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ISOLATION AND DIFFERENTIATION OF MUSE CELLS INTO INSULIN PRODUCING CELLS

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ABSTRACT

Mesenchymal stem cells (MSCs) are heterogeneous population. Multilineage differentiating stress enduring cells (Muse cells) are rare pluripotent sub-population within MSCs. Accordingly; we isolated and compared the differentiation ability of Muse and non-Muse cells into insulin producing cells (IPCs). The purity of the isolated fractions was evaluated by flow cytometric analysis then, the cells were differentiated into IPCs. At the end of differentiation, cells were evaluated by expression of pancreatic endocrine genes, as well as the release of human insulin and c-peptide in response to increasing glucose concentrations challenge and the percentage of generated IPCs. The percentage of generated IPCs by flow cytometric analysis was 10.2±1.7% in Muse cells and 3.9±2.1% in non-Muse cells.

In conclusion, Muse cells are a pluripotent subpopulation of MSCs capable of generating IPCs in higher percentage than non-Muse cells.

Key words: Muse; Mesenchymal stem cells; differentiation; insulin producing cells.

INTRODUCTION

Diabetes mellitus (DM) is one of the most endocrine and economical disorders worldwide, in 2016 according to the WHO, 422 million patients suffers from diabetes **(Organization, 2016)**. Insulin therapy is considered to be the safest and the most applicable method as a treatment of DM, while as a daily usage on the long-term it gives rise to several complications **(Cryer, 2013; Holman et al., 2008)**. The shortage of cadaveric donors, lack of oxygenation, vascularization of the graft and the immune rejection activity are considered as disadvantages for pancreas and islet transplantation as an efficient treatment methods for DM **(Fox et al., 2015)**.

MSCs are multipotent stem cells with the capacity to differentiate into the three germ layers and considered as an important branch in cell therapy and regenerative medicine without teratoma formation and immune rejection.

MSCs from various tissues showed the ability to differentiate and produce insulin and c-peptide *in vivo* and *in vitro*, bone marrow **(Gabr et al., 2014; Sun et al., 2007)** and adipose tissue **(Timper et al., 2006)**. The current issue with the usage of MSCs as a treatment of DM is the modest number of the generated IPCs after induction with different differentiation protocols **(Gabr et al., 2014)**.

Muse cells are rare pluripotent sub-population derived from adult MSCs which are double positive to mesenchymal surface marker

CD105 and to stage specific embryonic antigen-3 (SSEA-3) pluripotent surface marker with low telomeric activity and the capacity to express pluripotent markers such as, Nanog, Oct3/4, Sox2 and Rex1. Muse cells have the capacity of generating cell cluster derived from single Muse cell culture which is very similar to embryoid body and after transplanting Muse cells *in vivo*, it showed to have the ability of homing potential into injured organ with spontaneously differentiation potency to its own cell type **(Kuroda et al., 2010)**.

Unlike ESCs and iPSCs, Muse cells are nontumorgenic pluripotent cells with low level of cell cycle- related factors gene expression **(Wakao et al., 2011)**. Muse cells did not show any tumorogenic activity after transplantation into the testes of immunodeficient mice for up to six months **(Heneidi et al., 2013)**.

Muse cells comprised 0.003-0.004% of all mononuclear cells within bone marrow aspirate **(Kuroda et al., 2013)**, 1-5% in human dermal fibroblast **(Rada et al., 2011)**, 1% in human bone marrow-derived mesenchymal stem cells (BM-MSCs) **(Kuroda et al., 2013)**, and 1.9-8.8% in human adipose-derived mesenchymal stem cells (ASCs) **(Kinoshita et al., 2015)**.

Herein, we isolated Muse cells from ASCs to evaluate their capability of differentiation into IPCs compared with non-Muse cells.

MATERIAL AND METHODS

Retrieval of human adipose tissue

The required approvals for this study were obtained from the ethical committee of Mansoura University. Liposuction aspirates were obtained from three healthy volunteers during elective cosmetic surgeries after providing informed consent.

Isolation and expansion of MSCs derived from adipose tissue

Human liposuction aspirate was digested with 0.075% collagenase type I (Sigma Aldrish, St. Louis) in phosphate buffer saline (PBS) and was shacked in water-bath at 37□c for 30 minutes. An equal volume of αMEM supplemented with 10% hyclone fetal bovine serum was added to inactivate the chollagenase **(Yoshimura et al., 2006)**. Cells were then cultured in αMEM media supplemented with 10% FBS, then transferred to tissue culture flasks (BDbioscience, United States) coated with Laminin 521 (Biolamina AB, Sweden). The flasks were then incubated in CO_2 incubator. Aliquots were preserved in liquid nitrogen for subsequent expansion and examination. After 3 days, the nonadherent cells were discarded. The adherent MSCs were cultured to 80% confluence before passaging by trypsin. The cells were resuspended in complete αMEM and reseeded at a ratio of 1:2 and cultured for another 8 days to reach 80% confluence. This step was repeated for a second passage. At this point, the cells were spindle-shaped and displayed a fibroblast-like appearance.

Isolation of Muse cells by fluorescence activated cell sorting (FACS)

Isolation protocol of Muse cells by FACSAria III was previously introduced **(Kuroda et al., 2013)**. $1x10^6$ cells in a single tube was centrifuged at 210 g for 5 minutes, and resuspended in FACS buffer. 5 µl of anti-SSEA-3 (STEMCELL Technologies) was incubated at ice for 25 min in dark. The cells were then resuspended in 200 µl FACS buffer. SSEA-3 cells were isolated by SORP FACSAria III (Bekton Dickinson, Franklin Lakes, NJ). The isolated two fractions (Muse cells and non-Muse cells) were expanded in the same conditions as MSCs. And the purity of the isolated two fractions was determined by flow cytometer.

Differentiation of Muse and non-Muse cells into IPCs

Differentiation was performed according to a protocol previously reported **(Tayaramma et al., 2006)**. Initially, the cells were cultured for 3 days in serum-free DMEM supplemented with Trichostatin-A (TSA) at a concentration of 55 nanomoles (Sigma). Then, the cells were cultured for an additional 7 days in high-glucose (25 millimoles) medium containing a 1:1 ratio of DMEM:DMEM/F12 (Sigma). This mixture was supplemented with 10% fetal bovine serum and 10 nanomoles of glucagon-(GCG-) like peptide-1 (GLP-1, Sigma).

Gene expression by qRT-PCR

Total RNA was extracted from undifferentiated and differentiated cells according to the protocol employed using RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). 3 μg of total RNA was converted into cDNA using RT^2 First Strand kit (Qiagen Sciences, Maryland, USA). Custom gene arrays CAPH13024D were designed and supplied in 96-well plates for pancreatic endocrine genes (Qiagen Science,Maryland, USA) including; insulin, glucagon, PDX-1 and somatostatin. Human islets were included to serve as positive control for pancreatic endocrine genes and GAPDH as an internal control. Amplifications were performed in a 25 μL reaction volume in each well that contains 12.5 μL 2X SYBR Green Rox Master Mix (Qiagen Sciences, Maryland, USA), 1 μL of cDNA template, and 11.5 μL of nuclease-free water. The plate array was inserted in real time thermal cycler (ABI PRISM 7000, Applied Biosystem, California, USA) and programmed according to manufacturer instructions. A mathematical model introduced **(Pfaffl, 2001)** was used for the relative quantification of target genes.

Flow Cytometric analysis for generated IPCs

At the end of *in vitro* differentiation, the cells were fixed in 4% formaldehyde for 10 min at 37^{o}C, permeabilized by using chilled 90% methanol for 30 min and blocked in incubation buffer for 10 min at RT. Then, the cells were incubated in the conjugated antibody for 60 min at RT. The cells were washed with incubation buffer and, after centrifugation, were resuspended in 0.5 mL PBS. The labeled cells were evaluated using a 15 mW argon ion laser at a wavelength of 488 nm (FACSCalibur, Becton, Dickinson, United States). A total of ten thousand events were obtained and analyzed using CellQuest software (Becton, Dickinson). Mouse pancreatic islets served as a positive control **(Gabr et al., 2014)**.

Determination of *in vitro* insulin and c-peptide

Insulin and c-peptide release of differentiated Muse and non-Muse cells was performed according to the procedure described before **(Gabr et al., 2013)**. 1×10^6 cells of each sample were collected from the same batch of each donor at the end of the differentiation period for measurement of released insulin and c-peptide hormones. 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. The supernatant was collected at the end of each incubation period. The collected samples were frozen at -70^{o}C until assayed using an Elisa Kit with a minimum detection limit of 1.76 μIU/mL (IMMUNOSPEC, USA).

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° was considered significant. The mean values were used as a measure of variation **(Chinna et al., 2012)**.

RESULTS

Characterization of the undifferentiated cells

The cultured cells at the end of expansion phase became spindle-shaped, fibroblast-like cells arranged in monolayers, and without any differences in the morphology between Muse and non-Muse cells (**Figure 1**).

After FACS separation the purity of the isolated fractions was determined by flow cytometer and the results indicate the 82.2±10.6 of Muse cells fraction were positive to anti-SSEA-3 while only 3.6±3.9% in non-Muse cells fraction (**Figure 2).**

Functional evaluation of the differentiated IPCs

Gene expression by RT-qPCR

At the end of the *in vitro* differentiation protocol, the relevant endocrine genes, insulin, glucagon, PDX1 and somatostatin were expressed in Muse and non-Muse cells, these genes expression were multiplicated in Muse cells compared to non-Muse cells, by 5.5-fold in insulin, 5.4-fold in glucagon, 4.5-fold in PDX-1 and 6.9-fold in somatostatin, relatively to human islet gene expression as shown in **(Figure 3)**.

Flow cytometric analysis of generated IPCs.

At the end of differentiation, flow cytometer analysis indicated that the proportion of IPCs generates from Muse cells was 10.1±1.7 and 3.9±2.1 generated from non-Muse cells (P <0.05) **(Figure 4)**.

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

Insulin and c-peptide release from generated IPCs derived from Muse and non-Muse was gradually increased in response to increasing glucose concentrations ($P< 0.05$). The amounts of insulin and c-peptide release were significally higher in Muse cells compared to non-Muse cells. **(Figure 5a-b)**.

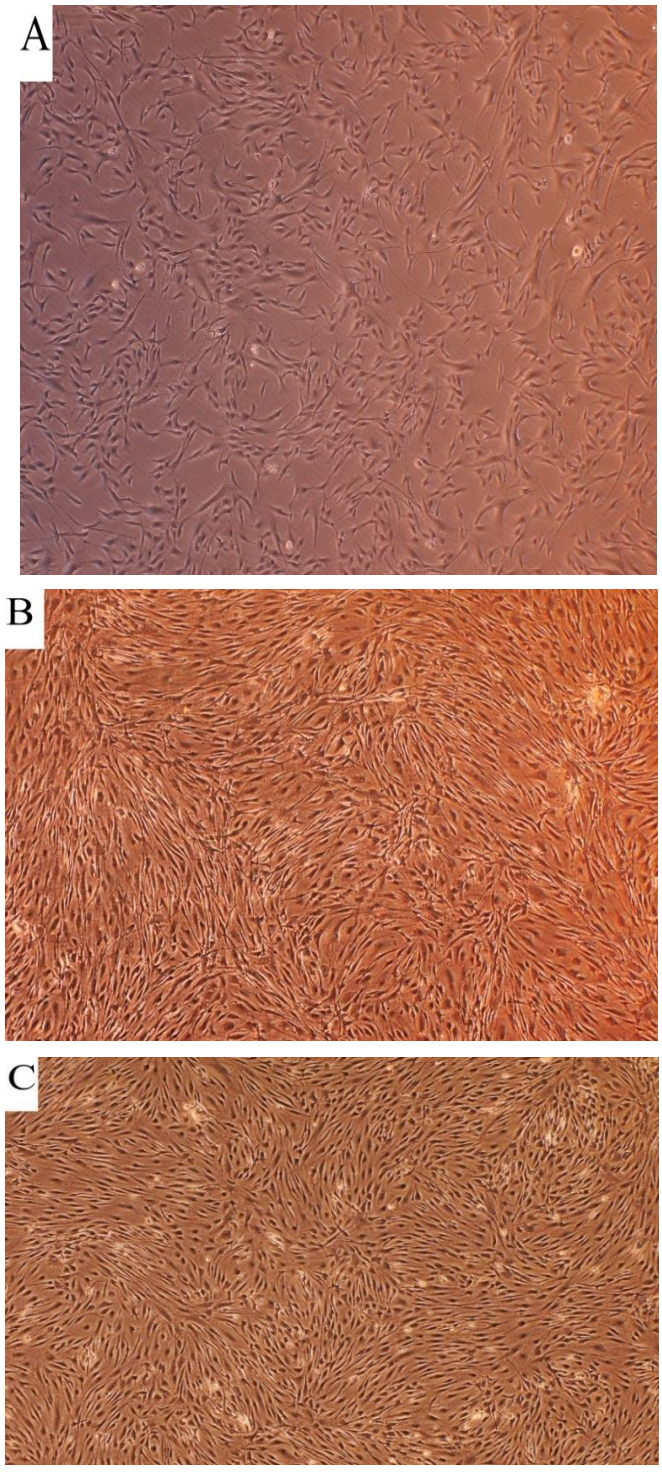

Figure (1): Morphological changes of Muse cells and ASCs during expansion and differentiation (A) Human ASCs during expansion. (B) Muse cells. (C) non-Muse cells.

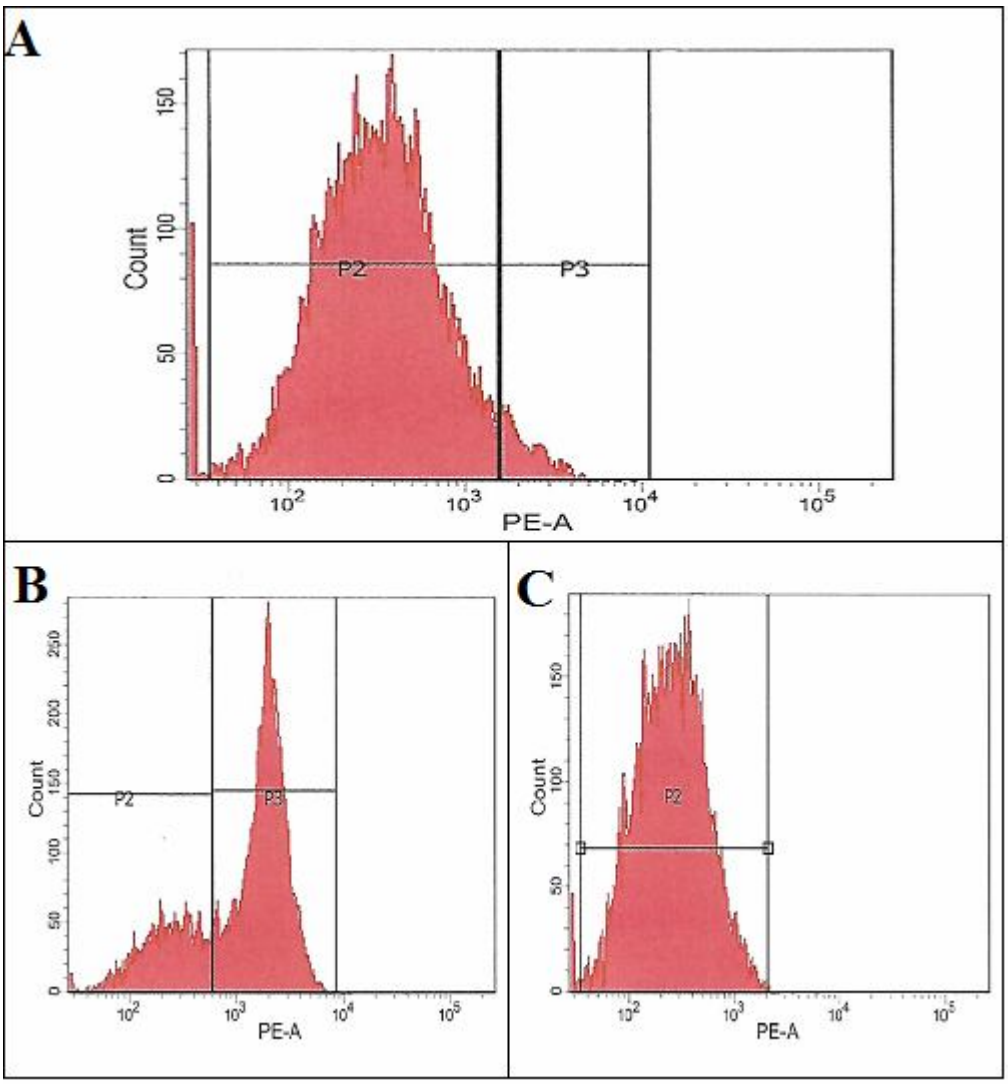

Figure (2): Verification of Muse cells after FACS separation.

A: Percentage of Muse cells among whole human ASCs.

B: Percentage of Muse cells within the positive fraction.

C: Percentage of non-Muse cells within the negative fraction.

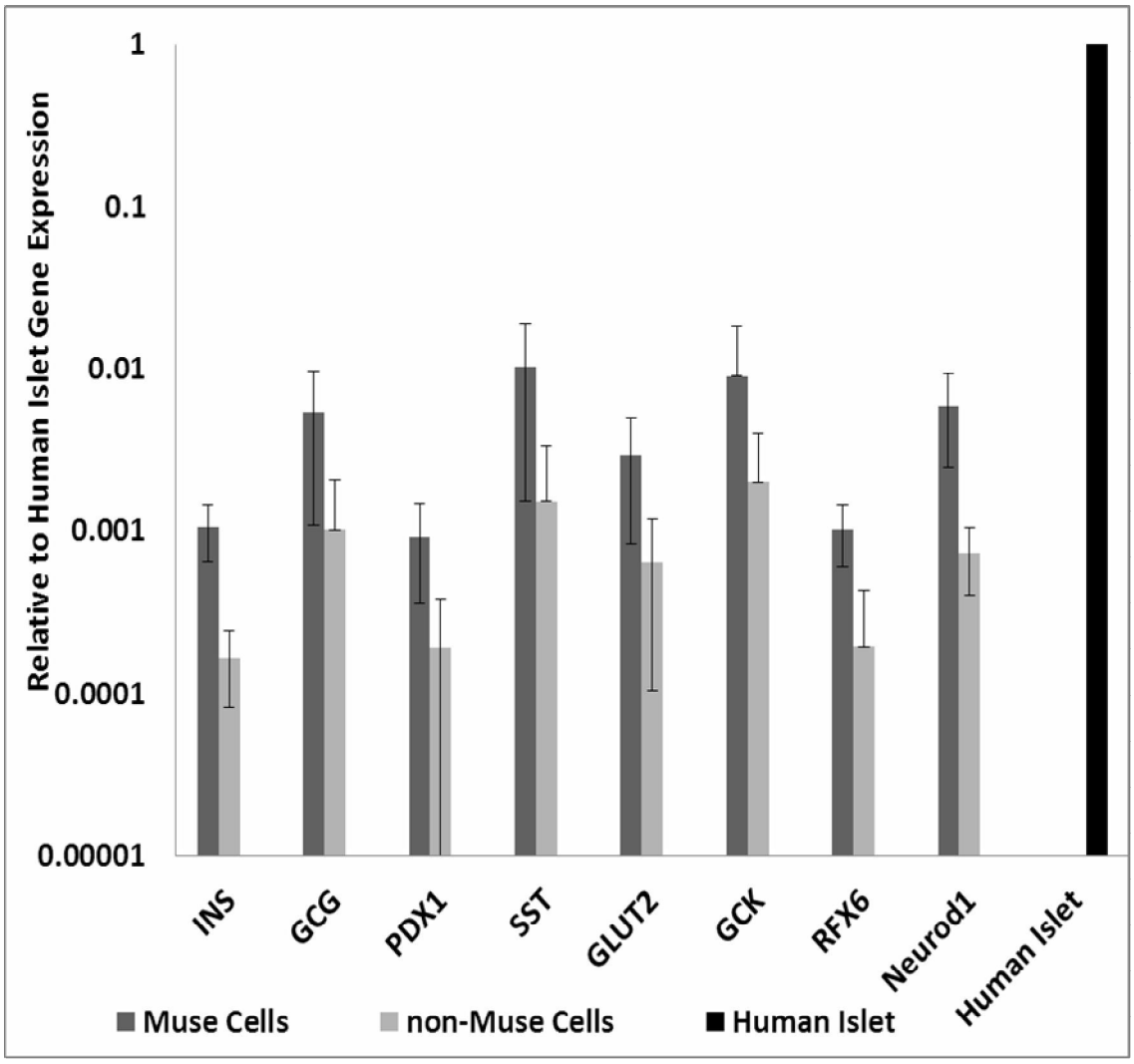

Figure (3): Relative gene expression of differentiated Muse and non-Muse cells to the human islets.

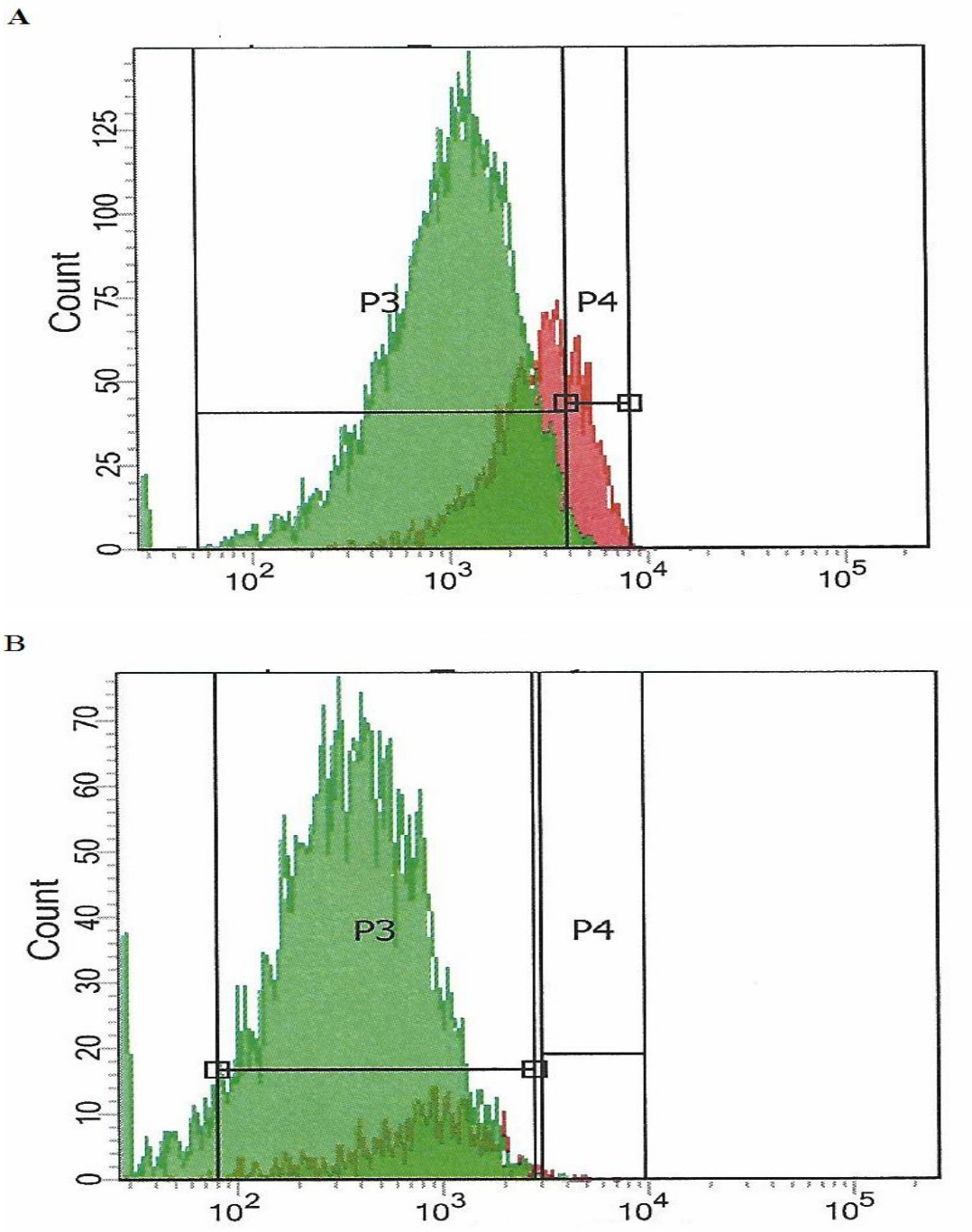

Figure (4): Flow cytometric analysis of the generating IPCs from differentiated Muse cells (A), and differentiated non-Muse cells (B).

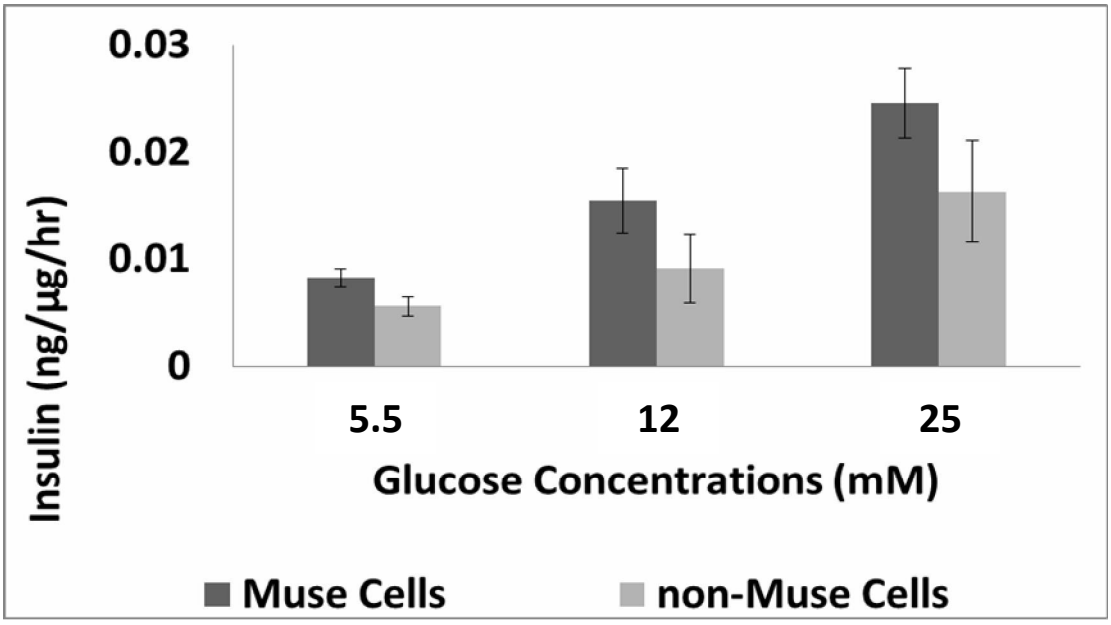

Figure (5a): *In vitro* human insulin release in response to glucose challenge.

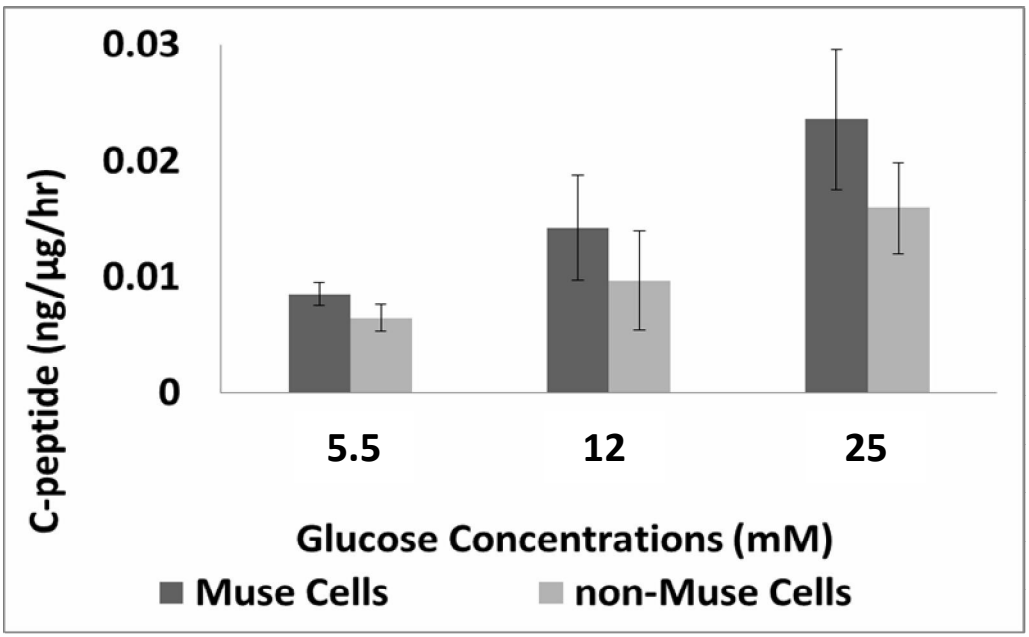

Figure (5b): *In vitro* human c-peptide release in response to glucose challenge.

DISCUSION

The ability to isolate and manipulate multipotent stem cell from non-embryonic origin has been considered as a revolutionary success in regenerative medicine and in stem cell therapy for many diseases especially DM, which had led the scientific community to investigate and apply several studies to identify and evaluate MSCs characteristics, although previous studies has shown that heterogeneity of MSCs population with several surface markers and functions **(Lv et al., 2014)**.

Adipose tissue had been shown to be a rich and easy source for multipotent MSCs and considered to be feasible source for autologous stem cell transplantation **(Yoshimura et al., 2006)**. Muse cells can be easily isolated by several methods including environmental stress; hypoxia, long term trypsinizatio (LTT), nonserum culture and and FACS separation **(Kuroda et al., 2010; Kuroda et al., 2013)**, but during our work, we had found that FACS separation is the most feasible and simple method for isolating Muse cells with high purity.

The poor insulin release in response to glucose challenge as showed in the present study were reported previously in both differentiated IPCs generated from ESCs and IPCs derived from adult stem cells **(Gabr et al., 2014)**. Also, in this study the results showed the poor of insulin and c-peptide release, while there was a step wise up-regulation in the release in Muse cells compared to non-Muse cells in exposure to different concentrations of glucose in Muse rich cells.

At the end of differentiation, genes including insulin, GCG, SST and PDX-1 were expressed and multiplicated in Muse cells compared to non-Muse cells.

SSEA-3 is a globoseries glycosphingolipid epitope surface marker, which is a well-known stemness marker for ESCs **(Kannagi et al., 1983)**. Previous report had reported that there is no relation between the expression of SSEA-3 and the expression of pluripotent markers after depletion of SSEA-3 and SSEA-4 expression in ESCs **(Wright and Andrews, 2009)**.

In conclusion, this study is regarded as the premiere study which had evaluated the ability of Muse cells to differentiate and to produce insulin and c-peptide *in vitro*. According to the isolation method of Muse cells, the negative fraction (non-Muse cells) may have to contain other pluripotent subpopulation. MSCs can be replaced by Muse cells for in-vivo investigations and in translation medicine.

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الملخص العربي
فصل و تحويل خلايا الميوز إلى خلايا منتجة للإنسولين

على محمد فؤاد، محمود محمد جبر، السيد قاسم عبد الهادى، محمود محمد زكريا و محمد أحمد غنيم

الخلايا الميزنكيمية هى مجموعة من الخلايا الغير متناجسة فى الوظيفة و الخصائص. خلايا الميوز هى فئة من الفئات المكونة للخلايا الميزنكيمية ولكن بنسبة قليلة جدا و تتميز بصفات مختلفة عن باقى فئات الخلايا الميزنكيمية. لقد قمنا بفصل الخلايا المزينكيمية الى خلايا ميوز و خلايا غير ميوز و تحويل هذة الخلايا إلى خلايا منتجة للإنسولين ثم قمنا بمقارنة قدرتيهما على إفراز الإنسولين. تم قياس جودة الفصل بجهاز التدفق الخلوى. كما تم قياس قدرة الخلايا المتحولة على إفراز الإنسولين بجهاز تفاعل البوليميريز المتسلسل و تقنية مقايسة الممتز المناعى المرتبط بالإنزيم و جهاز التدفق الخلوى.

وجد أن نسبة خلايا الميوز المتحولة إلى خلايا منتجة للأنسولين 10.2±1.7% بينما الخلايا المنتجة للأنسولين المتحولة من الخلاية الغير ميوز كانت 2.1% ±3.9.

فقد أظهرت خلايا الميوز كفائة عالية على إنتاج خلايا مفرزة للأنسولين مقارنة بالخلايا الغير ميوز.