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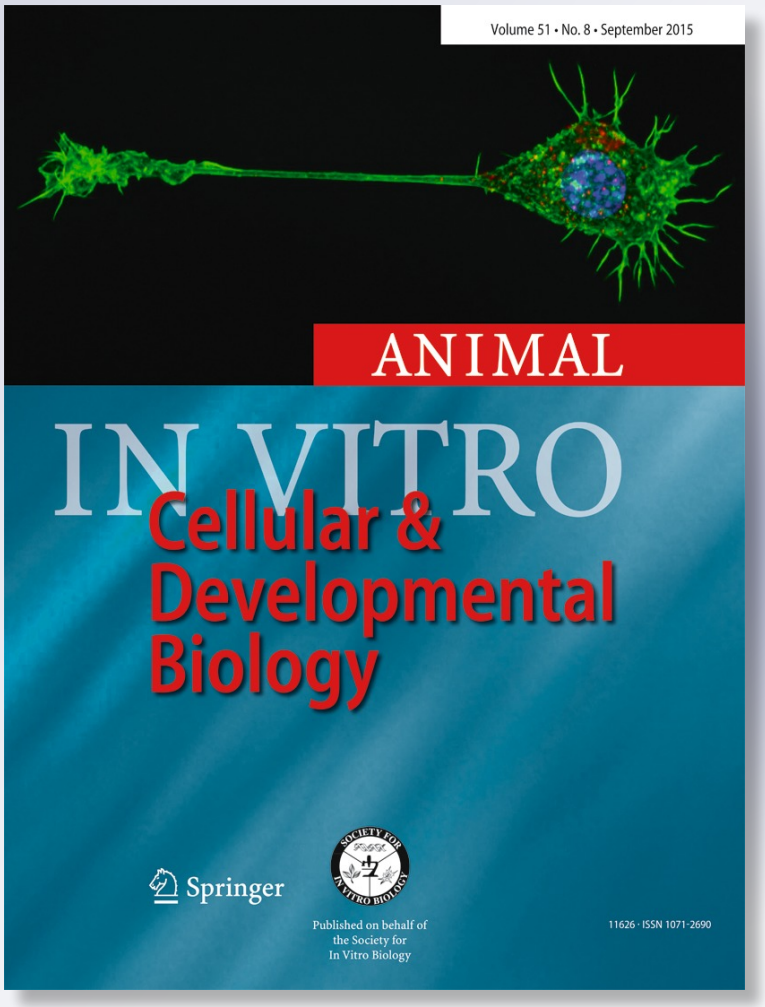

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In vitro evaluation of different protocols for the induction of mesenchymal stem cells to insulin-producing cells

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Abstract Stem cells therapy is a new promising approach for diabetes mellitus (DM) treatment, but the insulin secretion rate in differentiated cells is low when compared with pancreas beta cells embedded in Langerhans islets. In this study, we evaluated different protocols of insulin secretion to achieve the most appropriate protocol for in vitro insulin secretion. We differentiated human umbilical cord matrix-derived mesenchymal cells (hUCMs) into insulin-producing cell (IPC) by the aim of three previously reported protocols and a modified protocol. The insulin content was analyzed through gene expression and immunocytochemistry (IHC). Dithizone (DTZ) staining was done for identification of islet-like structures. Insulin and C peptide secretion was measured by chemiluminesence immunoassay (CLIA) and enzyme immunoassay (EIA) as well. Reverse transcription-PCR (RT-PCR) showed efficient expression of insulin genes in all the study groups. IHC analysis showed higher expression of insulin and proinsulin proteins in the modified protocol. DTZ staining exhibited variable islet-like clusters in the different protocols except control. This finding was confirmed by the higher response to glucose challenge test in this group. A modified protocol using an intermediate step that makes the cells vulnerable to nestin production in combination with inducing agent results in the higher differentiation of stem cells into insulin-producing cells and more insulin secretion in vitro.

Keywords Human umbilical cord matrix-derived ?mesenchymal cells (hUCMs) · Insulin-producing cell (IPC) · Nestin · Differentiation protocol

Introduction

The permanent destruction of insulin-producing cells results in type I diabetes mellitus (DM), a degenerative disease with worldwide distribution and increasing attacks (Himsworth 2011). Stem cells therapy is a new promising approach for DM treatment (Hussain and Theise 2004). Among the stem cells, mesenchymal stem cells (MSC) are highly recommended for cell-based therapies due to the safety and easy access compared with embryonic stem cells (Jahr and Bretzel 2003). MSCs have been defined by their subsequent expansion under appropriate culture conditions and their differentiation potential in vitro and in vivo (Javazon *et al.* 2004). MSCs are usually isolated from bone marrow, umbilical cord matrix and blood, periosteum, adipose tissue, synovium, skeletal muscle, and some other tissues (Barry and Murphy 2004). Phenotypically, MSC have been reported to be positive for CD73, CD44, CD90, and CD105 and

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negative for hematopoietic lineage markers such as CD45, CD34, and HLA-DR (Wang *et al.* 2004; Wagner *et al.* 2005). Under appropriate induction conditions, these cells can differentiate into a variety of cell types, including fat, bone, and cartilage cells (Lee *et al.* 2004).

Several investigations have shown that MSCs can successfully differentiate into insulin-producing cells (IPC), including pancreatic stem cell (Gao *et al.* 2003), human adipose tissue-derived mesenchymal stem cells (MSCs) (Timper *et al.* 2006), bone marrow-derived MSCs (Chen *et al.* 2004; Wu *et al.* 2009), human umbilical cord blood (Sun *et al.* 2007a, b; Van Phuc *et al.* 2011; Nguyen *et al.* 2014), human umbilical cord matrix (Chao *et al.* 2008; Wang *et al.* 2011), amniotic fluid (Carnevale *et al.* 2013), human periosteum (Kim *et al.* 2012), and dental pulp (Carnevale *et al.* 2013). However, the rate of insulin secretion is low when compared with the rate of insulin secretion of pancreas beta cells embedded in Langerhans islets (Serup *et al.* 2001; Chao *et al.* 2008; Wang *et al.* 2011). The frequently used extrinsic factors for the induction of stem cells towards IPCs include nicotinamide (Otonkoski *et al.* 1993; Gabr *et al.* 2008; Gao *et al.* 2008), exendin-4 (Gabr *et al.* 2008; Li *et al.* 2010), gastrin (Wang *et al.* 1993), HGF (Otonkoski *et al.* 1996), bFGF (Hardikar *et al.* 2003), activin A (Demeterco *et al.* 2000), betacellulin (Demeterco *et al.* 2000), and low concentration of glucose (Bonner-Weir *et al.* 1989). In addition to the different inducing substances being used so far, different cocktails have been used in the various studies. Some protocols have shown an increase in the insulin content (Sun *et al.* 2007a, b), while others amplified IPCs insulin secretion and their response to glucose challenge test (Chao *et al.* 2008; Gao *et al.* 2008).

Although different methods have been used for differentiation of stem cells into IPCs, but inadequate differentiation, insufficient insulin secretion, and low response to glucose challenge test are still challenging (Hussain and Theise 2004). Since expression of nestin, an intermediate filament, has been reported as a necessary intermediate stage for the IPC and neuronal differentiation (Hunziker and Stein 2000), some researchers have utilized neuronal-conditioned medium (NCM) at different stages of in vitro IPC differentiation to attain higher insulin production in the differentiated cells (Chao *et al.* 2008). It seems that using an intermediate step of nestin production for the induction of MSCs followed by the inducing agents may result in higher insulin secretion in vitro.

To examine this hypothesis, we have employed a new modified differentiation protocol and compared it with the three commonly reported protocols. Reverse transcription-PCR (RT-PCR), ICC, dithizone staining, chemiluminescence immunoassay (CLIA), and enzyme immunoassay (EIA) were used to evaluate the efficacy of different strategies for the in vitro insulin secretion.

Materials and methods

All the materials were purchased from Sigma Company (Sigma, Basel, Switzerland) unless those otherwise stated. Ethical approval has been issued by the Institutional Ethical Review Board (approval number 69-1780) at Kerman University of Medical Sciences, Kerman, Iran.

hUCMCs isolation The protocol for the isolation and culture of hUCMs has been described elsewhere (Salehinejad *et al.* 2012). Briefly, human Wharton Jelly (WJ), was cut into small pieces and cultured in DMEMF12 supplemented with 10% FBS (Gibco), 100 IU/ml penicillin, streptomycin, and 2 μg/ml amphotericin B until the hUCM cells were propagated from the boundary of the explants. hUCM cells at 80% confluence were harvested by trypsin and ethylenediaminetetraacetic acid (EDTA) digestion. The viable cells were either used for experiments or cryopreserved with conventional freezing protocols (Kaviani *et al.* 2014).

Flow cytometry To confirm the mesenchymal and stemness properties of the isolated cells, the cell surface markers were determined using flow cytometry method accordingly; 1×10^5 hUCMs after the first passage were harvested, and the following markers including CD34, CD45, CD73, and CD90 were analyzed. The cells were fixed with 4% paraformaldehyde for 15 min, washed with phosphate-buffered saline (PBS), and incubated with goat serum in PBS to block non-specific binding protein. The cells were then exposed to 3–5 μl PE-conjugated anti-CD34, CD45, CD73, and CD90 for 1 h at 4°C. At least 10,000 events were recorded with flow cytometer machine (BD Biosciences). The data were analyzed by WinMDI software.

Osteogenic and adipogenic differentiation Adipogenic and osteogenic differentiation was induced as previously described (Salehinejad *et al.* 2012). About 5×10^4 hUCM cells were initially cultured for 24 h in 3-cm culture plates, and the osteogenic medium consisted of DMEMF12 supplemented with 10% FBS, 10 mM β-glycerophosphate, 10 nM dexamethasone, and 82 μg/ml ascorbic acid was added for 21 d. Differentiated cells were stained with Alizarin red and observed with a light microscope (Olympus, Japan).

For adipogenic induction, after 24 h initial culture, the cells were cultured for 14 d in adipogenic medium (Invitrogen, Carisbad, CA, A1007001). The differentiated cells were stained by Oil red O and observed with a light microscope. The same protocols were used for negative control, except that the adipogenic induction was omitted.

Differentiation of hUCMs to IPCs In the present study, four different protocols were employed for IPCs differentiation. The number of 4×10^5 hUCMs was cultured in 25 cm culture

Table 1. Details of various protocols

Protocols	Stages	Compositions	Duration (d)	References
I	Stage 1	DMEMF12+10% FBS	3–6	Chao et al. (2008)
	Stage 2	NCM	7	
	Stage 3	DMEMF12 (25 mM glucose)+nicotinamide (10 mM)+2% FBS	7	
	Stage 4	DMEMF12 (25 mM glucose)+nicotinamide (10 mM)+2% FBS+ITS+SCM	14	
II		Serum-free DMEMF12+glucose (17.5 mM)+nicotinamide (10 mM)+exendin-4 (10 nM)+hepatocyte growth factor (100 pM)+pentagastrin (10 nM)+B27 serum-free supplement (Gibco)	14	Timper et al. (2006)
III	Stage 1	H-DMEM medium, 5% FBS+nicotinamide (10 mM)+exendin-4 (10 nM)	14	Wu et al. (2009)
	Stage 2	Addition of nicotinamide (10 mM)	7	
	Stage 3	Addition of exendin-4 (10 nM)	7	
IV	Stage 1	DMEMF12+10% FBS	3	New modified protocol
	Stage 2	NCM	5	
	Stage 3	Serum-free DMEM/F12+glucose (17.5 m M)+nicotinamide (10 mM)+exendin-4 (10 nM)+pentagastrin (10 nM)+hepatocyte growth factor (100 pM)+B27 serum-free supplement (Gibco)	9	

flask, and after 80% confluence, differentiation protocols were carried out according to Table 1. The medium was refreshed every 3 d except in the stage 2 from protocol I and also the modified protocol that the medium was changed every other day.

Preparation of the neuronal-conditioned medium Seven-day-old rats were killed, and their brains were removed. After thoroughly rinse in PBS, the brains were immersed in DMEM-F12 with 10% FBS, cut into small pieces, grounded and abraded, and passed through a 50-μm steel mesh to obtain single cells. The samples were then immersed in DMEM-F12 supplemented with 10% FBS and incubated at 37°C with 5% CO_2 in the air for 24 h. The supernatant containing neural cells and excluding oligodendroglia (adherent cells) was transferred to a new flask and incubated with 10% FBS-DMEM at 37°C and 5% CO_2 in the air for 4 d. NCM was prepared after two times centrifugation at 3000 rpm for 5 min.

Stem cell-conditioned medium preparation hUCMs were cultured in DMEMF12 supplemented with 2% FBS, 10 mM nicotinamide, and 1 mM glutamine for 3 d. The supernatant was then removed and used as stem cell-conditioned medium (SCM) (Chao *et al.* 2008).

NCM exposure time determination and anti-nestin immunocytochemistry For determining the appropriate time of NCM exposure, about 10×10^4 hUCM cells were cultured for 24 h in 6 cm culture plates and the NCM was mixed with an equal volume of complete culture medium. The medium was changed every other day. The culture dishes were analyzed for nestin expression by immunocytochemistry (ICC) at days 3, 5, and 7. For the detection of nestin expression, the cells in protocols I and IV, after stage 2 were fixed with 4% paraformaldehyde for 30 min and subsequently washed with PBS. The cells were stained using mouse anti-nestin antibody (Abcam) followed by a Dako Envision+Dual system-HRP kit (Dako-Cytomation Inc., Carpenteria, CA) as recommended by the manufacturer. The cell nuclei were counterstained with hematoxylin for 2 min and observed under a light microscope (Nikon TS100).

Reverse transcription-PCR At the end of the differentiation time, the cells were collected to examine the expression of insulin gene. Total RNA was extracted by Total RNA Purification Kit (Jena Bioscience). The RNA samples were treated with DNase I (Fermentas) to eliminate any DNA contamination; 100 ng total RNA was then used for complementary DNA (cDNA) synthesis with Accupower Cycle Script RT Premix kit (Bioneer). Primers in Table 2 were used for RT-PCR reaction. The PCR reactions were accomplished in 20 μl reaction mixtures containing 2 μl of cDNA, 2 μl forward and reverse primers, 10 μl master mix (Amplicon), and 6 μl Dnase/RNase-free water. Thirty-five cycles were carried out using the following program and a thermal cycler machine (Bio-Rad c1000 USA): 4 min denature step at 95°C, 35 cycles at 94°C for 30 s, 30 s of annealing step at 58–60°C, and elongation step at 72°C for 45 s with a final extension at 72°C for

Table 2. Primer sequences for RT-PCR

Gene	Size	Strand	Sequence
GAPDH	86	F	TGCACCACCAACTGCTTAGCT
		R	GGCATGGACTGTGGTCATGAG
Insulin	192	F	TTCTTCTACACACCCAAGAC
		R	CTAGTTGCAGTAGTTCTCCA

5 min. PCR products were transferred to 2% agarose gel and visualized with a gel documentation system (Bio-Rad USA).

Immunocytochemistry analysis of insulin and proinsulin The differentiated cells were fixed in 4% paraformaldehyde for 30 min, washed with PBS, and incubated in PBS containing 0.5% Triton X-100 and 10% goat serum for 45 min at 37°C to block non-specific banding protein sites. The primary guinea pig anti-insulin antibody (Ab7842, 1:100) and anti-proinsulin antibody (Ab7761, 1:200) were then added overnight at 4°C, followed by incubation with Alexa Fluor 488 Goat Anti-guinea Pig IgG (Ab150185) and Alexa Flour Goat Anti-mouse IgG (Invitrogen) as secondary antibodies for 45 min at room temperature. The cells nuclei were visualized by 5 μg/ml Hoechst 33258. The stained cells were observed under a fluorescence-inverted microscope equipped with a digital camera (DP71, Olympus, Japan).

Dithizone staining About 5×10^4 hUCM cells were cultured in 3 cm culture plates and were exposed to various protocols. After differentiation of the hUCMs into IPCs, dithizone (DTZ) staining was performed to confirm IPCs differentiation. A stock solution was prepared by dissolving 10 mg dithizone (D5130) in 1 ml dimethyl sulfoxide (DMSO); 10 μl stock solution was then mixed with 1 ml PBS, and the culture dishes were incubated in DTZ solution for 15 min at 37°C, washed subsequently three times with PBS, mounted with glycerol, and observed with an inverted microscope (Olympus, Japan) equipped with a digital camera. Undifferentiated hUCMs were used as negative control.

Biochemical assessment of insulin, C peptide secretion, and glucose sensitivity At the end of differentiation period, the cells were cultured overnight in low glucose DMEM medium supplemented with 2% FBS. The cells were then

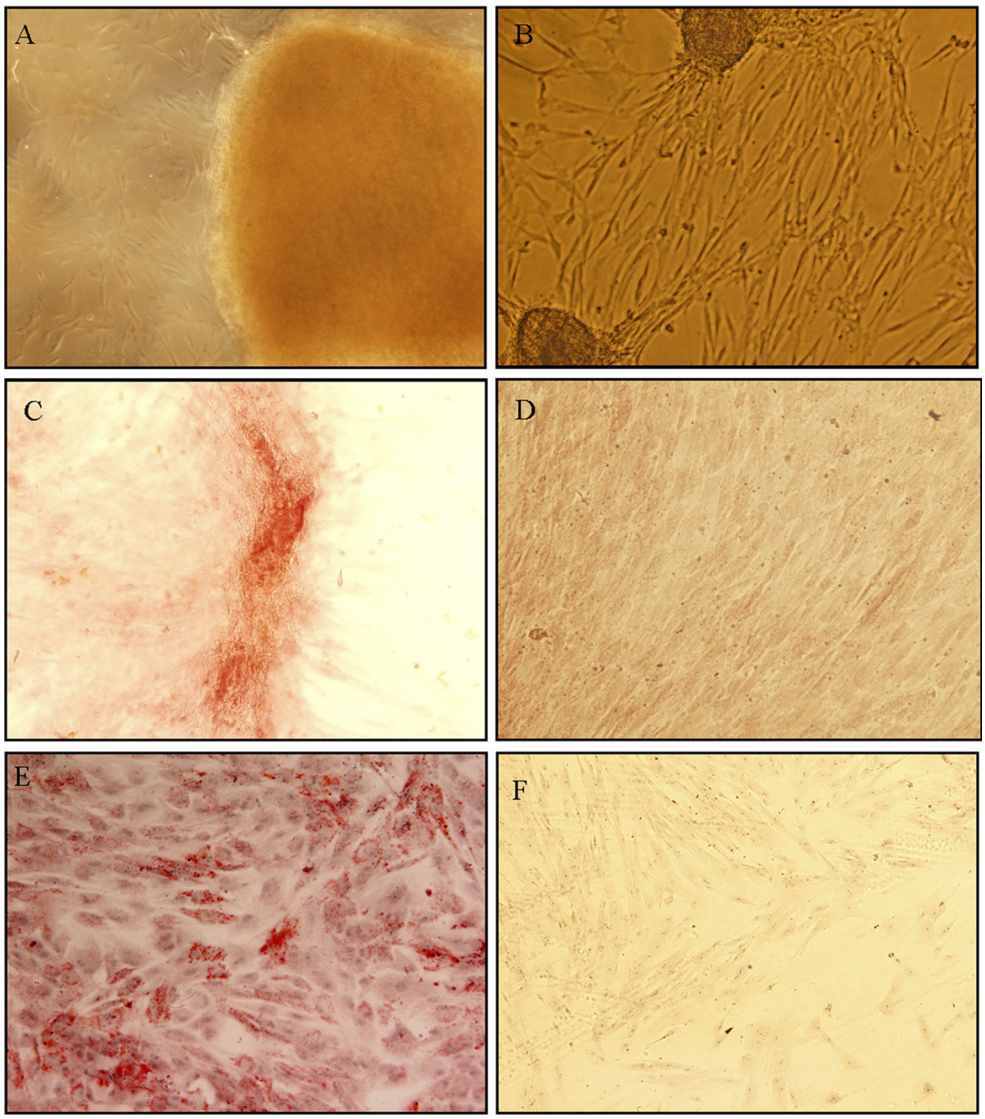

Fig. 1 Projection of hUCMs cells from human Wharton Jelly boundary (*A*). Colony formation in hUCM cells (*B*). *Alizarin red staining*, displayed Ca^{2+} aggregation in the extracellular matrix of induced cells (*C*) and lake of this in non-induced hUCM cells (*D*). *Oil red staining* exhibited lipid droplets in the cytoplasm of the induced cells (*E*) and lake of this in non-induced hUCMs cells (*F*). Magnifications *A* ×100 and *B*, *C*, *D*, *E*, and *F* ×200).

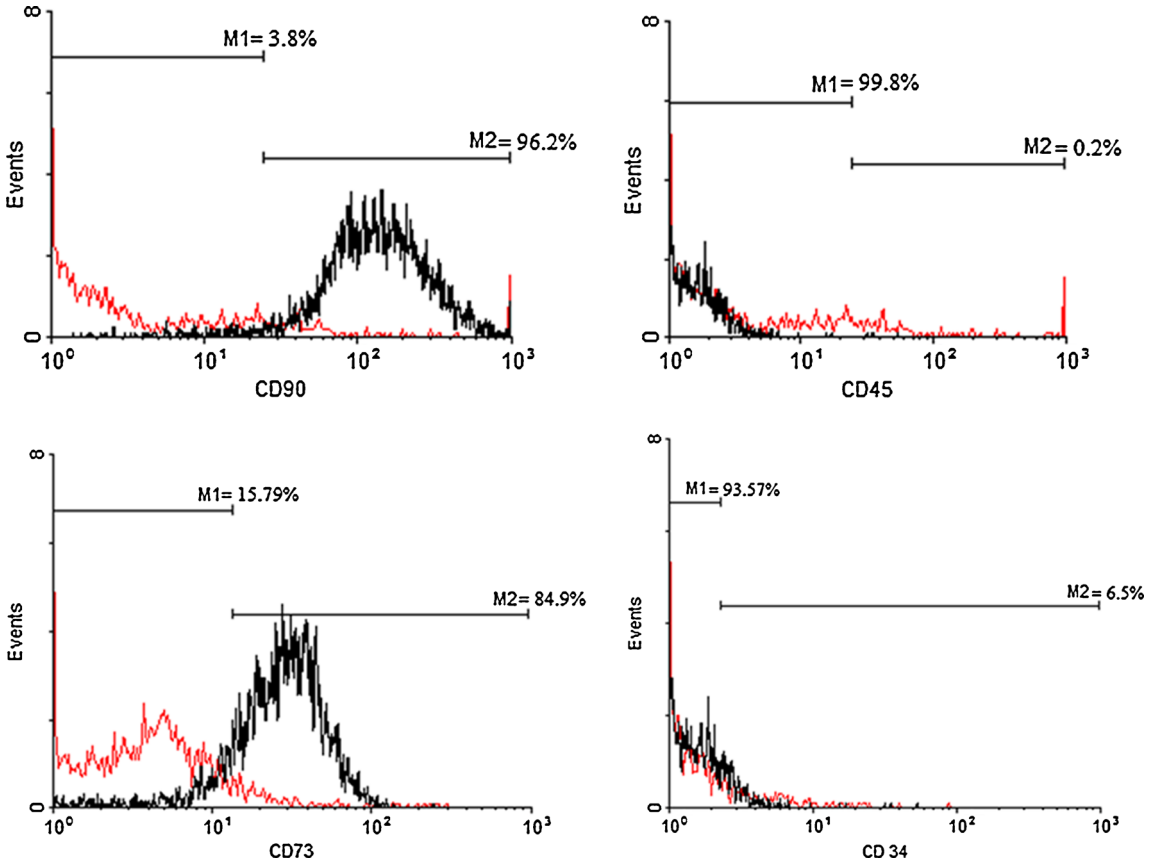

Fig. 2 Flow cytometry analysis of hUCM cells. *Red histograms* display the isotype control-stained cells, and the *black histograms* show the antibody-stained cells.

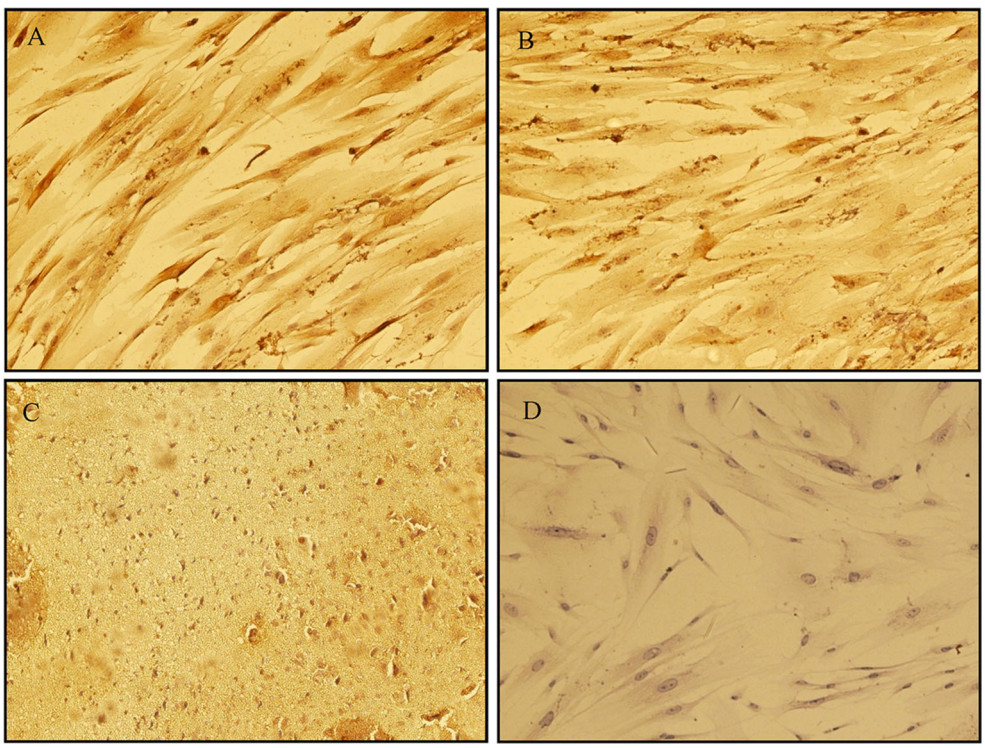

Fig. 3 Immunocytochemical analysis on nestin protein in protocol I (*A*), protocol IV (*B*), mouse brain (*C*), and hUCM cells (*D*). Magnification ×200.

exposed to high and low glucose medium for 1 h, respectively, to determine the insulin secretion. The supernatant was collected and kept frozen at −20°C for the assessment of insulin and C peptide secretion by CLIA and EIA, respectively.

Statistical analysis Data from different groups were analyzed by one-way ANOVA and tukey post- hoc test. A difference of $p \leq 0.05$ was considered statistically significant. The data were presented as mean±SE.

Results

hUCM cells isolation The isolated cells were observed 7–10 d after the initiation of explant culture at the boundary of the tissue fragments (Fig. 1*A*). The majority of cells were elongated with several cytoplasmic processes, while few of them exhibited round shape with small cell processes. By extending the culture period, cell colonies became visible in culture (Fig. 1*B*). Mitotic index was high in these cells (data not shown). At confluence of >80%, the cells were detached from the substratum with trypsin/EDTA and expanded for further experiments.

Flow cytometery and surface marker assessment The isolated cells at the first passage were analyzed for mesenchymal cell markers as well as hematopoietic markers. The majority of adherent cells were negative for CD34 and CD45, indicating that these cells were not of hematopoietic origin, while they were positive for CD73 and CD90, which is specific for cells of the mesenchymal origin (Fig. 2).

Osteogenic and adipogenic differentiation In the osteogenic induction medium, hUCM differentiated into osteocytes with numerous cell expansions. Alizarin red staining demonstrated calcium phosphate deposits in extracellular matrix (Fig. 1*C*). The presence of adipocytes with lipid droplets

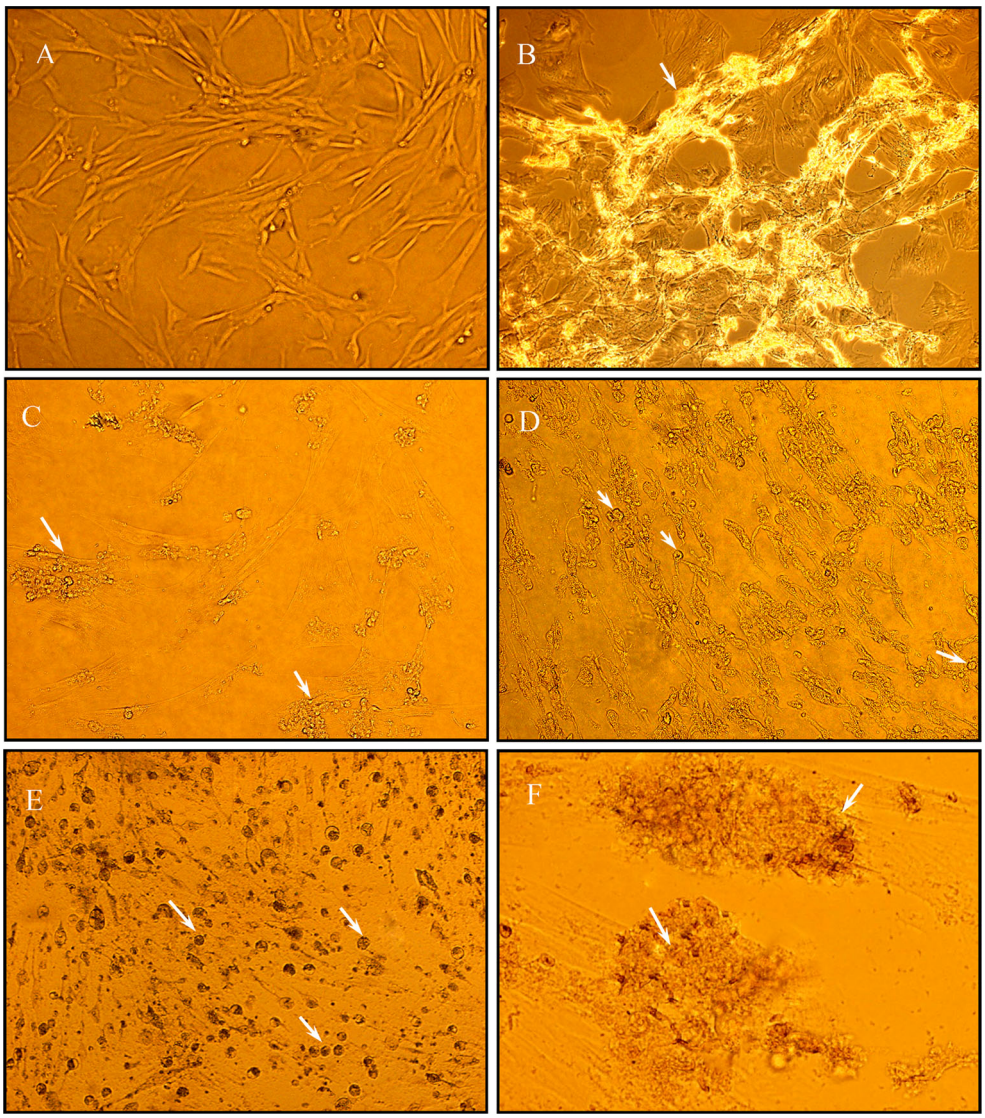

Fig. 4 Morphological changes in different groups of hUCM cells. Undifferentiated hUCM cells (*A*), differentiated cells in protocols I (*B*), II (*C*), III (*D*), and IV after 7 d (*E*) and 14 d (*F*). The *arrows* show round shape of the cells in (*D*) and (*E*) and cluster formation in (*B*), (*C*), and (*F*). Magnification ×200.

stained with Oil red O demonstrated the adipogenic differentiation of hUCMs (Fig. 1*E*). No spontaneous differentiation was observed in control cultures (Fig. 1*D*, *F*).

NCM appropriate exposure time determination Immunocytochemical analysis was carried out for the detection of nestin protein, after exposure of hUCM to NCM for 3, 5, and 7 d. Proportion of nestin positive cells was 30±3.7, 61±3.8, and 89±2.8 for 3, 5, and 7 d exposure times, respectively. The morphology of the NCM-exposed cells changed from day 1 onwards. By day 2, the exposed cells showed some dark particles inside the cytoplasm. After addition of the differentiation medium, the cell shape changed from spindle to round or oval form, for 1–2 d, after which they exhibited secretory-like cells morphology. The cell size increased gradually, while the attachment properties of the induced cells decreased. From day 5 on, some of the exposed cells gained neural-like morphology with long slender cell processes and small cell bodies.

Nestin assessment When the cells were exposed to NCM, some dark particles were detectable under inverted microscope from day 2 on (Fig. 3*A*, *B*). ICC staining demonstrated nestin expression in the majority of the NCM-treated cells in protocols I and IV (Fig. 3*A*, *B*), mouse brain as positive control (Fig. 3*C*), and no expression in untreated cells as negative control (Fig. 3*D*) were used.

Morphology of IPCs In protocol I, the induced cells initially passed through a nestin expression stage confirmed by ICC. During the 3rd stage, in which the cells were cultured in high glucose medium, the cell shape became round and oval (data not shown). Three to four days later, the cells started to

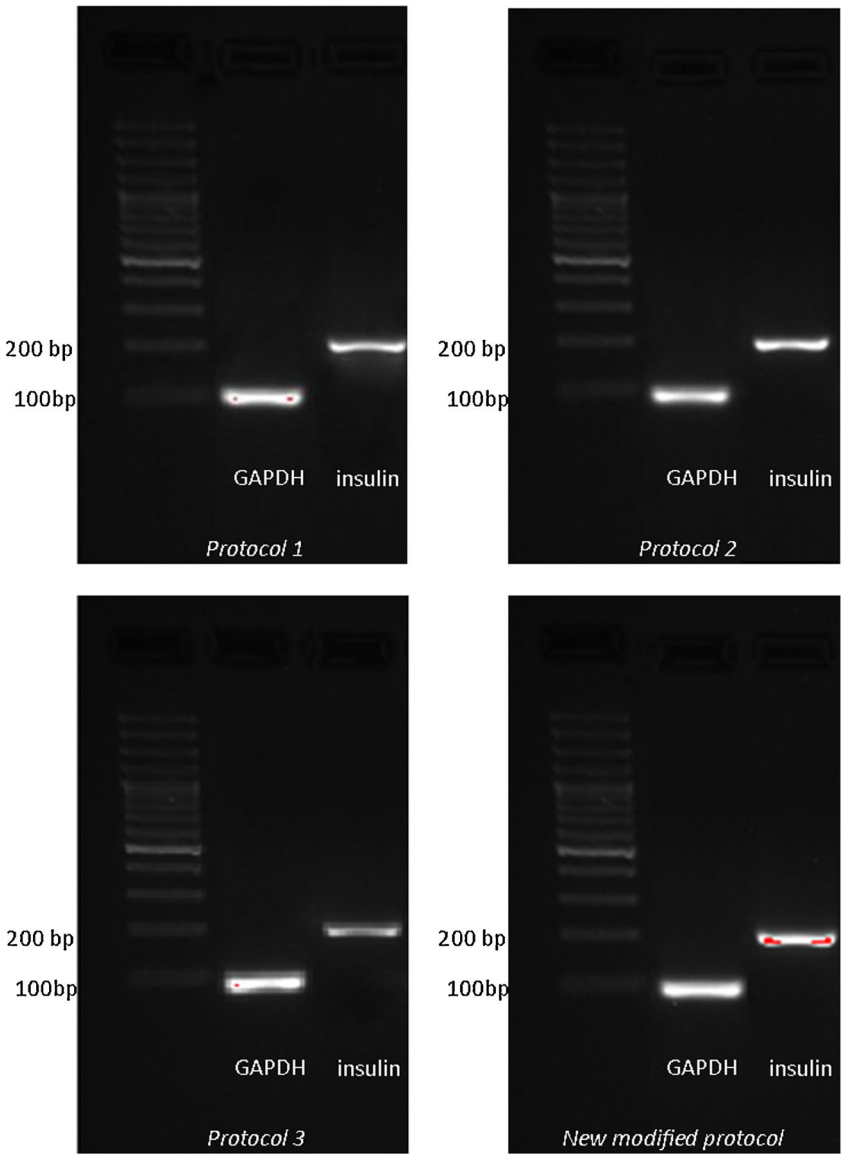

Fig. 5 GAPDH (87 bp) and insulin (192 bp) gene expression in the different protocols.

aggregate. At stage 4, the cells aggregation proceeded and resulted in islet-like structures (Fig. 4*B*).

In protocol II, after exposure of hUCMs to the induction medium, the spindle shape cells became round, aggregated, and formed islet-like cluster (Fig. 4*C*).

In protocol III, although the induced cells became round, no obvious cluster was observed (Fig. 4*D*).

In protocol IV, induced hUCM cells formed small dark particles in the cytoplasm after NCM treatment. During the 3rd stage, in which the cells were exposed to IPC-inducing medium, the cell shape changed from spindle to a round shape. During the following days of culture and induction, cell size increased while the cell attachment decreased (Fig. 4*E*). The cells in protocol IV formed more apparent clusters than the other protocols (Fig. 4*F*).

Reverse transcriptase-PCR Gene expression was evaluated by RT-PCR. GAPDH, as a reference gene was expressed significantly in cultured cells. *Insulin* gene was expressed in the induced cells collected from all four groups as well (Fig. 5).

Detection of insulin by immunocytochemistry Immunocytochemical analysis was carried out on the induced cells of either protocols. ICC revealed insulin (Fig. 6) and pro-insulin (Fig. 7) protein production in all the protocols, but it was higher in protocol IV compared with the other protocols.

Dithizone staining IPCs clusters in the different protocols were stained with DTZ. The intensity and the extent of DTZ-positive cells were more obvious in the new modified protocol IV than the others (Fig. 8).

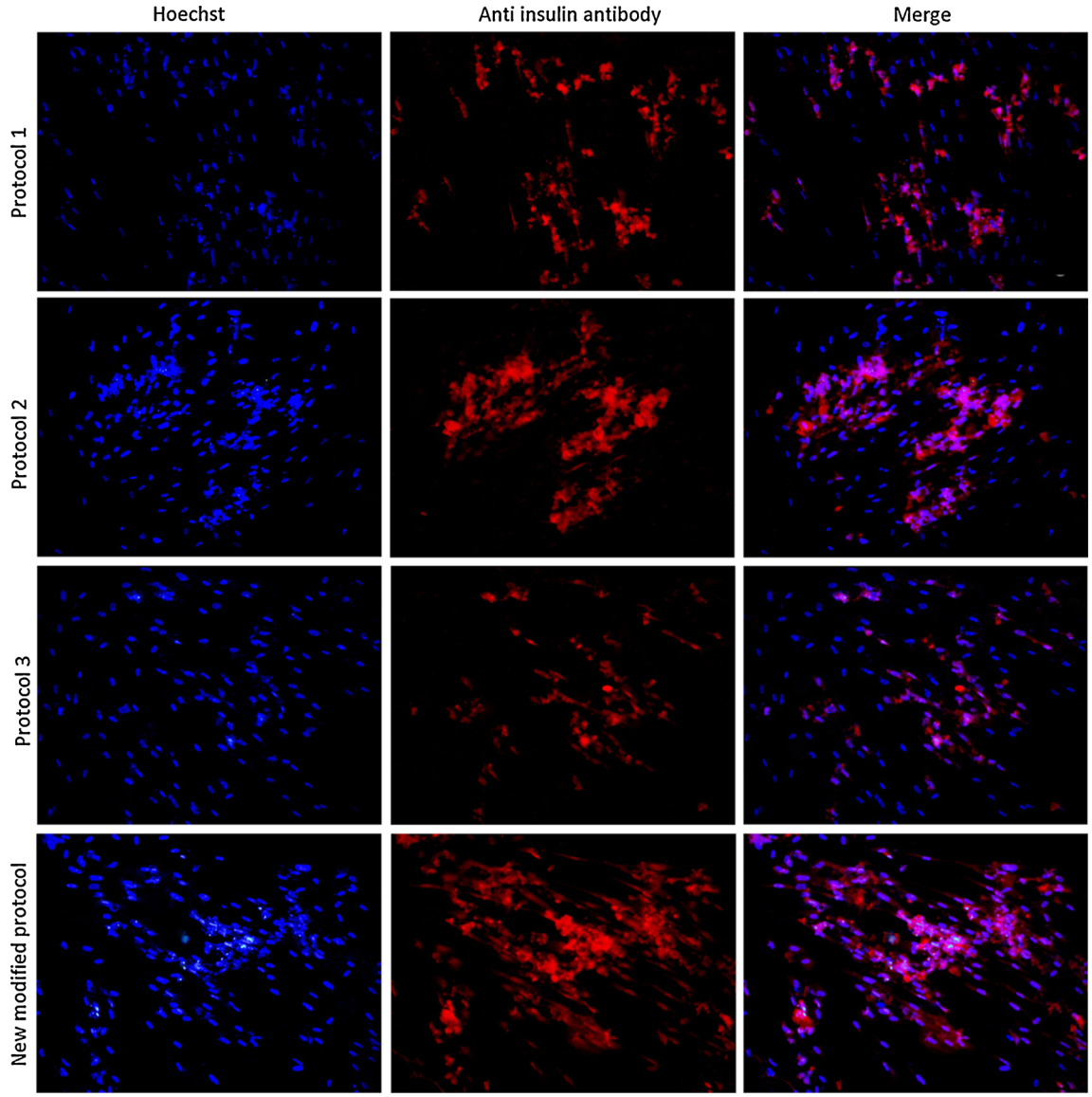

Fig. 6 Immunocytochemical analysis on insulin protein in the different protocols after pancreatic differentiation; the cell nuclei were counterstained with Hoechst. *Magnification* ×200.

Insulin, C peptide secretion, and glucose challenge test The insulin and C peptide secretion rate in either protocols was measured by using CLIA and EIA, respectively. The results demonstrated that insulin secretion rate in the new modified protocol IV was significantly higher than the other groups (Table 3).

Discussion

In the preset study, we compared three commonly used induction protocols used for IPCs differentiation with a modified protocol to achieve higher insulin production in vitro. Our study demonstrated that exposure of human umbilical cord matrix-derived mesenchymal cells to neuronal-conditioned medium followed by 9 d induction with inducing agents yields greater insulin and proinsulin intracellular protein production which was confirmed by immunocytochemistry and a sharper band of insulin gene in the modified protocol.

Considering DTZ staining results, the modified protocol IV exhibited greater differentiation potential when the size of clusters and dye intensity was considered among the groups. DTZ is a zinc-binding substance (Shiroi *et al.* 2002), which is used to visualize insulin granules of pancreatic β cells (Chausmer 1998; Shiroi *et al.* 2002; Moshtagh *et al.* 2013). Free ionized zinc serves as a reservoir for insulin granules in the extra-granular space (Chausmer 1998). These zinc pools are usually used to characterize IPCs (Shiroi *et al.* 2002; Moshtagh *et al.* 2013). Increase in DTZ staining intensity in protocol IV may indicate the higher number of differentiated cells as well as increase in secretory granules.

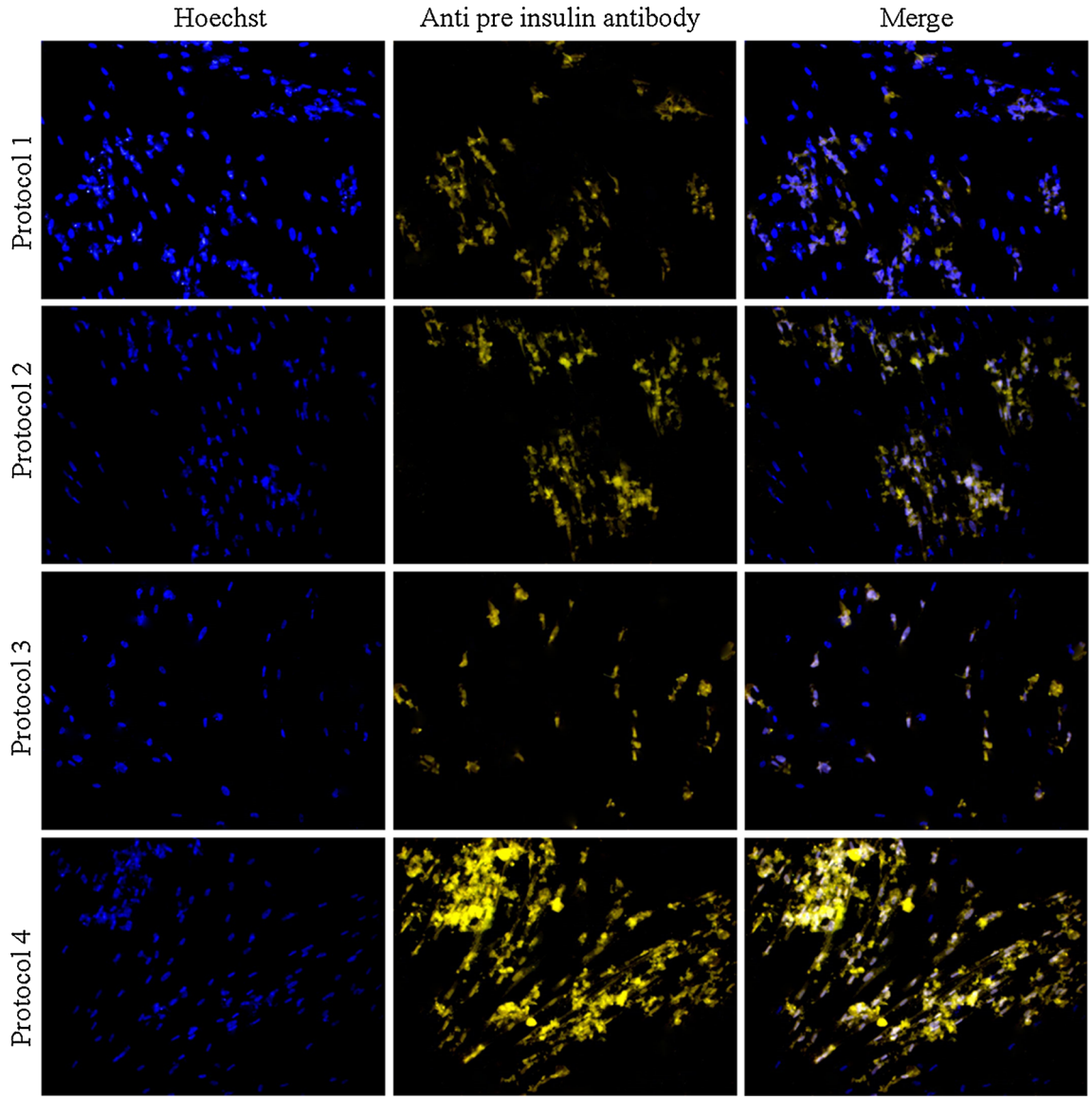

Fig. 7 Immunocytochemical analysis on proinsulin protein in the different protocols after pancreatic differentiation; the cell nuclei were counterstained with the Hoechst staining. Magnification ×200.

Higher rate of insulin secretion in new modified protocol paralleled the results obtained from ICC, DTZ, and RT-PCR. It was the highest insulin secretion rate in protocol IV compared with the other protocols. In addition when in vitro IPCs were challenged with higher glucose concentrations, induced cells in all the protocols responded by increase in insulin secretion but the cultured cells in protocol IV secreted significantly higher insulin amounts than the cells in the other protocols.

Nestin, an intermediate filament predominantly expressed in pancreas progenitor cells as well as neuron precursor cells, is an important molecule for IPC differentiation (Hunziker and Stein 2000; Zulewski *et al.* 2001). So we induced hUCM cells to undergo an intermediate stage of nestin expression by introduction of NCM at the initial stage of differentiation of hUCM cells towards IPCs. Some similarities have been reported between embryonic signaling pathways in IPCs and neuroepithelial cells (Edlund 2001). Endoderm-derived pancreatic β cells and ectoderm-derived nerve cells are similar in many features, including biosynthetic enzymes, transcription factors, secretory, and metabolic proteins (Zulewski *et al.* 2001). Passage of stem cells through a nestin-positive stage has been proposed as a necessary event for pancreatic differentiation (Hori *et al.* 2005; Chao *et al.* 2008; Wei *et al.* 2013). We first cultured hUCM cells in NCM containing-medium to alleviate the nestin-positive cell population followed by incubation of pretreated cells in IPC-inducing medium. The time of exposure to NCM also plays an important role in IPC differentiation. We examined three exposure times; 3, 5, and 7 d and found that cultivation of hUCM cells for 3 d in NCM results in a small number of nestin-positive cells, while extension of exposure time to 7 d, resulted in neuronal morphology of the NCM-treated cells and a presecretory small number of hUCMs. Considering these results, the most appropriate time

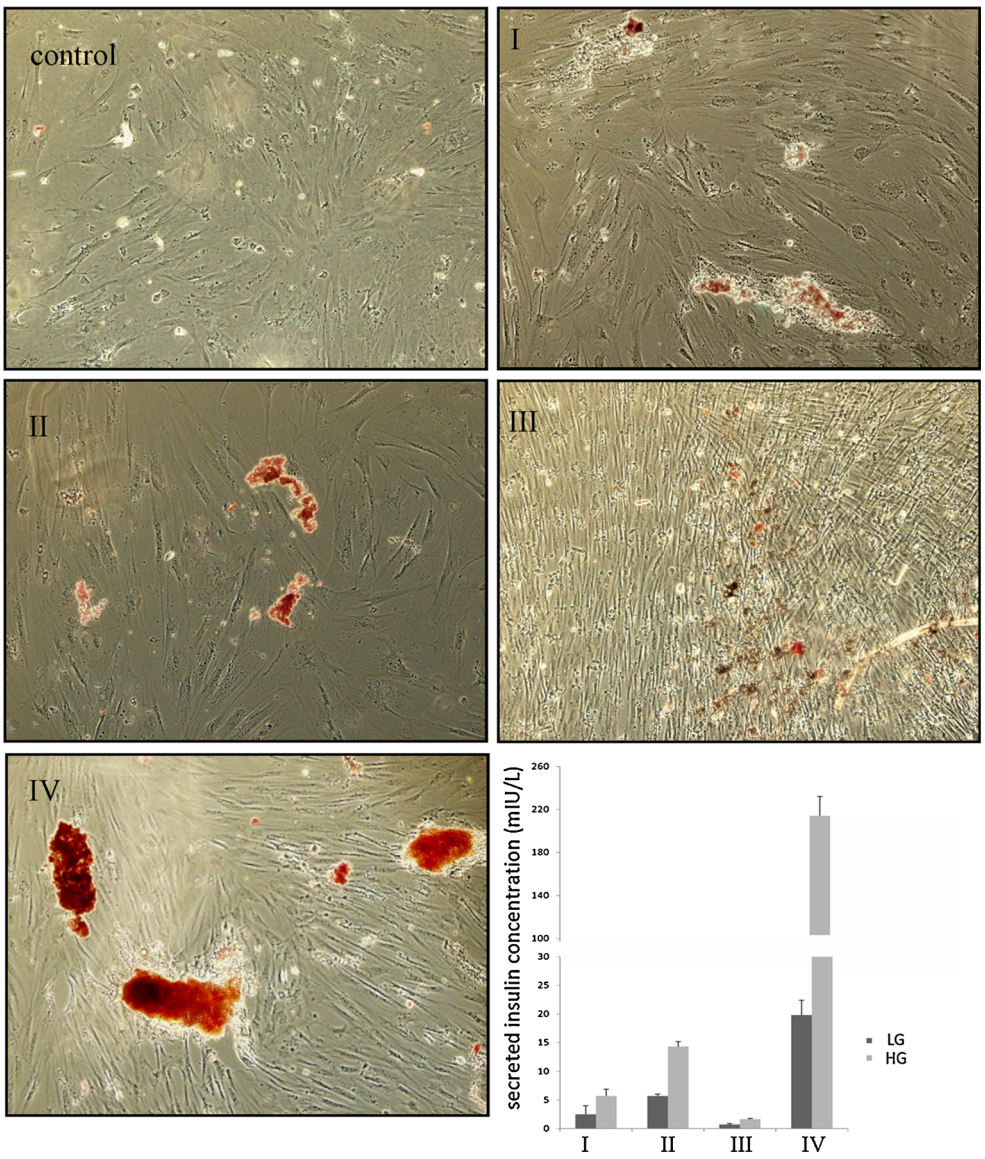

Fig. 8 Dithizone staning on control and induced cells in protocols I–IV. Magnification ×100. The diagram shows insulin secretion in I–IV protocols. *LG* low glucose medium, *HG* high glucose medium.

Table 3. Insulin and C peptide secretion rate in the different protocols

Groups	Insulin secretion		C peptide secretion	
	Low glucose	High glucose	Low glucose	High glucose
Protocol I	2.5±1.5	7.8±1.2	85±12.5	150±50
Protocol II	5.7±0.35	14.3±0.85	166.6±33	233±33.3
Protocol III	0.73±0.16	1.66±0.13	100	200
Protocol IV	19.8±2.59*	214.6±18.2**	106±6.6	233±33.33

Values were pooled from three independent experiments and were expressed as mean±SE

*$P \leq 0.00$

for the exposure of hUCMs to NCM was adjusted at 5 d, during which more nestin-positive cells capable of differentiating to IPCs were recognized. However, animal source of NCM should be considered when it is to be used for human regenerative procedures. Some sera (FBS, FCS) and components (BSA) are among the mostly used materials which are usually obtained from animals. Whether NCM can also be used without the fear of xenogenic transformation needs to be explored. Analysis of NCM components which may enhance differentiation of MSCs towards IPCs can reduce probable xenogenic effects of NCM.

After the initial stage of nestin expression in the cultured hUCM cells, they should undergo pancreatic induction by endocrine- and pancreatic-inducing materials. The previous studies have shown that several materials are critical or supportive for pancreatic differentiation (Wong 2011). Some of these inducing factors are: nicotinamide that promotes differentiation of various stem cells into IPCSs and increase the insulin and DNA content (Otonkoski *et al.* 1993; Gabr *et al.* 2008; Gao *et al.* 2008), exendin-4 that induces stem cell differentiation (Gabr *et al.* 2008) and increase insulin secretion from IPCs (Li *et al.* 2010), gastrin that stimulates islet differentiation and islet growth (Wang *et al.* 1993), HGF that stimulate the number of IPCs in cultured human islets (Otonkoski *et al.* 1996), bFGF that promotes beta-cell differentiation and may accelerate cluster formation (Hardikar *et al.* 2003), activin A that increases insulin content and beta-cell differentiation (Demeterco *et al.* 2000), betacellulin that plays some role in cluster formation and induction of beta-cell differentiation (Demeterco *et al.* 2000), and finally low concentration of glucose that raises insulin content and high concentration that increases beta-cell reproduction (Bonner-Weir *et al.* 1989).

The difference between new modified protocol IV and protocol II was the use of an initial stage of nestin preselection. An initial exposure of hUCMs to NCM followed by prolonged culture in the presence of inducing agents resulted in higher rate of pancreatic differentiation and more insulin secretion. Although nestin preselection is an important stage in the IPC differentiation procedure, exposure of hUCMs to the extrinsic materials has also a critical role. In protocol I, despite the nestin preselection of hUCM cells, insulin secretion was lower than protocol II that more components (Table 1) were used for IPC differentiation. In contrast, in protocol II, the majority of the inducing materials were used but the nestin preselection stage was omitted. The insulin secretion following glucose challenge test was higher than protocol I but lower than protocol IV. In protocol III, only some of common inducing materials were used without nestin preselection stage, and the insulin secretion was lower than the other protocols. As IPC differentiation is a rather complicated phenomenon, an initial stage of nestin preselection and appropriate inducing materials are both necessary for adequate pancreatic differentiation.

Culture period is another important issue when the differentiated stem cells are to be used in regenerative therapies. Shorter protocols will reduce expenses, decrease the probability of contaminations that are mostly generated in the environmental conditions, and increase the simplicity of the procedure. The new modified protocol IV was 2 wk shorter than protocols I and III but identical to protocol II. Nestin preselection stage of hUCM cells followed by 9 d differentiation in an induction cocktail promoted IPC differentiation and insulin secretion. However, insulin secretion is still lower than in vivo environment. Change in the inducing milieu such as 3D culture, co-culture of cells with appropriate somatic cells, and other inducing components may increase the efficacy of the models for IPC differentiation.

Conclusion

From the results, we may conclude that appropriate period of nestin preselection stage followed by induction with adequate inducing agents will enhance human umbilical cord matrix-derived mesenchymal cells to differentiate into greater number of insulin-producing cells and more insulin secretion in vitro.

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