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Transplantation of differentiated umbilical cord mesenchymal cells under kidney capsule for control of type I diabetes in rat

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ABSTRACT

Nowadays, stem cells have been introduced as an appropriate source of regenerative medicine for treatment of type I diabetes. Human umbilical cord matrix-derived mesenchymal cells (hUCMC) have successfully been differentiated into insulin producing cells. The isolated hUCM cells were characterized by the expression of stem cell surface markers and by differentiation into adipocytes and osteocytes. The hUCMCs were cultured with different concentrations of neural conditional medium (NCM) and were induced to differentiate into insulin producing cells (IPCs). As 60% NCM concentration resulted in higher nestin and PDX1 expression, the cells were first exposed to 60% NCM and were then induced for IPCs differentiation. PDX1 and insulin gene expression was evaluated in the treated cells. Also, the secretion capacity of the IPCs was assessed by glucose challenge test. IPCs were transferred under the rat kidney capsule. Blood glucose level, weight gain and immunohistochemistry assessments were done in the treated animals. hUCMC expressed mesenchymal cell surface markers and successfully differentiated into adipocytes and osteocytes. Higher NCM concentration resulted in higher PDX1 and nestin expression. The IPCs expressed insulin and PDX1. IPCs were detectable under the kidney capsule 2 months after injection. IPCs transplantation resulted in a sharp decline of blood sugar level and less weight loss. Differentiated hUCM cells could alleviate the insulin deprivation in the rat model of type I diabetes. In addition, higher NCM concentration leads to more differentiation into IPCs and more nestin and PDX1 expression. Kidney capsule can serve as a suitable nominee for IPCs transplantation.

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1. Introduction

The autoimmune destruction of insulin producing cells (IPCs) of pancreas eventuates in the ablation of pancreatic β cells. The outcome is insufficient insulin secretion and type I diabetes; a complex metabolic disorder which is increasing worldwide (Wild et al., 2004). Insulin is administered for the control of hyperglycemia but it cannot prevent long term complications such as vascular disorders, kidney failure, neuropathy, cardiovascular disease, blindness and stroke (Froud et al., 2005). A combination of treatments including drug administration and islet transplantation are applied to minimize the risk of further complications (Drucker et al., 2010). A new form of treatment known as cell transplantation which is based on pancreatic islet cells injection, has recently been innovated. However, the number of accessible donated pancreatic islets is very limited (Hussain and Theise, 2004). To unravel this problem, some studies have focused on finding a suitable cell sources, an appropriate differentiation protocol and on the route of cell transplantation with minimal complications (Soria et al., 2008). Till now, many types of stem cells have been introduced for the treatment of diabetes, which are classified into embryonic stem cells (ESC), adult stem cells and extra embryonic or infant stem cells which are isolated from cord blood and placenta (Van, 2011). Umbilical cord matrix-derived cells (Wharton's jelly cells) have the properties of

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mesenchymal cells (Anzalone et al., 2011). These multipotent cells (Fong et al., 2007) have an embryonic origin and they do not face any ethical precautions and teratoma formation following transplantation. Also transplantation of differentiated hUCMCs, has resulted in no immuno rejection complications (Weiss et al., 2006, 2008).

Although stem cell therapy in diabetes is still in its infancy period, by considering the hUCM cells accessibility and unique immunological characteristics, establishing an appropriate protocol for in vitro differentiation of these cells into IPCs and transplantation of induced cells into a suitable animal model would be a long step toward the type I diabetes treatment.

In the present study we assessed the effect of different concentrations of neural conditional medium (NCM) on nestin and PDX1 expression followed by a three steps protocol to induce hUCMCs to IPCs. The insulin and C-peptide secretion levels were evaluated by glucose challenge test. Also the insulin and PDX1 expressions were detected by semi quantitative RT-PCR. In the next step, we transplanted the induced cells under the kidney capsule of the rat models of type I diabetes and the blood glucose level and body weight were assessed in the treated animals within 8 weeks.

2. Materials and methods

All the materials were purchased from Sigma Company (Sigma–Aldrich, MO, USA) unless those otherwise stated. The institutional ethical review board (approval number 69-1780) of Kerman University of Medical Sciences, Kerman, Iran, issued ethical approval.

2.1. Isolation of human umbilical cord Wharton jelly mesenchymal cells

We used a previously reported protocol for harvesting hUCM cells (Salehinejad et al., 2012) with few modifications. Briefly, the umbilical cords (UC) were obtained from cesarean section of full-term pregnancy with no complications, after a written consent from the mothers. The UCs (Fig. 1A) were washed with PBS, the amnion and vessels were removed, the matrix was cut into small pieces and transferred into culture dishes (Becton Dickinson & Company Franklin Lakes) (Fig. 1B) with DMEM, 20% FBS (Gibco), 200 IU/ml penicillin, streptomycin and 2 μg/ml amphotericin B. The culture dishes were incubated at 37 °C with 5% CO_2 in the humidified air. The culture media was refreshed every 72 h. After the hUCM cells appeared at the margin of fragments, the matrix fragments were removed and the culture continued to reach 80% confluence. The cells were dissociated by trypsin and EDTA in PBS. Cells were either cultured or cryopreserved for further use.

2.2. Cells characterization

2.2.1. Immunocytochemistry of actin filament

To assess the myofibroblastic nature of the harvested cells, passages two of hUCMCs were immunostained for α-SMA (mouse monoclonal Clone 1A4; Sigma, A2547) to clarify the presence of actin filaments in isolated cells. The cells were cultured on a glass slide for 48 h. The cells were then rinsed with PBS and fixed in 4% paraformaldehyde for 5 min at 4 °C. The slides were then blocked with 10% normal goat serum for 30 min at room temperature and washed with PBS. The slides were incubated with primary antibody for 60 min, washed with PBS and incubated with secondary antibody (human anti-mouse IgG) for 60 min at room temperature. Finally, the cells were stained with 3,3′-diaminobenzidine and observed by a phase contrast inverted microscope (Olympus, IX71, Tokyo, Japan).

2.2.2. Alkaline phosphatase assay

Passage two cells were cultured in 35 mm culture dishes until colony formation by refreshing the medium every 72 h. Alkaline phosphatase activity was detected using an AP Kit (Sigma–Aldrich Chemie GmbH, Germany, Catalog No. 86-1) according to the manufacture's instruction. A red reaction product after exposure to alkaline dye mixture confirmed AP activity.

2.2.3. Osteogenic and adipogenic differentiation

For osteogenic and adipogenic differentiation of hUCM cells, 5×10^3 viable passage three cells were cultured in 35 mm culture plates for 24 h. The osteogenic medium; DMEM, supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate and 82 μg/ml ascorbic acid, was added to the culture plates for 21 days. The adipogenic medium consisted of DMEM-F12 supplemented with 15% equine serum and 100 nM dexamethasone was added to the culture dishes for 14 days. The same procedure was carried out for negative control group, except the inducing agents were removed. To visualize osteogenic and adipogenic differentiation, the induced cells were respectively exposed to Von Cossa to detect black calcium phosphate deposits and Oil-red-O to detect red lipid droplets in adipocytes.

2.2.4. Cell surface analysis by flowcytometry

1×10^4 viable cells at passages two were harvested by trypsinization. Suspended cell were fixed by 4% paraformaldehyde for 15 min and permeabilized with triton ×100 for 15 min at 4 °C. After washing in PBS, the cells were incubated with 10% goat serum in PBS at 4 °C for 15 min to block non-specific binding sites. The cells were then incubated for 1 h with FITC conjugated human anti mouse primary antibodies; CD34, CD44, CD45 and PE conjugated anti CD73, CD90 and CD105. At least 10,000 events were recorded with FACS Canto flow cytometer (BD Biosciences, San Jose, CA) machine. The data were analyzed on FACS Diva software (BD Biosciences). The cells of control group were stained with matched isotype antibodies (FITC- and PE-conjugated mouse IgG monoclonal isotype standards), which were confirmed by positive fluorescence of the limbal samples.

2.3. Differentiation of hUCM into insulin-producing cells

For differentiation to IPCs, 3rd passage cells were cultured for a week with basal medium containing DMEMF12 supplemented with 10% FBS, as pre treatment stage. This was for cells expanding and reaching confluence. Then the hUCMs were induced for IPC differentiation by a three steps protocol as follow:

Step 1: The cells were cultured in 20%, 40% and 60% NCM to determine nestin expression ratio according to the different concentrations of NCM. Then the cells of each concentration were immuno-stained for nestin.

Step 2: NCM pre-treated cells of each NCM concentration were cultured in DMEMF12 (5 mM glucose) supplemented with 2% FBS, 10 mM nicotinamide and 1/100 ITS (Insulin-transferrin-selenium, Gibco) for one week, and the cells' morphology was assessed daily. Then RT-PCR was done on cells of different concentrations of NCM to detect the PDX1 expression. After observing higher PDX1 expression with 60% NCM concentration, the other analyses of the cells of 2nd and 3rd steps were done with the cells, cultured in 60% of NCM.

Step 3: The cells, cultured with 60% NCM, were cultured for 2 weeks in DMEMF12 (25 mM glucose) plus 2% FBS, 10 mM nicotinamide, 1/100 ITS and 20% stem cell conditioned medium (SCM) to produce islet-like cell clusters.

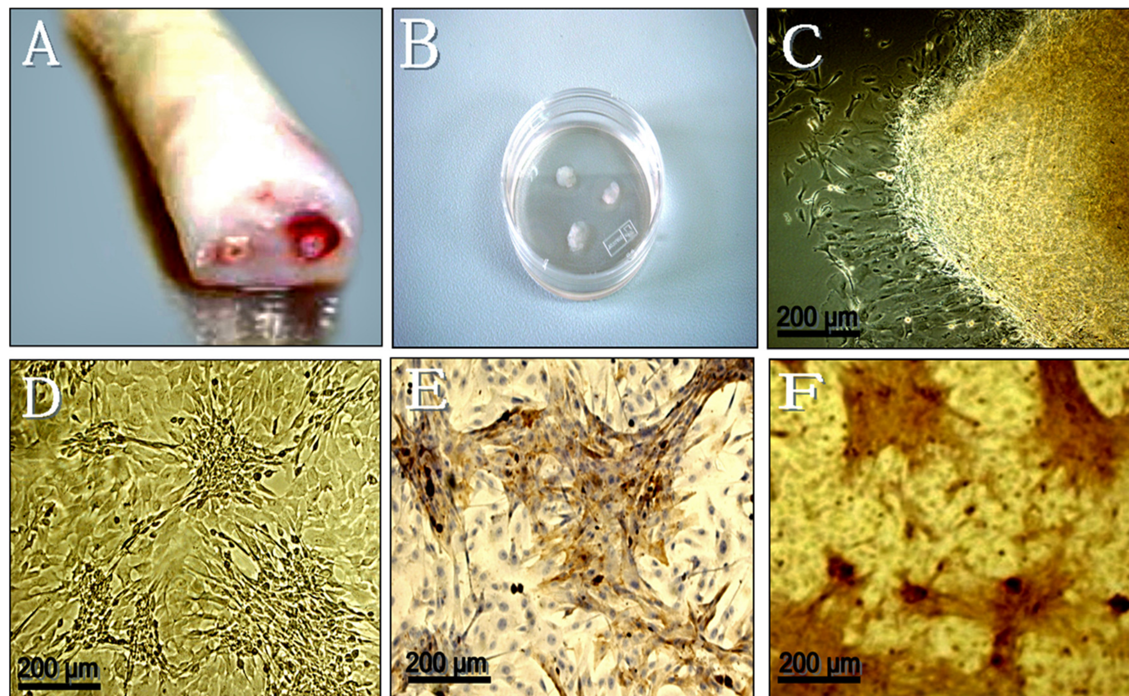

Fig. 1. Human umbilical cord (A). Wharton's jelly fragments explant culture (B). The immigrating cells at the boundary of the Wharton's jelly fragments with fibroblastic like shape, 7 days after culture (C). The confluent cells at passage one formed colony (D). Positive reaction of hUCMs to αSMA antibody (E). Positive alkaline phosphatase activity in the colonies of hUCMs (F).

2.3.1. Preparation of neuronal conditioned medium (NCM)

The brains of 7 days old male Wistar rats were removed and washed with PBS. The supernatant was removed and the precipitate was immersed in 10% FBS + DMEMF12. The precipitate composed of brain tissues was abraded 15 times to obtain single cells. The cells were cultured in DMEMF12 supplemented with 10% FBS at 37 °C and 5% CO_2 in the humidified air. The following day, 2 mM AraC was added and 3 days later the NCM medium was harvested for further use. It was used to produce nestin positive cells from hUCMCs (Fu et al., 2006).

2.3.2. Preparation of stem cell conditioned medium (SCM)

For the preparation of SCM medium, the hUCMs were cultured in DMEMF12 supplemented with 2% FBS, 10 mM nicotinamide and 1 mM glutamine. Three days later, the medium was removed and used as SCM medium.

2.4. Anti nestin immuno-staining

To detect nestin expression in hUCMCs incubated with different concentrations of NCM at step 1, we treated the slides of the induced cells with 10% FBS in PBS to block nonspecific binding sites of anti nestin antibody. The slides were washed with PBS and incubated for 1 h with mouse anti-human nestin antibody (Chemicon) at 1/200 dilution, A HRP system supplied by Dako was applied for 45 min to visualize mouse IgG (EnVision + system, HRP). The slides were washed 3 times with PBS at intervals. The cells were observed under a light microscope (Nikon TS100) to calculate the proportion of nestin positive cells.

2.5. Effect of various NCM concentrations on PDX1 expression

1×10^6 passage 2 cells were cultured in 20%, 40% and 60% NCM for 5 days. The cells were harvested to detect expression of PDX1 with RT-PCR (see infra for method). PDX1 RNA bands were scanned and quantified with scanning the optic densities by using ImageJ software (NIH, USA).

2.6. Reverse transcription-PCR (RT-PCR) of PDX1 and insulin gene

To determine the effect of low and high glucose medium and the medium with and without SCM on PDX1 and insulin gene expression, RT-PCR was carried out on harvested cells of 2nd and 3rd steps. 1×10^6 induced hUCM cells at stage two and three were harvested. Total RNA was extracted by 1 ml Trizol (Invitrogen), chloroform and isopropanol. RNA concentration was measured by a spectrophotometer at 260 nm. For elimination of DNA contamination, 1–2 µg of template RNA samples were exposed to 2.0 IU DNase I (Fermentas) at 37 °C for 30 min and inactivated with 2 µl 25 mM EDTA at 65 °C for 10 min. One microgram DNase-digested total RNA was reverse transcribed with Meloney murine leukemia virus (M-MuLV) reverse transcriptase enzyme in the presence of oligo-dT primer at 42 °C for 1 h. cDNA concentration was measured by a spectrophotometer at 260 nm. The cDNA product was amplified for 30 cycles using the following primer sequences: insulin (240 bp), forward: 5′-AGC CTT TGT GAA CCA ACA CC-3′, reverse: 5′-GCT GGT AGA GGG AGC AGA TG-3′, PDX1 (220 bp), forward: 5′-GGA TGA AGT CTA CCA AAG CTC ACG C-3′, reverse: 5′-CCA GAT CTT GAT GTG TCT CTC GGT C-3′). The PCR products were fractionated with electrophoresis by 1.5% agarose gel. Beta actin (321 bp) was used as housekeeping gene, sense: 5′-ATGGCCACGGCTGCt CCAGC-3′, Anti sense 5′-CAGGAGGAGCAATGATCTTGAT-3′.

2.7. Insulin immunocytochemistry

The cells of stage three, were fixed for 20 min with 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C. Following washing with PBS, they were permeabilized for 1 h with 0.5% triton ×100. After subsequent washing with PBS, the cells were treated with 10% normal goat serum in a humidified chamber for 30 min at room temperature to prevent nonspecific antigen–antibody

bindings. The cells were then washed with PBS and the induced cells at 2nd and 3rd stage were incubated at 4 °C with 1/200 dilution of anti insulin antibody (Guinea pig polyclonal anti insulin antibody) followed by goat anti guinea pig secondary antibody conjugated with Texas red (Abcam®) for 1 h and mounted with glycerol. The cells nuclei were visualized by 5 μg/ml Hoechst 33258 (Sigma) for 10 min. Induced cells were then observed with a fluorescence inverted microscope (Olympus IX71) equipped with a digital camera (DP71).

2.8. Glucose challenge test (insulin release and C-peptide secretion level)

To detect whether the IPCs at stage 3, induced with 60% NCM, could response to higher glucose concentrations, the culture medium was refreshed with DMEM (5.5 mM glucose) and 4 h later a sample was collected and stored at −20 °C as control. The cells were then washed several times with PBS, cultured for another 4 h in high glucose DMEM (25 mM glucose) with 0.5% BSA. A sample of culture medium was harvested and stored at −20° C. The insulin and C-peptide level was measured by enzyme-linked immuno-absorbent assay kit (ELISA), according to the manufacturer's protocol. The test was repeated three times.

2.9. In vivo analyses

2.9.1. Animal housing

Sixty male Wistar rats weighting 200–250 g and normal blood glucose level were obtained from animal house of Afzalipour School of Medicine (Kerman, Iran). They were kept under standard housing conditions with 12 h light/dark cycle and 21–25 °C temperature. They received ad libitum access to standard laboratory rat food and water. Also they were supported under human care according to the "Guide for the Care and Use of Laboratory Animals", approved by the Research Council of Afzalipour School of Medicine.

2.9.2. Diabetes induction in rats and cell transplantation

By intra-peritoneal injection of streptozotocin (STZ, 50 mg/kg) on two consecutive days, experimental type I diabetes was induced in Rats. The rats maintained deprived of food 12 h before STZ injection. Before injection, the animal blood sugar was measured with glucometer (Lifescan). Immediately after STZ injection, the rats received 20% sugar solution for 8 h. Three days after STZ injection and every week until the end of experiments, the animal blood sugar was measured. Animals with fasting blood sugar (FBS) of ≥300 mg/dl were considered as diabetic. In addition, clinical signs like polyurea, polypepsia and weight loss were noted as diabetes indicators. Diabetic animals were divided into 4 groups ($n = 8$). They were anaesthetized by intramuscular injection of 400 mg/kg chloride hydrate. In the differentiated cell treated group (Dif-hUCM), 2×10^6 step three cells were slowly injected under the right kidney capsule with a 22#G needle via laparotomy. In the other group of diabetic animals (hUCM), 2×10^6 undifferentiated cells were injected under the right kidney capsule. Cell treated groups received 10 mg/kg/day cyclosporine to suppress probable immune response. The vehicle group received the same volume of PBS and the sham group endured laparatomy and kidney capsule incision without any further manipulation.

2.9.3. Blood sugar and body weight assessment

Blood samples were taken from the tail vein and the glucose was measured by glucometer, the first day and the day after treatment and each week for 8 weeks. The animals' weight was recorded the first day and weekly for 8 weeks with a digital balance.

2.9.4. Immunohistochemistry

Rats were euthanized by chloroform inhalation 8 weeks latter and right kidneys were removed with a sharp scissors and fixed in 10% formalin. The paraffin embedded tissues were cut into 5 μm sections. The sections were incubated with primary guinea pig anti-human insulin antibody (1:100) for 24 h at 4 °C. The samples were then washed with PBS, incubated with FITC-conjugated goat anti-guinea pig IgG secondary antibody (1:300) for 1 h, mounted with glycerol and examined under a fluorescent microscope.

2.10. Statistical analysis

Data from different groups were analyzed by one-way ANOVA except for the analysis of C-peptide and insulin secretion that Student *T* test was applied. *P* value <0.05 was considered statistically significant.

3. Results

3.1. In Vitro analyses

3.1.1. Isolation of hUCM cells

The isolated cells were observed at the periphery of the explants around the day 7 of culture (Fig. 1C) and the Wharton's jelly segments were removed at day 10 of culture. The culture reached confluence 7–8 days later. The cells were passaged and transferred to new flasks. Cells colonies appeared (Fig. 1D) 8–10 days after first passage. These cells had a fibroblastic appearance with numerous cytoplasmic extensions. Mitotic activity was evident in the culture.

3.1.2. Alkaline phosphatase and actin detection

Immunocytochemical analysis of alpha smooth muscle actin protein demonstrated a positive reaction to actin antibody which confirmed the myofibroblastic nature of the cells (Fig. 1E). Also, alkaline phosphatase activity was detectable among the isolated cells and colonies by a red product (Fig. 1F).

3.1.3. Osteogenic and adipogenic differentiation

To evaluate the differentiation capacity of the isolated cells, their osteogenic and adipogenic potentials were assessed. The untreated control cells neither showed positive reaction to Van Cossa staining (Fig. 2A) nor oil-red-O staining (Fig. 2B). About 14 days after the onset of osteogenic induction, the cells demonstrated positive reaction to Von Cossa staining by black extra cellular deposits of calcium phosphate (Fig. 2C).

Adipogenic differentiation resulted in fat droplets in the cytoplasm of induced cells about 6 days after induction, the first oil droplets were observed in the induced cells. The oil-Red-O staining revealed the red fat droplets in the differentiated cells after 14 days (Fig. 2D). As positive control, in the section of bone, the calcium deposits were visualized as red to dark spots (Fig. 2E) and the fat vacuoles in the fat tissue section showed positive reaction to oil-red-O staining (Fig. 2F).

3.1.4. Flow cytometry

Mesenchymal surface markers expressed in hUCMs are shown in the diagrams (Fig. 3). Results showed that hUCM cells did not express CD34 and CD45 while they expressed CD44, CD73, CD90 and CD105 (as mesenchymal stem cells markers). Data are the average values obtained from three different umbilical cords.

3.2. Nestin expression

At the first stage of induction, the cells were cultured with 20%, 40% and 60% NCM and evaluated for nestin expression by

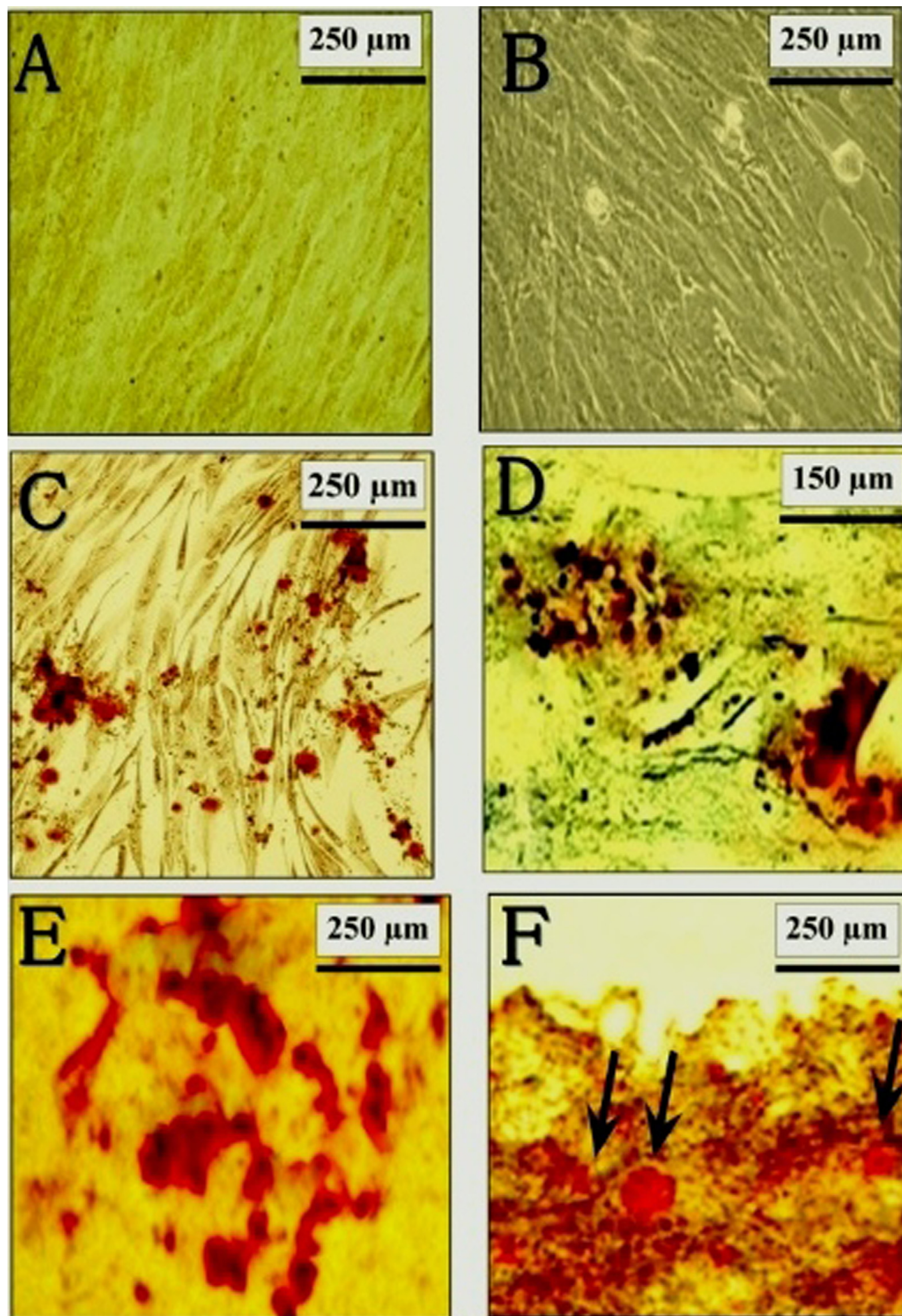

Fig. 2. hUCMs were used as control for osteogenic (A) and adipogenic (B) differentiation. Dark purple calcium phosphate deposits formed in the extracellular matrix of induced osteoblast cells (C). Oil droplets produced in adipocyte cells via adipogenic induction (D). A section of bone used as positive control for osteogenic differentiation (E). A section of fat tissue used as positive control for adipogenic differentiation. The arrows demonstrate the fat droplets (F). (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

immunocytochemistry. These cells exhibited a positive reaction to nestin antibody. The proportion of nestin positive cells was 17%, 31%, and 85%, in 20%, 40% and 60% NCM concentration, respectively (Fig. 4A–C). No reaction was observed in untreated cells (Fig. 4D), while brain slices (positive control) expressed nestin obviously (Fig. 4E).

3.3. The effect of NCM concentration on PDX1 expression

After initial culture of hUCM cells, the propagated cells were exposed to various concentrations of NCM then PDX1 expression was determined by RT-PCR. The outcome of ImageJ software analysis demonstrated that optical density of PDX1 expression was 0.44,

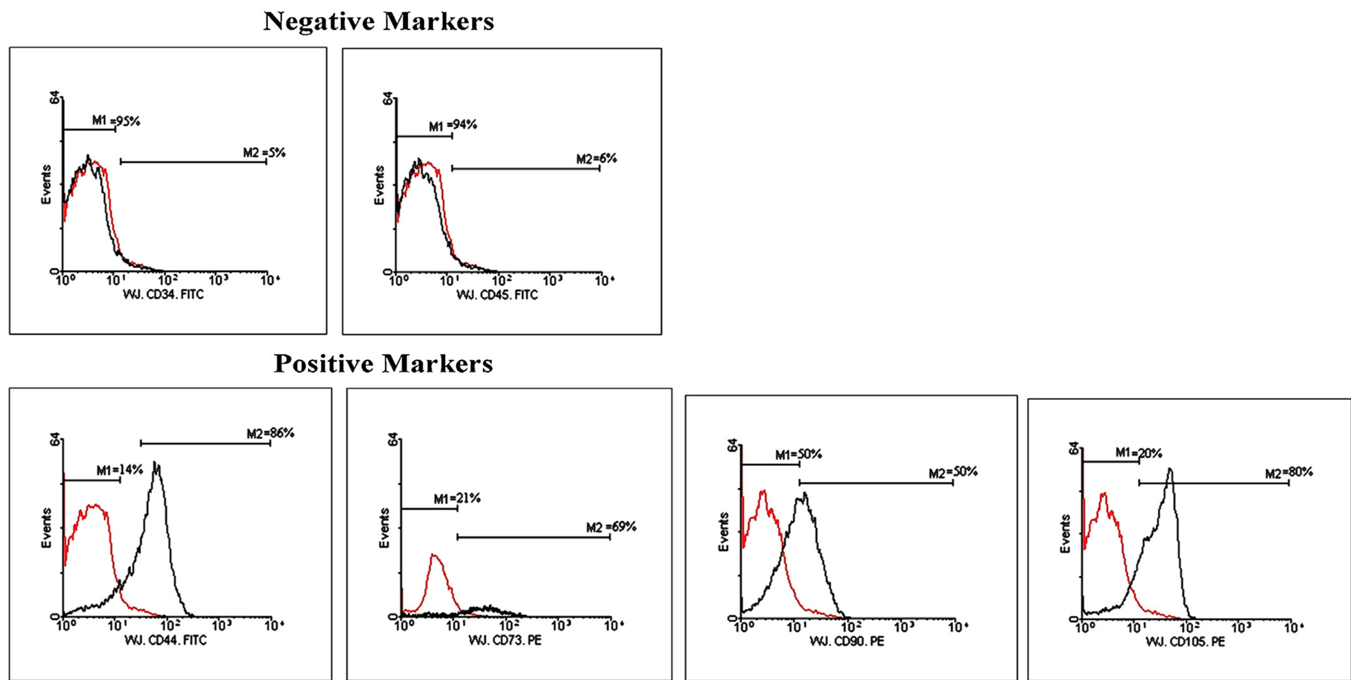

Fig. 3. Mesenchymal stem cell marker expression was detected by flowcytometry in hUCMs.

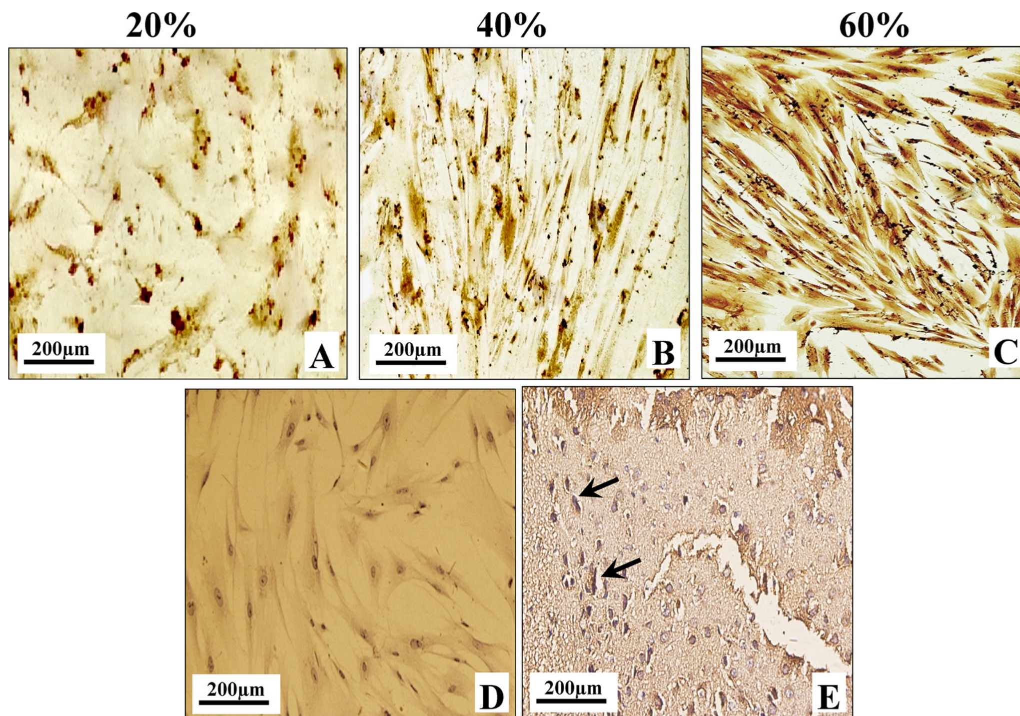

Fig. 4. Positive reaction to anti nestin antibody in hUCMs cultured with 20% (A), 40% (B) and 60% NCM (C). The number of nestin positive cells increased when NCM concentration increased. The percentage of nestin expression in 20%, 40% and 60% NCM concentrations was 17%, 31%, and 85%, respectively. hUCMs were used as negative control (D) and a section of brain, was used as positive control and the arrows show nestin positive cells of brain (E).

0.59 and 0.83 in 20%, 40% and 60% NCM concentrations, respectively. So, further experiments were carried out in the presence of 60% NCM (Fig. 5).

3.4. Differentiation of hUCM to insulin producing cells

After stage one (Fig. 6A), the cells were exposed to pancreatic inducing mediums through stages two and three. From stage two, some granule like structures formed and increased in the cells (Fig. 6B and C). Simultaneously, the cells shape changed from spindle to oval and round appearance (Fig. 6C). These cells started to aggregate (Fig. 6D) and accumulated to become more compact structures resembling islet like structures (Fig. 6E and F).

3.5. Insulin immunocytochemistry

The cells of the stage three (cultured with 60% NCM) were stained with anti-insulin antibody to detect insulin production

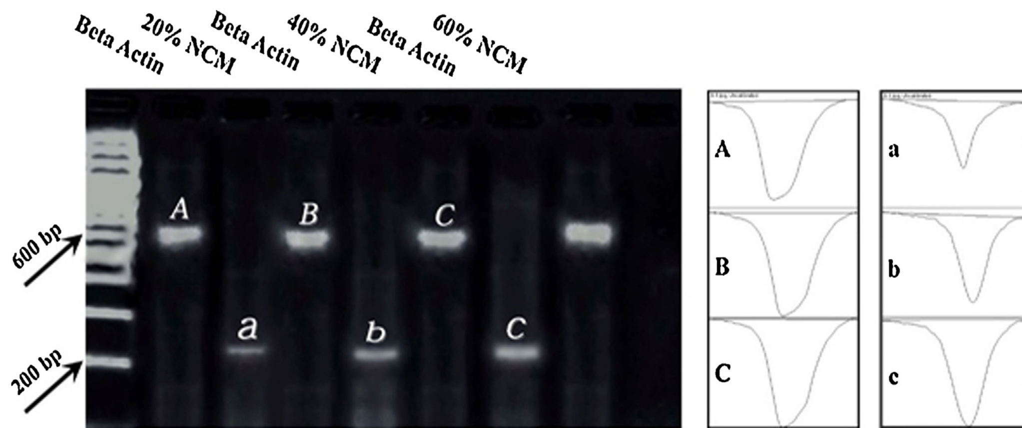

Fig. 5. RT-PCR of PDX1 in differentiated cells after exposure to different concentrations of NCM which demonstrates more PDX1 expression at higher NCM concentrations. Areas were A: 26,469.271, B: 28,505.927, C: 29,368.756, a: 11,878.279, b: 16,869.128, C: 24,604.513 and the proportionate of each PDX1 gene expression to its beta actin was: a/A: 0.448, b/B: 0.591, c/C: 0.837. So, the output of ImageJ analysis software demonstrated that the PDX1 expression in 20%, 40% and 60% NCM concentrations were 0.44, 0.59 and 0.83, respectively.

in the induced cells. Control slide of un-differentiated cells was stained with Hoechst (Fig. 7A) and anti-insulin (Fig. 7B). These slides demonstrated no positive reaction to anti insulin antibody (Fig. 7B and C). The treated cells were also stained with Hoechst (Fig. 7D) and anti insulin antibody (Fig. 7E). A positive reaction to anti-insulin antibody was demonstrated in non-aggregated differentiated cells (Fig. 7E and F). Also, the accumulated cells were stained with Hoechst (Fig. 7G) and anti insulin antibody (Fig. 7H) and they exhibited positive reaction to anti- insulin antibody (Fig. 7H and I).

3.6. PDX1 and insulin gene expression

To detect whether the induced cells progressed toward the IPCs, we analyzed PDX1 and insulin expression by RT-PCR. The RT-PCR analysis of induced cells at stages two and three demonstrated a 220 bp band for PDX1, a 240 bp band for insulin and 600 bp band for beta actin, as housekeeper gene (Fig. 8).

3.7. Estimation of insulin secretion and C-peptide release

The amount of insulin and C-peptide was in parallel with each other. Insulin secretion was more pronounced than C-peptide at low and high glucose concentrations (5.5 and 25 mmol) (Fig. 9A).

3.8. Blood sugar level in diabetic rats

The STZ-induced diabetes rats were treated with 3rd stage differentiated cells and with hUCM, as control. The body weight and blood sugar levels were evaluated as described earlier. Seven days after STZ injection, the blood sugar raised above the normal level and reached a plateau of about 400 mg/dl and remained high in consecutive weeks of experiments in control and sham groups. Diff-hUCM treated rats also showed a rapid increase in blood glucose at 1st week but at 3rd week it fall below 300 mg/dl and after 8 weeks it declined below 200 mg/dl, that was significantly lower than control and sham groups ($P < 0.05$). Undiff-hUCM treated rats also showed a rapid rise to 420 mg/dl in blood glucose in one week but during the succeeding weeks it decreased to 325 mg/dl. At the end of the final level, blood glucose was 325 and 186 in Diff-hUCM and hUCM groups, respectively. But blood glucose level in hUCM treated rats was not significantly different from control and sham groups, while in Diff-hUCM treated animals it was significantly lower than control and sham (Fig. 9B * and **).

3.9. Body weight gain

A considerable but not significant weight loss was observed in all groups, during the first week after treatment. Weight loss continued until the 4th week in all groups without any significant difference. From the 4th weeks, the Diff-hUCM treated animals started to gain weight gradually but hUCM group did not show a considerable weight gain after the 4th week. Control and sham animals continued to weight loss toward the end of the experiments (Fig. 9C). These gradually increase of weight in Diff group may be related to the time need for cells compatibility with kidney capsule tissues.

3.10. Immunohistopathology

The STZ treated animals were euthanized with cervical dislocation two mounts after cell transplantation and their kidneys were removed, fixed, sectioned and evaluated under fluorescent microscope and the transferred cells were detected in the treated animals beneath the kidney capsule after labeling with anti human specific antibody (Fig. 9D).

A graphical abstract has been presented to show the steps of the experiments and the main outcomes.

4. Discussion

In our study, treatment of DM rats with Diff-hUCM cells resulted in considerable decline of blood glucose one month after treatment followed by a higher decrease in the next month. Mesenchymal stem cells have been proposed as a suitable candidate for the treatment of diabetes and elimination of its complication (Volarevic et al., 2011). Up to now, bone marrow mesenchymal cells have been reported as the most preferred tissue in cell therapy procedures but due to the limited number of bone marrow mesenchymal cells, other stem cells sources is demanding (Kassem et al., 2004). Wu et al. (2009) reported that Wharton jelly mesenchymal cells have more advantages than bone marrow mesenchymal cells in cell therapy procedures. These cells are more primitive compared to other kind of adult mesenchymal stem cells. It has been shown that differentiation of Wharton's jelly cells to IPCs, resulted in larger clusters, higher PDX1 expression and more insulin and C-peptide production (Wu et al., 2009). These studies suggest that hUCM cells can probably serve as a suitable candidate for pancreatic tissue engineering and cell therapy (Godfrey et al., 2012).

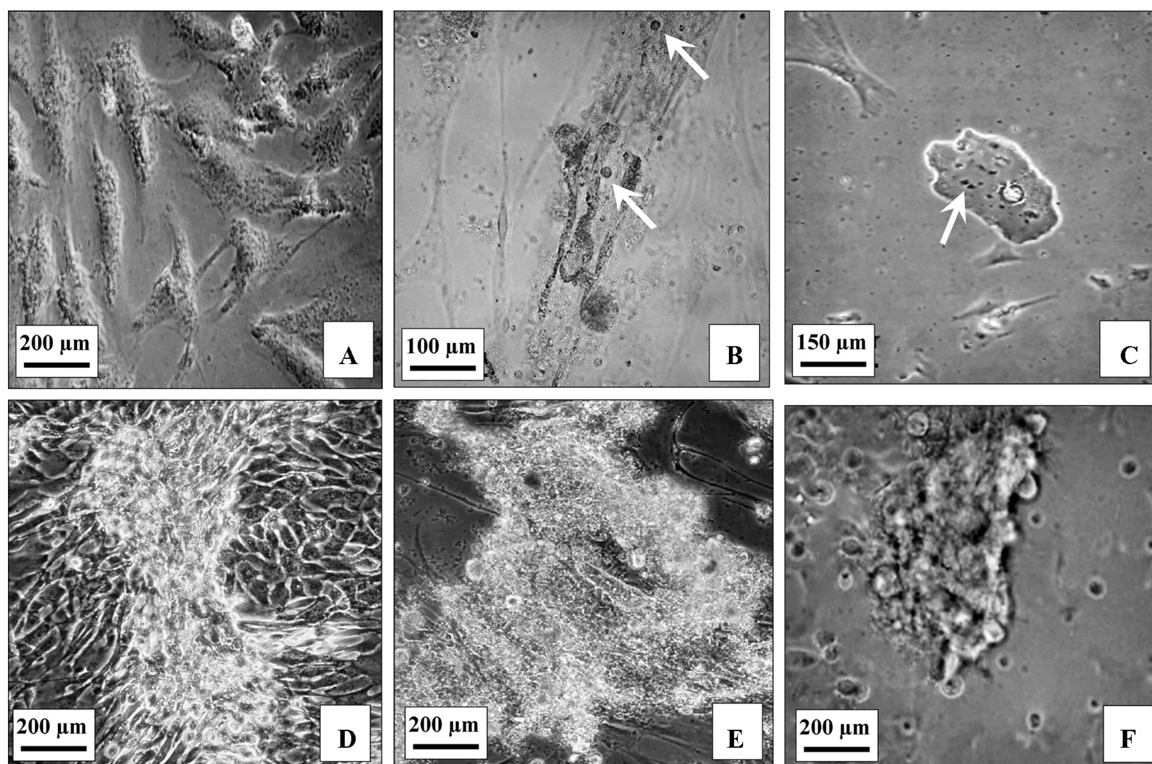

Fig. 6. The expanded hUCM cells at stage one (A). The arrows show the granule like structures formed in differentiated cells of 2nd stage (B). The spindle shape of induced cells changed to round shape at 2nd stage. The figure shows a round cell with granule like structures in its cytoplasm which the arrows indicate them (C). The cells of 3rd stage are accumulating (D) to make an islet like structure (E). A fully formed islet like structure with round cells at its margin (F).

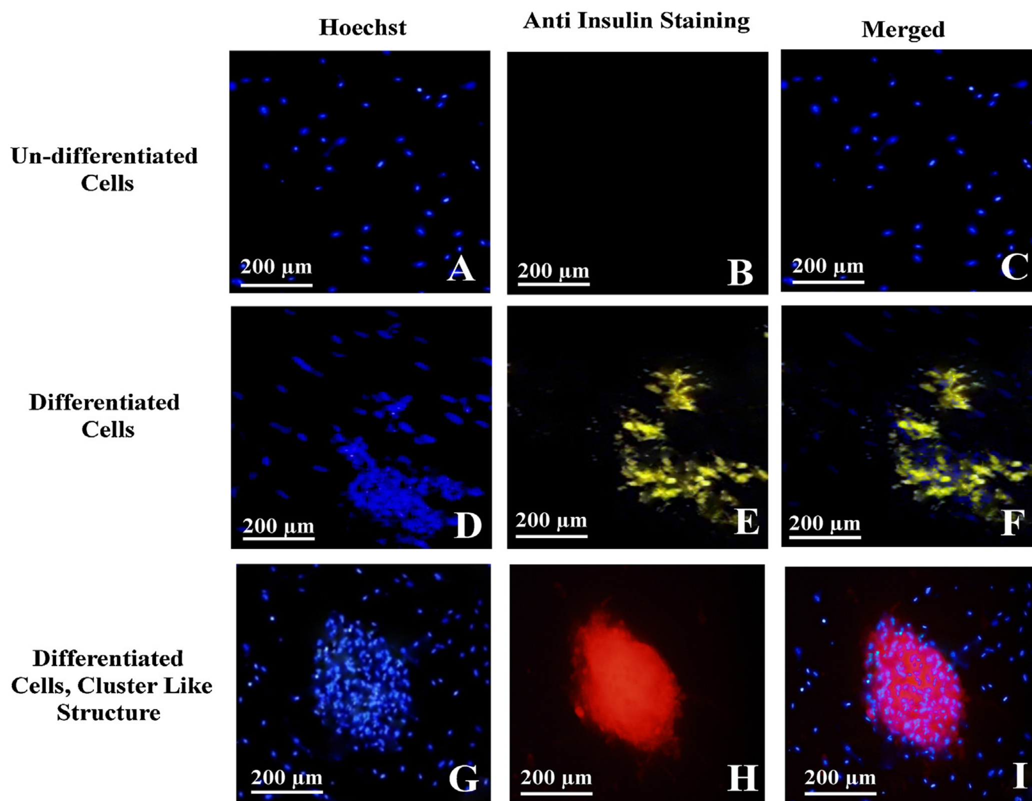

Fig. 7. Immuno florescent view of insulin antibody in cell clusters. Hoechst (A) and anti insulin staining of undifferentiated cells (B and C), used as negative control. Hoechst (D) and anti insulin staining (E and F) of differentiated cells. Hoechst (G) and anti insulin staining (H and I) of an aggregation of the differentiated cells which demonstrate positive reaction to insulin antibody.

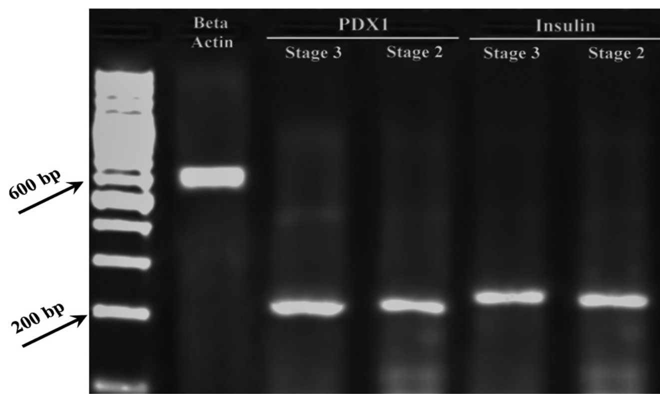

Fig. 8. The RT-PCR of PDX1 and insulin gene in differentiated cells of 2nd and 3rd stages.

Stem cell therapy faces some challenges. For example, the most efficient kind of cells without further complications for pancreas regeneration has not still been introduced (Chao et al., 2008; Godfrey et al., 2012). As hUCMs have been nominated as immuno modulator, they reduce the immune rejection probability of transplanted cells. Compare to other MSCs, they exhibit unusual immune properties (Anzalone et al., 2011) without expressing type II HLA (Turnovcova et al., 2009). They can alleviate immune reaction processes which in type I diabetes causes beta cells destruction (Anzalone et al., 2011). This fact in parallel to the replacement of the destructed beta cells, are the probable mechanisms by which hUCM cells may control diabetes signs.

In our study, both differentiated and un-differentiated cells altered the blood glucose level but in differentiated cells group, it reduced blood glucose to a considerable and significant level compared with control animals.

Nicotinamid is an endocrine differentiation factor and glucose is need for secretion of insulin, so from stage two, we applied mediums, containing glucose and nicotinamid. Nestin is a precursor marker for islet cells differentiation. Stage one was just an intermediate step between pancreatic and neural differentiation that are common in nestin expression (Horwitz et al., 2005). In our study we first evaluated the effect of different concentrations of NCM, as an inducer of nestin, on nestin and PDX1 expression. The results showed that higher NCM concentration could lead to more nestin and PDX1 expression.

Expression of nestin in stage one confirmed successful passage of hUCMs through the intermediate stage of neuronal and pancreatic differentiation. Inhibition of nestin expression has led to the decrease of pancreatic transcription factors in ESCs and pancreatic derived precursor cells (Lumelsky et al., 2001). Exposure of hUCMs to higher concentration of NCM resulted in higher PDX1 expression, as a marker of IPCs.

Glucose and amino acids (De Gasparo et al., 1978), are necessary for pancreatic differentiation, therefore at stage two and three we cultured hUCMs in a medium containing higher glucose concentration, to stimulate their differentiation. In addition, insulin is necessary for glucose absorption (Deane et al., 2011), so we

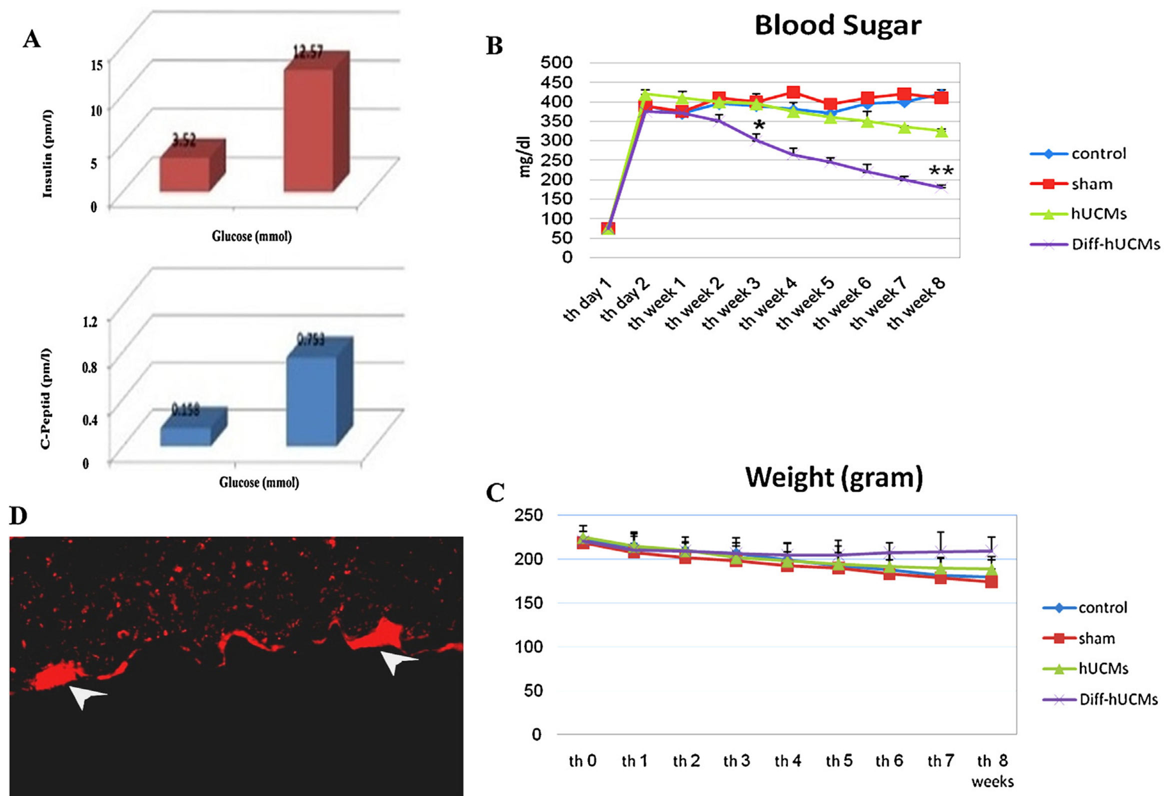

Fig. 9. Diagrams show C-peptide and insulin secretion levels, evaluated by ELISA (A). Both physiological glucose concentration (5.5 mM) and induced hyperglycaemia (25 mM) resulted in insulin and C-peptide secretions in culture medium, after 24 h. The insulin secretion levels were higher than those of C-peptide in either of glucose concentrations. Also, the insulin and C-peptide secretion levels were propitiate to glucose concentration. Blood glucose increased until 7–9 days of the induction of diabetes in the animals. Two weeks after cell transplantation, the glucose level in the Diff-hUCM and hUCM treated animals started to decrease significantly in Diff-hUCM group compared with sham and vehicle groups. Fall in glucose concentration continued toward the end of experiment at a final level of 325 and 186 in Diff-hUCM and hUCM groups, respectively. However, never it did not returned to normal values (B). Diagram shows a non-significant weight lose after cell transplantation in Diff-hUCM and hUCM groups. It changed significantly at the end of experiment in Diff-hUCM group compared with hUCM group (C). Presence of transplanted cells under the kidney capsule at the end of experiments, 2 months after cells transplantation (D).

used ITS, as an insulin containing mixture at stage two and three. Addition of ITS in culture medium enabled us to reduce FBS concentration as low as 2%. Higher glucose concentrations have been shown to inhibit IPC differentiation. It has been shown that when human neuronal progenitor cells, which produce nestin, are cultured in a high glucose medium, they start to aggregate and produce cell cluster (Hori et al., 2005). It should be noted that, due to the resistance to insulin sensitivity, exposure to glucose at stage two and three should not last for prolonged time. Similar to in vitro beta cells behavior, the IPCs initiate insulin secretion when glucose level elevates in the medium so that the induced cells started to secret insulin after glucose challenge test (Chao et al., 2008). We detected PDX1 expression at stage two and three, which show that the cells were passing through an intermediate stage toward pancreatic differentiation. PDX1 is expressed in the pancreas progenitor cells, which have the potential to differentiate into all kinds of pancreas cells (Chen et al., 2009). The expression of PDX1 discriminate these cells from the other pancreatic endocrine cell (Wang et al., 2001). Although, the expression of PDX1 lonely, is not sufficient for inducing insulin mRNA expression, its co-expression with other genes like NKX2.2 is necessary for insulin gene expression (Zhou et al., 2013). PDX1 activates glut2 which is essential for sensitivity to environmental glucose level (Gradwohl et al., 2000). It was also reported that PDX1 expression level is related to pancreas genes expression pattern and IPCs differentiation.

We assessed insulin and PDX1 at stage three because we expected insulin production and secretion at stage three (Gallo et al., 2007). Nicotinamid is essential for endocrine differentiation (Cho et al., 2008), especially for increasing the beta cells mass in human fetal pancreatic cells (Otonkoski et al., 1993; Cho et al., 2008). Also, it protects beta cells from glucose resistance, produced in long-lasting exposure to large amounts of glucose (Freeman et al., 2006). We used nicotinamide for stage two and three to promote IPC differentiation.

Animal blood sugar, as a critical complication in diabetes, also decreased considerably one month after transplantation of induced hUCM cells. This delay might be due to the time needed for cells compatibility with host body. However, still the long term activity of in vitro induced hUCMs as well as their physiologic roles in "in vivo" conditions requires to be investigated.

C-peptide is produced via converting pro insulin to insulin and its serum level is associated with blood insulin level. The in vivo amounts of insulin and C-peptide are balanced to blood glucose level (Kemmler et al., 1971).

One of the challenges of cell transplantation is the engrafted site. It should be accessible and sustain the microenvironment of transplanted cells for oxygen, nutrient supplies and long term survival of the cells. Also it should be either efficient for cell survival or for cell function. Portal vein, liver (Pepper et al., 2013; Carlsson et al., 2001), spleen (Gray, 1990), omentum, pancreas, muscle, gastric sub mucosa, subcutaneous, genitourinary tract, anterior eye chamber, thymus and testis are the suggested sites for animal islet transplantation (Cantarelli and Piemonti, 2011; Rajab, 2010). Renal sub capsular space can provide a better condition for transplanted islet cells (Mellgren et al., 1986; Si et al., 2001; Hawthorne et al., 2011) and it is the most widely used site in rodents (Pepper et al., 2013). Also, in human it was reported that kidney, liver, muscle, and omentum are respectively the best sites for islet transplantation (Kim et al., 2010). Transplantation of cells into the liver is technically challengeable as the liver is a fragile tissue and any manipulation in the parenchyma of liver may result in sever hemorrhage. In the present study we used renal sub capsular space for transplantation of the differentiated cells as an ideal site for cell survival and function. Via sub capsular site we could show long term survival of hUCMs and insulin secretion in diabetic rats. All together, we may conclude that hUCMs are capable of successful differentiating into insulin producing cells. Transplantation of these cells, as a good candidate for beta cell regeneration, into rat model of diabetes would control weight loss and blood sugar considerably. However, achieving higher insulin production in differentiated cells, longer survival of cell treated animals and improved cell therapy outcome with the least complications should be investigated.

Conflict of interest

None.

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