

Molecular and Biochemical Studies on Stem Cells in Relation to Diabetes MellitusManal A. Khaled¹, Hussein I. El-belbesi¹, Medhat F. Mahmoud¹ and Ahmed M. Balah²Departments of ¹Biochemistry and ²Histology, Faculty of Vet. Med., Zagazig University
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Abstract: Background: In recent years, islet transplantation for diabetes has shown signs of the treatment efficiency, but its application is limited due to lack of donor organizations, sources and immune rejection. Bone marrow mesenchymal stem cells have become a new resource of islet cell substitutes. **Aims:** we aimed to study the differentiation ability of adult rat bone marrow mesenchymal stem cells to form insulin-producing cells and their ability to treat chemically induced diabetic rats. **Methods:** Mesenchymal stem cells were derived from the bone marrow of female white albino rats. After their expansion and at the end of passage 3, flow Cytometric analysis of undifferentiated cells showed purified mesenchymal cells as they were negative for CD45, meanwhile they positive for CD90, CD146 and CD105. The cells were cultured for 14 days in high glucose medium, then in media containing nicotinamide and exendin-4 for another 7 days. After 21 days of differentiation culture, the cells formed islet-like clusters. They were positively stained for insulin. They were then transplanted into female rats which were made diabetic by IV injection of streptozotocin (STZ). The rats were divided into control, diabetic and treated group. Serum insulin and glucose were estimated and pancreas tissues were examined in all groups. **Results:** Diabetic rats which received differentiated mesenchymal stem cells showed significantly lower serum glucose and increased serum insulin levels compared with the diabetic group also treated group showed increasing in the size of Langerhans islets and regranulation of islet cells was appeared and undifferentiated stem cells with irregular nucleus appeared around blood capillaries compared with diabetic group. **Conclusions:** Mesenchymal Stem Cells (MSCs) can isolate from bone marrow, culturing, propagation and differentiated into insulin producing cells *in vitro* and when transplanted into diabetic rats it can reverse hyperglycemia and repair pancreatic damage this can provide a new strategies for diabetic treatment.

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1. Introduction

Diabetic mellitus is one of the major causes of death in advanced countries, and has been shown to adversely affect health and quality of life. It is associated with various severe or fetal complications, including blindness, kidney failure, heart disease, stroke, neuropathy and amputations (1).

For over 80 years the main therapeutic approach to insulin-dependent diabetes has been confined to treating the symptoms by insulin replacement (2), Even with meticulous glucose control, such therapy carries the risk of hypoglycemic episodes that can lead to anxiety, seizures, coma and even death (3), Extensive research has been dedicated to discovering new ways to prevent or treat type1 diabetes and eliminate the need for insulin injections (4).

Because diabetes is caused by the loss of a single cell type, in recent years, cellular replacement therapy for diabetes mellitus, especially type one diabetes mellitus, has received much attention. However, there are insufficient organ donors and the recipient has to take a risk of anti-rejection therapies (5). Cell therapy of diabetes mellitus has been mainly based on islet transplantation. Transplantation of pancreatic islet cells as a potential cure for diabetes has become a subject of

intense interest and activity over the past two decades (6), Islet transplantation involves isolation of islets and their transplantation through a simple injection into the umbilical vein, an operation devoid of the many potential complications of transplantating the pancreas (7).

Advances in cell-replacement therapy for type 1 diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest in developing renewable sources of insulin-producing cells appropriate for engraftment. Recent studies suggest that mouse embryonic stem (ES) cells can be manipulated to express and secrete insulin (8)

Recent work suggests that adult stem cells from one tissue or organ can differentiate into the cells of other organs, either *in vitro* or *in vivo* (9). Among them, bone marrow derived stem cells (hematopoietic or mesenchymal) carry the more significant implications for possible clinical development because they are routinely collected from adults without the ethical concern inherent to fetal embryonic tissues (10), Based on their ability to adhere to a plastic support, multipotential stem cells can be isolated from the bone marrow, expanded and cultured. Under appropriate experimental conditions they differentiate into multiple

mesenchymal cell types, including cartilage, bone, adipose and fibrous tissues, and myelosupportive stroma (11). This has led many investigators to explore the potentials of their therapeutic applications (12).

2. Material and Methods

Experimental animal and study groups:

A total of 50 female White albino rats 3 month old were housed and accommodated at the animal house in Faculty of Vet Med Zagazig University for 15 days before starting the experiment. The animals maintain on basal rat chow diet and free access to water.

The animals distributed into 4 groups as follow:

Groups	No. of rats
Animals donor for bone marrow	10
Animals non diabetic control	10
Animals diabetic control	10
Animals diabetic treated with differentiated mesenchymal stem cell	20

I- Isolation of bone marrow stem cells:

Isolation of bone marrow stem cells were done as described by (13) and (14). Bone marrow was obtained from femurs and tibias of 10 female white albino rats (170-180g). The bones were sterilized by immersion in 70% ethanol. The ends of bones were cut and bone marrow was extruded by inserting a needle in one end throw the bone shaft and injection of culture media [Dulbecco's modified Eagle's medium(DMEM, Lonza,Belgium) containing 10% fetal bovine serum (FBS, Sigma- Aldrich, Germany)]. The effluent was collected in sterile tubes. Gentle pipetting of Ficoll Paque (Biochrom AG, Germany) resulted in generation of a single cell suspension. Bone marrow cells were resuspended and cultured in 5ml DMEM low glucose supplemented with L-glutamine,10%FBS, 1% antibiotic(Lonza, Belgium) and cultured in culture flask, and then incubated at 37°C in a humidified atmosphere containing 5%CO₂.

II- Cultured of mesenchymal Stem Cells:

Culturing of Mesenchymal stem cells were done as describe by (15) and (16). After 4 days the non-adherent cells were discarded and adherent cells were cultured in Fresh complete medium {LG-DMEM with 10% mesencult growth enzyme (stem cell technologies, USA) and 1% antibiotics} which change twice a week. When mesenchymal stem cells reached 80-90% confluence they were passage by digestion with 0.25% trypsin (Lonza, Belgium) for 3-5 minutes at 37°C. After centrifugation, Cells were resuspended with complete DMEM and replated at ratio of 1:2 and referred to as first passage. These steps were repeated for second and third passages. At the end of third passage the cells became morphologically homogeneous.

III – Flow Cytometric Analysis:

The bone marrow-derived MSCs at passage 3 were released by trypsinization. The cells were centrifuged at 300 g for 8 minutes, and then were solved in PBS at the concentration of 1x10⁶ cells/ml. the antibodies against CD45, CD90 directly conjugated to phycoerthrin (PE) and CD 146, CD105 conjugated with fluorescein-isothiocyanate (FITC) were added. The fluorescent labeled direct antibodies (10 µl for each 100µ sample) and incubated for 30 minutes at room temperature. Labeled cells were thoroughly washed with two volumes of PBS and fixed in flow buffer (1% formaldehyde in PBS). The labeled cells were analyzed on a FACS Calibur. A total of 10 000 events were obtained and analyzed with the Cell Quest software program (Becton-Dickinson, USA).

IV- In Vitro differentiation culture:

In vitro differentiation cultures were done as described by (14) and (17). At the third passage, BM-MSCs with 80% confluence were induced to differentiate into insulin producing cells. Cells were induced with 5% FBS HG-DMEM (25 mmol /L glucose) for 14 days, and added 10 mmol/L nicotinamide (Sigma-Aldrich, Germany), and 10 nmol/L exendin-4 (Sigma-Aldrich, Germany) for 7 days.

V- Morphological Evaluation:

- 1- To evaluate the insulin producing cells in clusters, we stained the clusters with diphenyl thiocarbazon (DTZ). Stock solution was prepared as previously described (18) by dissolving 10 mg of Ditzone (Sigma-Aldrich, Germany) in 10 ml of dimethyl sulfoxide (Sigma-Aldrich, Germany) and was stored at - 20°C. At staining 10µ of stock solution was added to 1 ml of culture medium. Cells were incubated at 37°C for 30 minutes in DTZ containing medium.
- 2 – To further determine whether cluster turn to IPCs, the conditioned media was collected from flasks and frozen at -20°C until assayed for insulin content (19).

VI- Induction of diabetes in rats:

30 female rats 5-6 month old weighing between 185 and 200g were injected by STZ-Na citrate buffer which prepared immediately before injection to avoid degradation STZ. Rats were injected by STZ (Biomedical, France) at the dose of 60 mg / kg of the body weight intravenously according to (20). [7.5 mg of STZ was solvent in 1 ml of Na-Citrate buffer (Na-Citrate buffer was prepared by dissolve 1.47g of Na Citrate in 50 ml d H₂ O)]. Three days later, the blood glucose level was tested with glucose tester (Bionime GmbH, Switzerland), one week after injection of STZ stable hyperglycemia level was achieved and fasting glucose level was more than 250 mg/dl, rats were observed to have obvious symptoms such as illness, polyuria and weight loss.

VI – Transplantation of differentiated islet like clusters in rats:

All medium were removed and wash with PBS for 3 times. Clusters were detected with a solution of 0.25 % trypsin and 0.02% EDTA for 3-5 minutes at 37°C, then add 5 ml FBS, 5ml physiological saline were add and centrifuge at 1200 for 10 minutes, removed the supernatant, cells were resuspended with 0.5 ml of saline as medium (21) and transplanted into diabetic rats through a tail vein according to (22).

VII – Quantitative Determination of Glucose:

Glucose level was determinate according to the method described by (23). The kits Obtained from Spinreact Co, Spain.

VIII – Measurement of Insulin Secretion by ELISA:

Determination of plasma insulin has been done by using immuno enzymatic assay for Quantitative measurements according to the method described by (24). The kits obtain from (SPI bio, France).

IX- Histological examination:

Tissues were collected then immediately fixed in 10% neutral buffered formalin for 48 hrs. Five-micron-thick paraffin-sections were prepared, stained by

hematoxylin and eosin and then examined microscopically (25).

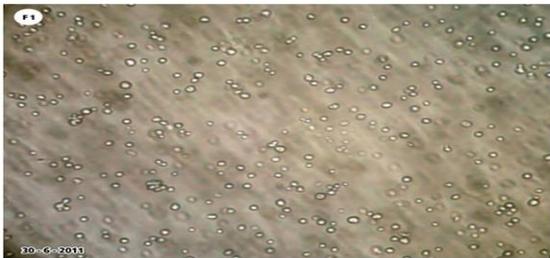
IX-Statistical analysis:

The obtained data were analyzed using one-way ANOVA (26). All the results expressed as mean \pm SE. Analysis of variance followed by Duncan test. Results were considered significant at $P < 0.05$. Means at the same column followed by different small letters were significantly different and the highest value was represented with letter (a).

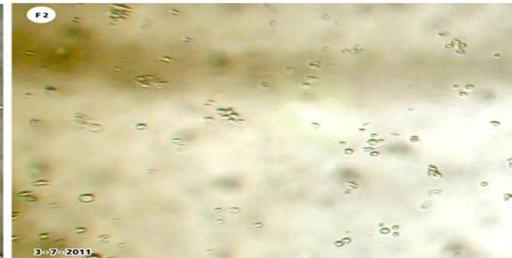
3.Results:

I- Morphological changes of MSCs during differentiation:

Bone marrow cells 1st day of culturing after isolation contain stem cells (Fig 1). After 4 days of culturing the media was change and the suspended cells were removed and adherent cells were cultured in new media (Fig 2). Under inverted microscope with magnification power (40x



Fig(1)



Fig(2)

Figure (3) after 7 days of culturing the first appearing of spindle-shaped adherent cells. After 28 days of culturing the spindle-shaped adherent cells reached 80% confluence. The cells then marked as first

passages culture. Figure (4) present cells after 3 passages. Under inverted microscope with magnification power (40x10).



Fig(3)



Fig(4)

II- Flow Cytometric Analysis:

Flow Cytometric analysis of the MSCs at passage 3 showed that these cells were negative for CD45 (3.4%) and positive for CD146 (53.33 %), CD90

(43.49 %) and CD105 (25.59 %). These results indicated that relatively purified bone marrow-derived MSCs were isolated.

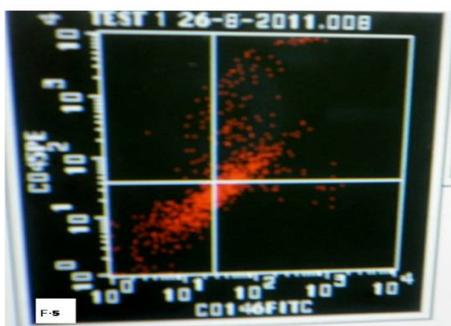


Fig (5) cells were negative to CD45 but positive to CD 146.

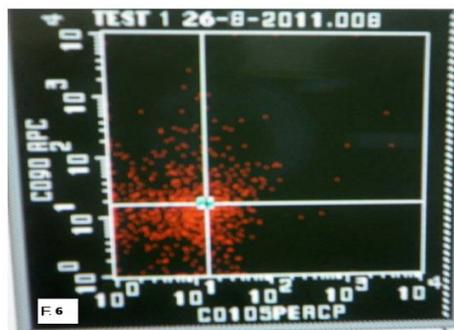


Fig (6) cells were positive to CD 90 and positive to CD105.

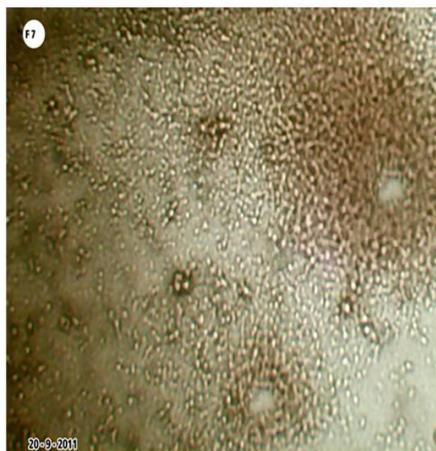
III- In Vitro differentiation culture:

Fig (7): Clusters after 21 days in differentiated media.

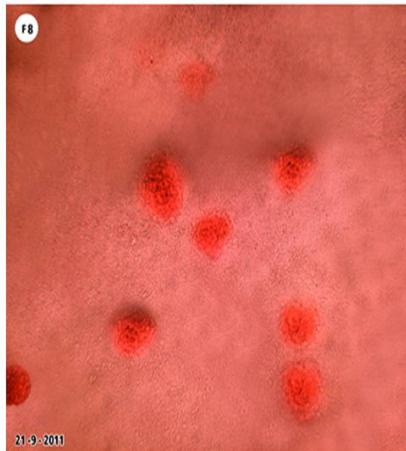


Fig (8): Cell clusters stained crimson red with DTZ

Under inverted microscope with magnification power (40x10).

2 – By measuring insulin released in media by insulin ELISA kit, found that the mean insulin secretion

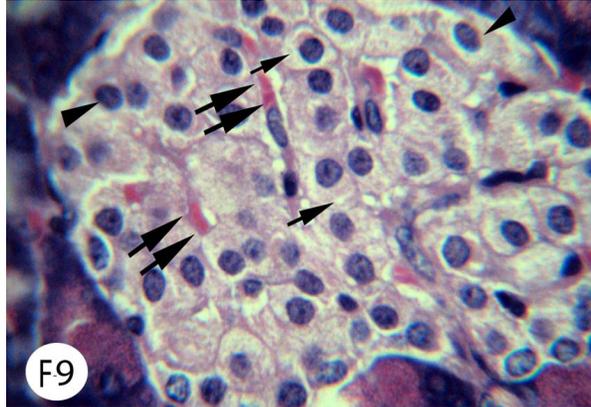
by 100 clusters was (3.68±0.16 ng/ml). This is the mean values of 10 experiments.

IV- Table (1): Showing changes in serum glucose and serum insulin (mean values ± SE) in 3 groups after 1, 2 and 3 weeks of treated by IPCs.

	Glucose (mg/dl)			Insulin(ng/ml)		
	1st week	2nd week	3rd week	1st week	2nd week	3rd week
1- Normal control rats	95.90 ± 2.88f	97.50 ± 2.98f	96.90± 2.64f	2.78 ± 0.22a	2.77 ± 0.15a	2.76± 0.13a
2- Diabetic rats	268.10± 3.79b	303.80 ± 4.88a	307.60± 7.21a	0.997± 0.057e	0.902± 0.010f	0.857± 0.011g
3-Diabetic treated rats	203.90± 3.07c	184.80± 3.64d	118.90± 3.13e	1.337± 0.038d	1.745± 0.033c	2.020± 0.017b

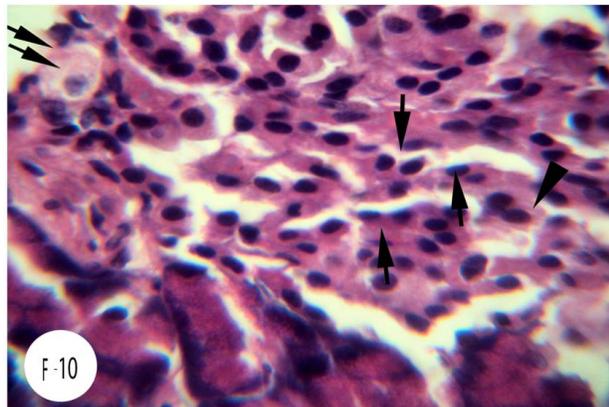
V- Pathological results:

Fig. (9) A photomicrograph of a section in pancreas of control rats showing α -cells "arrow head" at the periphery, normal β -cells "arrow" and blood capillaries" double arrow".



Fig(9) Obj.x100: Oc.x10.

Fig. (10) A photomicrograph of a section in pancreas of diabetic rats showing Clumping of β -cell "arrow" with mainly pyknotic nuclei and deeply acidophilia of the cytoplasm "arrow head" or marginal hyperchromasia "double arrow".



Fig(10) Obj.x100: Oc.x10.

Fig. (11) A photomicrograph of a section in pancreas of treated rats showing the increase in pancreatic islets size with stem cells around blood capillaries" arrow" and less acidophilia of the cytoplasm of β cells "double arrow".

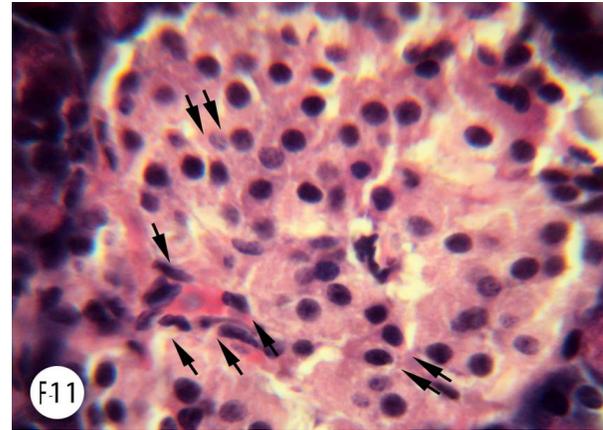


Fig (11) Obj.x100: Oc.x10

4. Discussion:

Type 1 diabetes mellitus (T1DM) is a single-cell disorder in which insulin-secreting β -cells in pancreatic islets of Langerhans are irreversibly destroyed. Because diabetes is caused by the loss of a single cell type, in recent years, cellular replacement therapy for DM, especially T1DM, has received much attention. However, there are insufficient organ donors and the recipient has to take a risk of anti-rejection therapies. More recently, additional efforts have focused on the use of both autologous and allogeneic stem cells as sources of new islets (27).

Bone marrow derived cells have been shown to differentiate into various lineages, such as hepatocytes, *in vivo* and *in vitro*. As stem cell research progresses, new methods for the therapy and treatment of diseases such as diabetes mellitus may be possible. In addition, stem cell research may advance our understanding of organ regeneration and the mechanism(s) thereof (19).

Previous studies have shown that MSCs are able to differentiate into several cell types, including cardiomyocytes, vascular endothelial cells, neurons, hepatocytes, epithelial cells, and adipocytes, making them a potentially important source for the treatment of debilitating human diseases. Such multipotent differentiation characteristics coupled to their capacity for self-renewal and capability for the regulation of immune responses, described MSCs as potentially new therapeutic agents for treatment of the complications of diabetes mellitus (28).

In the present study, bone marrow-derived mesenchymal stem cells were isolated from femurs and tibias of female rats, growing and characterized by their adhesiveness and spindle-shaped cells and by detection of CD90, CD105, CD146 (some of the surface markers of rat mesenchymal stem cells), rather than CD45 (leukocyte marker). This result agrees with many investigators who isolate MSCs from bone marrow and characterized it by different combination of CD markers, (29) isolated and cultured MSCs from murine bone marrow, which uniformly expressed stem cell

antigen-1, CD29, CD44, c-kit and CD105, while being negative for expression of CD45, CD31 and CD34. (16) Reported that by flow Cytometric analysis of the MSCs at the passage 3 showed that these cells were negative for CD34, CD45 and CD14. While expressed high levels of CD29, CD44 and CD106. These results indicated that relatively purified bone marrow-derived MSCs were isolated. (14) Found that BM-MSCs were typical of spindle and fibrocyte-like adherent monolayers with high CD90, CD29 positive rate and very low CD45 expression. (30) And (31) characterized MSCs by its fibroblastic morphology and by expressed CD29 and CD90, and less expression for CD45 (leukocyte marker) and CD34 (hematopoietic cell marker). While (32) characterized bone marrow-derived mesenchymal stem cells by their adhesiveness and fusiform shape and by detection of CD29.

Several recent studies indicate that under specific *in vitro* culture conditions, mesenchymal stem cells derived from bone marrow can be induced to differentiate into functional islet cells capable of normalizing hyperglycemia in a diabetic animal model (13 and 33). In the present study for *in vitro* differentiation of mesenchymal stem cells into insulin-producing clusters, cells from 3 passages were cultured in DMEM-HG with nicotinamide and exendine-4 then detected the insulin producing by staining the cluster with DTZ and media analysis. These agree with (14) and (34) reported that the use of nicotinamide and exendine-4 in high glucose media can induce mesenchymal stem cells into insulin producing cells. Where (12) used the low glucose media, nicotinamide and exendine-4 in presence of pancreatic extract to induce insulin producing clusters from hematopoietic stem cells and detected the producing of insulin by staining cells with DTZ. But (16) and (21) used the combination of β -mercaptoethanol, nicotinamide, IGF-1 and β -cellulin in the serum-free H-DMEM culture medium to induce insulin producing clusters from mesenchymal stem cells.

In the present study, after transplantation of IPCs to STZ- diabetic rats through a tail vein a significant decrease in glucose level and significant increase in insulin blood level of treated rats can achieved in comparison to diabetic untreated rats from the first week after transplantation and when we reach the third week after transplantation the values of serum glucose and insulin reached nearly to the normal control group. this agree with (22), (27) and (35) who reported that transplantation of mesenchymal stem cells throw tail vein can reverse hyperglycemia in STZ-treated mice. (19) reported that transplanted of BM-derived insulin-producing clusters into the renal subcapsular space of STZ-induced diabetic mice can normalize their blood glucose levels with 17 days post-transplantation. Where (13) reported that transplantation of murine bone marrow derived stem cells into the left renal

capsule and the distal tip of the spleen of diabetic mice can decrease and normalized blood glucose within 1 week following transplantation. (14) Reported that transplantation of differentiated mesenchymal stem cells via portal vein into STZ-induced diabetic rats decrease glucose levels at day 6 after transplantation and kept it below 15mmol/l during day 12 to day16 after transplantation. (31) Reported that transplantation of differentiated MSCs via the left cardiac ventricle of STZ-induced diabetic rats decreased random blood glucose especially at the early phase of treatment 3 days to 2 weeks. (21) Reported that administration of differentiated MSCs in peritoneum of diabetic rats can significantly reduced glucose level about 14 days after the injection when compared with the glucose level of rats before treatment.

In the present study the pathological results illustrated in Fig (9, 10), normal pancreatic islets of normal rats showing large pale area of pancreatic islets among the darker staining secretory acini and each islets consisted of β -cells in the central region and α -cells in the periphery with normal granular cytoplasm, where the STZ-induced diabetic rats showed shrinking of the islet of Langerhans and clumping of β -cells with mainly pyknotic nuclei and deeply acidophilic cytoplasm this finding agree with (36) and (20) who reported that a single injection of STZ into rats caused β -cells destruction with degenerative and necrotic changes, and shrinking of the islets of Langerhans. The nucleus of necrotic cells indicated either pyknosis or marginal hyperchromasia. In our study as illustrated in Fig (11) after treatment of diabetic rats by MSCs differentiated IPCs throw a tail vein the islets showed substantial recovery. The islets of Langerhans were distinctly increased in size, the severity of degenerative and necrotic changes in the islet cells of Langerhans were less than those in the diabetic group with less acidophilic cytoplasm. The stem cells with irregular nucleus appeared around blood capillaries. This agree with (14) and (22) who reported that transplantation of BM derived cells increase the number of β -cells in STZ-treated mice or rats. These also agree with (37) reported that Mesenchymal stem cells may play a dual role: they may help in tissue repair by endogenous factors and inhibit β -cell-specific T-cell response. The possibility of contribution to tissue regeneration emerges from the concept that MSCs have trophic effects inhibiting fibrosis and enhancing angiogenesis by secreting a variety of cytokines.

In conclusion, our findings present an evidence that MSCs can isolate from bone marrow, culturing, propagation and differentiated into IPCs *in vitro* and when transplanted in diabetic rats it can reverse hyperglycemia and repair pancreatic damage this can provide anew strategies for diabetic treatment. However there is much effort must be done to reduce

in vitro culturing time and watch the influence of stem cell therapy on pancreas for much longer time.

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