RESEARCH ARTICLE

Cellular and Glycemic Regenerative Effect of Systemic Mesenchymal Stem Cells in Animal Model with Diabetes

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Abstract

Umbilical cord blood is known as a rich source of hematopoietic stem cells, which makes it a valuable alternative to bone marrow transplantation in hematology and oncology.

Aim of work: The aim of this work was to detect the feasibility of generating insulin-producing cells obtained from progenitor cells of cord blood, and their success in reversing the hyperglycemia in rats with diabetes.

Materials and Methods: The current study was performed on 44 male rats and 6 umbilical cord blood samples collected from 6 normal vaginal deliveries from the obstetric ward of Kasr El Eini Hospitals.

Results: WThe present study confirmed that cord blood mesenchymal stem cells (CB-MSCs) are able to differentiate into islets that can secrete insulin in response to glucose *in vitro* and *in vivo*. The transplantation of culture-expanded, undifferentiated CB-MSCs in experimental mice with diabetes reversed hyperglycemia.

Conclusion: In conclusion, our data confirms that CB-MSCs are able to differentiate into islets that can secrete insulin in response to glucose *in vivo*.

Keywords: Diabetes mellitus; Human umbilical cord blood; Mesenchymal stem cells

Introduction

Diabetes mellitus (DM) is a chronic disease with great social and economic impact. It affects nearly 285 million people worldwide, corresponding to 6.4% of the world adult population. DM is reaching an epidemic state in Egypt, with a health burden of patients and annually diagnosed new cases. Type I Diabetes represents 10% of cases with diabetes. It is caused by autoimmune destruction of pancreatic islet β cells that reduces an individual's ability to regulate blood glucose, ultimately resulting in poor blood circulation and often premature death. Despite recent advances in diabetes care, chronic hyperglycemia persists causing serious long-term complications in most patients [1]. Pancreas or islet transplantation is the only methods available to date which are able to establish long-term normoglycemia or near-normoglycemia. Islet transplantation has been performed widely for 10 years. However, although approximately 70% of patients did not need daily insulin 1 year after transplantation, approximately 90% of patients required insulin after 5 years [2]. It also has been hampered by immune rejection and recurrent attacks against islets by the underlying autoimmunity [3,4]. Immunosuppressive regimens are capable of preventing islet failure for months to years, but these regimens may increase the risk for specific malignancies and opportunistic infections. Therefore, their use remains restricted to subjects in whom a simultaneous transplantation of the kidney is needed or to those with impaired glycemic control who are at a high risk of hypoglycemia on insulin therapy [5]. An alternative for islet transplantation would involve the use of a renewable source of stem cells capable of self-renewal and differentiation, as well as insulin production. The development of a simple, reliable procedure to obtain stem cells having 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 [6].

Human umbilical cord blood (HUCB) is known as a rich source of hematopoietic stem cells which makes it a valuable alternative to bone marrow transplantation in hematology and oncology Although cord blood is mainly used for the treatment of malignant

and genetic blood diseases, the therapeutic potential of these cells can go beyond blood system therapy [7-10]. Although it is not likely that HUCB contains pancreatic progenitors, it is possible that they are able to differentiate toward pancreatic cells. Since cord blood contains a large population of immature unprimed highly functional regulatory T lymphocytes, this may be the most important reason for therapeutic applications of umbilical cord blood (UCB) in Type 1 Diabetes [11,12]. Subsequent studies have demonstrated that these cells can be used to alter immune function and improve markers of type 1diabetes in non-obese diabetic mice (NOD) [13]. In this respect, the stem cell-rich human umbilical cord blood represents a promising source for the treatment of type 1 diabetes. MSCs are multipotent cells capable of self-renewal and differentiation into a various cell lineage. They are derived from many organs such as bone marrow, adipose tissue, skin, fetal liver, and umbilical cord blood [1,14]. Many researches have demonstrated that insulin-producing cells could also be differentiated from MSCs using different protocols and different sources for MSCs [15,16]. The aim of the present work was to evaluate the feasibility of generating IPCs obtained from MSCs of cord blood, and their success in reversing hyperglycemia in Alloxan induced rats with diabetes, and compares the results of undifferentiated, glucose primed, and Exendin-4 differentiated MSCs, in addition to pathological assessment of rat's pancreata two weeks after treatment to detect degree of regeneration.

Methods

Induction of animal model

44 rats: 5 controls, 39 diabetics. Ten of which left as positive control and 29 received cellular transplant. Positive controls are the rats with Diabetes mellitus proved by fasting and post prandial blood sugar assay, that were left diabetic without therapy as an initial state to compare them with the therapeutic changes made by the MSC injection in the other group (29 rats) The experiment was done on 44 male Wister rats (Laboratory Animal Center of Kasr El Eini, Cairo University), weight about 200-255 gm. Thirty-four were injected with Alloxan, (Sigma Chemical Co., St Louis, EUA) in a single intraperitoneal dose of 80 mg/kg of body weight. Five of them were considered as control group with diabetes received no treatment. The other 10 was considered as controls without diabetes. Their blood sugar was within the normal range (70-110mg/dl). Blood was drawn from the tail vein and fasting blood glucose was measured at 10:00 am, once a week for 2 successive weeks with a blood glucose meter (Life scan Inc.). Rats with blood glucose < 300mg/dl were excluded from the study.

The rats were maintained under sterile conditions, and all animal work was carried out under protocols approved by the Laboratory Animal Care and Use regulations of Kasr El Eini, Cairo University.

Preparation of MSC

Samples: Six umbilical cord blood samples collected from 6 normal vaginal, full term (± 40 weeks) deliveries from the obstetric ward of Kasr El Eini Hospitals after taking written informed consent from the mothers. Institutional research board approval was taken prior to the study.

Separation of mononuclear cell layer (MNC): Twenty ml venous cord blood (19 ml blood and 1 ml preservative free heparin) was collected under complete aseptic conditions from each cord. The MNC fraction from the cord blood was obtained by Ficoll-hypaque (Biochrom AG, Berlin) gradient separation, followed by washing twice with Dulbecco's Modified Eagle's Media (DMEM) (EuroLone).

MSC separation: Cells were plated in a density of 100.000 - 200.000/ ml into ten T25 Sterile Culture Flasks, in complete media (5ml DMEM (high glucose with stable L-glutamine, 2ml Penicillin/Streptomycin (10,000U/10,000μg/ml, Biochrom AG), 2ml Fetal Bovine serum (EuroLone) and incubated in 5% CO2 at 37 °C.

Harvest of Mesenchymal stem cell population: On day 3, non-adherent cells were discarded, media was changed every 3 days, when cells became 70- 80% confluent; media was discarded, 1 ml of 0.25% trypsin EDTA (Euro Lone) was added on each sterile culture Flask, shaken for 1 min then another 1 ml DMEM (containing 100 μl FBS) was added to counteract the action of trypsin. The mixture then was aspirated by a sterile Pasteur pipette and placed in a new 15 ml conical tube and washed twice with phosphate buffer saline (PBS) with twice centrifugation at 2000 rpm for 20 min. The cells were resuspended in DMEM and analyzed for count, viability and immunophenotyping.

Induction of MSC differentiation: Group I: Un manipulated MSCs; cells were sub cultured in the same culture condition with no extra growth factors. Third passage of MSCs was used for transdifferentiation and injection.

Group II: Glucose primed MSCs; 25% glucose was added to the complete culture medium.

Group III: Exendin-4; 10 nmol/L exendin (**Sigma, Aldrich**) beta-islet differentiating agent was added to the complete medium. MSCs were incubated in CO_2 incubator at 37 ºC for one week.

Evaluation of MSC Transdifferentiation: Morphological, immunocytochemical and flowcytometric detection of cytoplasmic insulin were done using Anti-human/bovine/mouse insulin-allophycocyanin (APC) monoclonal antibodies (R&D Systems).

Cell tracking: Cells were tagged with iron oxide particles (Feridex; Bayer HealthCare, Leverkusen, Germany). Cells were incubated 30 minutes with 25 μg iron/ ml then washed 3 times by PBS.

Preparation of cells for injections: Cells were suspended in saline (0.9% NaCl) in a dilution of 3×10^6/mL. Each rat was injected by 1 ml of cell suspension in the caudal vein.

Evaluation of the results of MSCs transplantation in rats with diabetes: Blood sugar was measured using Accuacheck. After 2 weeks from injection, sacrifice of the rats was done; specimens of pancreata were evaluated for regenerating islets and compared with normal control group.

Estimation of blood glucose level of rats was done 1 and 2 weeks after treatment.

Examination of rats' pancreata by the followings:

- Cell tracking was done using Pearl Prussian blue stain to detect iron particles on the regenerating pancreatic cells to insure its source from the transplanted MSCs.
- Immunohistochemistry (IHC), using Peroxidase-labelled antibodies against insulin and glucagon (provided by Dako, USA) were also done to evaluate the function of generated islet cells of pancreas.
- Histopathological examination by morphometry: Morphometric analysis was performed at the Pathology Department, National Research Center using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England). Formalin fixed paraffin embedded tissue blocks were obtained from pancreatic specimens. Paraffin sections of 5-micron thickness were prepared from each of the paraffin blocks. For routine histopathological examination and morphometry slides were stained with hematoxylin–Eosin stain. The major axis (length) and the minor axis (width) of the islets were measured on a real-time image from the microscope that we visualize on the video monitor. The reading of each measurement appears in micrometers.

Statistics

Statistical calculations were performed using Microsoft Excel version 7 (Microsoft Corp., Redmond, WA, USA) and Statistical Package for the Social Sciences (SPSS for Windows version 16) (SPSS Inc., Chicago, IL, USA) software. Results were reported as mean ± standard deviation (±SD) or frequency (%) when appropriate. Comparison of quantitative variables between the study groups was done using one-way analysis of variance test. A p-value less than 0.05 were considered statistically significant.

Post-hoc power analysis was conducted after a study has been completed, and used the obtained sample size and effect size to determine what the power was in the study. In our study, (alpha=.05, 2 tailed), "The sample size used had power of 80% (intermediate power) to detect a minimal difference of 5 % with an alpha level of 0.05. A probability value of <0.05 was determined as significant"

Results

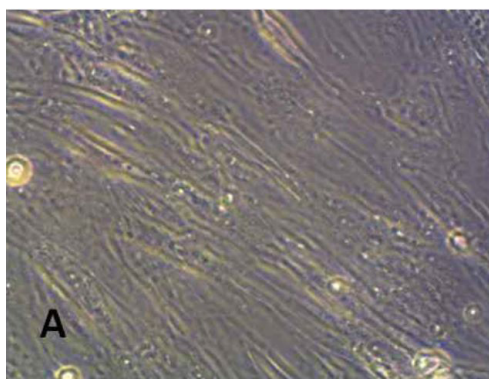### Verification of MSCs

Morphology

Immunophenotyping

Figure 1: A) Inverted microscopic picture of cord blood MSCs culture at 80% confluent x200

Verification of MSCs Transdifferentiation

Morphology

Intracytoplasmic insulin: Insulin expression in the three groups of cells was estimated by flowcytometry (results are summarized in Table I). There were highly statistically significant differences between the unmanipulated stem cells group and the other 2 groups (p value<0.0001), While no statistically significant differences were detected between the stem cells primed with glucose 25% and the stem cells primed with Exendin 4 (p value = 0.2363).

Stem cells (unmanipulated) mean±SD	Stem cells primed with glucose 25% mean±SD	Stem cells primed with Exendin 4 mean±SD	p-value
1.3 ± 0.34	21 ± 8.3		<0.0001[HS]
1.3 ± 0.34		15.5 ± 2.38	<0.0001[HS]
	21 ± 8.3	15.5 ± 2.38	0.2363 [NS]
1.3 ± 0.34	18.4 ± 2.2		<0.0001[HS]

Table 1: The mean values (%) of insulin expressing cells in the three groups of cultured cells

Correction of Hyperglycemia (Functional evaluation)

The 34 rats with diabetes included in the present study were treated as follows:13 cases were injected by unmanipulated stem cells,10 cases were injected by stem cells primed with glucose 25%, 6 cases were injected by stem cells primed with Exendin 4, and 5 cases acted as control received no MSC treatment. All treated rats showed reduction of hyperglycemia, with 59% reached normal glucose level (range 71-110mg/dl), while 41% did not reach normal levels (range112-180 mg/dl). While blood glucose ranges of the control rats with diabetes (received no cell treatment) was (395-404mg/dl) and reached (423-438mg/dl) at day 15. Blood glucose estimated of each group and response to therapy (fate) are summarized in Table 2.

	Stem cells (Unmanipulated) N=13	Stem cells primed with glucose 25% n=10	Stem cells primed with Exendin 4 n=6	Untreated Diabetic Controls n=5	p- value
Blood glucose after 2 weeks (mean±SD)	110.61±14.01	102±21.96	132.2±35.05	430.2±5.89 P value when compared with any of the 3 previous treated groups (<0.001=highly statistically significant)	0.0432[S]
Fate Partial recovery No (%) Complete recovery No (%)	5(38) 8(62)	1(10) 9(90)			0.2981[NS]
Partial recovery No (%) Complete recovery No (%)	5(38) 8(62)		6(100) 0(0)		0.0411[S]
Partial recovery No (%) Complete recovery No (%)		1(10) 9(90)	6(100) 0(0)		0.0028[S]

Table 2: Blood glucose levels and fate of diabetic rats (at day 15)

There was statistically significant difference between the unprimed stem cells treated group and the stem cells primed with Exendin 4 group (p value = 0.0411) as well as between the stem cells primed with glucose 25% group and the stem cells primed with Exendin 4 group (p value=0.0028). But no significant difference between the unprimed stem cells and the cells primed with glucose 25% group (p value=0.298).

Results of stem cell tracking

Iron stain of the pancreatic tissues showed the blue iron particles attached to the regenerating cells indicating its origin from the transplanted cells previously marked by iron particles (Figures 1B and 1C)
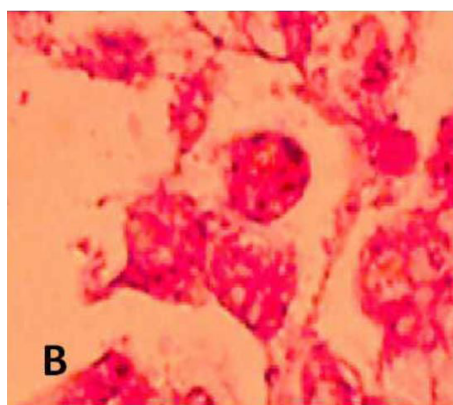

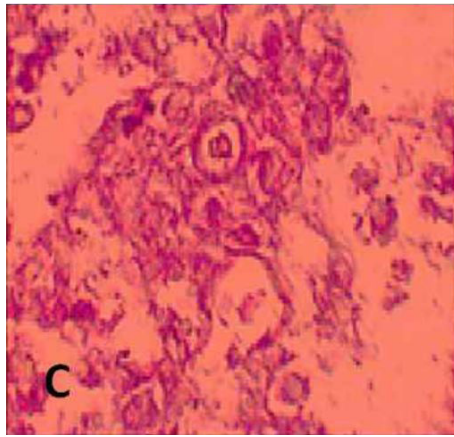**Figure 1: B)** Iron stain of the pancreatic tissues showed the blue iron particles on the regenerating cells

Figure 1: C) Iron stain of the pancreatic tissues showed the blue iron particles on the regenerating cells

Results of histopathological evaluation of the regenerated rats' pancreata

Results of IHC staining: the regenerating pancreatic cells were positive for both insulin and glucagon i.e. the regenerated cells gained the secretory function of islet cells of Langerhans (Figures 1D, 1E and 1F)
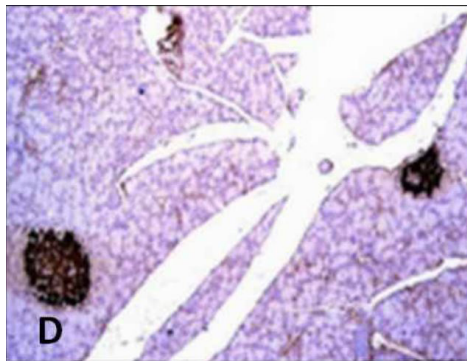

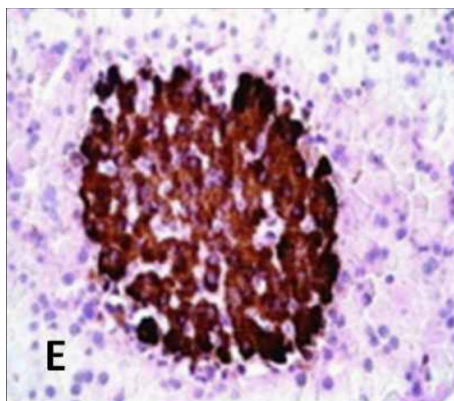**Figure 1: D)** Positive insulin in β islet cells; using peroxidase labeled antibodies

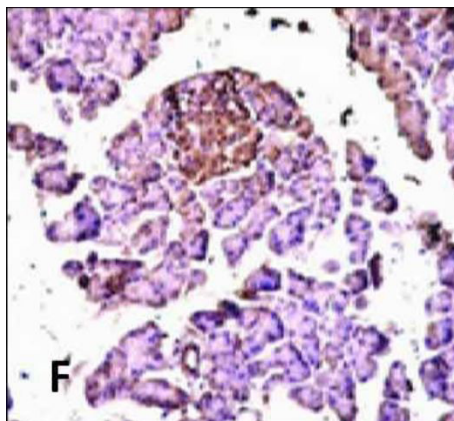**Figure 1: E)** Positive insulin in β islet cells; using peroxidase labeled antibodies

Figure 1: F) Positive glucagon in β islet cells; using peroxidase labeled antibodies-(x200)
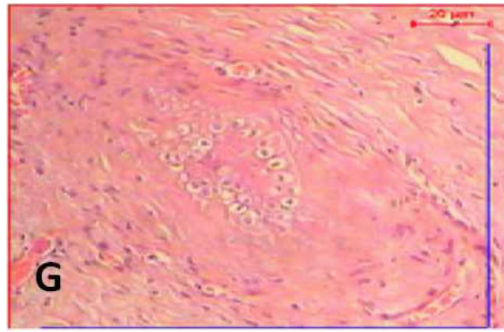
Figure 1: G) Section of connective tissue with a focus of regenerating cells with clear cytoplasm and central nucleus forming sheets (H&E, X100) i.e. regenerated cells without retaining the normal architecture of the pancreas
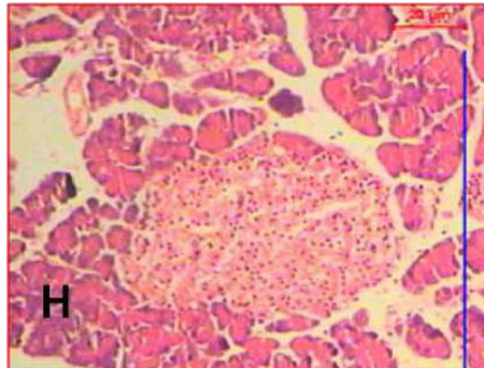

Figure 1: H) Section of pancreatic tissue with normal acini and normal islets of Langerhans
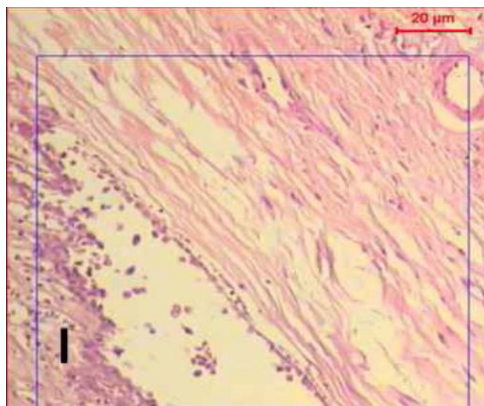

Figure 1: I) Section of pancreatic tissue retrieved from diabetic control rat showing focus of degenerated cells in the form of sheets (H&E, X100)

Results of morphometry: The examined pancreata showed different stages of regeneration. Some cases showed regenerated cells without retaining the normal architecture of the pancreas, others retained the normal architecture of the pancreas with normal islet cells of Langerhans. In comparison to the control diabetic untreated rats; their pancreata showed complete degeneration with no evidence of regeneration (Figure 1G, 1H and 1I). Comparison between the morphometric measurements of the areas of regenerated islet cells are summarized in Tables 3 and 4.

	Stem cell (Unmanipulated)		Stem cells primed with glucose 25%		Stem cells primed with Exendin 4	
Normal architecture	5/13 (38.5%)		6/10 (60%)		6/6 (100)%	
Islets measures	Range μm²	Mean±SD μm²	Range	Mean±SD	Range	Mean±SD
	973.23-1919.10	1298.50±451.91	445.92-1286.72	900.98±358.74	169.35-1153.66	590.48±400.37

Table 3: Pathological assessment of regenerated pancreata

	Stem cells and glucose	Glucose and Exendin	Stem cells and Exendin
P value	0.1179[NS]	0.2594[NS]	0.0211[S]

Table 4: Comparison between morphometric measurements of every 2 groups (by independent T test)

Discussion

Generation of insulin-producing cells remains a major goal for cellular replacement therapy in treatment of diabetes. BM-MSCs are multipotent stromal cells which can provide a potential therapy for diabetes mellitus. Lee and co-workers reported that

undifferentiated hBM-MSC infused in streptozotocin (STZ) induced mice with diabetes normalized hyperglycemia over 1 month by increasing blood insulin levels. In addition, when these cells were engrafted into kidneys, the researchers observed an improved glomerular morphology. That was the first documented evidence that hBM-MSC could be useful per se not only to enhance insulin secretion but also to improve the outcome of the renal lesions that develop in patients with diabetes [17]. Although bone marrow represents the most common source of adult MSC, the yield of MSC is substantially low and decreases progressively with the advancing age of the donor [11]. Many researchers have therefore searched for alternative sources of MSC, such as adipose tissue, umbilical cord blood, amniotic fluid, placenta and Wharton's Jelly [12,13]. All of these populations share a high similarity with the BM-MSC phenotype.

HUCB as a source of stem cells has significant advantages over other stem cell sources. First of all, non-invasive collection and a real abundance with more than 100 million births annually. In addition, HUCB stem cells are among the youngest types of cell that can be isolated from a human being with better quality of DNA which could be impaired by environmental and endogenous factors during their lifetime. Moreover, the HUCB cells carry a lower risk of viral contamination. UCB cells are also considered good substitutes for BM-derived hematopoietic progenitors due to the immaturity of newborn cells that is associated with lower immunogenicity; therefore, UCB reduces graft-versus-host reactivity when compared with adult-derived marrow grafts [18]. Furthermore, UCB raises no ethical issues for basic studies and clinical applications [19,20]. Pessina, *et al.*, were the first to state that naïve un-manipulated UBC-MSC expressed a panel of typical markers in the pancreatic differentiation pathway (Ngn3, Nestin, Cytokeratin-18 Cytokeratin-19, Isl-1 and Pax-4) [21]. Parekh, *et al.*, indicated that such hUCB-derived MNC population contains a subset of "pancreas-committed" cells that have the potential to differentiate into insulin-producing cells *in vivo* [22].

In the present study CB- MSCs were isolated, cultured, and characterized. We aimed to compare the ability of un-manipulated stem cells, stem cells primed with glucose 25% and stem cells cultured with Exendin 4 to differentiate into insulin producing cells. The presence of insulin production before cell transplantation was confirmed by flow cytometry. *In vivo* insulin production was tested by correction of hyperglycemia in experimentally induced mice with diabetes and immunohistochemical stains. The severe diabetes produced by alloxan results in blood sugar levels equivalent to a total pancreatectomy.

International Society for Cell Therapy has defined MSCs by the followings, (i) plastic adherence of the isolated cells in culture and (ii) expression of CD29, CD44, CD73, CD90, and CD105 in greater than 95% of the culture, and lack of expression of markers such as CD34, CD45. We verified MSC by its appearance as spindle shaped cells adherent to the plastic flasks, detection of strong positive CD44, negative CD34 in addition to positive oct3/4 which is considered a master regulator of hESC pluripotency and self-renewal capacities. The insulin expression was highest in glucose primed cells, followed by exendin-4 primed cells. Unprimed stem cells expressed only 1-2% insulin. These results are in accordance with Gao, *et al.*

As regard results of MSC transplantation, after only 2 weeks of transplantation, all grafts exhibit human insulin-producing cells. All cases also showed reduction of glucose level indicating insulin production. Cells primed with Glucose gave the best results with 90% of rats achieved normoglycemia, followed by unprimed stem cells (62%). All rats treated by cells allowed for differentiation by exendin-4 showed partial recovery, although they showed regenerated pancreata with normal architecture; their response to hyperglycemia was not by enough insulin to reach normal levels. The rats with diabetes of the control group showed no changes in hyperglycemia and complete degeneration of the pancreatic tissues caused by alloxan on pathological assessment. So the chance of spontaneous recovery in our studied groups was excluded.

In vitro studies did not achieve great success like the study of Gao, *et al.*, who induced UCB-MSCs *in vitro* to differentiate into islet-like cell clusters using a 15-day protocol based on a combination of high-glucose, retinoic acid, nicotinamide, epidermal growth factor, and exendin-4 [4]. These clusters appeared about 9 days after pancreatic differentiation; expressed pancreatic beta-cell markers, including insulin, glucagon, Glut-2, PDX1, Pax4, and Ngn3. The insulin-positive cells accounted for (25.2-3.36) % of whole induced cells. However, insulin secretion of those insulin-producing cells did not respond to glucose challenge very well. These results suggested the important role of the extracellular matrix to induce the formation of three-dimensional structures in pancreatic endocrine cell maturation. Our results are being in agreement with that of Parekh, *et al.*, who studied MNCs isolated from 270 human umbilical cord blood samples [22]. They observed that freshly isolated MNCs grown out by *in vitro* culture express key pancreatic transcription factors: pdx1, ngn3, isl1, brn4 and pax6. However, after 32-fold expansion, MNCs showed decreased abundance of pdx1 and ngn3, indicating that islet/pancreatic progenitors detected in freshly isolated MNCs died or were diluted out during *in vitro* expansion. Therefore, they transplanted freshly isolated MNCs in non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice to check their ability to differentiate into insulin-producing cells. Human insulin-producing cells were detected 9 weeks after transplantation. These results indicated that such hUCB-derived MNC population contains a subset of "pancreas-committed" cells that have the potential to differentiate into insulin-producing cells *in vivo*.

Yoshida, *et al.* also detected insulin-producing cells of human origin following the intravenous transplantation of HUCB into NOD/SCID mice [23]. The authors suggested that the generation of HUCB-derived insulin-producing cells can be mediated through both fusion-dependent and -independent mechanisms.

Abdi, *et al.* suggested that MSC could be used "per se", even as undifferentiated cells, in the therapy of type 1 diabetes, for their intrinsic ability to negatively modulate the immune effectors by acting on dendritic cells, T-cells and NK-cells [24]. As Type 1 diabetes is a T-cell driven disease, in which the role of CD8+ cells is assuming a growing importance, the promising immune

modulatory features of MSC could therefore be of key importance for the resolution of the disease [25]. The partial recovery of hyperglycemia in 41% of rats with diabetes in our research may be referred to low number of transplanted cells or short period of follow up (2 weeks). Higher doses of stem cells and longer time in immune-compromised rats may be needed for complete regeneration of islet cells and avoid allograft rejection. Endeet, *et al.*, examined the dose-dependent effect of early (prediabetic stage) intravenous administration of HUCB mononuclear cells on blood glucose levels, survival, and insulitis in NOD mice with autoimmune type 1 diabetes [26]. Their results showed that mice treated with HUCB cells significantly lowered their blood glucose levels and increased their lifespan. Also, a significant reduction in insulitis was observed in treated than in untreated mice. The mice that received the highest dosage (200×10^6) of cells had greater reduction in blood glucose levels and the degree of insulitis than the mice that received lower dosage (100–150×10^6) of cells.

Conclusion

In conclusion, our data confirms that CB-MSCs are able to differentiate into islets that can secrete insulin in response to glucose *in vivo*. The transplantation of culture-expanded, undifferentiated CB-MSCs in experimental mice with diabetes reversed hyperglycemia. Thus, these cells offer another non-pancreatic, readily available, noninvasive, and inexhaustible source of allogeneic stem cells for cell replacement therapy in diabetes. Further work is required to examine the curative effects on larger animal models, longer follow up time and on humans.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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