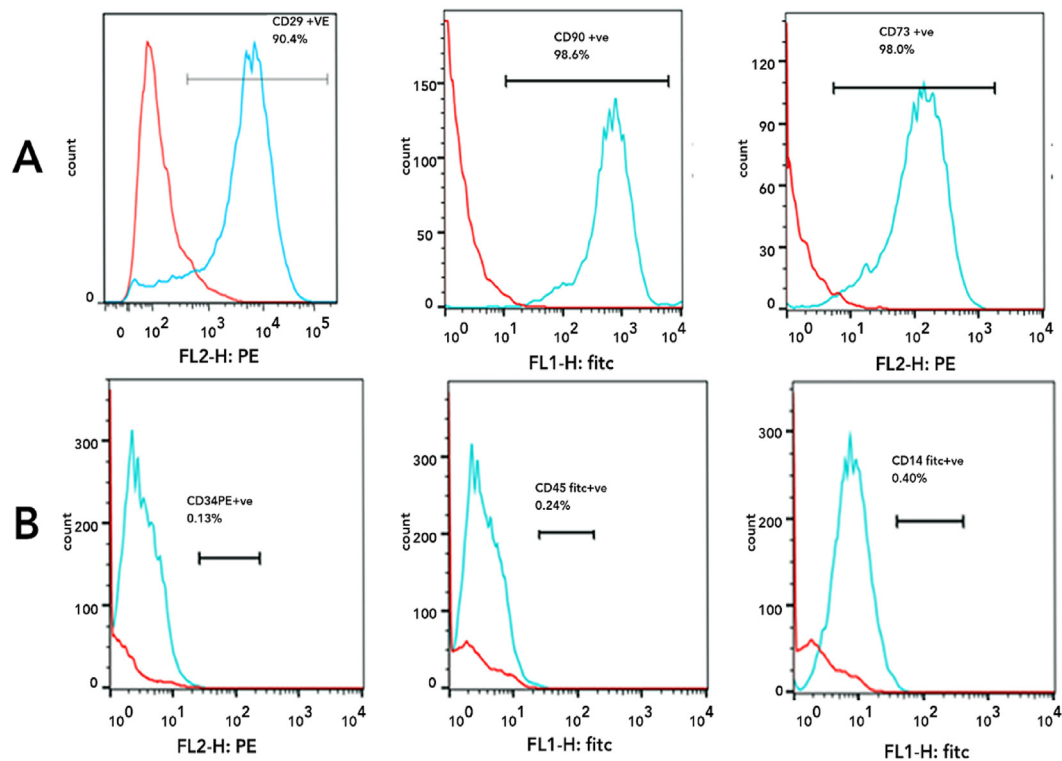
Figure 1. Surface staining flow cytometry for AT MSCs showed that the cells (blue) with highly expression of CD29, CD90 and CD73 (panel A), but, neglected the expression of CD34, CD45 and CD14 (panel B), when compared to their isotype control (red).
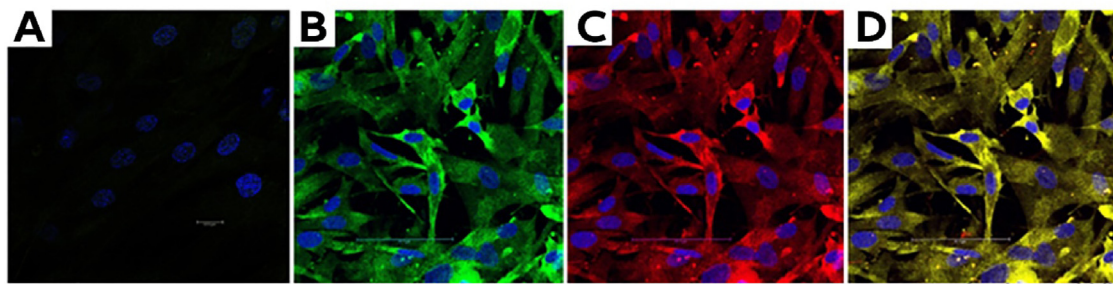

Figure 2. Immunocytochemistry of rat AT-MSCs. A) Undifferentiated rat AT MSCs negative for both of insulin and c-peptide. (B) Differentiated rat AT-MSCs positive for Insulin (green), (C) Differentiated rat AT-MSCs positive for c-peptide (red). (D) Differentiated rat AT-MSCs with Insulin and c-peptide co-expression in the same time (yellow). 40, 6-diamidino-2-phenylindole was used to counterstain the nuclei (blue).

3.6. Semen evaluation

There was a highly significant (p < 0.001) decrease in the mean value of the sperm count and the sperm motility in the diabetic group compared to that of the normal group, but, the abnormal sperm morphology mean value of the diabetic group showed a highly significant (p < 0.001) increase when compared to the mean value of the abnormal sperm morphology obtained from the normal group. It was found that the mean value of the sperm count exhibited a highly significant (p < 0.001) exceed in the IPCs treated group in comparison with that of the diabetic group, Motility increased significantly (p = 0.001) in the treated group compared to that of the diabetic group, meanwhile, abnormality highly significant (p < 0.001) decreased in the treated group compared to that of the diabetic group. In addition, the mean value of sperm count and motility in the treated group showed a highly significant reduction compared to that of the normal group, while, the abnormal sperm morphology mean value of the treated group showed a significant (p = 0.001) increase when compared to that of the normal group as shown in Table 2.

3.7. Relative gene expression by RT-qPCR

The results of the real-time quantitative PCR showed that the expression of Bax increased in the STZ-diabetic group compared to that of the normal group, meanwhile, showed a highly significant (P < 0.001) increase compared to that of the treated group. Contrary to expected, the Bax expression in the testis of the treated group was lower than that of the normal group. The Bcl-2 and GPx4 gene expression was down regulated in the testis of the diabetic group compared to that of the normal group. In contrast, IPCs treated group significantly increased the expression of both Bcl-2 (P < 0.01) and GPx4 (P < 0.05) genes compared to that of the STZ group. Also, it was recognized that the Bcl -2 and GPx4 genes expression in the testis of the treated group were slightly higher than that of the normal group (Figure 5).
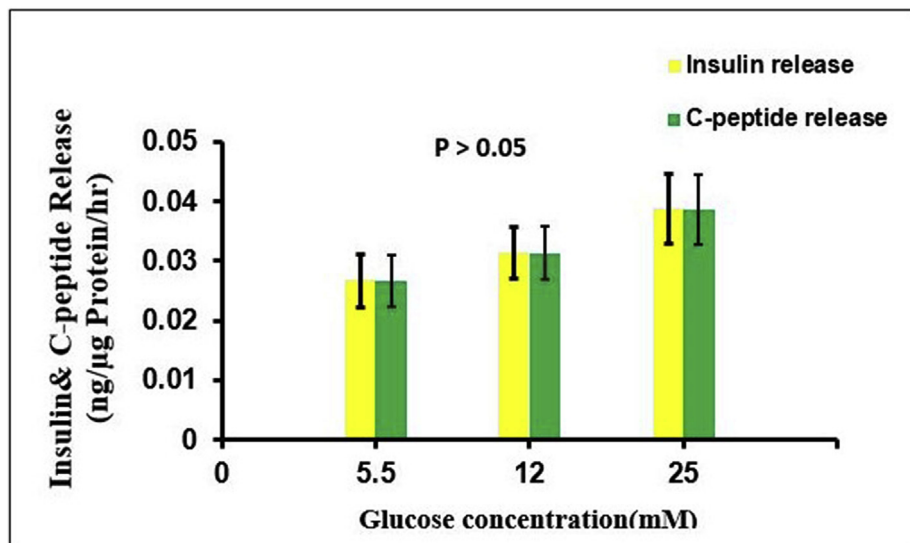
Figure 3. Responding to the glucose challenges, the rat insulin and rat c-peptide release by in vitro study using ELISA. The release of the insulin and c-peptide gradually exceeded with the exceeding in concentration of the glucose ($P > 0.05$). Results showed that AT MSCs (differentiated) have sensitivity toward glucose and ability to secrete insulin as needed.
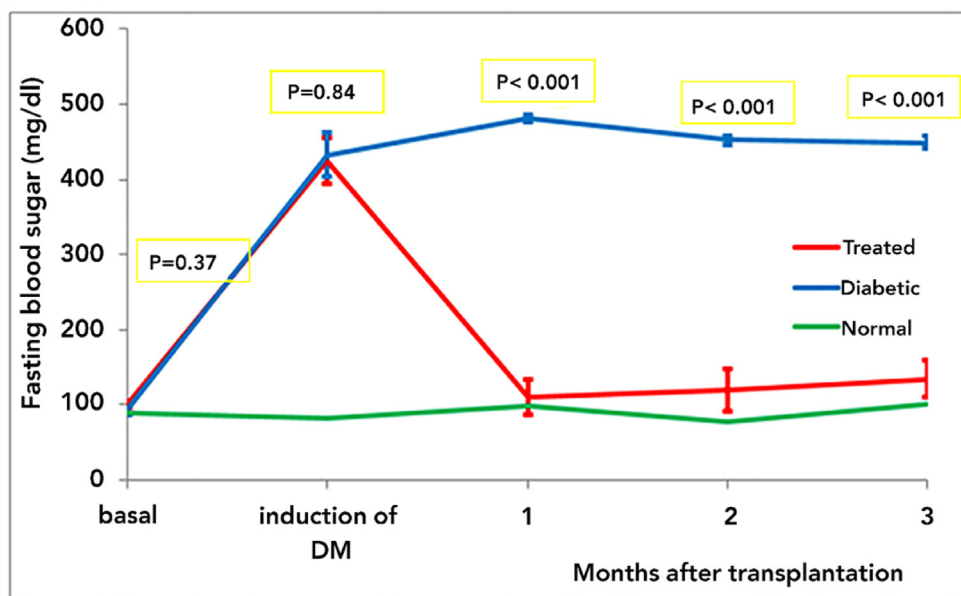

Figure 4. Fasting blood sugar levels of the treated group were improved within a month from transplantation and became nearly normoglycemic throughout the 3 months of the observation period. Data expressed as Mean $\pm$ S.E.M.

3.8. Immunofluorescence of the retrieved capsule and immunolabeling of the pancreas

Immunocytochemistry for the differentiated AT MSCs inside the capsules; applied after the completion of the follow-up period (3 months); showed that the extracted AT MSCs were positively stained for insulin and c-peptide proteins. The proportion of differentiated IPCs was 6.3% in the TheraCyte capsule. These proteins were co-expressed by the same cells (Figure 6A1-A3). Moreover, the immunolabeling for the native harvested pancreata of the treated animals showed atrophied islets and was negative for insulin contrary to the normal pancreata that showed normal islets and were positive for insulin (Figure 6B1 and B2).

3.9. Histological studies of testes

The sham group (normal control) showed smooth regular seminiferous tubules and thin basement membrane with no invaginations. The spermatogenic series cells and Sertoli cells were monitored with no abnormalities. No intraluminal multinucleated giant cells were recognized. The interstitial compartment was intact showing average amount of Leydig cells with their characteristic eosinophilic cytoplasm, vesicular nuclei, and conspicuous nucleoli. Also, edema and inflammation were not observed (Figure 7A).

On the other hand, the diabetic group showed irregular contour seminiferous tubules, with mild to marked invaginations in the basement membrane. The spermatogenic series cells presented a significant

Table 2. The changes in biochemical and semen analysis of normal, diabetic and treated groups determined at the end of the experiment. Data expressed as Mean ± SEM, (n = 10).

parameters		Normal group	Diabetic group	Treated group
Hormonal profile	Testosterone (ng/ml)	13.11 ± 1.2	1.508 ± 0.155 [+]	4.606 ± 0.502 [•] [$]
	FSH (miu/ml)	4.393 ± 0.201	0.317 ± 0.0408 [+]	1.905 ± 0.274 [•] [°]
	LH (miu/ml)	21.84 ± 2.5	0.716 ± 0.072 [+]	4.33 ± 0.589 [•] [$]
Oxidative stress enzyme	MDA (nmol./mg)	2.126 ± 0.346	8.432 ± 0.86 [+]	4.399 ± 0.247 [#] [°]
Antioxidant status	GSH (mg/g)	10.549 ± 0.258	1.679 ± 0.253 [+]	4.657 ± 0.174 [•] [°]
	CAT (U/g)	1.046 ± 0.008	0.643 ± 0.043 [+]	0.865 ± 0.007 U/g [•] [°]
Sperm parameters	Sperm count	$1.280966 \times 10^8 \pm 3.95 \times 10^6$	$2.975256 \times 10^7 \pm 3.55 \times 10^6$ [+]	$7.204600 \times 10^7 \pm 9.200 \times 10^5$ [•] [°]
	Sperm motility (%)	84.95 ± 2.776	46.513 ± 0.612 [+]	60.73 ± 2.189 [•] [$]
	Sperm abnormality (%)	26.95 ± 0.719	59.515 ± 1.215 [+]	37.85 ± 2.44 [#] [°]

Note:
+ Highly significant ($p < 0.001$) compared to diabetic group.
• Highly significant ($P < 0.001$) compared to treated group.
Significant ($p < 0.05$) compared to treated group.
° highly significant ($p < 0.001$) compared between treated and diabetic group.
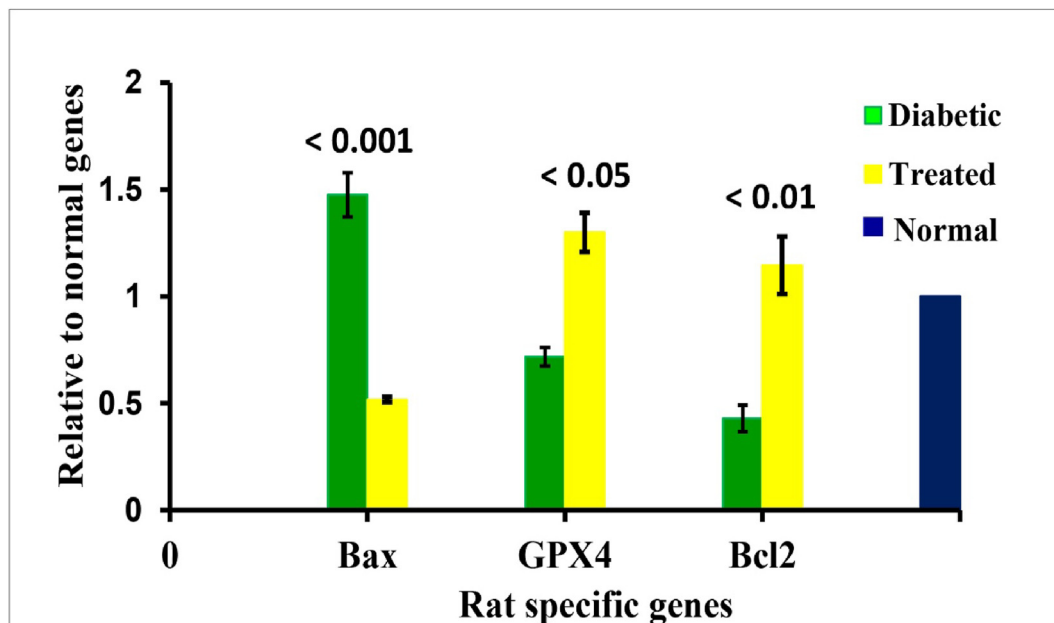$ Significant ($p < 0.05$) compared between treated and diabetic group.

Figure 5. RT-qPCR for relative gene expression in the rat testicular tissue showed that; after the graft removal at the end of the experiment; the apoptotic genes (Bax and Bcl-2) and antioxidant enzyme gene (GPx4) were expressed in all samples. After transplantation, the gene expression of Bax decreased, but the gene expression of GPX4 and Bcl-2 increased. Results expressed as Mean ± SEM with p value.

reduction of the germinal epithelium thickness, which sometimes was completely atrophic. Sertoli cells showed variable vacuolization with detached spermatogonia, lost spermatogenesis inside the lumen in addition to focal areas showing primary spermatogonial proliferation. Some damaged seminiferous tubules were atrophic with intraluminal multinucleated giant cells. The interstitial compartment integrity was affected with variable degrees of atrophic Leydig cells with patchy edema. Affected capillaries and arteriolar walls showed mostly moderate thickening and consequent interference with the nourishment of Leydig cells and seminiferous tubules (Figures 7B-F).

While the treated group showed improvement in the contour of the seminiferous tubules and basement membrane with mild invaginations. The spermatogenic series cells presented mild and focal reduction of the germinal epithelium thickness but totally was near normal. Sertoli cells showed scattered vacuolization with mild detached spermatogonia and lost spermatogenesis inside the lumen in very rare scattered tubules. No primary spermatogonial proliferation was shown. Occasional damaged

seminiferous tubules were detected. The interstitial compartment was sparsely affected with a mild degree of atrophic Leydig cells and patchy edema. Affected capillaries and arteriolar walls showed mostly mild thickening. In general, the morphology was improved than the diabetic group (Figures 8 A-D).

4. Discussion

Diabetes mellitus (DM) is a pathological condition of hyperglycemia that has a subversive impact on the male genital system. It is well known that the main cause of hyperglycemia occurred in diabetes is the insulin deficiency in the blood which leads to negative outcomes on the body [22]. Our results revealed that diabetic rats had lower testosterone levels. This attenuation in the level of testosterone might occur as a result of the reduced insulin as it had been reported that there was a direct relationship between insulin, gonadotropins, and testosterone. Reduced insulin secretion inhibited gonadotropin secretion (FSH and LH) which were
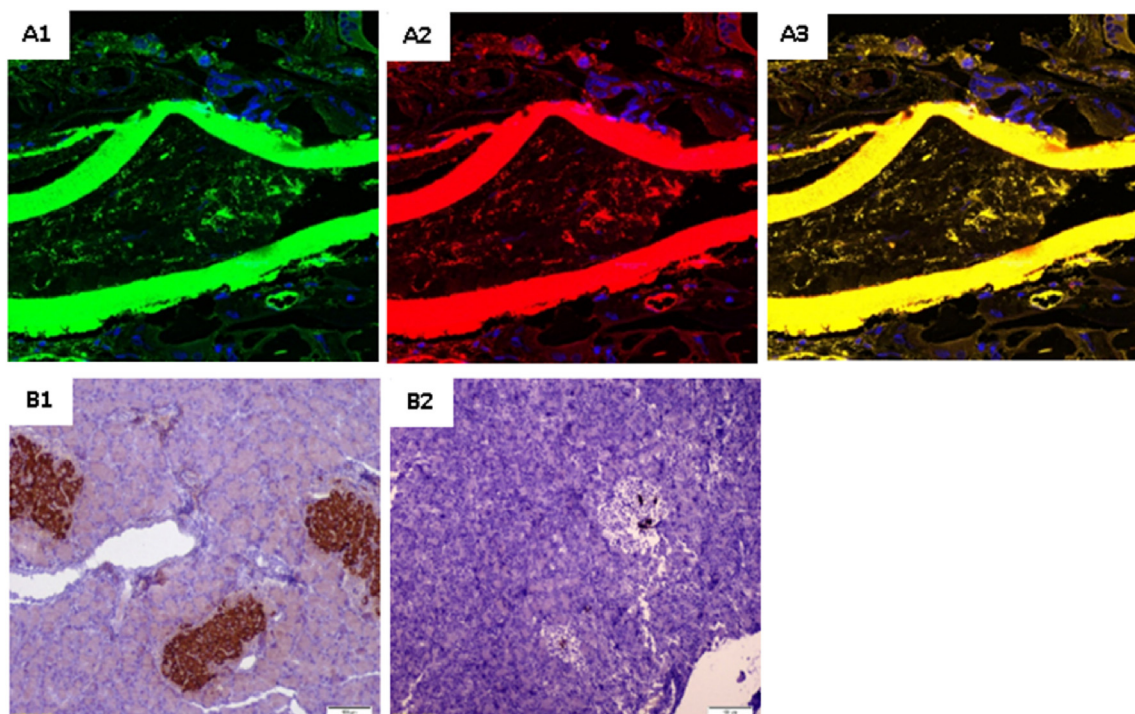
Figure 6. (A) Harvested capsules histology at the end of the experiment. (A1) immunofluorescence staining for insulin expression (green) of the encapsulated AT-MSCs was positive., (A2) Immunofluorescent staining for c-peptide expression revealed that the encapsulated cells was positive (red), (A3) Immunofluorescent staining also demonstrated that there was a merge (yellow) emphasized that the same cells co-expressed both of the insulin and c-peptide, (B) Rat pancreas immunostaining, (B1) The pancreatic islets collected from normalized un treated rats positively stained for insulin and (B2) The pancreatic islets of treated group were atrophic and negatively stained for insulin.

responsible for promoting the androgens secretion such as testosterone [23]. Subsequently, the attenuated levels of FSH and LH lessened the testosterone secretion in the blood of diabetic animals. Another cause for the reduction in serum testosterone level was the changes in the body composition that occurred as a result of diabetes; such as the increase in the body lipids and the decrease in the antioxidant levels as reported in a previous study [24].

Testosterone is important for the occurrence of spermatogenesis and the activity of the Sertoli cells [23]. So, the deficiency of testosterone level in the blood was responsible for defects in the sperm parameters. The sperm count decreased in our diabetic group as a result of severe hyperglycemia; which exceeded the oxidative damages in the body. The outcomes of such oxidative damage and lipid peroxidation could also include disruption of spermatozoa membrane matrix structure and integrity of the membrane which contribute to increasing the sperms with defective morphology in the diabetic rats of our study. Also, the reduction in the sperm motility of our diabetic rats was observed which may be resulted from the sudden decrease in the intracellular ATP concentration that occurred as a result of diabetes; as observed previously in another study [25].

In a previous study [26], it was reported that hyperglycemia could induce apoptosis in addition to oxidative stress by inducing the emergence of reactive oxygen species (ROS) and imbalance between oxidant and antioxidant species. In the current study, the STZ induction of diabetes in animals significantly increased oxidative stress in the testis by augmenting MDA levels. Furthermore, the induction lessened the activity of antioxidant enzymes such as CAT and GSH in the testicular tissue of our diabetic rats. ROS are well known to cause reproductive dysfunction by causing testicular injuries and apoptosis [27]. As a result of these biochemical disruptions; apoptosis induction was occurred and evidenced by the pro-apoptotic gene (Bax) upregulation, the anti-apoptotic gene (Bcl-2), and the antioxidant enzyme gene (GPx4) downregulation.

Previous work suggested that STZ-induced DM causes vast tissue damages, resulting in a series of reproductive disruptions [28]. The abnormalities in the reproductive system of diabetic rats were triggered by the diabetes induction rather than the STZ toxicity or any of its byproducts [4]. In our results; when concerning the histopathological alterations in the seminiferous epithelium; it was found that diabetic animals exhibited an existence of cellular debris as well as germ cell detachment in the tubular lumen. Diabetic animals also displayed Sertoli cell vacuolization, scattered intraluminal giant cells, and a significant reduction of the germinal epithelium thickness. Moreover, one of the diabetic cases showed spermatogonial proliferation. In this study, it was suggested that IPCs treatment could be used as a powerful tool for controlling diabetes; as demonstrated in previous studies [29, 30, 31]; and avoiding the induced reproductive impairments as a result of diabetes.

At the *in vitro* differentiation protocol end, we had found that the AT MSCs were well-differentiated into IPCs. The differentiation was confirmed by the immunocytochemistry, Insulin, and c-peptide release assay. The treatment occurred by transplantation of encapsulated IPCs in the TheraCyte capsule at the subcutaneous site in the chemically-induced diabetic rats. Because the TheraCyte capsule was easily retrievable, had a rough external membrane that promoted neovascularization, and an internal membrane with a semipermeable characteristic that allowed for free glucose and insulin exchange, so, this commercial encapsulation device "TheraCyte capsule" was used in this experiment [32]. The device's efficacy in controlling STZ induced diabetes in animals was observed. The effect of transplantation of encapsulated IPCs was compared to that of the untreated diabetic group. The retrieved capsules were subjected to immunofluorescence study after the completion of the follow-up period of our experiment. Insulin and C-peptide-positive cells were observed by immunofluorescent studies and both of them were co-expressed within the same cells. Furthermore, the percentage of cells positively stained for insulin in the TheraCyte capsule augmented when compared with that before the transplantation due to the further
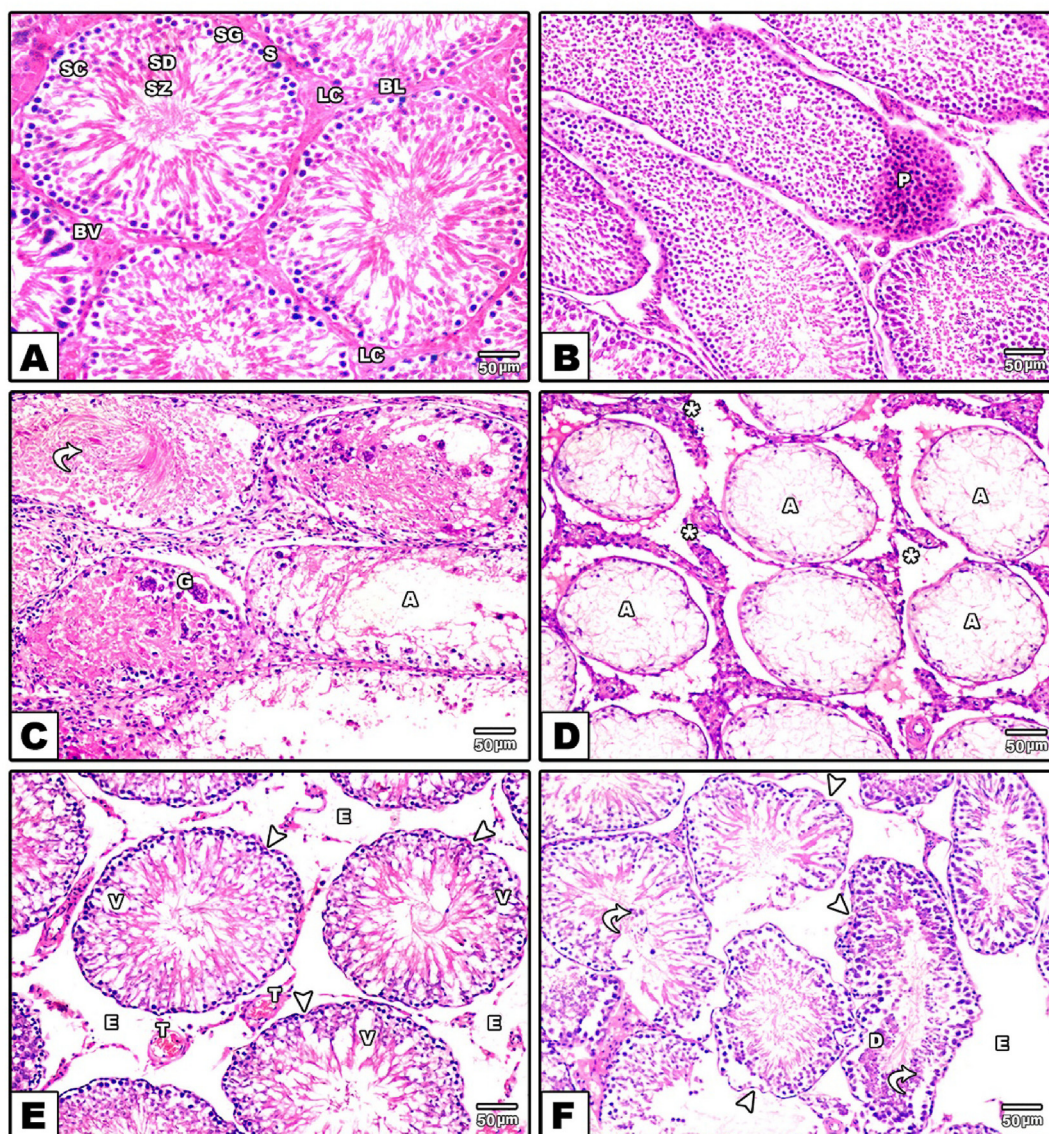
Figure 7. Photomicrographs of testicular tissue cross-sections in the normal and diabetic groups stained with H and E stain (200X). A: Testis of the normal group; intact seminiferous tubules with smooth thin walled basal lamina (BL) showing Sertoli cells (S) and regular spermatogonial stages; spermatogonia (SG), Spermatocyte (SC), Spermatids (SD)and Spermatozoa (SZ).The interstitial compartment showed active leydig cells (LC) with thin walled blood vessel (BV).B–F Testicular tissue section in the diabetic group. B: Primary spermatogonial proliferation (P). C: Many scattered intraluminal giant cells (G) with atrophic tubules (A) showing attenuated germ cell epithelial thickness and lost germ cell (curved arrow) into lumen. D: Diffuse marked tubular atrophy with damaged Leydig cells (asterisk). E: Diffuse basement membrane invagination (head of arrow) with Sertoli cell vacuolization(S), interstitial edema (E) and thickened capillary (T) basement membrane. F: Marked tubular basement membrane invaginations in addition to mild detached spermatogonia (D) with detached moderate intraluminal loss of spermatogenesis.

maturation that occurred to the differentiated cells after transplantation; as mentioned before in our previous study [33].

Improvement in fasting blood glucose of the transplanted group was observed monthly and maintained near the normoglycemia throughout 3 months of the observation period. Moreover, it was important to mention that in our previous study [34], we found that diabetic animals received no cells or undifferentiated MSCs remained hyperglycemic.

The improvement in the fasting blood glucose that occurred in our treated group was due to the insulin source of the transplanted cells as the immunolabeling of the harvested pancreata in the transplanted group showed negligible insulin expression compared to the normal pancreata. So this means that the encapsulation achieved some success because the IPCs induced normoglycemia after their transplantation in the diabetic rats. However, several problems had to be solved. Not only should the used material of the capsule be biodegradable and biocompatible, but it

should also be incapable of triggering a foreign body reaction. It was rational to undergo the transplantation of the encapsulated device in the subcutaneous site as it offered a large surface area, was easily accessible, and permitted minimal invasiveness for both transplantation procedures and graft retrieval. But, the subcutaneous site showed low vascularization and poor oxygenation, so, it was necessary to induce early vascularization and to prevent late fibrosis [35].

Fortunately, at the end of our experimental period (after 3 months of the observation period), IPCs transplantation showed an elevation in the testosterone, FSH, and LH levels in the treated group compared with that of the non-treated STZ-diabetic group. Also, treatment markedly improved the sperm parameters (count, motility, and abnormality) by trapping the ROS (MDA) and increasing testicular antioxidant enzymes status (GSH and CAT). Meanwhile, treatment attenuated the testicular tissue apoptosis by downregulating (Bax) and upregulating (Bcl-2 and GPX4).
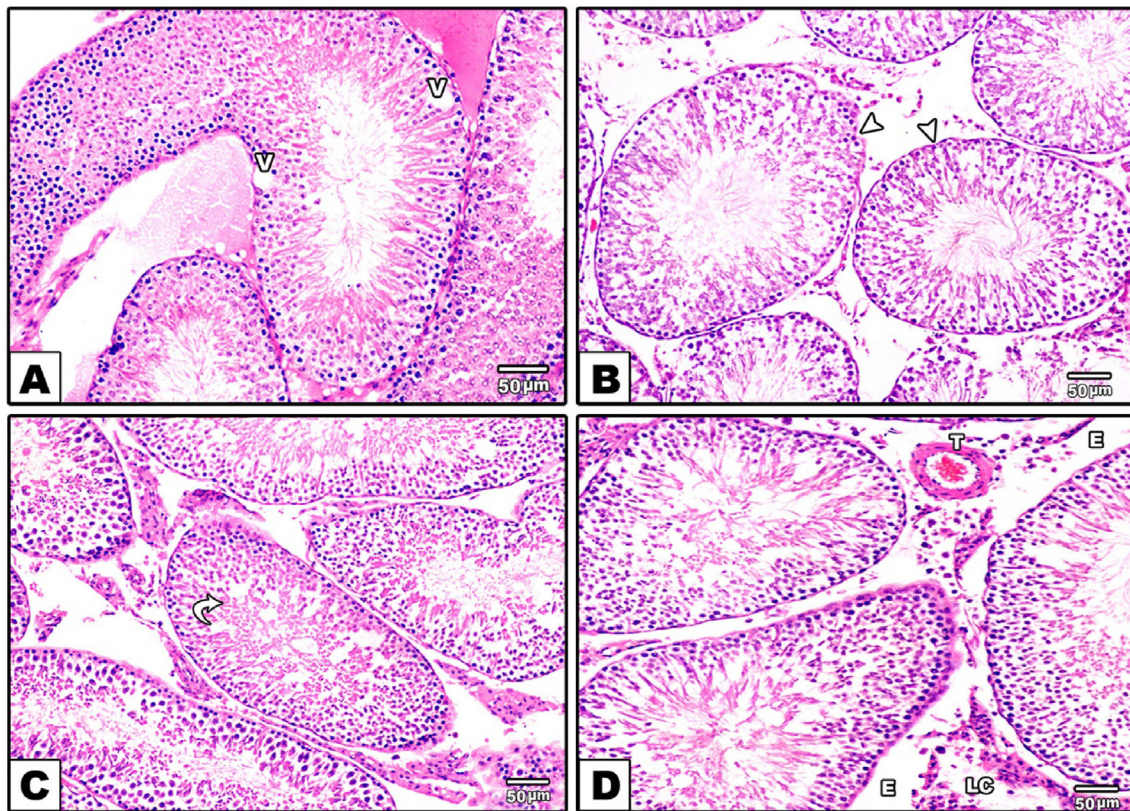
Figure 8. Photomicrographs of the testicular tissue cross-sections stained with H and E stain (200X) in the treated group (A–D). A: Minimal Sertoli cell vacuolization amidst near normal tubules. B: Near normal tubule with mild basement membrane invaginations. C: One tubule showing mild detachment and intraluminal loss of spermatogonia with near intact remaining seminiferous tubular and interstitial compartments. D: Mild interstitial edema with mildly thickened arteriole with near normal presence of Leydig cells.

Contrary to expected, It was noticed that the Bax expression in the treated group was lower than that of the normal group, moreover, the expression of Bcl2 and Gpx4 in the treated group was higher compared to that of the normal group. This result may confirm the fact that the expression of certain genes had been elevated in response to oxidative stress in a compensatory manner to defend against tissue insult, as the treated group was firstly diabetic and diabetes may trigger several changes in the molecular levels of cells and tissues [36], and followed by the IPCs transplantation that improved the antioxidant status and decreased apoptosis. In agreement with the concept that diabetes could participate in the elevation of GPX4 expression, a previous study showed that diabetic rat's mRNA levels of renal Sod-1, Cat, and Gpx4 augmented as compared to controls [37]. And in agreement with our results, a previous study demonstrated that mRNA levels of testicular Bax in treated rats slightly declined as compared with normal and mRNA levels of testicular Bcl2 in treated rats somewhat enhanced compared to normal [38].

At the same time, the treatment by using IPCs significantly attenuated the atrophy and reduction in seminiferous tubules diameter specifically through its excessive free radical scavenger and anti-apoptotic characteristics. Also, the spermatogenic series cells in the testes histology of all the treated group were totally near normal.

5. Conclusions

The results of this study emphasized that diabetes stimulates extreme biochemical changes and histopathological variations in the STZ-diabetic rat's testes. Transplantation of IPCs in the diabetic group could inverse the impact of diabetes. Also, IPCs treatment was considered an effective remedy for diabetes-related pathologies and their complications especially testicular dysfunction by decreasing oxidative stress and apoptosis. However, the underlying mechanism is still very complex.

So, before clinical applications; many questions have to be answered; those questions are:

How long can the transplanted cells maintain their function?
How many cells are required for transplantation for each kilogram of the body weight?
What is the perfect site for cell transplantation?
So, further investigations must be done in the future.

Institutional review board statement

The study was regulated in accordance with the NIK Guide and licensed by the Ethics Committee of department of the animal house in the research unit of the urology and Nephrology; Mansoura University.

Informed consent statement

Not applicable.

Declarations

Author contribution statement

Mai A. Abd El Kader: Performed the experiments; Wrote the paper.
Mahmoud M. Gabr: Conceived and designed the experiments.
Sherry M. Khater: Performed the experiments; Analyzed and interpreted the data.

Reham A. Ghanem, Amoura M. Abou El Naga: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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