Current Topics in Biochemical Research
Vol. 14, *2*, 2012

Original Communication

Novel evidence of restoring and augmenting antioxidant defense after treatment of diabetic rats using stem cells

Mohamed A. El-Far[1,*], Mahmoud M. Gabr[2], Sawsan M. El-Halawani[2], Rana Y. Ibrahim[2], Sherry M. Khater[2], Ayman F. Refaie[2] and Mohamed A. Ghoneim[2]

[1]Division of Biochemistry, Faculty of Science, [2]Division of Biotechnology, Urology and Nephrology Center, Mansoura University, Mansoura, Egypt

ABSTRACT

The main objective was to verify the differentiation ability of adult bone marrow hematopoietic rich stem cells (HSCs) to form glucose-regulating insulin-producing cells for treatment of diabetic rats and then to study the role of it in augmenting the antioxidant defense system. Differentiation was carried out. Cells formed cell aggregates that were tested for insulin secretion *in vitro* and were then subjected to transplantation into the testes of diabetic rats. We evaluated effectiveness of this treatment on making changes for concentrations of glucose, insulin, and some blood antioxidants. Results: 1- Transplanted differentiated HSCs in the testes of diabetic rats induced euglycaemia. Highly significant increase in blood insulin levels was observed, concurrently with a highly significant decrease in glucose levels. All levels were remonitored and found to be within normal ranges during four weeks of follow up. 2- Highly significant improvement in levels of antioxidants (TAC, GSH, SOD and CAT) as well as oxidative stress biomarker (MDA) were observed after treatment with differentiated HSCs. 3- Following orchidectomy of the engrafted testes, the blood glucose levels showed a highly significant increase concurrently with a highly significant decrease in insulin levels in same group of animals. Differentiated cells transplanted in the testes were positively stained for insulin and c-peptide. Conclusion: Transplanted HSCs may include pancreatic progenitor cells. They were efficiently capable of treating diabetes. We report a novel evidence that differentiated HSCs transplantation can correct and reverse the imbalance between ROS and antioxidant defense by restoring and augmenting the capacity of antioxidants.

KEYWORDS: diabetes, insulin secreting cells, stem cells, antioxidant, malondialdehyde, antioxidant enzymes, oxidative stress, antioxidant therapy

INTRODUCTION

Diabetes has become a global epidemic in the present century [1]. Recent success achieved by transplanting insulin-producing pancreatic islets of Langerhans into the livers of diabetic patients has provided hope that diabetes might eventually be cured [2]. However, the practical benefits of this therapy are limited by a severe shortage of donor pancreata relative to the needs of potential transplant recipients. This situation has prompted a search for alternative sources of insulin-producing tissues, such as β-cell progenitor or stem cells. Adult stem cells have been shown to differentiate into a wide variety of cell types such as osteoblasts, chondrocytes, endothelial cells, skeletal myocytes, neurons and cardiac myocytes and insulin-producing cells [3-8].

It has been reported that diabetic patients have significant defects of antioxidant protection and

*Corresponding author:
elfarma2002@yahoo.com
profelfarma@yahoo.com

generation of reactive oxygen species (oxidative stress) which may be important in the aetiology of diabetic complications [9]. Decreased superoxide dismutase (SOD), catalase (CAT), ceruloplasmin and glutathione peroxidase (GPx) activities, as well as a decrease in the glutathione dehydrogenase (GSH) level and an increase in the concentration of glutathione disulphide (GSSG), were observed in erythrocytes of diabetic patients and in tissues from diabetic animals [10]. It has been also shown that free radicals and lipid peroxide are easily formed in the diabetic state using streptozotocin-induced diabetic rats and elevated levels may also play an important role in the development of diabetic complications [11].

The aim of this research was to isolate adult bone marrow hematopoietic stem cells (HSCs) and induce them to differentiate into insulin-producing cells *in vitro*. Then, the differentiated cells will be tested for their ability to treat chemically induced diabetes using streptozotocin-induced diabetic rats. Furthermore, the aim was also to verify their ability to correct the diabetic complications including possible defects of antioxidant protection.

MATERIALS AND METHODS

The experimental animals and the study groups

Adult inbred Sprague Dawley (SD) rats weighing an average of 150 g were utilized. These were divided into four experimental groups of ten animals each:

Group I : Normal non-diabetic animals.
Group II : Diabetic non-treated animals.
Group III : Diabetic animals treated with undifferentiated HSCs.
Group V : Diabetic animals treated with differentiated HSCs.

Diabetes was chemically induced using an intravenous injection of streptozotocin (STZ) at 50 mg/kg body weight [12]. The blood glucose levels were monitored using an Accutrend glucose detector (Boehringer Mannheim GmbH, Mannheim, Germany). Rats with two successive blood glucose levels higher than 350 mg/dl were considered diabetic. Animal care and protocols were in accordance with and approved by the institutional animal ethics committee.

Isolation and differentiation of hematopoietic cells

Bone marrow was obtained from long bones (femurs and tibias) of Sprague Dawley rats. HSCs cells were cultured, isolated and differentiated to insulin- producing cells according to the method we have previously described and published [13, 14].

Determination of insulin secretion

As previously described [13, 14], undifferentiated and differentiated bone marrow cells were initially incubated for three hours in glucose free-Krebs-Ringer bicarbonate buffer (KRB) containing 0.5% bovine serum albumin (BSA). This was followed by incubation in KRB containing 5.5, 12, or 25 mM glucose concentration for additional two hours. The KRB was collected and frozen at -70°C until assayed. Insulin assay was performed by enzyme-immunoassay (Linco Research Inc. Missouri, USA) according to the instructions from the manufacture.

Determination of intracellular C-peptide content

The formed clusters were washed three times with PBS. They were then suspended and dispersed in 50 mM HCl/70% ethanol. After centrifugation at 8000 rpm for 5 minutes, the supernatant was collected from the cell lysate and neutralized by the addition of 50 mM NaOH. C-peptide concentrations in the supernatants were determined by rat C-peptide ELISA Kit (Gentaur. Molecular products BVBA, Legerlaan, Brussels) according to the instructions from the manufacturer as we described before [13].

Transplantation of differentiated cell clusters in rats

The differentiated cell clusters were transplanted in the abdominally placed testis as 5000 clusters per rat (10 rats). Through a lower abdominal incision, the testes were pulled through the inguinal canal to the abdominal cavity. Clusters were injected into the testis through a 26-gauge needle. The grafted gonad was anchored to the

abdominal wall by suturing the gubernaculums to the abdominal muscles.

Follow-up evaluation

Urine output of experimental animals was measured daily, and their weights were determined every week. Random blood glucose levels and serum insulin assays were determined every week after transplantation. Insulin assay was done by enzyme-immunoassay (Linco Research Inc, St. Charles, MO, USA) according to the instructions from the manufacturer.

Evaluation of the antioxidant role

Preparation of blood samples and lysates

Blood samples were collected from all animal groups four weeks after transplantation of undifferentiated and differentiated cells into heparinized glass fine Pasteur pipette from the orbital sinus and centrifuged for 15 min at 1000 g. Plasma was carefully removed and the cells were then washed three times with 0.9% NaCl solution. The washed cells were lysed by ice-cold water (1:4) and mixed thoroughly.

Determination of lipid peroxide (Malondialdehyde)

Plasma malondialdehyde (MDA) concentration was determined using a lipid peroxide (malondialdehyde) assay kit (Bio-diagnostic. Giza, Egypt) according to the instructions from the manufacture. This method is based upon the reaction of thiobarbituric acid (TBA) with (MDA) in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product, then the absorbance of the resultant pink product was measured by spectrophotometer at 534 nm.

Determination of superoxide dismutase (SOD) activity

This assay relies on the ability of the superoxide dismutase enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. SOD activity in the erythrocyte lysate was determined using superoxide dismutase kit (Bio-diagnostic. Giza, Egypt) according to the instructions from the manufacture expressed as units/ml.

Determination of total antioxidant capacity (TAC)

Total antioxidant capacity was assayed exactly as mentioned in the instructions from the manufacture using a total antioxidant capacity (TAC) kit (Bio-diagnostic. Giza, Egypt).

Determination of catalase (CAT) activity

CAT activity in plasma was determined according to the manufacture instruction, using catalase assay kit (Bio-diagnostic. Giza, Egypt). This method is based upon the principle that catalase reacts with a known provided quantity of H_2O_2. The remaining H_2O_2 reacts with 3, 5-Dichloro-2-hydroxybenzene sulphonic acid (HDBS) and 4-aminophenazone (AAP) to form a chromophore with color intensity inversely proportional to the amount of catalase in the original sample. This color can be measured spectrophotometrically at 510 nm.

Determination of reduced glutathione (GSH)

Erythrocyte lysate GSH activity was measured spectrophotometrically using glutathione reduced kit (Bio-diagnostic. Giza, Egypt). It relies on the reduction of 5, 5' dithiobis (2- nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Orchidectomy of the engrafted testes

The gonads of rats that had undergone a transplant were surgically removed four weeks after transplant. Further measurements of glucose and insulin levels were done weekly in animals.

Immunofluorescence study

The excised gonads were frozen sectioned at 3 μm, fixed with 2-4% formaldehyde in PBS for 15 minutes at room temperature and rinsed with PBS for three times, 5 minutes each. The tissue was then incubated with ice-cold 100% methanol for 10 minutes followed by blocking the specimen in 5% of rat normal serum in PBS/Triton (BDH Laboratory Supplies Poole, England), for 60 minutes. The tissue was incubated overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-insulin 1:200 (Cell Signaling

Technology, Danvers, MA, USA), rabbit polyclonal anti-glucagon 1:200 (Cell Signaling Technology) and rabbit polyclonal anti-c-peptide 1:100 (Cell Signaling Technology). Subsequently, the tissue was incubated with the secondary antibody; polyclonal swine anti-rabbit immunoglobulin labelled with FITC (DakoCytomation, Glostrup, Denmark) at 22°C for two hours. The nuclei were counter-stained with DAPI (Invitrogen, UK). The negative controls were obtained by eliminating the step of the overnight incubation with the primary antibodies. Specimens were immediately examined and photographed using a fluorescence microscope and the blue filter (Olympus, Japan).

Immunohistochemical studies

For immunohistochemical analysis testes transplanted with differentiated clusters were processed to paraffin blocks. Sections were dewaxed in xylene and rehydrated in descending grades of alcohol (Ethanol anhydrous denatured, histological grade 100% & 95%). Microwave antigen retrieval in 10 mM sodium citrate at pH 6 was carried out for 20 minutes. Sections were subsequently covered with 3% hydrogen peroxide for 10 minutes. After sections were covered by blocking solution for one hour, the diluted primary antibody was applied to slides. Primary antibody concentrations were as follows: 1/100 monoclonal rabbit anti rat insulin (Cell Signalling Technology) and 1/100 rabbit anti rat C-peptide (Cell Signalling Technology). Primary antibodies were incubated overnight at 4°C. Avidin-Biotin complex reagent was applied to slides for 30 minutes in room temperature. For visualization, 3, 3' diaminobenzidine tetrahydrochloride (DAB) reagent was used to cover the slides for 5 minutes. Haematoxylin was used as a counter stain. Finally, sections were dehydrated, cover-slipped and examined. Positive results were identified as a brown colour at the antigen site.

Post-mortem evaluation

At the end of the observation period, the rats were euthanized and their pancreata were harvested and immunohistochemically stained for insulin and C-peptide as previously described.

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, 15.0 version). Differences were considered statistically significant when $P < 0.05$, and highly significant when $P < 0.001$.

RESULTS

Results are summarized in Table 1 and different figures.

Differentiation of hematopoietic-rich stem cells in culture

Figure 1 demonstrated the morphologic changes of hematopoietic-rich stem cells during differentiation. This indicated that we obtained compact formed islet-like clusters. The mean number of clusters on each cover slip was 500 ± 45.

Insulin release and c-peptide content in the differentiated cells

Figure 2 demonstrates that differentiated islet-like clusters when exposed to increasing glucose concentrations, they secrete increasing amount of insulin in a glucose concentration-dependent manner. The amount of insulin secreted in 25 mM glucose by 100 clusters was more than four times that of the 5.5 mM glucose concentration. When the glucose concentration was 25 mM, the content in the same number of clusters ranged between 150 and 190 ng/mL for insulin and 960 and 1038 pg/mL for C-peptide.

Transplantation of differentiated cell clusters in rats

The profiles of the four experimental groups relative to blood glucose and serum insulin levels throughout the observation period are expressed in Figure 3. Hyperglycemia found to be reversed in all diabetic rats under investigation that received differentiated insulin-producing cells. The blood glucose began to decrease within 2-3 days after transplantation. Throughout the observation period, the determined blood glucose levels were highly significantly lower ($P < 0.001$) and serum insulin levels were highly significantly higher ($P < 0.001$) in animals of the differentiated group than they were

Table 1. Effect of undifferentiated and differentiated HSCs transplantation on MDA, SOD, TAC, CAT and GSH as compared to diabetic and normal Control groups.

Parameters	Normal control group	Diabetic group	Undifferentiated HSCs group	Differentiated HSCs group
a) Oxidative: MDA (nmol/ml)	1.4 ± 0.1	3.6 ± 0.05*	3.59 ± 0.06*	1.5 ± 0.06●
b) Antioxidant: SOD (U/ml)	246.1 ± 3.8	162.1 ± 8.1*	158.5 ± 4.07*	246.4 ± 8.6●
TAC (mM/L)	1.29 ± 0.003	0.52 ± 0.01*	0.51 ± 0.014*	0.99 ± 0.006●
CAT (U/L)	244.9 ± 8.2	366.9 ± 3.9*	364.2 ± 3.08*	256.9 ± 1.7●
GSH (mg/dl)	9.6 ± 0.22	5.1 ± 0.16*	4.9 ± 0.16*	8.7 ± 0.12●

The results are expressed as Mean ± SEM. The mean is an average of 10 values.
*: highly significant ($P < 0.001$) compared to normal control.
●: highly significant ($P < 0.001$) compared to diabetic or undifferentiated HSCs group.

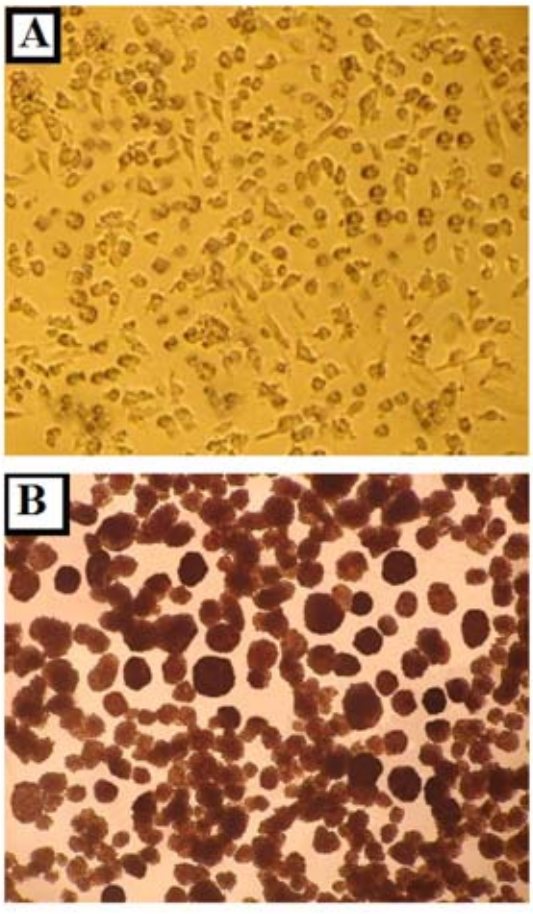

Figure 1. (**A**) Undifferentiated HSC one day after isolation (X 200). (**B**) Collected cell clusters after treatment with nicotinamide and exendin-4 for 7 days (X 100).

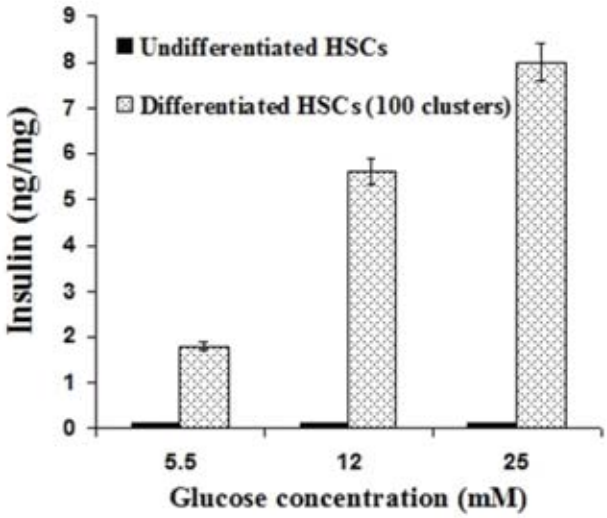

Figure 2. Insulin release in response to glucose stimulation as detected by immunosorbent assay. One hundred clusters treated *in vitro* with 25 mM glucose have secreted insulin whose mean value was nearly six times higher than that observed when clusters were treated with 5.5 mM glucose.

in animals in diabetic group and undifferentiated group. An increase in body weight and a reduction in the urine volume of the treated animals (differentiated group), also was noted. After orchidectomy, these rats became hyperglycemic again with a steep highly significant ($P < 0.001$) reduction in the serum insulin levels.

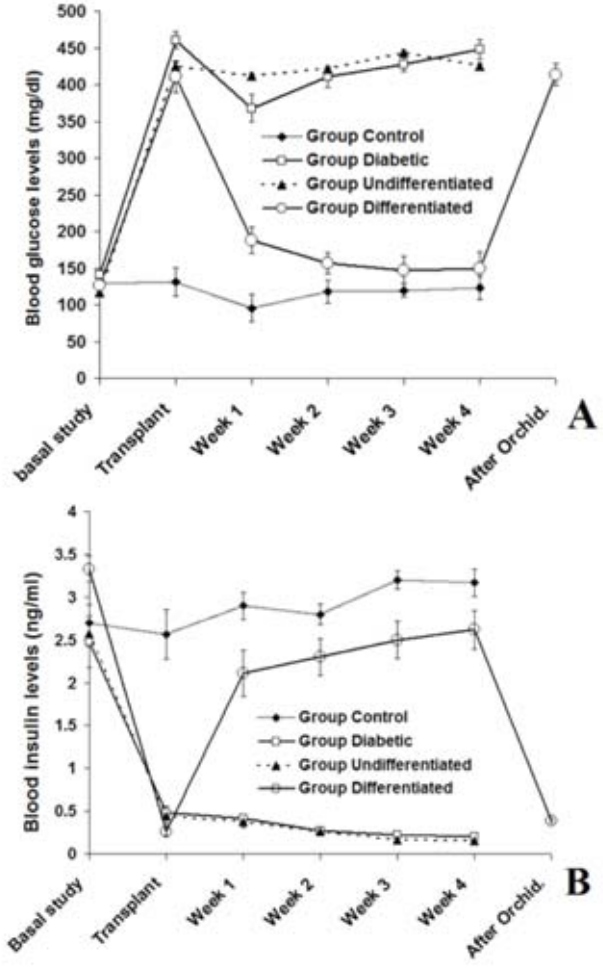

Figure 3. (**A**) Changes in the glucose concentration among the 4 studied groups: normal control group, diabetic group, undifferentiated group and differentiated group. (**B**) Changes in the insulin levels in clusters of rats that had undergone a transplant. Blood glucose began to decrease within two to three days. Throughout the observation period, the determined blood glucose levels were highly significantly decreased ($P < 0.001$) and serum insulin levels were highly significantly ($P < 0.001$) increased of animals in the differentiated group than they were in diabetic group animals or undifferentiated group animals.

Changes among different antioxidants as well as lipid peroxidation following implantation

The effect of undifferentiated and differentiated HSCs transplantation on MDA, SOD, TAC, CAT, and GSH as compared to diabetic and normal control groups is shown in Table 1.

In this study, the mean value of MDA content in plasma in untreated STZ-diabetic group (3.6 ± 0.05 nmol/ml) and undifferentiated HSCs group (3.59 ± 0.06 nmol/ml) exhibit a highly significant ($P < 0.001$) increase as compared to that of normal control animals (1.4 ± 0.1 nmol/ml). On the other hand, the mean value of plasma MDA content of differentiated HSCs rat group (1.5 ± 0.06 nmol/ml) revealed a highly significant ($P < 0.001$) decrease as compared with that of the untreated diabetic or undifferentiated rat group.

Table 1 demonstrates and summarizes the effect of undifferentiated and differentiated HSCs transplantation on antioxidant enzymes as compared to diabetic and normal control groups.

The mean value of erythrocyte lysate SOD activity in diabetic group (162.1 ± 8.1 U/ml) and in undifferentiated HSCs group (158.5 ± 4.07 U/ml) were significantly ($P < 0.001$) decreased as compared to that of normal control animals (246.1 ± 3.8 U/ml), while the mean value of differentiated HSCs rat group (246.4 ± 8.6 U/ml) was significantly increased ($P < 0.001$) as compared to the mean value of the diabetic or undifferentiated rat group. In addition, it is in the same range of the normal control group. The transplantation of differentiated HSCs resulted in an increase in SOD levels in blood.

Also, as apparent from Table 1, the mean value of plasma TAC of untreated diabetic rat group (0.52 ± 0.01 mM/L) and undifferentiated group (0.51 ± 0.014 mM/L), both revealed a high significant decrease ($P < 0.001$) as compared with that of normal control group (1.29 ± 0.003 mM/L). TAC levels of transplanted rats transplanted with differentiated cells (0.99 ± 0.006 mM/L) showed a highly significant increase ($P < 0.001$) as compared to that of diabetic or undifferentiated rat groups.

In this study, the mean value of plasma CAT activity in diabetic untreated group (366.9 ± 3.9 U/L) and undifferentiated group (364.2 ± 3.08 U/L) were significantly increased ($P < 0.001$) as compared with mean value of CAT activity of the normal control rat group (244.9 ± 8.2 U/L). In contrast, the plasma CAT activity of transplanted rats with differentiated cells (256.9 ± 1.7 U/L) was significantly decreased ($P < 0.001$) as compared to mean value of CAT activity in the diabetic or undifferentiated rat groups.

After estimation of reduced glutathione concentration in the four rat groups, the results showed that the mean value of erythrocyte GSH concentration in the untreated diabetic group (5.1 ± 0.16 mg/dl) and in the undifferentiated group (4.9 ± 0.16 mg/dl) exhibit a highly significant decrease ($P < 0.001$) as compared to the mean GSH concentration in the normal control group (9.6 ± 0.22 mg/dl). The mean value of GSH concentration in the differentiated group (8.7 ± 0.12 mg/dl) was significantly increased as compared with the diabetic or undifferentiated rat group; it is also in the same range obtained in the normal control group.

Immunohistochemistry and immunofluorescence studies

Histology of the testes of rats that received the differentiated clusters revealed that the cell aggregates were arranged within the interstitial tissue. There was no lymphocytic infiltrate within the testicular tissue or in the transplanted clusters. In addition, the transplanted clusters were positively stained for insulin and C-peptide by immunohistochemical and immunofluorescent staining (Figure 4&5).

In contrast to the findings in the normal pancreas, harvested pancreata from the treated animals showed atrophy of their islets and negative for insulin by immunohistochemistry staining (Figure 6).

DISCUSSION

Previous studies have shown that bone marrow-derived stem cells can be directed to become functionally insulin-producing cells [8, 15]. On the basis of this approach, we also succeeded in efficiently directing hematopoietic stem cells into insulin-producing cells which were able to maintain a stable *in vivo* glucose response in STZ-induced diabetic rats.

These cells were not only capable of insulin production but also of its release in a dose-dependent fashion according to the glucose concentration (Figure 2). Some investigators suggested that, part of insulin detected may have been derived from insulin added to the culture media in certain protocols or insulin present in serum. The stepwise increase in the insulin release as a function of the glucose concentration does not support these contentions. In addition, detection of C-peptide in the islet-like clusters, by immunofluorescence study as well as by chemical assay, indicates that insulin release was the result of endogenous synthesis.

Persistent hyperglycemia during diabetes showed to cause increased production of free radicals, especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation [16, 17, 18].

In our experiments, insulin-producing cells were obtained from adult bone marrow hematopoietic-rich stem cells and were grafted into the testes of inbred rats. Inbred rats were used to overcome any immunologically related issues. The rats that received the differentiated insulin-producing cells became euglycemic during all times of the experimental period for four weeks. This improvement in blood glucose was associated with a gain in body weight and reduction in urine volume. The diabetic rats that received undifferentiated cells showed no improvement in their diabetic status during the observation period (Table 1, Figure 3). This is evidence that there was no recovery or regeneration of the pancreatic islet cells during this experiment, which was confirmed by the negative staining for insulin in the harvested pancreata of the treated animals (Figure 6).

The testes as a site for transplant, was chosen for its accessibility and ease of orchidectomy for monitoring the biochemical changes following removal of the transplanted differentiated cells. Following orchidectomy, the previously euglycemic rats became hyperglycemic again with steep reduction in the serum insulin levels (Figure 3). Our results presented in different figures and in Table 1 showed evidence that HSCs provide pancreatic progenitor cells capable of differentiating into functioning endocrine hormone-producing cells i.e, insulin, which in turn is capable of treating diabetes when transplanted to chemically induced diabetic rats. The Immunoflurescence study of the removed testes revealed viable insulin as well as C-peptide staining cell aggregates. Furthermore, there was no evidence of a lymphocytic infiltration, which indicates a lack of immune response.

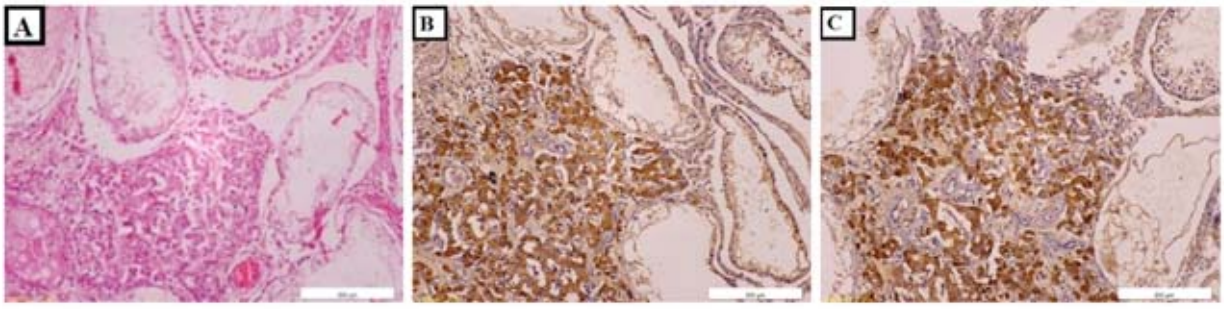

Figure 4. Histology of the grafted testes. **(A)** Hematoxylin and eosin staining of the engrafted clusters shows intact cells within the interstitial tissue. No lymphocyte infiltration was observed (X 200). **(B)** Immunohistochemistry for insulin expression of the engrafted clusters was positive (X 200). **(C)** Immunohistochemistry for C-peptide expression reveals that most cells of the grafted clusters were positive (X 200).

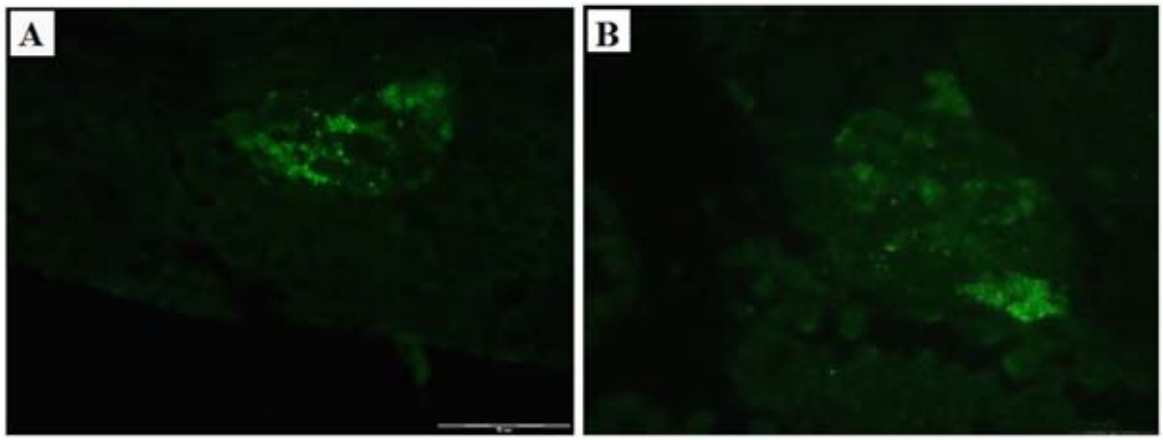

Figure 5. Histology of the grafted testes. **(A)** Immunofluorescent staining for insulin expression of the engrafted clusters was positive (X 200). **(B)** Immunofluorescent staining for C-peptide expression reveals that most cells of the grafted clusters were positive (X 200).

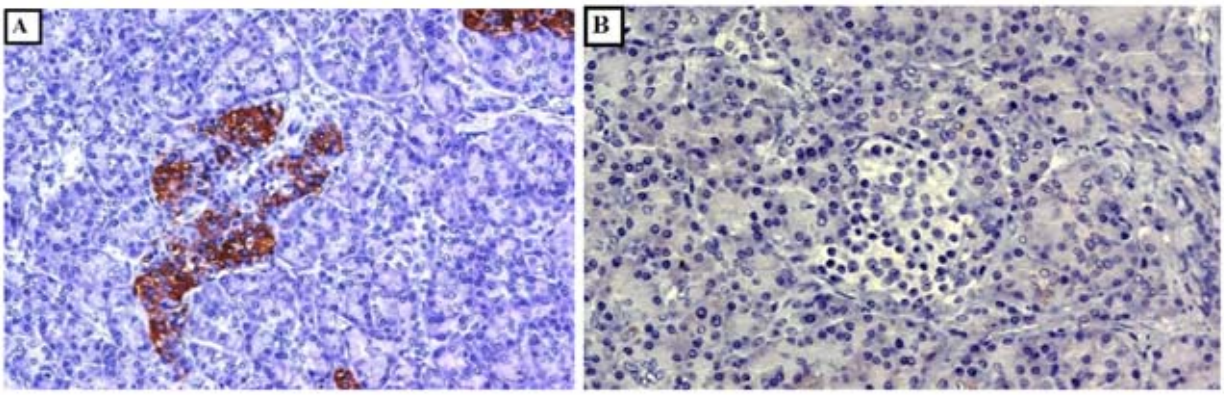

Figure 6. Immunostaining of rat pancreas. The islets from pancreas harvested from normal untreated rat stained positive for insulin (**A**, X 100). The islets from pancreas of diabetic rat treated with clusters were atrophic and negatively stained for insulin (**B**, X 100).

The present study is designed to examine biochemical changes that occur after transplantation of insulin producing clusters derived from bone marrow stem cells as a treatment modality for diabetes. To the best of our knowledge, it is the first study to confirm our hypothesis that this treatment modality would greatly attenuate this increase in oxidative stress due to diabetes, also reverse and eliminate the biochemical disturbance seen in oxidative defense system and modulate lipid peroxidation. The levels of MDA, CAT and TAC, also SOD and GSH were measured up to 4 weeks after transplantation of HSCs and were compared to that obtained from the diabetic group as well as normal control group animals (Table 1). The effects of HSCs transplantation to diabetic rats on antioxidants and lipid peroxidation were not reported before under same experimental conditions provided here.

The malondialdehyde is a short chain aldehyde and is an example of oxidative damage in cell membrane, lipoproteins and other lipid containing structure [19]. Researchers have reported a marked rise in the extent of lipid peroxidation in erythrocyte in uncontrolled diabetes in rats [20]. Lipid peroxidation in erythrocytes is known to cause decreased cell survival, altered membrane lipid asymmetry, hypercoagulation and increased adhesivity to endothelium [21]. In our present study, the mean values of MDA content in plasma of untreated diabetic group and treated diabetic animals with undifferentiated HSCs were found to be significantly increased as compared with that of the normal control group. Qujeq *et al.*, [22] indicated that MDA level is a marker of lipid oxidation, and was significantly increased in the erythrocytes of STZ-induced diabetic rats; this indeed is in agreement with our results. The observed increase in MDA content may be due to an increase in oxygen free radicals that could be due to either increased production or decreased destruction. Increased lipid peroxidation in diabetic rats was attributed to chronic hyperglycemia, which causes increased production of ROS due to autoxidation of monosaccharides, which leads to the production of superoxide and hydroxyl radical [23], which cause tissue damage by reacting with polyunsaturated fatty acids in membranes [24].

In differentiated group animals (Table 1), the mean plasma MDA showed no significant change in the mean values as compared with that of the normal control group. This indicates that transplantation of differentiated HSCs in diabetic rats is effective and efficient against the harmful action of lipid peroxidation.

On the other hand, SOD is the first line of defense against oxygen toxicity and its function is to provide a defense against the potentially damaging reactivity of superoxide. It catalyzes the dismutation of superoxide anion producing hydrogen peroxide [25]. In our study, the levels of erythrocyte lysate SOD in the diabetic non-treated group and undifferentiated group revealed a highly significant decrease as compared with that of the normal control group (Table 1). This is in agreement with others [20, 26]. Sindhu *et al.*, [27] also reported a decrease of the hepatic SOD activity in diabetic rats as compared to that of the control group. This decrease in SOD activity in the diabetic group may be due to increased level of superoxide anion radical. SOD may have an important role in combating this process, since it can catalyze the dismutation of two superoxide radicals into H_2O_2 [20]. So, the decreased SOD levels in diabetes may be due to its consumption in conversion of superoxide anions into H_2O_2 protecting the cell from a harmful effect of superoxide anions.

In our present study, the transplantation of differentiated HSCs into diabetic rats caused not only increasing, but also restoring of SOD levels. Our results (Table 1) showed highly significant SOD increase in the treated diabetic animals with differentiated HSCs as compared to the diabetic non-treated animals or to diabetic animals treated with undifferentiated HSCs. This indicates that transplantation of HSCs can increase highly significantly the levels of the free radical scavenging enzyme SOD, which indicates clearly the role of transplanted differentiated HSCs in providing not only insulin, but also provide an evidence of antioxidant and free radical scavenging characteristics of insulin generated by differentiated HSCs transplantation. Figure 3 show that, following orchidectomy of the engrafted testes which had undergone differentiated HSCs transplantation, the blood glucose levels immediately elevated

significantly (Figure 3A), concurrently with a high significant decrease in blood insulin levels (Figure 3B).

We demonstrated that, the mean value of erythrocyte lysate SOD activity in differentiated HSCs group was returned to normal following the transplantation of differentiated HSCs in the testes of diabetic rats (Table 1). Van Dam *et al.*, [28] reported that insulin treatment in diabetic rats restored the decreased hepatic SOD activity. This supports our finding. Our results are the first of its kind, which indicate that transplantation of differentiated HSCs in diabetic rats can restore SOD activity.

The highly significant increased CAT activity we demonstrated in both diabetic group and undifferentiated HSCs group as compared with the normal control group (Table 1), may be attributed to known oxidative stress of H_2O_2 formed in the diabetic state as CAT is considered as an inducible enzyme [29]. Oxidative stress mostly exists when free radical formation is in excess relative to protective antioxidants. It is worth mentioning that the balance between ROS production and antioxidant capacity is delicate [30]. The group of ROS includes superoxide (O_2^-), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen and hydrogen peroxide (H_2O_2). Consequently, by removing (H_2O_2) by an observed increase of CAT activity, would mostly and partly prevent the formation of ($OH^{\bullet}$). The reported increase of CAT in both groups would be to compete the increased oxidative stress by increased ROS which includes H_2O_2. This increase in free radicals and oxidative stress in the diabetic state was previously reported in STZ-induced diabetic rats [31]. They also reported significant increased erythrocyte catalase activity in STZ-diabetic rats when compared to control group. This indeed would support our findings of increased catalase activity in plasma. On the other hand, our novel reported observation of a highly significant decrease of plasma CAT activity in transplanted differentiated HSCs group compared to diabetic or undifferentiated HSCs group would strongly indicate that transplantation of differentiated HSCs reactivate the antioxidant defense mechanism and create an evidence to reverse serious side effect of excessive ROS in non-treated diabetes through reverse SOD and CAT plasma levels. This was not reported before under same experimental conditions as we described here.

In addition to enzymatic antioxidants, reduced glutathione was estimated as an example for non-enzymatic antioxidants. Reduced glutathione is an essential tripeptide with many important functions and is necessary for preventing lipid peroxidation and has the ability to detoxify hydrogen peroxide very effectively through glutathione peroxidase enzyme; it plays the role of intracellular radical scavenger so it is an important antioxidant molecule [32]. Our results indicate that the mean value of erythrocyte lysate GSH concentration in diabetic group and in diabetic undifferentiated HSCs group were significantly decreased compared with the mean value in the normal control group. Our findings agree with another study, Moussa, [33] that reported marked significant decreased levels of GSH in erythrocytes and plasma obtained from diabetic patients. He confirmed and explained the link between hyperglycemia and GSH depletion. So, hyperglycemia is therefore indirectly the cause of GSH depletion and as the GSH is an important antioxidant molecule, its depletion leads to increase of oxidative stress. On the other hand, we reported here that diabetic animals treated with differentiated HSCs showed a highly significant increase in levels of GSH as compared to diabetic or undifferentiated HSCs group, levels were found to be within a normal range when compared to the normal control group (Table 1). This clearly indicates that transplantation of differentiated HSCs increases insulin production and can ameliorate both blood glucose level (Figure 3 A&B) and also oxidative stress status consequently after our treatment modality. Following orchidectomy, results of the engrafted testes which underwent a differentiated HSCs transplantation, indicated the potential new evidence in restoring activation of oxidative defense in a reverse manner. Many researchers reported that hepatic GSH content was lower in STZ-diabetic rats, which were restored by insulin treatment [11, 34, 32]. This strongly supports our findings.

It has been suggested that TAC is capable of serving as a parameter to monitor diabetes of patients with type I diabetes mellitus [2]. Earlier reports [35] showed depletion in TAC estimated

in both the first and the second type of diabetic patients. In our present study, TAC levels were significantly decreased in the diabetic group as well as in the treated diabetic group with undifferentiated HSCs as compared to the normal control group (Table 1). This finding agrees with Celik *et al.*, [36], who reported blood TAC being significantly lower in STZ-diabetic wistar albino rats when they compared it to normal rats. This TAC decrease we have observed in our present study is logical in view of low activity of SOD and lower GSH concentrations in diabetic animal groups without treatment or treated with undifferentiated HSCs. The situation was reversed in case of diabetic animals treated with differentiated HSCs; we reported here highly significant increase in TAC level as compared to diabetic or undifferentiated HSCs group and found within a normal range of the normal control group. We propose that this increase in TAC is due to the marked elevation of levels of antioxidants which we reported in SOD and GSH in the same group, as elevations in the antioxidants are partially involved in elevation of TAC. Furthermore, elevated TAC in the differentiated group gives evidence that transplantation of differentiation HSCs may enhance the antioxidant status of STZ-diabetic rats under treatment, which is due to insulin production and its protective effect against oxidative stress. Work of Sindhu *et al.*, [27] showed that insulin treatment of STZ-diabetic rats normalized the activities and protein expression of all antioxidant enzymes. This finding supports our recent findings and conclusions. We suggest using TAC in monitoring diabetes of patients under treatment together with estimating other antioxidants as SOD, CAT and GSH. Earlier work [37] has suggested that measurement of TAC in diabetic patients can be a marker of glycemic control, which would strongly support our data and postulation.

Evidence from our results and demonstrated collected research information, support the hypothesis that free radicals play a significant role in causation and complications of diabetes mellitus due to alteration in the endogenous free radical scavenging defense mechanisms, which may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury. The use of antioxidant therapies in the treatment of several illnesses was also considered by us [38]. Also, another evidence and novel clinical application by El-Far *et al.*, 2007; 2009; 2009 [39, 40, 41] demonstrated that intravaginal sildenafil citrate tablets is safe antiabortive option in the treatment of threatened miscarriage in patients with a history of explained recurrent spontaneous miscarriage, and attributed this to the antioxidant effect of sildenafil citrate and its ability to attenuate greatly the increase in the oxidative stress in patients and by increasing the enzymatic antioxidant systems as SOD, CAT, and TAC. Most recently, El-Far *et al.*, [42], showed that cancer treatment with novel synthesized derivative of chitosan led to an augmentation of the antioxidant defense system.

CONCLUSION

The results of the present study show evidence that HSCs provide pancreatic progenitor cells capable of differentiating into functional endocrine hormone-producing cells. These cells were efficiently capable of treating diabetes when transplanted into diabetic rats by amelioration of the blood glucose level concurrently with a markedly significant increase in blood insulin levels as well as marked highly significant increase of antioxidant status. The differentiated cells also caused a significant decrease in MDA as biomarker of lipid peroxidation and thus attenuated oxidative stress.

We here reported novel evidence that transplantation of differentiated HSCs can correct and reverse the imbalance between reactive oxygen species and antioxidant defense in favour of antioxidant defense by restoring and augmenting its capacity as well as modulating lipid peroxidation. This may indicate and suggest a novel synergistic effect for possible use of antioxidant therapies for treatment of diabetes.

In conclusion, treatment of diabetic rats with transplantation of differentiated HSCs alleviates both hyperglycemia and oxidative stress in diabetic rats. These results encourage us to start with stem cells derived from human bone marrow mesenchymal stem cells to treat chemically induced diabetes in nude mice [43].

REFERENCES

1. Yajnik, C. S. 2001, Nutritional Rev., 59, 1.
2. Ryan, E. A., Lakey, J. R., Paty, B. W., Imes, S., Korbutt, G. S., Kneteman, N. M., Bigam, D., Rajotte, R. V. and Shapiro, A. M. 2002, Diabetes, 51, 2148.
3. Aejaz, H., Aleem, A., Parveen, N., Khaja, M., Lakshmi, M. and Habibullah, C. 2007, Transpl. Proc., 39, 694.
4. D'Ippolito, G., Diabira, S., Howard, G. A., Roos, B. A. and Schiller, P. C. 2004, J. cell. Science., 117, 971.
5. Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schiwartz, R. E., Kneene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T. Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A. and Verfaillie, C. M. 2002, Nature, 418, 41.
6. Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Shakis, S. J. 2001, Cell, 105, 369.
7. Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G. D. and Grain, B. 2003, Pro. Nat. Acad. Sci. USA, 100, 1364.
8. Oh, S. H., Muzzonigro, T. M., Bae, S. H., Laplante, J. M., Hatch, H. M. and Petersoen, B. E. 2004, Lab. Invest., 84, 607.
9. Opara, E. C. 2002, J. R. Soc. Health., 122, 28.
10. Abou-Seif, M. A. and Youssef, A. A. 2001, Clin. Chem. Lab. Med., 39, 618.
11. Mano, T., Shinohara, R., Nagasaka, A., Nakagawa, H., Uchimurak, K., Hayashi, R., Nakano, I., Tsugawa, T., Watanabe, F., Kobayashi, T., Fujiwara, K., Nakai, A. and Itoh, M. 2000, Metabolism., 49, 427.
12. Qujeq, D., Habibinudeh, M., Daylmkatoli, H. and Rezvani, T. 2005, Int. J. Diabetes & Metabolism., 13, 96.
13. Gabr, M. M., Sobh, M. M., Zakaria, M. M., Refaie, A. F. and Ghoneim, M. A. 2008, Exp. Clin. Transplant, 3, 236.
14. Gabr, M., El-Far, M., Ibrahim, R., El-Halawani, S., Ismail, A., Zakaria, M., Khater, S. and Ghoneim, M. 2012, Current Topics in Biochemical Research, 14(1), 45.
15. Jahr, H. and Bretzel, R. G. 2003, Transplant. Proc., 35, 2140.
16. Aragno, M., Tamagno, E., Gato, V., Brignardello, E., Parola, S., Danni, O. and Boccuzzi, G. 1999, Free Radic. Biol. Med., 26, 1467.
17. Bonnefont-Rousselot, D., Bastard, J. P., Jaudon, M. C. and Dellattre, J. 2000, Diabetes. Metab., 26, 163.
18. Ropertson, R. P. 2004, J. Biol. Chem., 279, 42351.
19. Girotti, A. W. 1998, J. Lipid. Res., 39, 1529.
20. Andallu, B. and Varadacharyulu, N. C. 2003, Clin. Chim. Acta., 388, 3.
21. Jian, S. K., Levine, S. N., Duett, J. and Hollier, B. 1990, Metabolism, 39, 971.
22. Qujeq, D., Aliakbarpour, H. R. and Kalavi, K. 1979, Diabetologia, 17, 371.
23. Wolff, S. P. and Dean, R. T. 1987, Biochem. J., 245, 243.
24. Das, S., Vasisht, S., Snehalata Das, N. and Srivastava, L. M. 2000, Curr. Sci., 78, 486.
25. Norman, I. and Kreinshy, N. I. 1992, Proc. Soc. Expt. Biol. Med., 200, 248.
26. Vural, H., Sabuncu, T., Arslan, S. O. and Aksoy, N. 2001, J. Pineal. Res., 31, 193.
27. Sindhu, R. K., Koo, J. R., Robert, C. K. and Vaziri, N. D. 2004, Clin. Exp. Hypertens., 26, 43.
28. Van Dam, P. S., Bravenboer, B., Van Asbeck, B. S., Van Oirschot, J. F., Marx, J. J. and Gispen, W. H. 1996, Eur. J. Clin. Invest., 26, 1143.
29. McCord, J. M., Keele, B. B. and Fredovich, I. 1971, Proc. Natl. Acad. Sci. USA., 68, 1024.
30. Valdivia, A., Pérez-Alvarez, S., Aroca-Aguilar, J. D., Ikuta, I. and Jordán, J. 2009, J. Physiol. Biochem., 65, 195.
31. Qujeq, D. and Rezvani, T. 2007, Int. J. Diabetes & Metabolism., 15, 22.
32. Peuchant, E. Carbonnean, M. A., Dubourg, L., Thomas, M. J., Perromat, A. and Clerc, M. 1994, Free Radic. Biol. Med., 16, 339.
33. Moussa, S. A. 2008, Rom. J. biophys., 18, 225.
34. Okawa, H., Ohishi, N. and Yagi, K. 1979, Anal. Biochem., 95, 351.
35. Satoh, K. 1978, Clin. Chim. Acta, 90, 37.
36. Celik, S. and Akkaya, H. 2009, J. Anim. Vet. Adv., 8, 1503.

37. Rahbani-Nobar, M. E., Rahbani-Pour, A., Rahbani-Nobar, M., Adi-Beig, F. and Mirhashemi, S. M. 1999, Medical Journal of Islamic Academy of Sciences., 12, 109.
38. Abou-Seif, M. A., El-Naggar, M. M., El-Far, M., Ramadan, M. and Salah, N. 2003, Clinica. Chemica. Acta., 337, 23.
39. El-Far, M., El-Sayed, I., El- Motwally, A., Hashem, I. and Bakry, N. 2007, Clin. Chem. Lab. Med., 45, 879.
40. El-Far, M., El- Motwally, A., Hashem, I. and Bakry, N. 2009, Clin. Chem. Lab. Med., 47, 1433.
41. El-Far, M., El-Sayed, I., El- Motwally, A., Hashem, I. and Bakry, N. 2009, J. Physiol. Biochem.,65, 175.
42. El-Far, M. A., El-Shal, M., Refaat, M. and El-Sherbiny, I. 2011, Drug Development and Industrial Pharmacy, 37, 1481.
43. Gabr, M., Zakaria, M., Refaie, A., Ismail, A., Abou-El-Mahasen, M., Ashamallah, S., Khater, S., El-Halawani, S., Ibrahim, R., Uin, G., Kloc, M., Calne, R. and Ghoneim, M. 2013, Cell Transplantation, 22, 133.