

## Comparison Between Bone Marrow Derived Mesenchymal Stem Cells And Hematopoietic Stem Cells In B-Islet Transdifferentiation

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**Abstract:** Bone marrow is an abundant source of adult stem cells that can differentiate into numerous cell types. It could provide a potentially unlimited source of islet like cells for transplantation and thus a promising therapy for diabetes mellitus. We studied the in vitro differentiation capacity and the efficiency of the therapeutic potential of human bone marrow derived hematopoietic stem cells (BM-HSCs) in comparison to bone marrow derived mesenchymal stem cells (BM-MSCs) into insulin-producing cells (IPCs) through culturing in high glucose medium containing exendin-4 and 20% fetal calf serum. Their differentiation capacity were assessed by insulin expression analysis using RT-PCR, intracellular insulin expression analysis using flow cytometry and the study of their therapeutic potential in alloxan-induced diabetic rats. We found that both BM-HSCs and BM-MSCs are capable of differentiation into IPCs as evidenced by positive insulin expression using RT-PCR. However, the mean value of insulin positive rate of differentiated BM-HSCs (40%) was significantly higher than that of differentiated BM-MSCs (19%). Also the transplanted differentiated BM-HSCs into the diabetic rats induced faster and better alleviation of fasting blood glucose level than the differentiated BM-MSCs. These results indicates better differentiation capacity of BM-HSCs into IPCs than BM-MSCs. In conclusion, bone marrow stem cells offer a promising tool in providing autologous transplants of IPCs for the treatment of diabetes. However, researches must continue to improve the differentiation capacity of these cells.

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**Key words:** Bone marrow, mesenchymal stem cells, hematopoietic stem cells, in vitro differentiation, exendin-4, insulin producing cells.

### 1. INTRODUCTION

Diabetes mellitus (DM) is a chronic disease with great social and economical impact. It affects nearly 285 million people worldwide, corresponding to 6.4% of the world's adult population. The number is expected to grow to 438 million by 2030, corresponding to 7.8% of the adult population<sup>(1)</sup>. Type 1 DM represents 10% of all diabetes cases. Because of the increase in obesity in developed countries, the prevalence of type 2 diabetes has been rising very rapidly, affecting children and adolescents.

Type 1 DM and advanced type 2 DM are caused by a progressive loss of functional pancreatic  $\beta$ -cell mass<sup>(2)</sup>. This loss is due to T cell-mediated autoimmune destruction of pancreatic  $\beta$  cells in type 1 DM. While insulin replacement represents the current therapy for type 1 diabetes and type 2 insulin dependant DM, its metabolic control remains difficult, as exogenous insulin cannot exactly mimic the physiology of insulin secretion, which is tightly regulated for maintaining the optimum level of blood glucose nor is safe as it often causes hypoglycemic coma<sup>(3,4)</sup>. Therefore, considerable interest has developed in finding

mechanisms to increase  $\beta$  cell mass by donor pancreatic or islet transplantation, in vitro-differentiated islet-like cells transplantation<sup>(2,4,5)</sup> or stimulating endogenous regeneration of islets<sup>(6)</sup>. Pancreatic or islet transplantation can provide exogenous insulin independence, but is limited by immune rejection and the limited number of organ donors<sup>(2,4,5)</sup>. Stem cells, which have the ability to differentiate into insulin-producing cells (IPCs) either in vivo or in vitro, would provide a potentially unlimited source of islet cells for transplantation and alleviate the major limitations of availability and allogeneic rejection. Therefore, the utilization of stem cells is becoming the most promising therapy for DM<sup>(2,4,5,6)</sup>.

Recent investigations on insulin producing cells (IPCs) regeneration revealed that in addition to primary source i.e., pancreatic beta cells, IPCs can be derived from several alternative sources of stem / precursor cells including embryonic stem cells, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), pancreatic stem cells,  $\beta$ - cells through dedifferentiation, expansion and redifferentiation, nuclear

reprogramming induced pluripotent stem cells, hepatocytes and acinar cells through transdifferentiation, and duct cells by neogenesis<sup>(4)</sup>.

Bone marrow (BM) is an abundant source of adult stem cells. BM-derived stem cells include HSCs, MSCs, and endothelial progenitor cells<sup>(7)</sup>. The aim of present work is to compare the differentiation capacity and the efficiency of the therapeutic potential (in alloxan-induced diabetic rats) of bone marrow-derived mesenchymal stem cells (BM-MSCs) and bone marrow-derived hematopoietic stem cells (BM-HSCs), when subjected to transdifferentiation potential in vitro.

## 2. MATERIAL AND METHODS

### I- Preparation of differentiated BM-MSCs and BM-HSCs

#### 1- Isolation and preparation of BM- MSCs and BM-HSCs

##### • Isolation of the mononuclear cell layer from human BM:

Under aseptic conditions, 10 ml of human BM was collected from the iliac crest of each individual after written informed consent into 15 ml Falcon tubes containing 200U/ml heparin (Biochrom AG, Germany). Then the mononuclear cell layer was separated by Ficoll density gradient centrifugation and then washed with phosphate buffered saline (PBS) followed by centrifugation for 10 minute at 1800 rpm at 20°C. and the precipitate then completed with 1 ml PBS to be tested for viability using trypan blue staining and counted by hemocytometer. BM samples were taken from 12 individuals and 6 of them were used to culture the MSCs and the other 6 were used to isolate the HSCs.

##### • Culturing and subculturing of MSCs:

Expansion of human MSCs was done using StemXVivo MSC Expansion Media supplied by R&D Systems. Here, Penicillin-Streptomycin solution (10,000:10,000)-Invitrogen containing 10,000U/ml of penicillin and 10,000µg/ml of streptomycin was added to the Media at a 1:100 dilution to prepare Completed StemXVivo MSC Expansion Media. Therefore the final concentration of penicillin was 100U/ml and of streptomycin 100µg/ml.

BM cells ( $3.5 - 4.0 \times 10^5$ ) were cultured in 20 ml of the pre-warmed completed media in a T75 flask at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Every three days spent media were discarded and replaced with 20 ml of prewarmed completed Media. When cells became 80 - 90% confluent, they were subcultured in 20 ml of the prewarmed

completed Media for each T75 flask, after detaching the cells with 0.25% Trypsin and 0.02% EDTA for 5 minutes at 37°C, centrifugation and counting and testing the cells for viability. Cells took about 2-3 weeks to reach 80-90% confluence and multiple passages were done.

##### • Isolation of BM-HSCs:

Cells of the mononuclear cell layer were cultured in Low glucose Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (All provided by Invitrogen). After 3 days of incubation, non-adherent cells were collected and washed with fresh serum-free medium and counted.

#### 2- Flow cytometric analysis for cell surface and intracellular markers of BM-MSCs and BM-HSCs

Analysis of cell surface molecules (CD34, CD44) and intracellular molecules (Oct 3/4) was performed on stem cell preparations to assess purity. The cells were incubated at room temperature for 20 minutes with monoclonal antibodies labeled with fluorescein isothiocyanate (FITC) against one of CD34 or CD44 (Beckman Coulter, France) and acquired onto FACSCalibur (Beckman Coulter, USA). Also the cells were incubated for 30 minutes with phycoerythrin (PE)-conjugated anti-human/mouse Oct 3/4 monoclonal antibodies after fixation and permeabilization (R&D Systems) and acquired onto FACSCalibur (Beckman Coulter, USA).

#### 3- In vitro differentiation of BM-MSCs and BM-HSCs into functional IPCs

BM-MSCs with 80% confluence and BM-HSCs were induced to differentiate into insulin producing cells as follows: The cells were cultured (37°C, 5% CO<sub>2</sub>) for 2 weeks in basic medium composed of high glucose (25mmol/L) Dulbecco's modified Eagle's medium (HG-DMEM), 20% fetal calf serum (FCS) (Invitrogen), 10 nmol/l exendin-4 (Sigma-Aldrich), amphotericin B as antimycotic (5µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Invitrogen). (modified from the technique of Tang and associates)<sup>(8)</sup>.

## II - Functional assessment of differentiated BM-MSCs and BM-HSCs

### 1- Detection of insulin gene expression by RT-PCR

To determine whether the BM- MSCs and BM-HSCs had undergone pancreatic differentiation, gene expression profiles of insulin for both undifferentiated and differentiated BM-MSCs and BM-HSCs were assessed using RT-PCR.

- **RNA extraction:**

Total RNA was extracted from the undifferentiated and differentiated BM-MSCs and BM-HSCs using Gene GET™ RNA purification Kit supplied by Fermentas, Germany, according to the manufacturer instructions. 50 µl of total RNA was eluted. Total RNA was quantified by UV spectroscopy and stored at - 20°C for 1 day until further processing.

- **Two step RT PCR:**

A) *cDNA synthesis:* It was done using RevertAid™ H Minus First Strand cDNA Synthesis Kit. 1 µg of total RNA was mixed with 1µl of Oligo(dT)18 primer and completed to 12µl by nuclease free water then incubated at 65°C for 5 min. and put on ice. This was followed by adding 4µl of 5X reaction buffer, 1µl of RNase inhibitor, 2µl of dNTPs and 1µl of reverse transcriptase. The following mix with a total volume of 20µl was incubated at 42°C for 60 min. then at 70°C for 5 min. to obtain cDNA.

B) *PCR:* Amplification was done using Dream Taq Green PCR Master Mix (2x) supplied by Fermentas, Germany. Gene β-actin was used as positive internal control. Negative control composed of nuclease free water instead of cDNA was used. The PCR mix contained 25 µl of Taq PCR master Mix 2x, 2.5 µl (0.5 µM) of each primer, 2 µl of the cDNA and 18 µl of nuclease free water to reach a final volume of 50 µl. Amplification was done according to the following program: initial denaturation at 95°C for 3 min., 35 cycles of denaturation at 95°C for 1 min., annealing for 1 min. and extension at 72°C for 2 min., followed by final extension at 72 °C for 10 min. then hold at 4 °C. The annealing temperature for insulin program was 56 °C, while that for β actin program was 51 °C.

The primers sequences for amplification of insulin mRNA were as follows; the forward primer GCA GCC TTT GTG AAC CAA CA and the reverse primer GTT GCA GTA GTT CTC CAG GTG, while that for β-actin was as follows; the forward primer GTC AGG TCA TCA TCG GCA AT and the reverse primer AGA GGT CTT TAC GGA TGT CAA C GT<sup>(9)</sup>.

- **Agarose gel electrophoresis:**

10 µl of each amplified DNA & 50bp DNA ladder were separated on 2% agarose gel containing 0.3 µg/ml of ethidium bromide. The bands were visualized using UV transilluminator (254nm) & photographed.

## 2- Analysis of insulin expression by flow cytometry

Differentiated BM-MSCs and BM-HSCs (6 samples for each as the cultures

### Statistical analysis

belonging to the same patient were collected in one tube) were tested for differentiation by quantitating the cells expressing cytoplasmic insulin through flow cytometric analysis using Anti-human/bovine/mouse Insulin-Allophycocyanin Monoclonal Antibody supplied by R&D Systems. Isotypematched antibodies served as controls for autofluorescence. The simultaneous fixation/permeabilization procedure was used. Measurement of positive insulin rate was done on FACSCalibur (PowerMacintosh, USA). Also, the corresponding undifferentiated BM-MSCs and BM-HSCs (6 samples for each) were tested for differentiation. The Mean values was calculated for the results of the undifferentiated and differentiated BM-MSCs and BM-HSCs.

## 3- Transplantation of differentiated bone marrow- derived stem cells to alloxan – induced diabetic rats

- **Experimental animals study groups:**

Thirty female rats with weight ranging from 55-85 gm were obtained from the animal house of the faculty of medicine-Cairo university. They were divided into 2 major groups : 1- Group I (control group); including 5 healthy rats. 2- Group II (Alloxan induced-diabetic group); including 25 rats [5 rats received no treatment, 10 rats received intravenous injection of differentiated BM-MSCs into the tail vein ( $3 \times 10^6$  cells/rat) and 10 rats received intravenous injection of differentiated BM-HSCs into the tail vein ( $3 \times 10^6$  cells/rat)]. Care of animals was in accordance with the institutional guidelines.

- **Induction of DM:**

The rats were fasted for 24 h and blood was collected for baseline glucose determination. Fresh solution of alloxan monohydrate (LOBA CHEMIE, India) for DM induction was prepared just prior to injection by dissolving it in normal saline. Then it was given intraperitoneal to the rats at a dose of 80 mg/kg<sup>(10)</sup>. After alloxan Injection, fasting blood glucose (FBG) levels were measured every morning. DM was confirmed by the presence of continuous fasting hyperglycemia about one week following alloxan injection. FBG levels were measured using Roche ACCU-CHEK glucose

- **Assessment of diabetic rats response to differentiated BM-MSCs and BM-HSCs Transplantation :**

This was done by measuring the FBG level every 3 days in the normal rats, diabetic rats without treatment and in the rats receiving differentiated cells. Then the mean FBG levels was calculated for group I and subgroups of group II of rats.

The collected data were computed and statistically analyzed using SPSS version 9 software for windows (SPSS inc., Chicago). Quantitative values are expressed as mean  $\pm$  S.D and were compared using Student's t-test and paired t test. P value  $<0.05$  was considered a significant.

### 3. RESULTS

#### I- Assessment of cell viability :

The viability of the cells was confirmed by trypan blue exclusion. The live cells were not stained and the dead cells were stained blue (Figure 1).

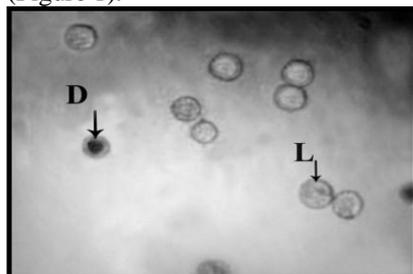


Figure 1: A photomicrograph of mononuclear cells after separation. L, live cells (non-stained); D, dead cells (stained). (Trypan blue x 1000) .

#### II-Morphological changes of the undifferentiated and differentiated BM-MSCs and BM-HSCs

##### 1-Morphological changes of expanded undifferentiated BM-MSCs

The primary culture of BM-MSCs was examined under inverted microscope showing the change of the rounded mononuclear cells into fibroblast like cells (Figure 2-1).

##### 2- Morphological appearance of undifferentiated BM- HSCs

Examination under the inverted microscope revealed crowded rounded cells (Figure 2-2).

##### 3- Morphological changes of differentiated BM- MSCs and BM-HSCs

Examination under the inverted microscope revealed cell clusters that look like islet like structure (Figure 2-3).

#### III-Phenotype characteristics of the undifferentiated BM-MSCs and BM-HSCs

##### 1- Phenotype characteristics of expanded undifferentiated BM- MSCs

Flow cytometric analysis of the immunophenotype of the MSCs showed that these cells expressed high levels of CD44 and were negative for CD34 cell marker. Oct 3/4 showed positive results indicating that they were stem cells (Figure 3-1,2).

##### 2- Phenotype characteristics of undifferentiated BM- HSCs

Flow cytometric analysis of the immunophenotype of the HSCs showed that

these cells expressed high levels of CD34 and were negative for CD44 cell marker indicating that BM-HSCs were isolated (Figure 3-3).

#### IV- Functional assessment of differentiated BM-MSCs and BM-HSCs

##### 1- Detection of insulin gene expression by RT-PCR

As illustrated in Figure 4, no expression of insulin was detected in undifferentiated BM-MSCs or BM-HSCs. On the other hand, insulin expression was detected in the differentiated BM-MSCs and BM-HSCs. As a positive internal control, the expression of  $\beta$ -actin gene was detected indicating our experimental system being intact.

##### 2- Analysis of insulin expression by flow cytometry

The Flow cytometric analysis quantitating cells expressing cytoplasmic insulin revealed that the insulin positive rates of the 6 samples of the undifferentiated BM-HSCs were 0.3%, 0.6%, 1.0%, 0.5%, 0.8% and 0.7% with mean value  $\pm$  SD of  $0.65\% \pm 0.24$ . It also revealed that the insulin positive rates of the 6 samples of differentiated BM-HSCs were 37%, 42%, 40%, 38%, 44% and 39% with mean value  $\pm$  SD of  $40\% \pm 2.61$ . On the other hand, the insulin positive rates of the 6 samples of undifferentiated BM-MSCs were 0.4%, 0.9%, 1.1%, 0.5%, 0.6% and 1.0% with mean value  $\pm$  SD of  $0.75\% \pm 0.29$ . However, those of the 6 samples of the differentiated BM-MSCs were 21%, 23%, 18%, 16%, 19%, 17% with mean value  $\pm$  SD of  $19\% \pm 2.61$  (figure 3-4, 5 & 6) .The difference between insulin positive rates of the differentiated BM-HSCs and the differentiated BM-MSCs was significant ( $P < 0.05$ , student t test = 13.95). Also the difference between the differentiated and undifferentiated cells was significant ( $P < 0.05$ , paired t test = 38.9 for BM-HSCs and paired t test = 16.9 for BM-MSCs). However, the difference between the undifferentiated BM-MSCs and BM-HSCs was non- significant ( $P > 0.05$ , student t test =0.65)

##### 3- Transplantation of differentiated bone marrow- derived stem cells to alloxan-induced diabetic rats

Measurement of blood glucose level of the diabetic rats revealed alleviation of hyperglycemia by the transplanted differentiated BM-MSCs and BM-HSCs. The mean FBG level of the diabetic rats that received differentiated BM-HSCs was 407.3 mg/dl just before transplantation. It started to decrease at day 3 post transplantation and reached minimum of 178.9 mg/dl at day 15 then it started to rise again at day 18 and reached to the pre transplantation level at day 30. On the other hand, The mean FBG level of the

diabetic rats that received differentiated BM-MSCs was 399.9 mg/dl just before transplantation. It started to decrease at day 6 post transplantation and reached minimum of 236.7 mg/dl at day 18 then it started to rise again at day 21 and reached to the pre transplantation level at day 27. On the other hand the diabetic rats maintained high FBG throughout the study with 3 rats died at days 18, 21, 27 respectively. The control maintained normal blood glucose throughout the study

The mean FBG levels of all the diabetic rats with or without stem cell transplantation were significantly higher than those of the control group ( $P < 0.05$ ). Moreover, the mean FBG levels of the diabetic rats with stem cell transplantation were significantly

lower than the diabetic rats without stem cell transplantation ( $P < 0.05$ ). In addition, the mean FBG level of the diabetic rats with BM-HSCs transplantation was significantly higher than that with BM-MSCs transplantation just before transplantation, but was significantly lower after transplantation except when it returned back to the pre transplantation level where it was significantly higher ((Figure 5 & Table 1).

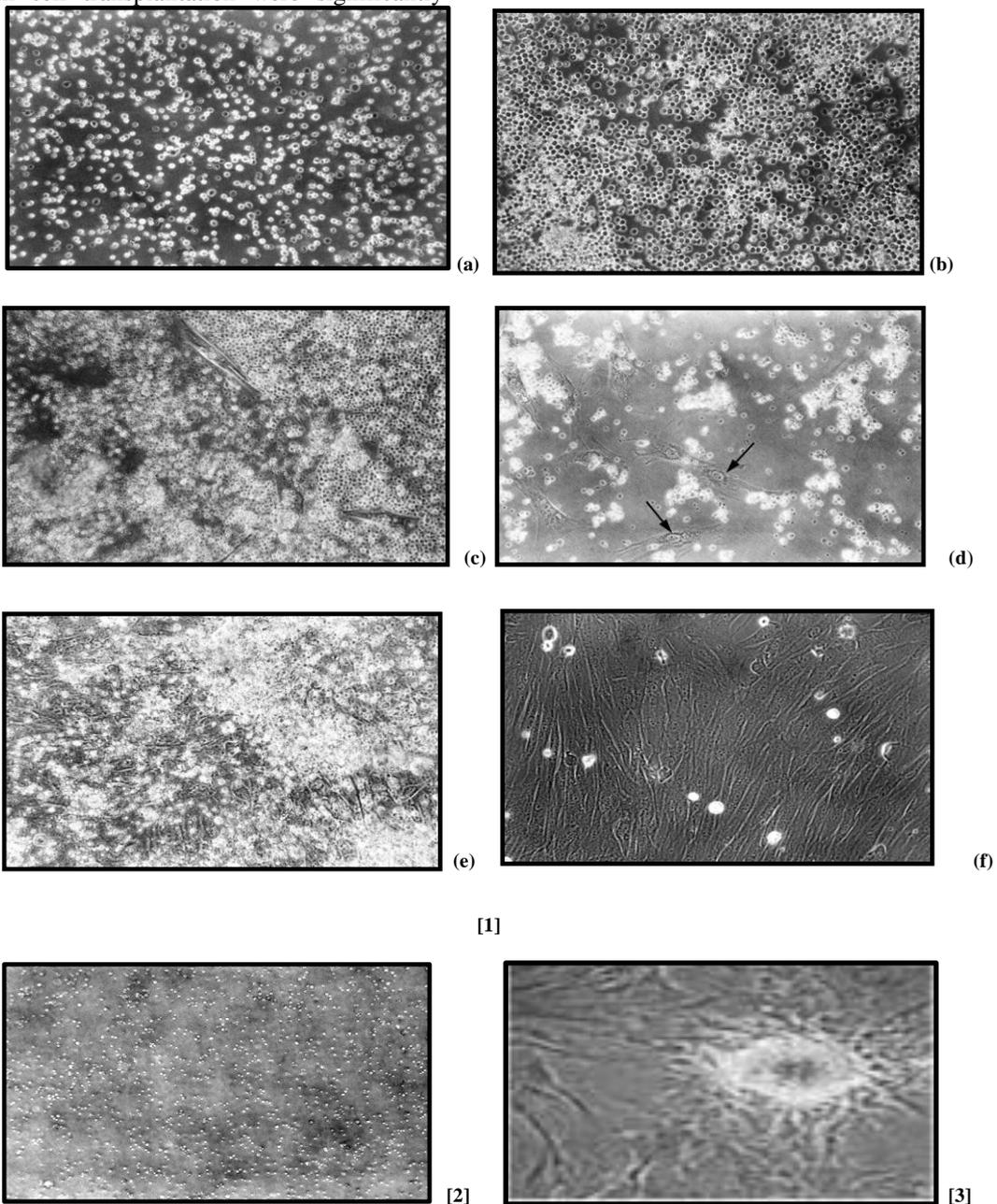


Figure (2):

[1] Inverted microscopic pictures of a primary culture of BM-MSCs x200 . (a) On day one of isolation and culture, showing cultured cells crowded and variable in size and shape. Most of the cultured cells appear rounded. (b) On day four of isolation and culture, showing crowded cultured cells having variable size and shape. Notice, some cells showing evidence of changing their shape and start to form processes. (c) On day seven of isolation and culture and before removal of the supernatant and changing the media showing adherent cells with long processes partially masked by crowded cultured cells. (d) On day seven of isolation and culture and after removal of the supernatant and changing the media showing the appearance of star-shaped cells with long processes. (e) On reaching about 80-90 % confluence before removal of the supernatant and changing the media showing the appearance of confluent adherent cells that are occasionally masked by non-adherent cells. (f) On reaching about 80-90 % confluence after removal of the supernatant and changing the media showing the appearance of confluent adherent cells consisting of dense population of spindle-shaped fibroblast-like cells with long processes that appear very close to each other.

[2] Inverted microscopic picture of the isolated BM-HSCs x100. It shows crowded cultured cells, that are variable in size and shape and most of them appear rounded.

[3] Inverted microscopic picture of the differentiated BM derived stem cells showing islet like cluster.

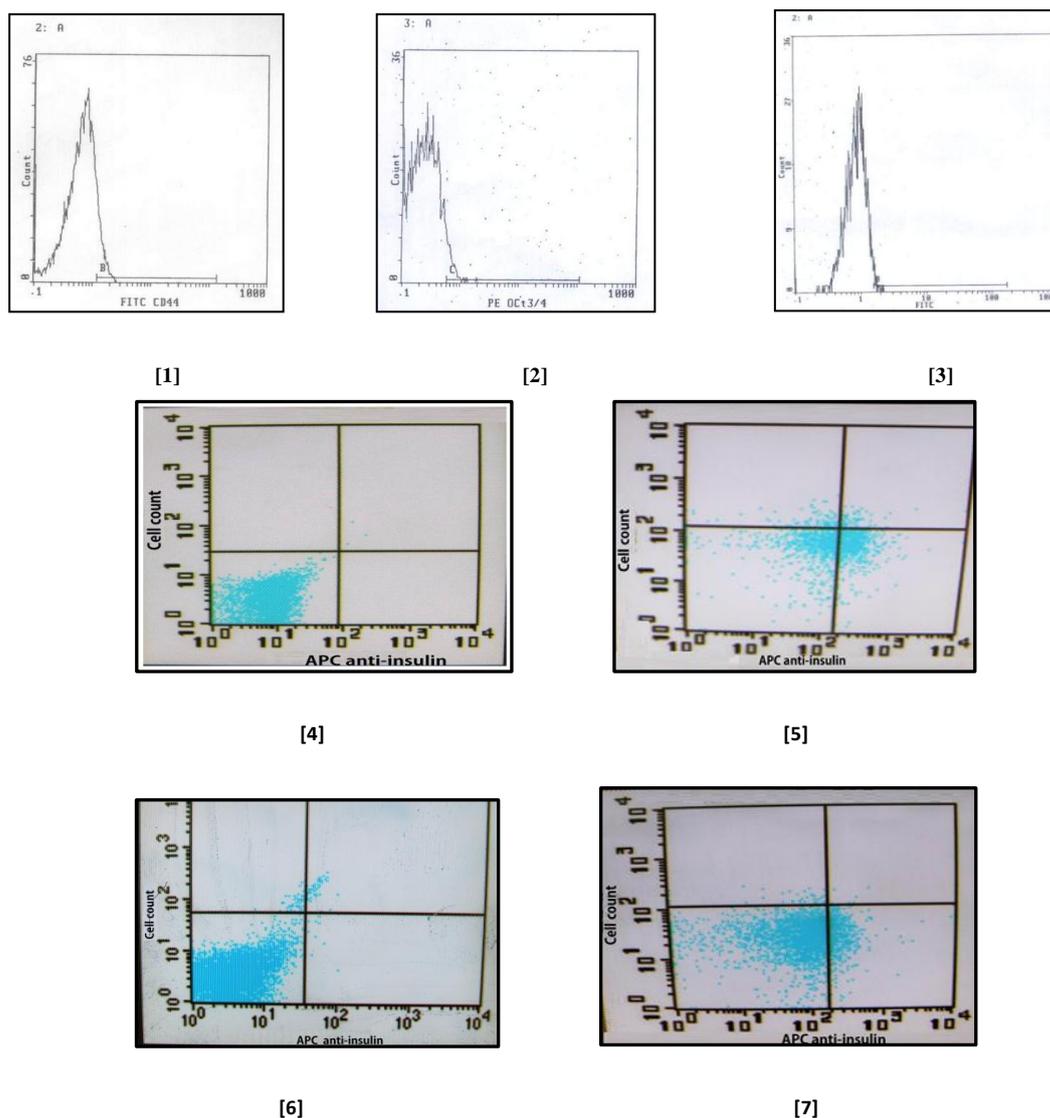


Figure 3: 1- Flow cytometric analysis of BM-MSCs, showing that MSCs expressed high levels of CD44  
 2- Flow cytometric analysis of BM-MSCs, showing that MSCs expressed oct 3/4  
 3- Flow cytometric analysis of BM-HSCs, showing that they expressed high levels of CD34  
 4 Flow cytometric analysis of undifferentiated BM-HSCs showing insulin positive rate of 0.5%  
 5- Flow cytometric analysis of differentiated BM-HSCs showing insulin positive rate of 40%.  
 6- Flow cytometric analysis of undifferentiated BM-HSCs showing insulin positive rate of 1.1%  
 7- Flow cytometric analysis of differentiated BM-MSCs showing insulin positive rate of 19%.

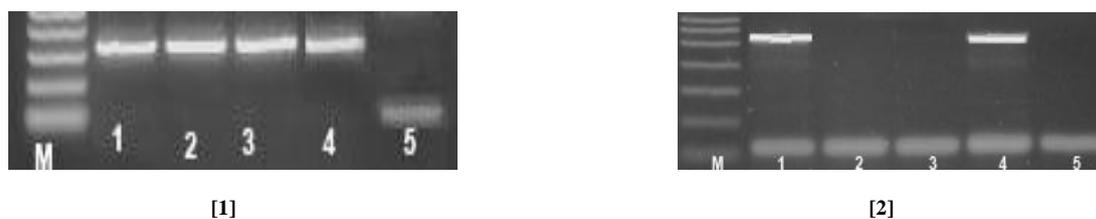


Figure 4: [1] The gel electrophoresis of the RT-PCR products of the  $\beta$  actin gene which are 160 bp in length indicating our experimental system being intact. Lane 5 shows the negative control showing absence of the 160 bp band Lane M shows 50 bp DNA marker. [2] The gel electrophoresis of the RT-PCR products of the insulin gene which are 263 bp in length. Lanes 1&4 show positive insulin expression of the differentiated BM-HSCs & BM-MSCs respectively, while Lanes 2,3&5 show negative insulin expression of the undifferentiated BM-HSCs, undifferentiated BM-MSCs & negative control respectively. Lane M shows 50 bp DNA marker.

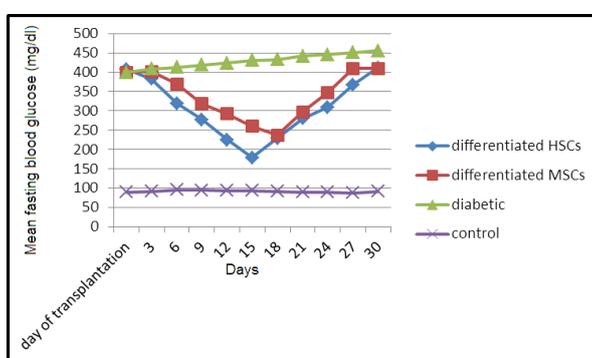


Figure (5): Showing the Changes in the mean fasting blood glucose level in the control rats, diabetic rats without treatment & diabetic rats receiving either differentiated BM-HSCs or BM-MSCs.

Table (1): Mean  $\pm$  SD and P values of FBG level in all groups compared with each other

| days group                                 |          | D0          | D3          | D6         | D9         | D12        | D15         | D18        | D21        | D24         | D27        | D30        |
|--|----------|-------------|-------------|------------|------------|------------|-------------|------------|------------|-------------|------------|------------|
| Control                                    | Mean     | 89.8        | 92.2        | 96.2       | 95.2       | 93.6       | 93.6        | 91.6       | 89.4       | 90.2        | 87.8       | 91.8       |
|  | $\pm$ SD | $\pm 10.66$ | $\pm 10.89$ | $\pm 5.93$ | $\pm 7.64$ | $\pm 6.43$ | $\pm 10.83$ | $\pm 7.92$ | $\pm 8.50$ | $\pm 7.19$  | $\pm 8.17$ | $\pm 5.63$ |
| Diabetic without treatment                 | Mean     | 399.6       | 408.2       | 412.8      | 418.6      | 423.6      | 430.2       | 432.3      | 442.3      | 445.3       | 451        | 455.5      |
|  | $\pm$ SD | $\pm 3.51$  | $\pm 5.4$   | $\pm 4.21$ | $\pm 4.34$ | $\pm 4.28$ | $\pm 5.89$  | $\pm 4.65$ | $\pm 5.69$ | $\pm 4.16$  | $\pm 2.83$ | $\pm 2.21$ |
| Diabetic receiving BM-MSCs transplantation | P        | <0.05       |             |            |            |            |             |            |            |             |            |            |
|  | Mean     | 399         | 401.9       | 369.5      | 318.8      | 292.2      | 260.6       | 236.7      | 297.5      | 347.8       | 409.9      | 409.6      |
|  | $\pm$ SD | $\pm 5.88$  | $\pm 5.88$  | $\pm 7.23$ | $\pm 7.49$ | $\pm 9.19$ | $\pm 7.3$   | $\pm 6.7$  | $\pm 7.88$ | $\pm 6.86$  | $\pm 5.49$ | $\pm 6.8$  |
| Diabetic receiving BM-HSCs transplantation | P        | <0.05       |             |            |            |            |             |            |            |             |            |            |
|  | P1       | <0.05       |             |            |            |            |             |            |            |             |            |            |
|  | Mean     | 407.3       | 382         | 319.5      | 277.6      | 225.3      | 179.8       | 229.1      | 280.3      | 309.1       | 367.8      | 412.4      |
|  | $\pm$ SD | $\pm 8.49$  | $\pm 8$     | $\pm 7.26$ | $\pm 7.71$ | $\pm 8.49$ | $\pm 7.35$  | $\pm 8.25$ | $\pm 7.51$ | $\pm 30.13$ | $\pm 8.34$ | $\pm 5.6$  |
| Diabetic receiving BM-HSCs transplantation | P        | <0.05       |             |            |            |            |             |            |            |             |            |            |
|  | P1       | <0.05       |             |            |            |            |             |            |            |             |            |            |
|  | P2       | <0.05       |             |            |            |            |             |            |            |             |            |            |

P=probability versus control

P1= probability versus diabetic rats without treatment

P2= probability versus diabetic rats receiving BM-MSCs transplantation

#### 4. DISCUSSION

BM is as a safe and important abundant source for large quantities of adult stem cells that are easily obtainable from each patient and thus easily accessible as an autograft, allowing potential circumvention of allograft rejection<sup>(7,11)</sup>. Also BM is an attractive source of Stem cells avoiding the ethical issues surrounding embryonic stem cell research<sup>(12)</sup>. BM-derived stem cells are capable of proliferation and differentiation into multiple, although not all types of, cell lineages<sup>(2,7)</sup>.

BM includes at least two distinct populations of cells, both with high plasticity: hematopoietic CD34<sup>+</sup> stem cells and mesenchymal CD34<sup>-</sup> stem cells<sup>(2)</sup>. BM-MSCs are of great multiplication potency. It was reported that BM-MSCs could be expanded in culture for more than 60 doublings, with cell-doubling time of 48-72 h<sup>(13)</sup>. In contrast, undifferentiated HSCs has a limited ability to grow and expand in vitro. However various expansion techniques have been developed for such purpose based on either the use of recombinant cytokines or transfer of transcription factor genes, trying to overcome the problem of decline of population capacity of HSCs in long term ex vivo culture<sup>(14,15,16)</sup>. In our study we used StemXVivo MSC Expansion Media provided by R&D Systems for ex vivo expansion of BM-MSCs. However we did not expand the BM-HSCs as the isolated number of HSCs from the BM were enough.

Several in vitro studies have shown that BM-derived stem cells could be reprogrammed to become functionally IPCs under certain culture conditions<sup>(8,17,18,19)</sup>. The mammalian pancreas develops from the embryonic foregut of the endodermal layer. Differentiation into IPCs is induced by a cascade of gene events controlled by several transcription factors such as PDX-1 and PAX-6<sup>(20)</sup>. Induction of bone marrow stem cells to differentiate into insulin-producing cells is similar to that process. In our study, BM-HSCs and the expanded BM-MSCs were induced to differentiate into IPCs through culturing in a glucose-rich medium (25mmol/l), exendin-4, and 20% FCS.

Supporting the importance of high glucose medium in the transdifferentiation of BM derived stem cells into IPCs is a study showing higher number and considerably larger size of islet like clusters obtained by culturing BM-HSCs in high glucose medium (25mmol/l) versus culturing them in low glucose medium (5.5 mmol/l)<sup>(18)</sup>. Another study reported that insulin secretion was 6

times higher in BM-HSCs cultured in high glucose medium (25mmol/l) compared to those cultured in low glucose medium (5.5 mmol/l)<sup>(19)</sup>. Moreover, it was found that there was no significant release of insulin from control BM-MSCs cells cultured in low glucose concentrations (5.5 mmol/l) even in the presence of exendin. This is in contrast to those cultured in high glucose concentrations (23 mmol/l) which expressed multiple genes characteristic of endocrine cell development including insulin<sup>(8)</sup>.

It was reported that glucose is a growth factor for  $\beta$ -cell replication in vitro and in vivo at 20- to 30-mM concentrations. Glucose has been shown to increase the insulin content in cells derived from embryonic stem cells at a 5-mM concentration. In effect, glucose could have a dual role. In the proliferation phase, the high glucose content may support the extra energy needed for cell division. In the differentiation stage, it could modulate specific gene programs linked to glucose sensing and insulin secretion<sup>(21,22)</sup>.

Exendin-4 is a glucagon-like peptide-1 (GLP-1) receptor agonist. GLP-1 receptor agonists could stimulate  $\beta$  cell proliferation and decrease  $\beta$  cell apoptosis<sup>(23)</sup>.

The importance of high concentrations of FCS was evidenced by a study reporting intense cell propagation and the formation of numerous cell clusters of 100-200 $\mu$ m in diameter similar to that of pancreatic islets from BM-MSCs in media containing 20% FCS within 4 -5 days. This is in contrast to 1% FCS containing media in which clusters were not formed and 90% of the cells died within 5 days<sup>(17)</sup>. However, it appears that the high concentration of FCS does not provoke insulin secretion by the cell clusters, since homogenates for cells cultured in 20% FCS were negative for insulin.

Our study confirmed the in vitro generation of functional IPCs from BM-MSCs and BM-HSCs by insulin gene expression analysis by RT-PCR, insulin expression analysis by flow cytometry and in vivo transplantation of differentiated BM-MSCs and BM-HSCs into alloxan induced-diabetic rats via the tail vein.

Insulin gene expression analysis by RT-PCR revealed positive insulin expression in the differentiated compared to negative results in the undifferentiated BM-MSCs and BM-HSCs indicating differentiation. These results are in agreement with other studies on BM-HSCs<sup>(18,21)</sup> and BM-MSCs<sup>(8,22,24,25)</sup>.

However, on flow cytometry analysis quantitating the cells expressing cytoplasmic insulin, BM-HSCs showed more

differentiation capacity into IPCs than BM-MSCs as the mean value of insulin positive rate of differentiated BM-HSCs was 40% and that of differentiated BM-MSCs was only 19%. Similar result about the differentiation capacity of BM-MSCs was shown by another study<sup>(22)</sup>. However, to our knowledge there were no previous reports about the differentiation capacity of BM-HSCs using flow cytometric analysis of insulin positive cells. Moreover our study revealed small percentages of insulin positive rates for the undifferentiated BM-HSCs & BM-MSCs, significantly lower than the differentiated cells indicating small amount of spontaneous differentiation. Therefore this intra cytoplasmic insulin expression was small to be detected by PCR performed in our study. This is in agreement with Wu and associates<sup>(22)</sup>.

Previous studies have shown that maturation to functional islet cells in vitro requires implantation into an in vivo environment<sup>(18)</sup>. Also blood glucose control is the most important target in the management of diabetes mellitus<sup>(12)</sup>. Therefore, we monitored the blood glucose level after in vivo transplantation of the differentiated BM-MSCs and BM-HSCs into alloxan induced-diabetic rats.

Alloxan induces diabetes through selective destruction of pancreatic islet beta cells (IPCs) with the resultant of a state like human type I diabetes as  $\beta$ -cells are destroyed with a lack of insulin<sup>(26)</sup>. In this study the pancreatic beta cells were destroyed with the help of alloxan. Alloxan and streptozotocin are the most usual substances to induce DM<sup>(10)</sup>

The results of in vivo transplantation revealed better and faster alleviation of blood glucose level by the transplanted differentiated BM-HSCs than by differentiated BM-MSCs. However the blood glucose level did not return to the normal level. Improvement of blood glucose level following in vivo transplantation of differentiated BM-HSCs was reported in other studies<sup>(18,21)</sup>. Also, this improvement was reported following in vivo transplantation of differentiated BM-MSCs<sup>(8,22,24,25)</sup>.

In our study, allograft rejection lead to the loss of the function of both types of the transplanted cells and the FBG increased to reach again the pretransplantation levels. This agrees with Wu and associates who injected the BM-MSCs into the portal vein of diabetic rats<sup>(22)</sup>. On the other hand this disagrees with Gabr and associates who injected the BM-HSCs into the testis of the diabetic male rats and revealed no allograft rejection. This is because the testis is an immunological privileged tissue. The immune privilege of the

testis is not attributed only to the isolation of neo-antigens from the immune system behind the blood-testis barrier, but is based on a complex multifaceted interplay between cells and factors that are essential for the reproductive function of the testis and the testicular immune system as there is accumulating evidence regarding the role of Sertoli cells, androgens, and selected population of leukocytes in the maintenance of immune privilege<sup>(27)</sup>.

### Conclusion:

In conclusion BM stem cells offer a promising tool in providing autologous transplants of IPCs for the treatment of diabetes and providing researchers with the ability to study pancreatic islet development and function. However, researches must continue to improve the differentiation capacity of these cells.

### CORRESPONDANCE TO

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