Insulin producing cell clusters derived from very small embryonic like stem cells are potent to treat diabetics mice

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Abstract: Considerable effort has been expended to isolate stem cells from a number of different tissues to use in regenerative medicine. In this study, we purposed to reckon up the ability of mouse very small embryonic like stem cells (VSEL) differentiation into insulin producing cell clusters(IPCCs), in order to treatment of diabetes. Mouse Bone marrow VSEL stem cells, isolated with Fluorescent activating cell sorting (FACS) method by using Fluorescent antibodies against CD45, CXCR4 and Sca1markers. After differentiation of VSEL stem cells into IPCCs with Activin A and nicotinamide as main factors of differentiation, RT-PCR and immunocytochemistry revealed that newly IPCCs could express Ins1, Ins2, PDX1 and Glut2. Invitro insulin secretion by IPCCs was confirmed by ELISA kit. For treatment, we injected IPCCs into thigh muscle of diabetic mice. Results showed that newly derived IPCCs were capable to produce insulin in vivo. There was a significant decrease in mean blood sugar of diabetic mice after passing 3 weeks from cell therapy. This study provides a strategy for using autologous VSEL stem cells for curing diabetes and other regenerative disease, as an alternative for other stem cell types.
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Key words: Diabetes 1, Very small embryonic like stem cells (VSEL), Fluorescent activating cell sorting (FACS) method, Insulin Producing cell clusters (IPCCs).

1. Introduction

Diabetes is a lifelong metabolic disease in which there are high levels of sugar in the blood. Now there are 150 million diabetic people in the World, According to World Health Organization (WHO) data, the number of diabetic person is expected to grow to 300 million by 2025 (Wild,2004).

One of the efficient ways to cure diabetes is cell therapy. The origins of cell therapy can perhaps be traced to the nineteenth century. Nowadays the cells that have the capacity to release soluble factors such as cytokines, chemokines, and growth factors which act in a paracrine or endocrine manner are used more specially than before (Niehans, 1960). Treatment of a patient with lowest side effects is the main goal of cell therapy. For this purpose, researchers have used different intact stem cells with diversified potency (Boyd, 2008; Uyanikgil, 2009; Dong-Myung, 2009; Kucia, 2006).

The capability of the cells is a key factor to reach to better results. .pluripotency trait is an eminent property for using stem cells in cell therapy to cure regenerative disease. But we know that Capability of mouse embryonic stem cells (ESCs) to generate insulin producing cell clusters (IPCCs) remains highly contentious (Miyanishi, 2013). Until recently, the differentiation profiles of stem cells were believed to take place within an organ and thought to be confined to considerably specific environments in an isolated way. However, it is well known that somatic stem cells exhibit a higher degree of differentiation capacity (Grossman, 2010). Very small embryonic like stem cells (VSEL) has been proposed by some researchers as an alternative to embryonic stem cells. Because they're said to exist in the bone marrow of adult humans and mice, they could obviate the ethical issues surrounding the use of human embryos. These cells don't express MHC1 and MHC2 markers and besides don't form teratomas (Bartunek, 2007). In 2006, Ratajczak and his colleagues, identified (VSEL) in adult murine bone marrow and proved that these cells are pluripotent and express Oct4, SSEA1 and CXCR4 (Kucia, 2008). Until now there is a doubt about the existence of these diminutive stem cells in adult tissues (Kaomei, 1999). In the present study, to prove that these tiny cells are present in the mice bone marrow and for comparison with other stem cells in cell therapy to cure regenerative disease like diabetes, we analyzed the potential of VSEL stem cells to differentiate into IPCCs.

2. Materials and Methods

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the Tehran University medical sciences.

Type of study:

In this experimental study, we had 2 groups with

6 female mice (2 month old/NMRY)) in each group. First group was control, second was diabetic experimental group that treated with IPCCs.

2.1 Induction of diabetes in mice:

Diabetes was induced in 12 mice by intra peritoneal high single dose (150mg/kg) of streptozocin (sigma85882), and after one week the mice that their non fasting blood glucose was more than 400mg/dl interred into the research. The mice were fasted for almost 4 hour prior to injection and Supplied mice with 10% sucrose water overnight to avoid sudden hypoglycemia Post-injection. Body weight and nonfasting blood glucose levels were monitored weekly. Blood samples were obtained from tail vain under general anesthesia by ketamine and Xylazine (Grossman, 2010).Blood glucose was measured by Bionime glucometer, model GM110.

2.2 VSEL stem cell sorting

20 female mice (2month old/NMRI) sacrificed by cervical dislocation and under sterile conditions bone marrow of femurs and tibias flushed with KO/DMEM and gathered media centrifuged for 5min in 1400rpm.after that, Adjust cell suspension to a concentration of 1-5x10^6 cells/ml in ice cold PBS, 10%FCS, 1%sodium azide. Then we added 0.1-10 µg/ml of the primary labeled antibody and incubated it for at least 30 min at 4°C.in the next step Washed the cells 3X by centrifugation at 400 g for5 minutes and resuspended them in 500ul to 1ml of ice cold PBS, 10% FCS,1% sodium azide. Kept the cells in the dark on ice or at 4°C and analyzed as soon as possible (Kucia, 2008).Based on our information, we decided to sort a population of CD45-CXCR4+Sca1+ cells from murine bone marrow with BD FACS AriaII cell sorter devise. First we separated CD45- and CD45+ cells by using anti-mouse CD45 (APC/Cy7 anti-mouse CD45 Catalog Number: 103115) and then from CD45- population, we isolated CXCR4+Sca1+ cells by using Anti-mouse Sca1 FITC (Catalog Number: ab 25031) and Anti-Mouse CXCR4 PE (Catalog Number: 12-9991) antibodies(Fig1).

\2.3 VSEL proliferation

Mouse ES cells can be maintained in a proliferative, undifferentiated state *in vitro* by growing them on feeder layers of MEF cells. An alternative to culture on feeder layers is the addition of leukemia inhibitory factor (LIF) to the growth medium.

For best results, VSEL stem cells were cultivated with two different ways. In one group, we seeded 1×10^6 cells on a feeder layer of primary mouse embryonic fibroblasts (MEF) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS,L-glutamine(2mM,Gibco),ß-mercaptoethanol (final concentration 5×10^5M) and non essential amino acid (NEAA, stock solution diluted 1:100, Gibco)and another group proliferated on a coated dishes with the same culture media, plus LIF(10ng/ml) (Sigma, L5158) (Kaomei, 1999). The dishes (25mm flask) coated with 0/1%gelatin solution (Fig2), and we seeded 1×10^6 cells in each dish.

2.4 Differentiation of VSEL stem cells into IPCCs:

Before the onset of differentiation, 5×10^6 VSEL stem cells were transferred and grown on gelatinized culture dishes (25mm flask) for two rounds to deplete feeder cells. With two step protocols we could differentiate VSEL stem cells into beta like cells. In first step we used DMEM/F12 with 4mM/L glutamine, 4.5g/l glucose, 1%heat in activated FBS and 50ng/ml of recombinant h Activin A (R&D systems, Catalog Number: 338-AC) .In second step, after 72h we used DMEM/F12 with 4mM/L glutamine, 4.5g/l glucose, 5%heat in activated FBS in the presence of N2 supplement-A, B27supplement, and 10mM nicotinamide(Sigma,N3376). Medium was changed every other day and differentiation process last for 3 weeks (Kucia, 2006) (Fig3).

2.5 RT-PCR analysis:

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Courtaboeuf Cedex, France), and genomic DNA was digested with deoxyribonuclease I (Invitrogen). Equal amounts (1 µg) of RNA were used for reverse transcription (Invitrogen or Qiagen).One twentieth of the cDNA mixture was used for all genes. The cycling parameters were as follows: denaturation at 94°C for 1 min, annealing at 52-60°C for 1 min (depending on the primer), elongation at 72°C for 1 min (35 cycles), and a final extension for 20 min at 72°C .

Product sizes and cycle numbers are summarized in Table I For beta like cells, we assessed PDX1, insulin1, insulin2, ngn3 (positive markers of beta cells), Glucagon (negative markers of beta cells), and Amylase (Exocrine marker) markers (Boyd, 2008) (Tab1).

Table1. RT-PCR for gene detection of newly formed beta like cells*

Primer	Sequence	Product
Amylase	F: TGCCATCACTGCCAACATTCA	889
	R: CCCACGGGCACATGTTTTG	
Glucagon	F: ACCAAGCCGTCATCTTCCAG	390
	R: CGTATAGGGCACGTAGCAGG	
Insulin1	F: GGCCAAACAGCAAAGTCCAG	86
	R: CACTAAGGGCTGGGGGTTAC	
Insulin2	F: CACCAGCCCTAAGTGATCCG	261
	R: GCTTGACAAAAGCCTGGGTG	
Ngn3	F: TGGATCCCTAGCCCTCTCAC	334
	R: TGCATGTAGCGGGCAGTAAA	
PDX1	F: GAGCGTTCCAATACGGACCA	315
	R: CAACCAGTTTTGCCCTGCTC	
Beta actin	F:CGGTTCCGATGCCCTGAGGCTCTT R: CGTCACACTTCATGATGGAATTGA	

* Expression of PDX1, Ngn3, Amylase, Ins1, ins2 and glucagon Genes mRNA was analyzed by the quantitative reverse transcription-polymerase chain reaction

2.6 Assessment of insulin and proinsulin release by differentiated beta like cells in cell culture media with use of insulin ELISA kit (ab100578). We used two different concentrations of glucose for this purpose, Low dose (3mM) and high dose (22mM) glucose(Sigma,G8270) (Boyd, 2008). Each 60-mm² dish of cells was washed with PBS ant the plate incubated at 37º for one hour first with 2 ml 3.3 mmol/l glucose solution and then subsequently with 25 mmol/l glucose solution. The supernatants were harvested after each times stimulation and subjected to ELISA (Fig6).

2.7 Immunocytochemistry of newly formed IPCCs

Cells in culture dishes were fixed with 4% paraformaldehyde in phosphate-buffered solution and immunocytochemistry was carried out using a standard protocol. For this purpose we used two primary and secondary antibodies against each antigen of newly formed IPCCs: The first was Rabbit polyclonal to PDX1primary antibody (ab47267) and Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (FITC) (ab97050) for PDX1 detection. The second was Anti-Glucose Transporter GLUT2 primary antibody (ab54460) and Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (FITC) (ab97050) for GLUT2 detection (Fig4).

2.8 Injection of differentiated beta like cells into thigh muscle:

After 21 days of invitro differentiation when island like colonies appeared in the bottom of flasks, newly differentiated IPCCs, labeled with DiI (CellTrackerTM CM-DiI; C7000, C7001, N22883, V22888).
based on data sheet information, Stock solutions of lipophilic tracers prepared in dimethylsulfoxide (DMSO) at 1–2 mg/ml. Immediately before labeling, dilute the 1–2 mg/mL solution stock (see Preparing Stock Solutions) into a suitable medium such as Hanks' balanced salt solution (HBSS). Suitable working concentrations generally range from 1–2 μM but may vary considerably depending on the application.

Labeled IPCCs were transplanted into the thigh muscle of 3 diabetic mice. Briefly, mice were anesthetized by intra peritoneal Ketamine (100mg/kg) and Xylazine (10mg/kg) injection, then the thigh region was scribed. Insulin syringes (a 0.3-ml syringe with a 29-gauge needle) with 5×10^6 cells in 50 μl of HBSS were injected into the thigh muscle. The plunger was slowly pushed, and the needle was retracted gently. Following injection of cells mice were placed on a heating pad, and their condition was observed until they were fully recovered (Ju Lan, 2013).

2.9 Invivo tracking of injected labeled IPCCs in thigh muscle:

After 4 weeks, the mice euthanized and the thigh muscle cut and fixed in 4 percent formaldehyde overnight, and then transferred into 10percent formalin for subsequent day. Then the tissues prepared for sectioning and further assessments to detecting labeled IPCCs.

For DAPI nuclear counter staining, the paraffin blocks were deparaffinized in xylene, rehydrated in graded ethanol and washed in phosphate buffered saline (PBS). Evaluation of the labeled cells in tissue slides was performed with invert microscope and recorded with image. Viability of the cells in the muscle was important (Fig7).

2.10 Statistical Analysis:

Statistical analysis was performed using Student's t test. Values of P=0.0002 were considered statistically significant. We used Graphpad prism5 and excel software.

3. Results

Cell sorting: In multicolor flow cytometric sorting analysis (FACS) of mice bone marrow cells, from CD45 negative population we gated Sca1 and CXCr4 Positive cells. In 3 color analysis, We used APC-Cy7 anti-mouse cd45 antibody for isolation of CD45 negative and positive cells then form CD45 negative population we sorted Sca1 and CXCr4 positive cell with anti-mouse Sca1 FITC and anti-mouse CXCr4 PE antibodies(Fig1).

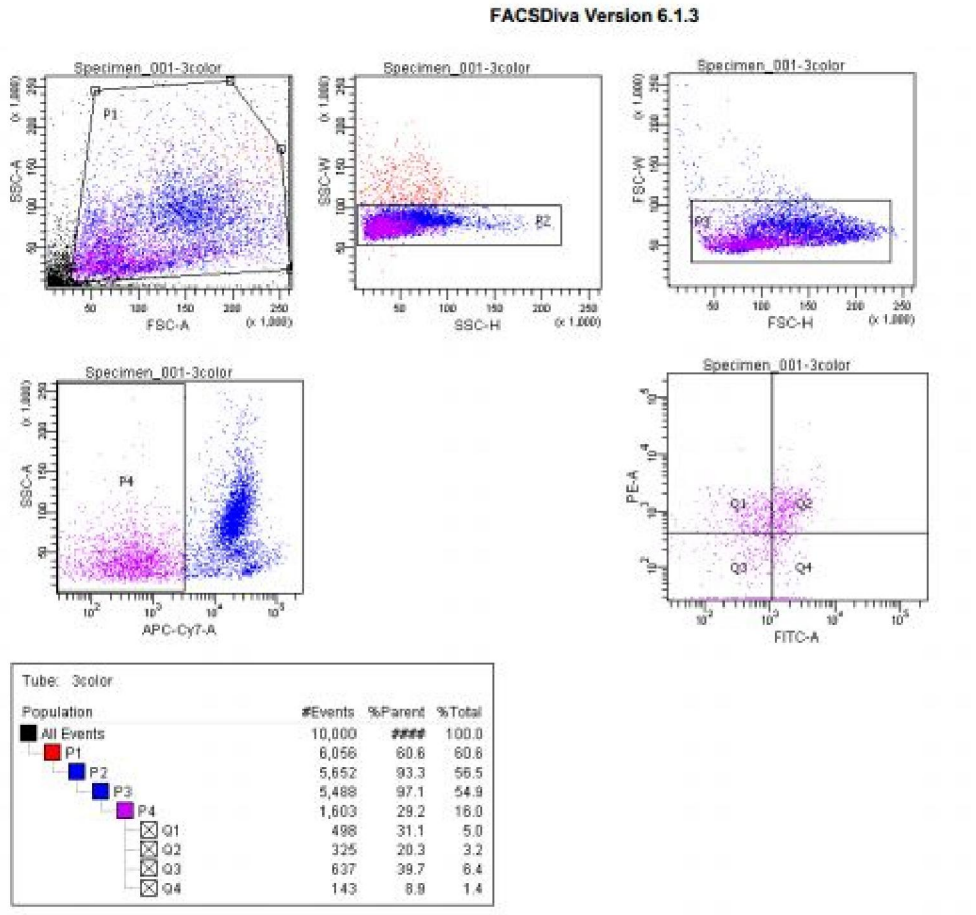

Figure 1: mice Bone marrow derived VSEL stem cell sorting by FACS. VSELs were sorted by BD FACS Aria II cell sorter, following immunofluorescence staining for CD45, Sca-1 and CXCr4.Panel **A**: gated cell population of interest. Panel **B** and **C**: Bone marrow mono nuclear cells visualized on dot plots showing their FSC and SSC signals related to the size and granularity of the cell , respectively.Penel **D** : separation of CD45 negative and positive cells. Panel **E:** CD45 negative cells gated based on Sca1 and CXCr4 positivity (Q2 area).

Proliferations of VSEL stem cells:

VSEL stem cells had a strong self-renewal capacity and to avoid spontaneous differentiation LIF was crucial. To prevention of colony formation, we changed cell culture media every day **(**Fig2**)** with every other day passages**.**

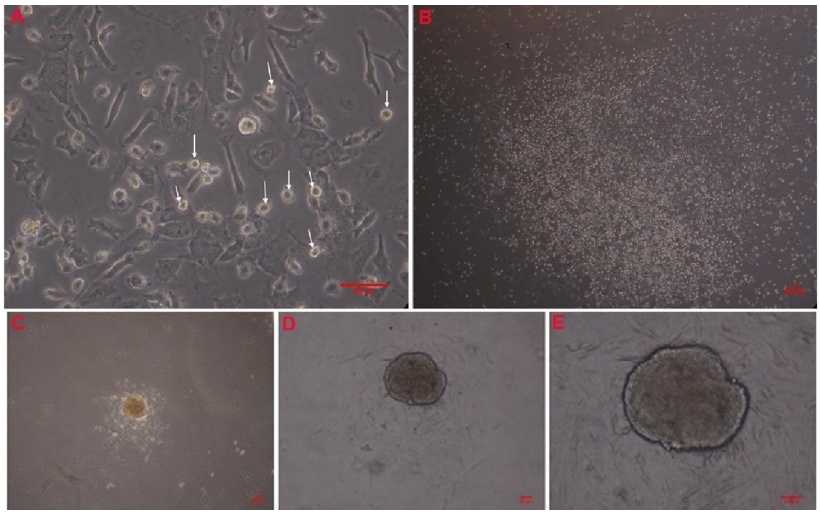

Figure 2: VSEL stem cells on MEF cells **(A)**, White arrows show VSEL cells (40 X).
VSEL on MEF cells (4X); Round bright small cells are VSEL **(B)**. Colony of VSEL stem cells (4X) **(C),** colony of VSEL stem cells (10X) **(D)**, colony of VSEL stem cells (20X) **(E)**

Images of IPCCs in different stages: VSEL stem cells of passage 3 were cultured in differentiation medium for 21 days. The induced cells were examined daily for morphological changes. After 5 days, these cells could self assemble to form cell clusters (Fig3).

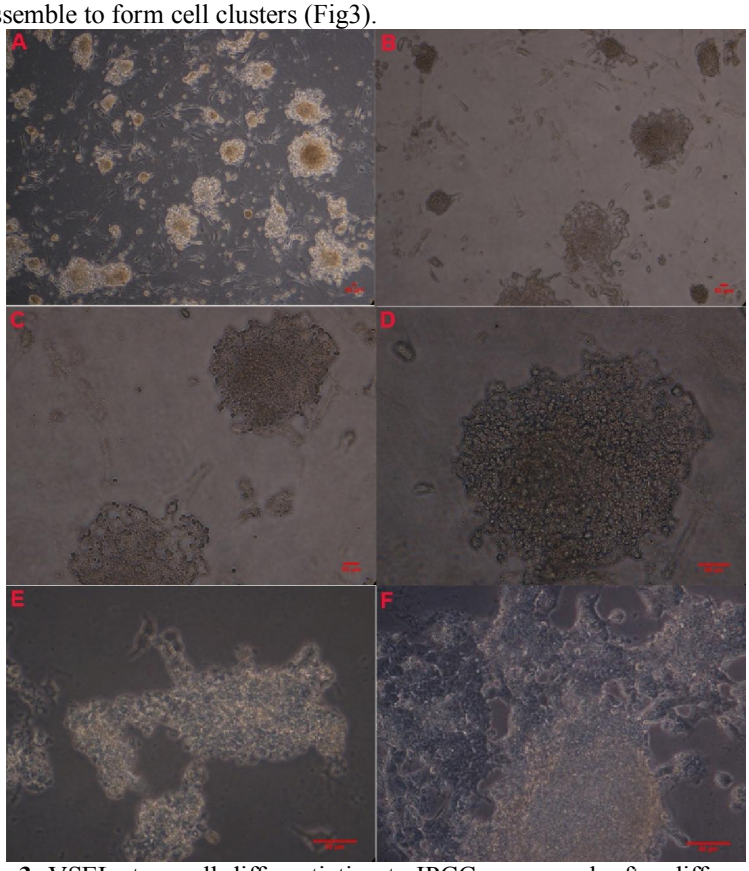

Figure 3: VSEL stem cell differentiation to IPCCs, one week after differentiation of VSELs into IPCCs (**A**: 4X-**B**: 10X-**C**: 20X-**D**: 40X). **E**: Two weeks after Differentiation of VSELs into IPCCs (40X) **F**: three weeks after Differentiation of VSELs into IPCCs (40X).

ICC of Differentiated IPCCs in different stages:

To determine whether the cells from clusters could express genes of beta cells (PDX1- GLUT2), IPCCs were subjected to immunocytochemistry (Fig4).

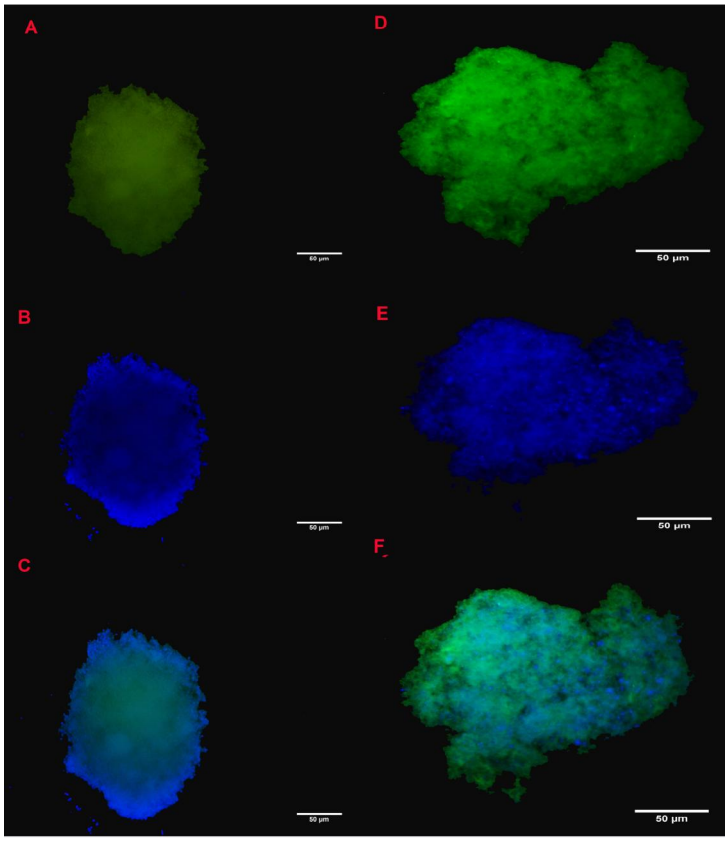

Figure 4: ICC for Glut2 and PDX1 markers in differentiated IPCCs, Glut2 expression was indicated by green fluorescence **(A)**. DAPI nuclear counterstain in blue **(B),** merged picture (40X) **(C)**.
PDX1 was indicated by green fluorescence **(D)**, DAPI nuclear counterstain in blue **(E),** Merged picture (40X) **(F)**.

RT-PCR analysis of IPCCs:

To confirm that VSEL stem cells had undergo pancreatic differentiation, the expression of genes related the pancreatic endocrine development and function was evaluated by RT-PCR. Total RNA extracted from freshly isolated VSEL stem cells for gene expression comparison (Fig5).

The RNA for these genes was not detected in freshly isolated VSEL stem cells. As an internal control, the expression of Beta-actin was detected, indicating that our experimental system was intact.

The newly formed IPCCs, strongly expressed PDX1 (definitive endoderm), insulin1 and insulin2 (beta cell) markers.PDX1 is a key factor for endoderm derivation, Inslin1 and insulin2 genes are beta cell markers.Amilase is a exocrine marker that are expressed weekly.

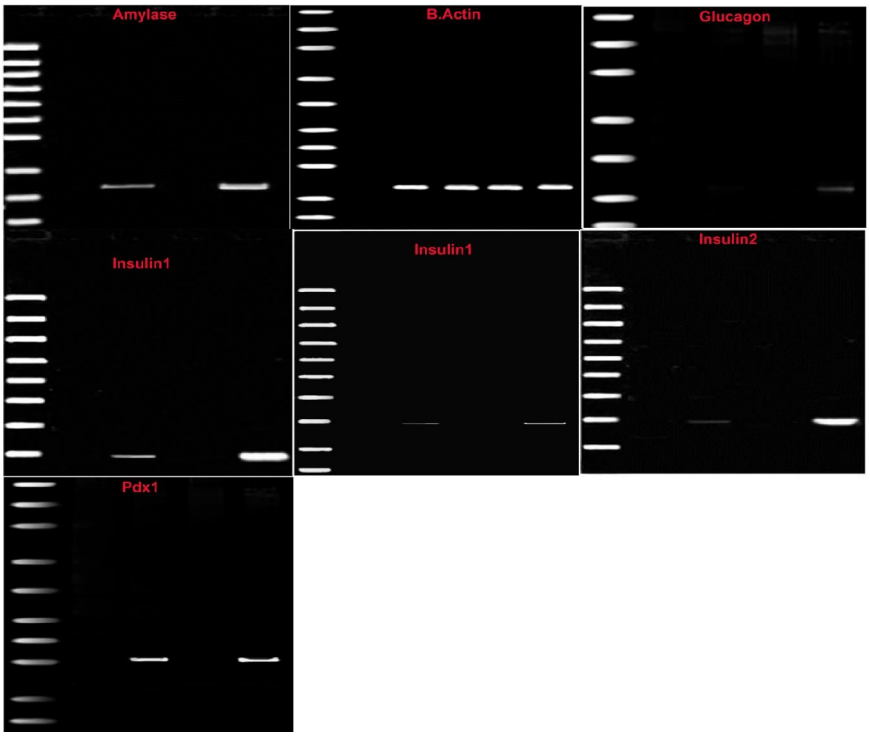

Figure 5: RT-PCR of differentiated IPCCs, first column is ladder, second column is H2o, third column is differentiated IPCCs, forth column is negative control (VSEL), and fifth column is positive control (mice pancreas).

Glucose stimulation assay:

To designation whether IPCCs could release insulin and were able to act manner a kin to pancreatic beta cells, we stimulated these cells with low (3.3mol/l) and high (25mmol/l) concentration of glucose. The highest levels of insulin secretion were observed in response to the low dose glucose concentration (Fig6).

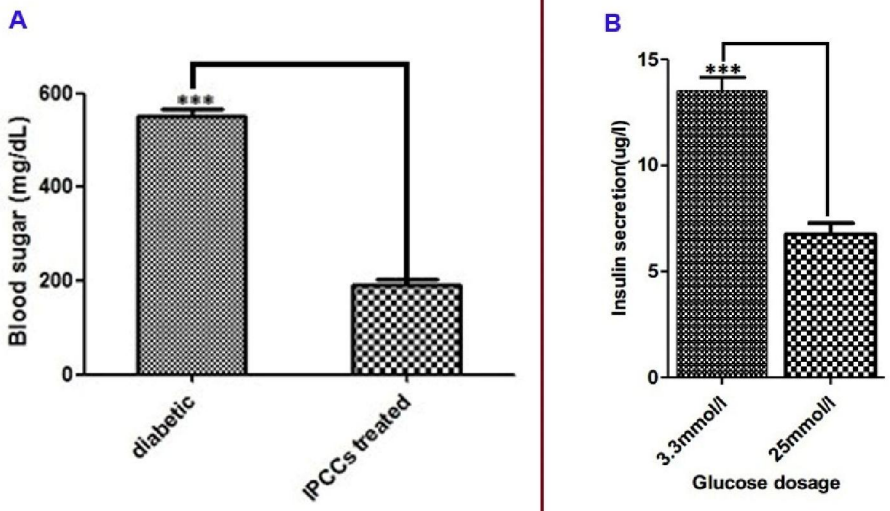

Figure 6: Chart of average none fasting glucose levels of IPCCs treated mice, before and after treatment **(A)** (the mean blood sugar of treated diabetic mice is related to, 3 weeks after cell therapy with IPCCs).
Chart of insulin released by IPCCs in response to glucose stimulation. Asterisks in figure denote statistical significance **(B)**, (p=0.0002).

Immunohistochemistry of thigh muscle, after 4 weeks:

To identify the survival of grafted cells, paraffin sections were analyzed for detecting of DIL labeled cells (Fig7) all 3 mice that grafted, survived throughout the experiment and was evaluated for immunocytochemistry.

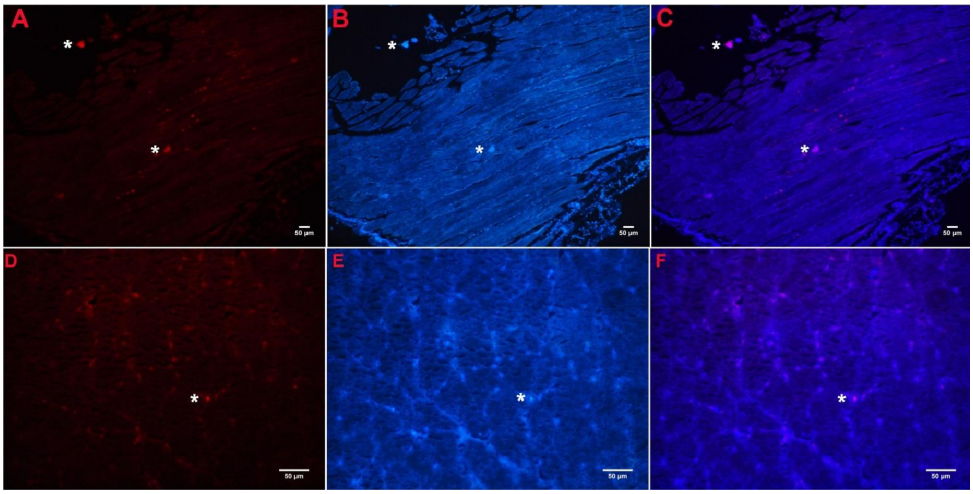

Figure 7: sections of formalin-fixed paraffin embedded thigh muscle of grafted mice. Labeled IPCCs injected in thigh muscle of diabetic mice after 4weeks, DiI-labeled cells localized between muscle fibers **(A)** (asterisks). DAPI nuclear counter staining **(B)**. Merged**(C)** (20X), DiI-labeled cells localized between muscle fibers **(D)** (asterisks), DAPI nuclear counter staining **(E)**, Merged **(F)** (40X).

The results of mean body weights assessment showed that the differences between mean body weights Of mice before and after diabetes induction and even before and after treatment were significant. The results analyzed with graphpad prism5 software, and were significant (fig8) (P=0.0002).

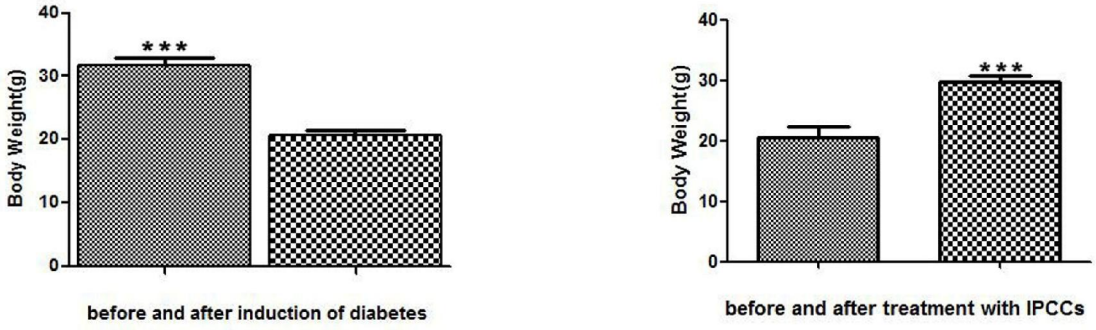

Figure 8: Mean body weight of mice before and after (3weeks) induction of diabetes (**A**), mean body weight of diabetic mice before and after (3 weeks) treatment with IPCCs (**B**). Body weight of diabetic mice differed significantly before and after treatment. Asterisks in figure denote statistical significance (p=0.0002).

4. Discussion

Today, cell replacement therapy for both type I and type II diabetic patients has become a promising scenario that could be achieved in the near future.

Pluripotent embryonic stem cells (ESCs) can spontaneously differentiate or be induced to differentiate into IPCs. For the first time, Soria et al. (Soria, 2000) reported that IPCCs derived from mouse ESCs normalized blood glucose levels when they were transplanted into STZ-induced diabetic mice.

Hori (Hori, 2002) and Lumelski (Lumelski, 2001) with almost same protocol succeeded to differentiate ESCs into IPCCs, but in this 5step protocols, generated IPCCs were incapable of de novo insulin production and had absorbed their insulin from insulin-rich culture medium. Both of these researchers used nestin positive cells in their protocol. Blyszczuk (Blyszczuk, 2003) with another method could differentiate ESCs into IPCCs in 19 days. In contrast to the Hori and Lumelski protocols, the Blyszczuk's method was able to Generate IPCCs with the functionality to induce short term and long-term rescue of experimental diabetes. Some researchers have recently identified bone marrow residing VSEL stem cells in both human and mouse as a putative non embryonic source for pluripotent stem Cells (PSCs).

Although many stem cell types has been used in cell therapy for treatment of type I Insulin-dependent diabetes mellitus (IDDM), the use of autologous stem cells like VSEL stem cells to produce IPCs is a promising approach for the treatment of diabetes. Ratajczak and his colleagues succeeded to differentiate murine VSEL stem cells into pancreatic cells that expressed Insluin1 and insulin2 markers, but they didn't explain that the differentiated cells can produce insulin in response to glucose stimulations. Besides they didn't examine function of these newly beta like cells in vivo (Kucia, 2006).

To clarify whether VSEL stem cells and insulin producing cell clusters (IPCCs) that derived from them can relief signs of diabetes, this study was accomplished. We encountered with two different challenges. First, we obliged to prove that VSEL stem cells are exist in mice bone marrow and then analyzed the capability of these cells in differentiation to IPCCs for cell therapy.

The use of Activin A and betacellulin either alone or in combination, has resulted differentiation of stem cells into IPCCs (Wong, 2011). More recently, many studies almost inevitably used nicotinamide in their induction protocols in one or more stages together with other extrinsic induction factors (Segev, 2004; Yu, 2007; Feng, 2008; Li-Bo, 2004).

As early as the 1990s Otonkoski et al. had used nicotinamide as an inducer of endocrine differentiation in cultured human foetal pancreatic cells. It was shown that treatment of human foetal pancreatic cells with 10 nM nicotinamide resulted in a twofold increase in DNA content and a threefold increase in insulin content associated with development of beta cell outgrowths from undifferentiated epithelial cell clusters. There was also an increase in the expression of the insulin, glucagon, and somatostatin genes (Otonkoski, 1993).

We succeeded to sort VSEL stem cells with multicolor FACS method. We used antibodies against CD45 marker then from CD45 negative groups; we isolated Sca1+ and CXCR4+ cells. We used recombinant human Activin A and nicotinamide as main factors for differentiation of VSEL stem cells into beta like cells and succeeded to differentiate these cells to IPCCs.

We know that PDX1 is needed for growth and branching of both pancreatic buds while Insulin1 is important for the development of dorsal pancreatic bud and mesenchyme (On the Development of The Islets of Langerhans).

The results of RT-PCR revealed that newly formed IPCCs expressed PDX1, Insulin1 and Insulin2 that proved that differentiation was occurred.

After ICC of IPCCs we observed that GLUT2 and PDX1 has expressed, that vigorously showed that differentiation has accomplished. Functional assessment of IPCCs with ELISA kit showed that newly derived IPCCs are capable to secret insulin in response to glucose stimulation and VSEL stem cells are potent for using as a source of stem cell invitro and in vivo.

3 weeks after cell therapy of diabetic mice with IPCCs, we observed that blood sugar had a significant reduction that last for almost 45 days (The time of blood sugar controlling).

Ethical concern in related to embryonic stem cells and doubt about the capability of these stem cells in differentiation into IPCCs can relief by using VSEL stem cells in future. VSEL stem cells are autologous and don't express MHC1 and MHC2 and are appropriate for intravenous injection.

In **conclusion**, the results of this paper showed that VSEL stem cells are capable to treat diabetes and can be regarded as a substitute for other stem cells in cell therapy.

These cells, mimic embryonic stem cell's behavior and can differentiate into IPCCs but they don't have any ethical concerns. Producing cells were able to secrete insulin invitro, and in the living body were also effective too.

Future study:

Further consideration is needed to evaluate the capability of these newly IPCCs for producing insulin in vivo for a longer period. Intact VSEL therapy should be done in future for treatment of diabetes.

Conflict of Interests:

The authors declare that there is no conflict of interests regarding the publication of this article.

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