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DIFFERENTIATION OF NESTIN-POSITIVE MESENCHYMAL STEM CELLS INTO INSULIN PRODUCING CELLS

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ABSTRACT

Diabetes constitutes a worldwide epidemic affecting all ethnic groups. Stem cell therapy provides an effective way to regenerate insulin-producing cells lost during the disease. Accordingly, we isolated and compared the differentiation ability of nestin rich bone marrow mesenchymal stem cells (MSCs) into insulin producing cells (IPCs) with the nestin poor counterpart. The purity of the isolated populations was evaluated by immunofluorescence then, cells were differentiated into IPCs. At the end of differentiation, cells were evaluated by expression of pancreatic endocrine genes, immunofluorescence as well as the release of human insulin and c-peptide according to increasing glucose concentrations that showed a significant difference. Under confocal microscope the mean percentage of IPCs was 14.3% for nestin rich MSCs while nestin poor MSCs showed 2.17 %.

In conclusion, nestin positive cells is a sub-population of MSCs, capable of generating IPCs in higher percentage than its negative counterpart.

Key words: Nestin; Mesenchymal stem cells; differentiation; insulin producing cells

INTRODUCTION

Diabetes mellitus (DM) is a widespread devastating disease affecting millions of people worldwide. Maintaining good glycaemic control with exogenous insulin imposes a burden on patients **(Sherwin and Jastreboff, 2012)**.

For diabetes, maintaining an appropriate glycemic control using exogenous insulin is possible but represents a load on patients. Transplantation of an intact pancreas as well as isolated pancreatic islets is ideal alternative. However, the shortage of cadaveric organs and the need for immunosuppression are limiting factors **(Ryan et al., 2005)**.

The progress in the field of regenerative therapies provides the potential for the generation of surrogate β-cells from stem cells derived from various sources. Mesenchymal stem cells (MSCs) display a high capacity for self-replication, thereby providing a large number of autologous cells while avoiding the limitations of ethical issues, organ availability, and allogenic rejection. MSCs derived from various tissues were utilized in an attempt to differentiate them into IPCs. Bone marrow **(Karnieli et al., 2007; Sun et al., 2007; Gabr et al., 2013)** and adipose tissue **(Timper et al., 2006)** are among several other tissues that have also been used to generate IPCs. Although the use of MSCs as a source for surrogate β-cells is very attractive, the most successful

differentiation protocols have produced only a modest number of functional IPCs **(Evans-Molina et al., 2009)**.

Nestin, which is a marker for neural stem cells **(Matsuoka et al., 2002)**, has been reported in regenerating pancreas and also in early embryonic stem (ES) cell derivatives **(Blyszczuk et al., 2003)**. The mechanism of nestin action in ES and adult pancreatic ductal stem (PDS) cells was investigated in regard to the neogenesis of insulin-secreting β-cells. Accordingly, these data may demonstrate that nestin is a stem cell marker constituting a functional factor at differentiation of stem cells **(Kim et al., 2010)**. It was suggested that nestin has a vital role as an intermediate regulator controlling differentiation of stem cells during their differentiation into insulin-producing cells **(Zulewski et al., 2001; Maria-Engler et al., 2004)**.

It has been shown for the first time that rat bone marrow multipotent nestin-positive stem cells can be differentiated in vitro into pancreatic ductal and insulin-producing β-cells **(Milanesi et al., 2011)**.

Herein, we isolated nestin-positive mesenchymal stem cells from adult human bone marrow to evaluate their efficiency to differentiate into insulin-producing cells (IPCs) as compared with nestin negative fraction.

MATERIALS AND METHODS

1. Retrieval of human bone marrow cells

In the present study, the approvals required for all the procedures were obtained from Mansoura University ethical committee. The aspirates of bone marrow were collected in heparin from the iliac crests of three type II, insulin-requiring diabetic donors.

2. Isolation and expansion of MSCs derived from bone marrow

Isolation and expansion of MSCs was carried out as previously described **(Gabr et al., 2014)**. Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, Missouri, United States), low-glucose was used to dilute the obtained BMAs by the ratio 1:1 then, the mixture was added dropwise on Ficoll-Paque, 1.077 g/mL (Pharmacia, Uppsala, Sweden) by the ratio of 2:1 respectively in order to form 2 distinct layers. Then a centrifugation at 600 g for 10 min was applied. Thereafter, the layer of mononuclear cells was collected from the DMEM/Ficoll interface then, phosphate-buffered saline (PBS) was used for washing cells twice. After that, cells were resuspended in 10 mL low-glucose complete DMEM (provided with100U/mL penicillin, and 100U/mL streptomycin (Sigma Aldrich) in addition to fetal bovine serum (10%) (HyClone, Logan, Utah, United States)). The cells were then cultured in 25 cm^2 tissue culture flasks at a density of 5×10^5 nucleated cells/mL and incubated in a 5% CO_2 incubator at 37^oC. Discarding the non-adherent cells was performed after 3 days of culture.

The subculture using trypsin was performed for the adherent MSCs when they reached 80% confluence where the cells resuspended in complete culture media (DMEM) and recultured at a ratio of 1:2 till reaching 80% confluence and so on for the next passage. The samples from each donor were examined in duplicate for the in vitro components of this study.

3. Isolation and expansion of nestin cells by magnetic cell separation method

Nestin cells were isolated by magnetic cell separation method (Easysep magnetic cell separator, Stemcell Technologies, Vancouver, British Columbia, Canada) using EasySep®

"Do-It-Yourself" Selection Kit (Stem cell Technologies) and nestin monoclonal antibody (ThermoFisher Scientific, Waltham, Massachusetts, United States) **(Dobbin and Landen, 2013)**.

Cell suspension was prepared at a concentration of $1x10^7$ cells/100 µL recommended medium (PBS with 2% FBS and 1 mM EDTA, Ca^{+2} and Mg^{+2} free), the cocktail assembled for positive selection was added to the cell suspension at 10 µL/100 µL cells, then the suspension was incubated for 15 min at RT after mixing well, then magnetic nanoparticles were added at 5 µL/100 µL cells and mixed well then incubated for 10 minutes at RT. Afterthat, the cell suspension was completed to reach a total volume of 2.5 mL by adding recommended medium.

The tube with the cell suspension was then put into the magnet for 5 minutes, then the supernatant was poured off. The tube was then removed from the magnet and 2.5 mL of recommended medium were added, the cell suspension was mixed gently then the tube was then placed back in the magnet and for 5 minutes, the last 2 steps were repeated, for a total of three 5-minute separations; The isolated nestin rich and nestin poor cells were then cultured in the same method as MSCs.

4. Differentiation of nestin rich and nestin poor MSCs into IPCs

Differentiation was performed according to a protocol previously reported by Tayaramma and his team **(Tayaramma et al., 2006)**. At the first stage, a serum-free media (DMEM provided with Trichostatin-A (Sigma Aldrich) at a concentration of 55 nanomoles) was used for cell culture for 3 days. At the second stage, the cell culture lasted for another 7 days using high-glucose (25 millimoles) medium composed of DMEM:DMEM/F12 (Sigma Aldrich) with a ratio of 1:1 which was provided with fetal bovine serum (10%) and 10 nanomoles of glucagon-like peptide-1 (GLP-1, Sigma Aldrich).

5. Gene expression by qRT-PCR

Total RNA was extracted from viable MSCs by RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). Then, the concentration and the purity of the extracted total RNA was measured by Nanodrop 2000 instrument (Thermo Fisher, USA). Conversion of three micrograms of total RNA into cDNA was performed by using RT First Strand kit according to manufacturer's instruction (Qiagen Sciences, Maryland, USA). Primer sequences of endocrine custom gene arrays were designed on line at National Center of Biotechnology Information (NCBI) and supplied in 96-well plates (CAPH13024, Qiagen Science). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene for mathematical calculation **(Pfaffl, 2001)**. Human islets were also included to serve as positive control. Each reaction of the RT-PCR was performed with 25μL total volume which contains 12.5 μL of 2X SYBR Green Rox Master Mix (Qiagen Sciences), 11.5 μL of nuclease-free water and1 μL of cDNA template in a Qiagen custom plate (CAPH13024,Qiagen Science). This technique was performed by BioRad CFX96 thermal cycler (BioRad, USA) and the program was designed according to manufacturer instructions.

6. Immunofluorescence

Cell preparations were cultured on chamber slides (Nunc, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Then, the cells were fixed for 10 min at RT using paraformaldehyde (4%), permeabilized by chilled methanol (100%) for

10 min and blocked with normal goat serum (5%) at RT for 60 min, then incubated in the primary antibodies at 4°C overnight. These included mouse monoclonal anti-nestin (Thermo Fisher Scientific), mouse monoclonal anti-insulin, rabbit polyclonal anti-c-peptide (cell Signaling Technology, Danvers, Massachusetts, USA), Thereafter, a wash using PBS was applied for the cells, followed by an incubation at RT for 2 hours using the secondary antibodies (Alexa Fluor 555-conjugated antirabbit IgG (H + L) and Alexa Fluor 488-conjugated antimouse IgG (H + L) (Cell Signaling Technology)).The nuclei of these cells were counterstained with DAPI (Invitrogen, UK). Performing negative controls was achieved by avoiding treatment with the primary antibody **(Wang et al., 2014)**. Leica TCS SP8 microscope (Leica Microsystems, Mannheim, Germany) was used for capturing confocal images.

7. Determination of in vitro insulin and c-peptide release in response to increasing glucose concentrations

Insulin and c-peptide release of differentiated MSCs was performed according to the procedure described before **(Gabr et al., 2013)**.Three different samples (1×10^6) cells were collected from the same batch of each donor at the end of the differentiation period of nestin rich and nestin poor cells for measurement of released insulin and c-peptide. Cells were initially incubated for 3 hours in glucose-free Krebs-Ringer bicarbonate buffer (KRB). This was followed by incubation for 1 hour in 3.0 mL of KRB containing 5.5, 12, or 25 mM glucose concentrations. At the end of incubation, the supernatant was collected and samples were assayed by using an Elisa Kit (Diagnostic Automation / Cortez Diagnostics, Inc.) according to the manufacturer's instructions. Undifferentiated HBM-MSCs served as negative control.

8. Statistical analysis

Data were evaluated using SPSS 16.0; Independent samples t-test was used to evaluate P value for comparison between continuous data, P value of $< 0.0^{\circ}$ was considered significant. The mean values were used as a measure of variation **(Chinna et al., 2012)**.

RESULTS

1. Characterization of the undifferentiated cells

The cultured cells at the end of expansion phase became spindle-shaped, fibroblast-like cells arranged in monolayers. At passage 5, there was no difference in morphology between nestin rich and nestin poor cells (**Figure 1**).

Nestin rich and nestin poor cells were stained with anti-nestin antibody and analyzed under confocal microscope (**Figure 2**). The mean percentage of nestin positive cells was 91.1±3.33 for nestin rich cell population while it was 1.28±0.26 for the nestin poor one (**Table 1**).

2.Functional evaluation of the differentiated cells

2.1. Gene expression by RT-qPCR

At the end of differentiation protocol, the relevant endocrine genes including INS, GCG and SST in addition to the transcription factor PDX1 were expressed in nestin rich and poor cells. The results were calculated relative to the expression of human islets genes. Worthwhile, the expression of insulin gene in nestin rich cells was increased by 2.6 folds more than nestin poor cells as well as GCG and SST by 2.24 and 2.83 folds, respectively. The transcription factor PDX1 was also up

regulated in nestin rich cells by 2.49 folds more than nestin poor cells **(Figure 3)**.

2.2. Immunofluorescence

The presence of insulin granules within the cytoplasm of the insulin-positive cells of nestin rich cells was detected. In addition, immunostaining for c-peptide was also positive. Coexpression of insulin and c-peptide within the same cells was observed following electronic merging **(Figure 4)**. The mean percentage of IPCs detected by confocal microscope was 14.3±5.9 for nestin rich cells while it was 2.17±1.2 for nestin poor cells.

2.3. In vitro human insulin and c-peptide release in response to glucose challenge

The differentiated nestin rich and nestin poor cells released increasing amounts of insulin and c-peptide in response to increasing glucose concentrations (p value < 0.01). The released insulin and c-peptide amounts at different concentrations of glucose were comparable between the two populations **(Figure 5)**.

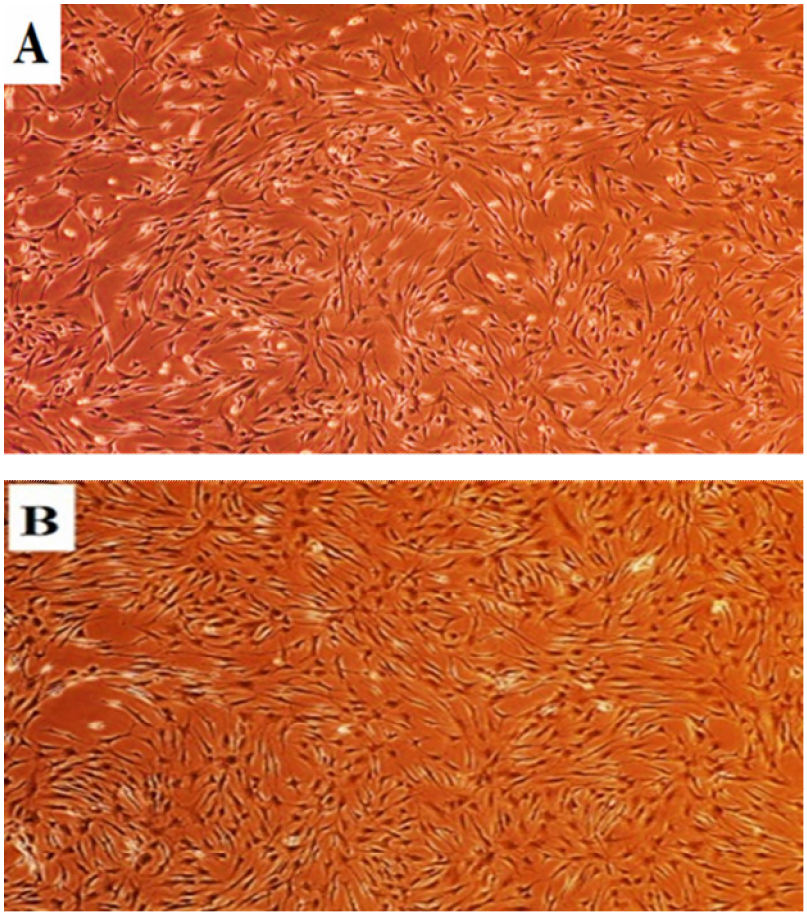

Figure (1): Morphology of the undifferentiated cells. (A) Nestin rich HBM-MSCs. (B) Nestin poor HBM-MSCs.

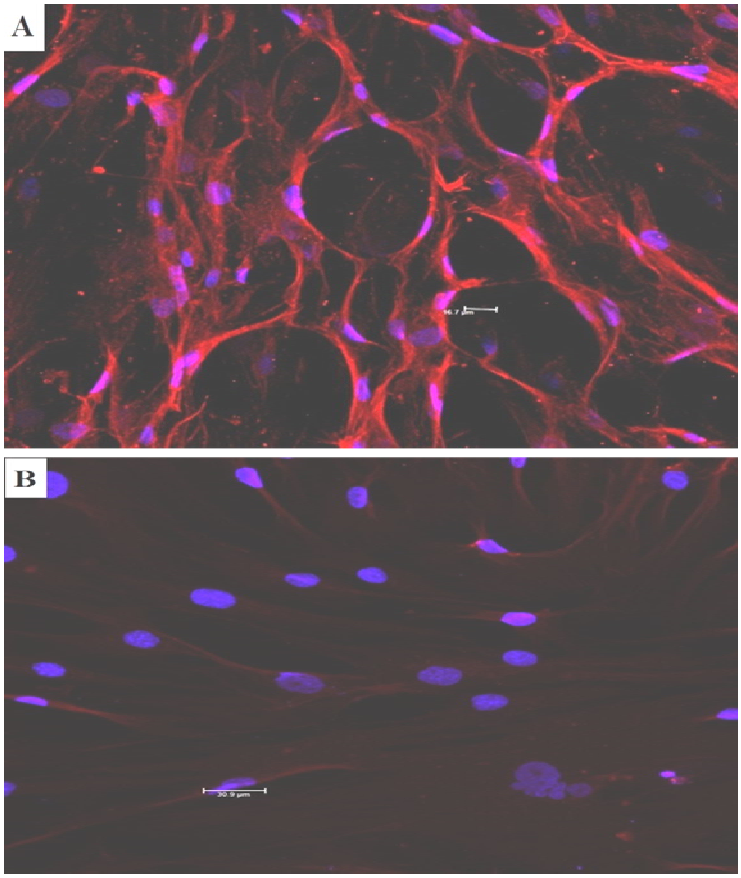

Figure (2): Immunofluorescence staining of the undifferentiated cells for Nestin detection. (A) Nestin rich HBM-MSCs, positive for nestin (red). (B) Nestin poor HBM-MSCs, negative for nestin.

Table (1): The positivity for nestin marker in nestin rich and nestin poor cells by immunofluorescence

	Nestin rich	**Nestin poor**
	90 %	1.5 %
	85 %	1 %
	90 %	1.5 %
	90 %	1 %
	90 %	1 %
	95 %	1.5 %
	95 %	1.5 %
	90 %	1.5 %
	95 %	1 %
Mean ± S.D.	91.1±3.33	1.28±0.26
P value	< 0.01	

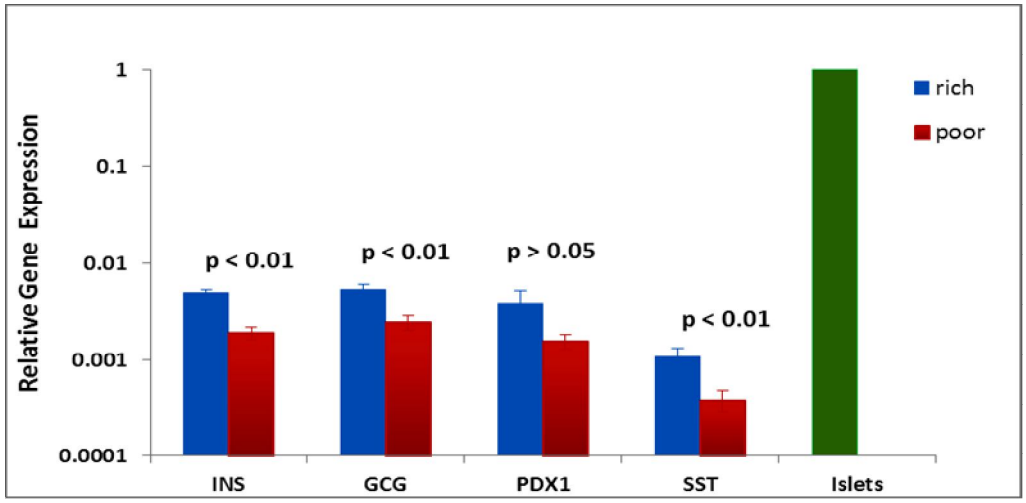

Figure (3): Relative expression of differentiated nestin rich and nestin poor cells to the human islets.

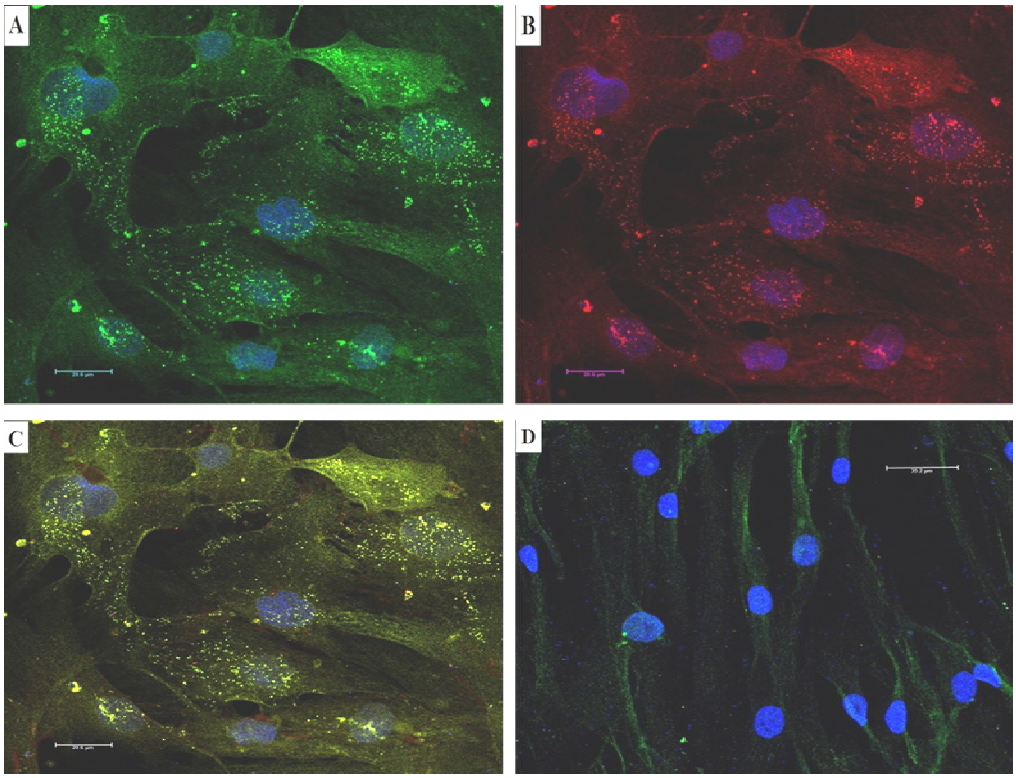

Figure (4): Immunofluorescence staining of differentiated cells. (A) Nestin rich HBM-MSCs, positive staining of intracellular insulin (green) with counterstaining for DAPI (blue). (B) Nestin rich HBM-MSCs, positive staining of intracellular c-peptide (red) with counterstaining for DAPI (blue). (C) Electronic merging of the insulin and c-peptide staining; The coexpression of insulin and c-peptide (yellow) was detected in the same cells. (D) Nestin poor HBM-MSCs, negative staining of intracellular insulin with counterstaining for DAPI (blue).

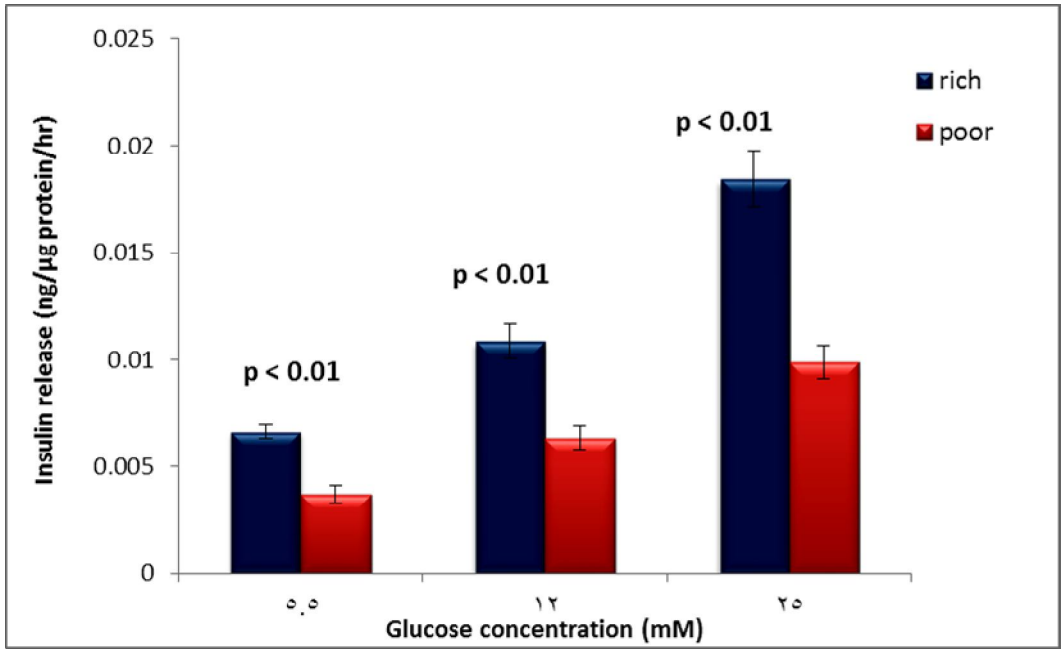

Figure (5a): In vitro human insulin release in response to glucose challenge.

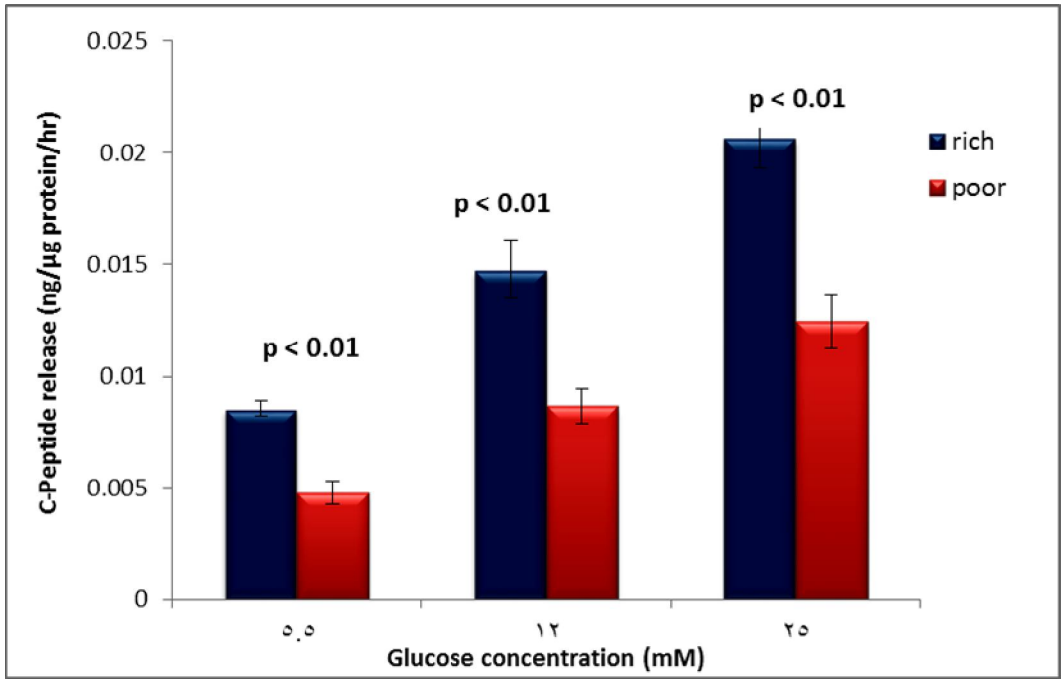

Figure (5b): In vitro human c-peptide release in response to glucose challenge.

DISCUSSION

Diabetes mellitus is a typical disease connected with marked elevation in morbidity and death rate. This disease can be characterized by raised blood glucose levels due to defects in insulin release, insulin activity or both of them, causing dysfunction in metabolism of lipid, carbohydrate and protein **(Jiang et al., 2011)**.

Recent research in regenerative therapies has focused attention on using stem cells as a prospective cure for diabetes mellitus **(Shapiro et al., 2000; Gabr et al., 2008; Wang et al., 2012)**. Bone marrow has been known to be a rich and accessible source for adult stem cells. It was reported that Mesenchymal stem cells derived from bone marrow are better than adipose tissue MSCs in terms of differentiation into IPCs **(Marappagounder et al., 2013)**. Other studies had provided data indicating that human bone marrow-derived MSCs can express insulin in addition to key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro **(Jiang et al., 2002; Moriscot et al., 2005)**.

The intermediate filament protein "nestin" has been detected in several cellular phenotypes during embryonic and adult life. Nestin expression would indicate multipotent and regenerative characters of cells **(Wiese et al., 2004).** In pancreas, the expression of nestin has been reported to be a marker for pancreatic stem cells and for islet progenitor cells **(Kim et al., 2004)**. Previous literature have reported that insulin-producing cells are generated by the isolation of nestin expressing mouse **(Soria et al., 2000)** and human **(Assady et al., 2001)** ES cells by the selection of progenitor cells expressing nestin **(Lumelsky et al., 2001; Blyszczuk et al., 2003)**.

In pancreatic cells, nestin expression has been suggested to be an essential process for stem cell differentiation into insulin-producing cells **(Lumelsky et al., 2001; Zulewski et al., 2001; Kim et al., 2004; Maria-Engler et al., 2004)**. It was suggested that nestin expression is induced in stem cells and could trigger an insulin cell differentiation surrogate for cell therapy in diabetes **(Kim et al., 2010)**. Milanesi and his colleagues were the first to report the capacity of nestin positive cells to differentiate into IPCs **(Milanesi et al., 2011)**.

In the present study, mesenchymal stem cells were isolated successfully from human bone marrow aspirates. Nowadays, there are two different techniques to isolate cellular subpopulations; they are fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS). It has been shown that FACS separation represents a physical stress on cells and can affect proliferation capacity of the cells while this can be avoided by using MACS because of biodegradability of the magnetic microbeads used **(Kerényi et al., 2016)**. Nestin positive and nestin negative human bone marrow-derived mesenchymal undifferentiated cells were isolated, cultured and characterized by their adhesiveness and fibroblast shape.

The protocol used in the present study consisted of two steps **(Tayaramma et al., 2006)**, Trichostatin-A was used at the first step as it is a natural product having a functional role in chromatin remodeling **(Otoguro et al., 1988)** and induce the expression of PDX-1. At the second step we used GLP-1 that plays a master role in differentiating intestinal epithelial cells into functional IPCs **(Suzuki et al., 2003)**, glucose was also added in this step as it is an essential growth factor for β cells and promotes β cells replication in vitro as well as in vivo **(Bonner-Weir et al., 1989)**.

Induction of PDX-1 expression is considered the initial step in directed

differentiation in order to form IPCs where PDX-1 is a transcription factor which is critical for β cell function and development **(Soria, 2001)**, it can also control pancreatic islet cell maturation. It has been demonstrated that PDX-1 expression is switched on firstly followed by the other genes related to IPCs **(Sun and Ji, 2009)**.

At the end of differentiation, the relevant endocrine genes including insulin, GCG, and SST, were expressed; their relative values for nestin rich cells were significantly higher than those for nestin poor cells.

In the present study, at the end of in vitro differentiation, immunofluorescence by confocal microscope was used to determine the insulin percentage of differentiated nestin rich cell population which was ١٤.٣% while it was ٢.١٧%. In contrast, several investigators have reported poor insulin release in response to glucose challenge **(Rajagopal et al., 2003; Boyd et al., 2008)**. The usage of different sources of cells & measurement units for reference, causes a difficulty in comparing between data **(Kim et al., 2012)**. While the present investigation indicated that, the increase in insulin release with increasing glucose concentration was significantly higher for nestin rich MSCs than nestin poor MSCs.

The field of regenerative medication is quickly developing, making ready for novel helpful mediations through cell treatments and tissue building methodologies that are remodeling the biomedical field. The striking pliancy of distinctive cell subsets got from human embryonic, and grown-up tissues from different sources (such as bone marrow, amniotic liquid, placenta, and adipose tissue) has started examination tries assessing utilization of these cells for various conditions, including diabetes and its complications **(Pileggi, 2012)**. It was concluded that nestin positive cells are supposed to be the population that is able to generate IPCs.

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الملخص العربي
تميز الخلايا الجذعية الميزنكيمية الموجبة للنستين الى خلايا منتجة للانسولين

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يشكل مرض السكري وباءا" عالميا يؤثر على جميع الفئات العرقية و يعد العلاج بواسطة الخلايا الجذعية هو واحد من أفضل البدائل للعلاج من خلال توفير وسيلة فعالة لتجديد الخلايا المنتجة للأنسولين المفقودة خلال فترة المرض. لذلك تعمل عدة مجموعات بحثية على تطوير طريقة قادرة على التمييز بين الخلايا الجذعية الوسيطة الى لخلايا المنتجة للإنسولين.

نستين هو دليل للخلايا الجذعية العصبية وقد اقترح أن نستين يلعب دورا محوريا كمنظم يحكم التمايز بين الخلايا الجذعية لتصبح منتجة للانسولين. و قد قمنا في هذه الدراسة بعزل الخلايا الجذعية الوسيطة الغنية بالنستين من نخاع العظم البشري لتقييم كفاءتها للتمييز الى خلايا المنتجة للأنسولين بالمقارنة مع الخلايا الفقيرة للنستين.

طريقة العمل

تم الحصول على عينات نخاع العظم من المتطوعين المصابين بداء السكري من النوع ٢ ثم عزل الخلايا الموجبة للنستين و الخلايا السالبة للنستين من الخلايا الجذعية الميزنكيمية لنخاع العظم عن طريق فصل الخلايا مغناطيسيا. و بعد ذلك تم تقييم نقاء المجموعتين باستخدام الأجسام المضادة الخاصة بالنستين وفحصها بواسطة المجهر متحد البؤر ثم تحويل الخلايا الجذعية المفصولة إلى خلايا منتجة للأنسولين خلال بروتوكول مكون من مرحلتين و بعد ذلك تم تقييم الخلايا عن طريق التعبير الجينى، استخدام الأجسام المضادة الخاصة بالانسولين وفحصها بواسطة المجهر متحد البؤر ، وكذلك قياس افراز الأنسولين البشري و السى ببتيد استجابة لزيادة تركيزات الجلوكوز.

النتائج

أظهر تقييم نقاء المجموعتين أن متوسط نسبة الخلايا الغنية بالنستين ٩١,١٪ في حين كانت النسبة ١,٢٨٪ للخلايا الفقيرة للنستن.و فى نهاية التمايز، تم التعبير عن جينات الغدد الصماء، ومن الجدير بالذكر أن التعبير عن جين الأنسولين في الخلايا الغنية بالنستين زاد بمقدار ٢,٦ ضعف عنه فى الخلايا الفقيرة للنستين أيضا، أظهر استخدام الأجسام المضادة الخاصة بالانسولين أن نسبته كانت ١٤,٣٪ فى الخلايا الغنية بالنستين و ٢,١٧٪ فى الخلايا الفقيرة للنستين.

ومن خلال النتائج التي توصلنا إليها يمكننا القول أن الخلايا الغنية بالنستين قادرة على التحول الى خلايا منتجة للانسولين بنسبة أعلى من نظيرتها الفقيرة للنستين.