

Isolation and Pancreatic Endocrine Differentiation of the Mesenchymal Stem Cells From Human Umbilical Cord

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Abstract

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia which resulting from defects in insulin secretion, insulin action, or both. Stem cells are group of cells which have the ability of self-renewal and differentiation into many types of cells such as insulin like producing cells. The aim of this study is to isolate mesenchymal stem cells from Wharton's jelly of umbilical cord from healthy babies born to healthy mothers and differentiation to insulin like producing cells which give good result using trichostatin-A-based (two- step protocol) . Flow cytometry analysis showed that MSCs were positive for CD90 and CD105 and negative for CD45 and CD14. These data show that the majority of the human umbilical cord derived cells were mesenchymal stem cells. Differentiation of mesenchymal stem cells to insulin like producing cells were evaluated by ELISA. We found that stepwise increase in insulin release in response to increase of glucose concentration.

Key words: Umbilical cord; Wharton's Jelly ; Diabetes Mellitus; flow cytometry; Insulin Like Producing Cells.

1. 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. Transplantation of whole intact pancreas or isolated pancreatic islets is an alternative treatment for patients with type 1 DM. However, the shortages of cadaveric organs and the necessity to use immunosuppression are limiting factors [1]. Recent progress in the field of regenerative therapies has focused attention on generation of surrogate cells from stem cells derived from embryonic, umbilical cord blood, Wharton's jelly, and a variety of adult tissues [2]. Human embryonic stem cells (h-ESCs) can be expanded and differentiated to all cell types including insulin-like producing cells (ILPCs) [3–5]. So far, the use of these cells is burdened by ethical considerations as well as by practical problems: the lack of available embryos, difficulties with the generation of immunocompatible cells, and the risk of uncontrolled malignant proliferation of residual undifferentiated cells. ILPCs can also be obtained by directed differentiation of cells from Wharton's jelly of human umbilical cord [6]. Mesenchymal stem cells are considered adult stem cells. They can produce many types of cells as bone cells and cartilage cells [7]. This source provides an opportunity for generation of large numbers of autologous cells without the major limitations of organ availability or allogeneic rejection [8]. Here in our study, we investigate the efficacy of trichostatin-A-based (two novel differentiation protocol) to generate ILPCs from human Wharton's jelly derived mesenchymal stem cells.

2. Material and Methods:

This study include 50 Human Umbilical Cord obtained from mothers undergoing caesarian section in Obstetric

and Gynecology Department, Mansoura university hospitals.

Mothers age preferred between 17 and 36 years, free from any diseases. Route of delivery (Cesarean section), full term infants and umbilical cord was in a good condition and its length between 15 to 20 cm.

Mothers younger than 18 or older than 36 years, gestational age was less than 37 weeks, umbilical cord was not in a good condition as too short and thin, Fever (> 38°C) and mothers

had Serologic positive results for blood transmitted diseases. (syphilis, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus) were excluded from the study.

Consent & Ethical committee

According to the policy approved by the Mansoura Faculty of medicine Institutional Research Board (IRB) - Mansoura University. All tissue samples were collected after written informed consent from all mothers prior to being included in the study .

Sample Collection

Collecting of umbilical cord: Cords were collected from caesarian sections at full term infants. Pour 2 tubes of sterile saline (0.9% NaCl) with antibiotic into sterile cord tissue container, use alcohol swabs to remove debris or fluid from the umbilical cord, use sterile scissors to cut apart of the umbilical cord near to the placenta 15 -20 cm in length, Place cord tissue segment into the cord tissue container, ensure that the cord is fully submerged in the saline. Cap container tightly to prevent leakage, finally transferred immediately to the Mansoura Research

Center For Cord Stem Cells (MARC-CSC) ,Mansoura Faculty of Medicine.

The steps of this method were done in biological safety cabinet (BSC) to reduce the risk of contamination.

A)Extraction of cells:

Spray down the human umbilical cord container with alcohol and bring it into the BSC.Wash the Human Umbilical Cord (HUC) with sterile normal phosphate buffer saline (PBS) (Lonza-BE17-516F) from any blood and place it on the sterile petri dish.Ensure that the HUC collected was from 15 to 20 cm in length, remove the vein and arteries and cut into pieces each is 5-10 mm³ in length using sterile scalpel and forceps or scratch Wharton's Jelly with a sterile slide .Transfer the cut HUCT into 2 falcon tube.Add in enzymatic solution (equal volume of collagenase(Sigma – C1639-50 MG) and Hyaluronidase solution(Sigma-H3757-100 MG)) into the tube , Ensure that the enzymatic solution covers the HUCT.Add in 2 ml of Antibiotic Solution(penicillin, streptomycin) (Sigma Aldrich -A5955) into the tube. Mix well with the mixture of enzymes and HUCT.Put for 1 hour at 37°C in a water bath with gentle agitation every 15 minutes.After incubation period, spray down the tube with alcohol and placed it into the BSC.Dilute the digested suspension with 1:2 volume of PBS to reduce the viscosity of the suspension.Place a sterile 70 µm cell strainer on a new sterile tube.Pass digested suspension through the 70µm cell strainer to obtain a single cell suspension.Remove the strainer and cap the tube.Centrifuge the cell suspension at 1100g for 20 minutes at 37°C and the pellet was resuspended in PBS for a second centrifugation .Aliquot 2ml for cell viability test with trypan blue.After centrifugation , remove the supernatant then resuspend the pellet in 6ml complete media.Then put it into a sterile 25 cm² tissue culture flask.Incubate in a CO₂ incubator at 37°C.Feeding by changing media every 3 days.

B) Subculture and expansion of cells:

After 3 days, the nonadherent cells were discarded. The remaining adherent MSCs were cultured to 80% confluence by changing media every 3 days before passaging with 0.25% trypsin(Gibco-15090).Cells were resuspended with complete Dulbecco's Modified Eagle's medium low glucose (DMEM)(Gibco-11885-084) and cultured for 21 days , to reach 80% confluence. This step was repeated for a second passage. The cells then had the appearance of fibroblast-like cells.

Trypsinization of the cells to be ready for characterization and differentiation.

C)Phenotyping Characterization of cells:

To ensure exclusion of hematopoietic stem cells and purity of MSCs.

Principle of test:

We used Stem-Kit™ reagents (Beckman coulter, USA) which contain monoclonal antibodies to CD14 , CD45, CD 90(FITC) and CD105(PE) conjugated to specific fluorochrome. The samples were incubated with specific antibody. In another tube isotype control was incubated with same antibodies to avoid nonspecific binding.

D)Differentiation of mesenchymal stem cell to insulin like producing cells:

When the entire surface of the culture flask was covered by cells, the process of differentiation was started.Differentiation of MSCs into Endocrine Cells at passage 2 , MSCs were cultured at a density of 1x 10⁵ cells/ml.

Two-Step Protocol. Differentiation was carried out according to the method previously reported by (Tayaramma et al.,2006).Initially, cells were cultured for 3 days in serum-free DMEM supplemented with Trichostatin-A (TSA) at a concentration of 55 nM.The cells were then cultured for additional 7 days in high glucose (25 mM) medium containing 1: 1 ratio of DMEM: DMEM/F12 (Sigma).This mixture was supplemented with 10% fetal bovine serum and 10 nM glucagon-like peptide-1 (GLP-1, Sigma).

E)Determination of in vitro insulin release in response to increasing glucose concentration :

Three different samples of 5x10⁴ cells are collected from different umbilical cord at the end of the differentiation period for measurement of released insulin hormone.Cells are cultured in 9 wells , we remove media and addition of media with different concentration of glucose 5.5 ,12 ,25 mM .(This is recommended concentration of glucose). Leave cells for 1 hour then the supernatant is collected at the end of each incubation period .The collected samples are assayed using an Elisa Kit of insulin .Undifferentiated MSCs used as control .

Data processing and statistical analysis:

The statistical analysis of data was done by using excel (Microsoft Office 2013) program and SPSS (statistical package for social science) program (SPSS, Inc, Chicago, IL) version 20. Qualitative data were presented as frequency and percentage .Quantitative data were presented as mean and standard deviation .

RESULTS

Table (1): Demographic data of studied groups:

		FREQUENCY	PERCENTAGE
FETAL GENDER	MALE	25	50.0%
	FEMALE	25	50.0%
GESTATIONAL AGE	FULL TERM	50	100%

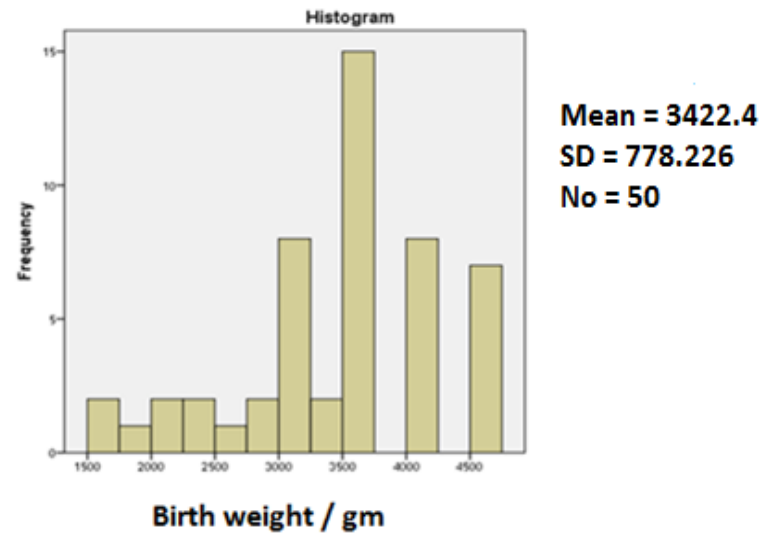


Figure (1): Distribution of birth weight / gm.

	MEAN ± SD	RANGE
MATERNAL AGE / YEARS	24.14 ± 5.725	17 – 36
GESTATIONAL AGE / WEEKS	38.84 ± 2.103	37 – 40
BIRTH WEIGHT / GM.	3422.4 ± 778.22	1500 – 4600

Table (1) demonstrates demographic characteristics of the studied groups. Half of infants were male (25, 50%) and other half were females (25, 50%). All infants were born by cesarean delivery with majority between 38-40 weeks of gestation. The mean of gestational age / weeks and birth weight / gm. were (38.84 ± 2.103) and (3422.4 ± 778.22) respectively. The maternal age / years was (24.14 ± 5.725).

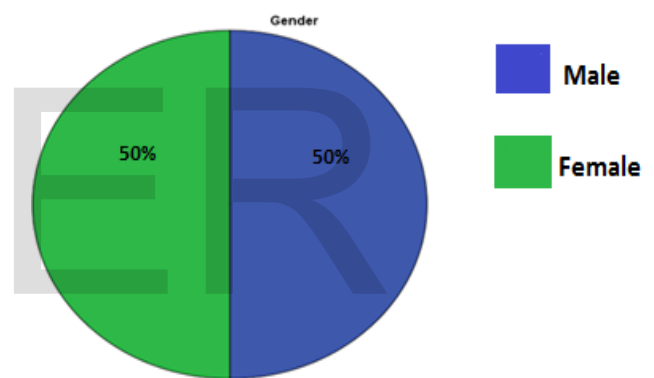


Figure (2): Distribution of fetal gender.

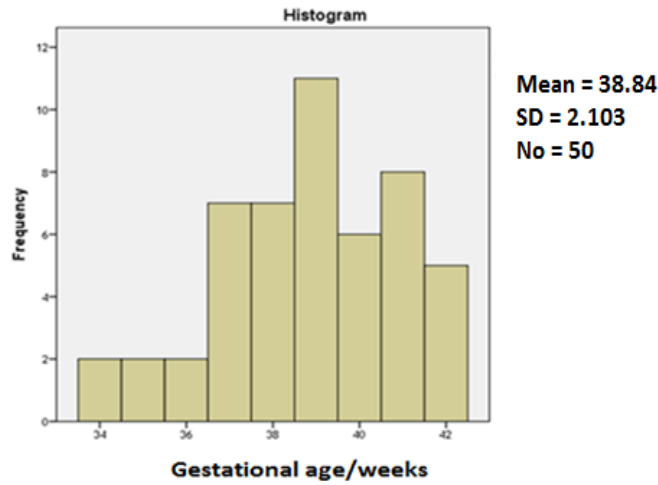


Figure (3): Distribution of gestational age / weeks.

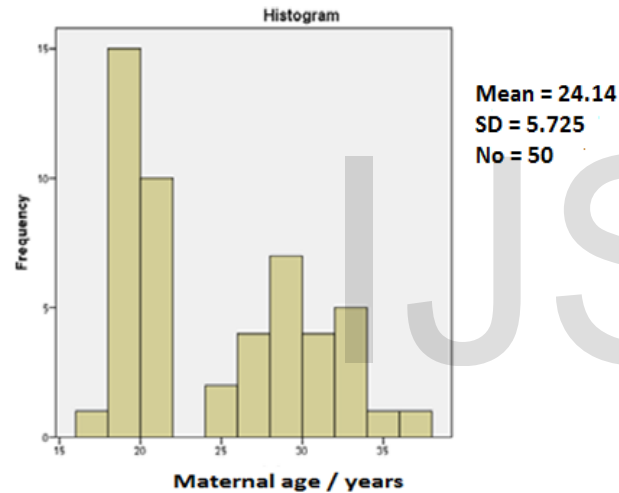


Figure (4): Distribution of maternal age / years.

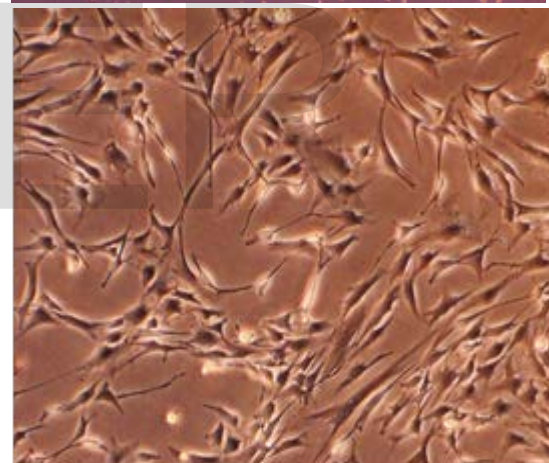
Table (2): Umbilical Cord characteristics:

	MEAN ± SD	RANGE
CORD LENGTH / CM	18.7 ± 1.42	15 – 20

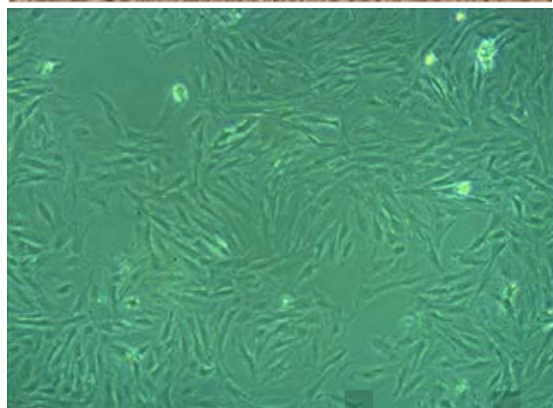
Table (2) shows umbilical cord characteristics: the mean of cord length / cm was (18.7 ± 1.42) .

These photomicrograph show different stages of stem cells:

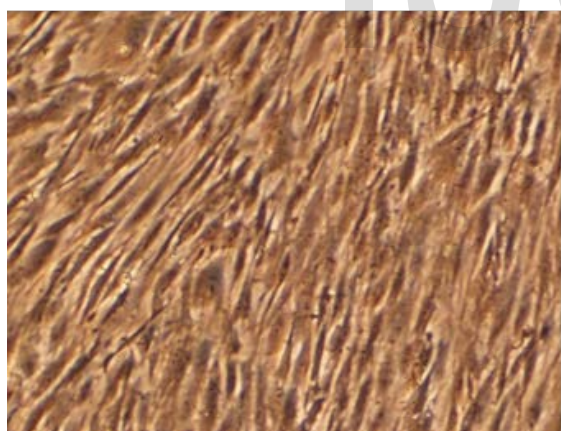
- 1-round stem cells just after isolation .
- 2-stem cells after one week .
- 3-spindle shape fibroblast like cells at the end of expansion .
- 4-stem cells after differentiation.



(a)
(b)



(c)



(e)

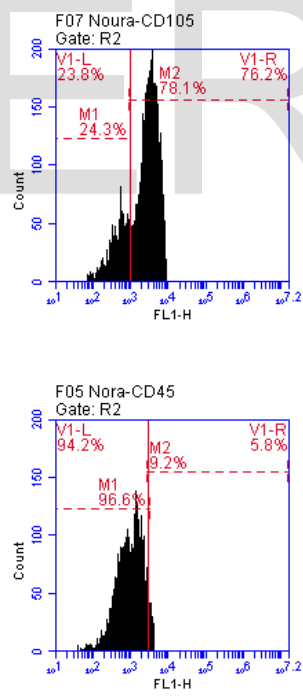
Figure(5):(a)This photomicrograph illustrates round stem cells just after isolation 4X.(b)This photomicrograph show stem cells after one week10X.(c): This photomicrograph shows spindle shape fibroblast like cells at the end of expansion 4X.(d): This photomicrograph shows untreated mesenchymal stem cell used as control 10 X.(e): This photomicrograph show stem cell after differentiation10X.

The International Society for Cellular Therapy (ISCT) has proposed a set of standards to define MSCs. A cell can be classified as an MSC if it shows plastic adherent properties under normal culture conditions and has a fibroblast-like morphology. And also we found these results :Mesenchymal stem cells obtained from Wharton’s Jelly grew as flat monolayer after being cultured in vitro for five to seven days. When reach confluence, at the end of the expansion phase, the cultured human cells become homogenous, spindle shaped, and fibroblast-like arranged in monolayers.Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin.The cell body contains a large, round nucleus .The cells proliferated with a doubling time of approximately 24 hours .The duration of primary culture was 10-14 days.

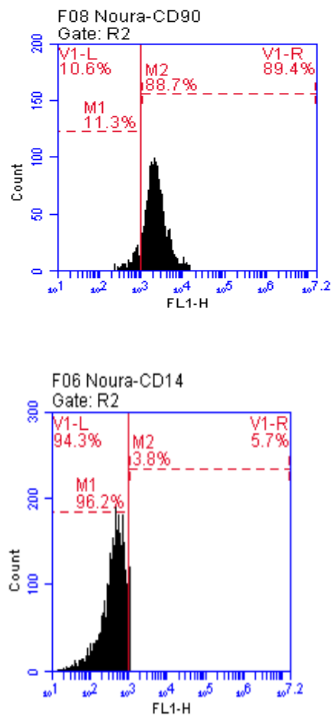
Detection of MSCs:

Flow cytometric analysis showed that stem cells expressed higher levels of CD105(78.1%) and CD 90(88.7%) but negligible levels of CD45(9.2%) and CD14(3.8%) , which are surface markers for hematopoietic stem cells. These data show that the majority of the human umbilical cord derived cells were MSCs.

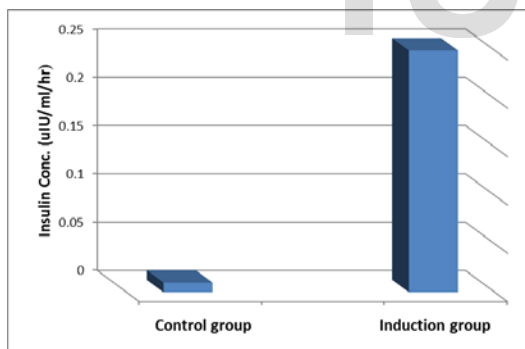
(d)



Morphological and Phenotypical Characterization of the cultured WJ-MSCs:



Figure(6): Flow cytometric detection of surface markers of the undifferentiated mesenchymal stem cells.



Figure(7): Insulin concentration of WJ-MSCs with and without induction.

Table (4) :In Vitro Human Insulin Release in response to a Glucose Challenge:

Glucose Conc.(mM)	5.5	12	25
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Insulin Conc. µIU/ml/hr	0.175	0.225	0.3
	0.15	0.175	0.25
	0.2	0.225	0.275
Mean±SD	0.175±0.025	0.208±0.029	0.275±0.025

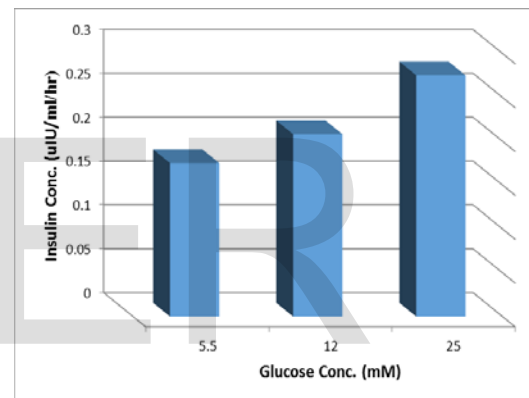


Figure (8): comparison between insulin release in response to different concentration of glucose.

The differentiated cell released increasing amounts of insulin in response to increasing glucose concentration . The amounts of insulin released at different concentrations of glucose were comparable among the three samples. There was no insulin release by undifferentiated MSCs .My comment is that there was stepwise increase in insulin release in response to increase of glucose concentration. So this means good differentiation of WJ-MSCs to insulin like producing cell.

DISCUSSION

Type 1 diabetes is characterized by destruction of pancreatic -cells, which is the result of autoimmune response. Both genetic and environmental factors also play a role in the development of the disease.

So far, the most commonly used treatment is insulin application, but it has its disadvantages. It seems that the solution for this problem could be pancreas or islet transplantation[10] .Islet transplantation is considered to be an efficient therapy for type 1 diabetes. But however its clinical usefulness has been restricted by immune rejection

of transplanted islets and the lack of donor islets cells[11].It has been suggested that regeneration therapy overcome the challenges in islet transplantation. MSCs are promoted as an appropriate population in differentiation of insulin like producing cells (IPCs) for autologous transplantation [12].Autologous transplantation prevents autoimmune rejection and so there is no need of immunosuppressants' use [13]. Mesenchymal stem cells are selfrenewing cells with a multilineage differentiation potential[14] .Their ease of isolation and high number and expansion from different adult human tissues such as(bone marrow, adipose tissue ,dental pulp, and umbilical cord which is easily obtained and processed) has considered them a potentially very important source of stem cells for the use in regenerative medicine and tissue engineering including a therapeutic potential for DM [12] .

MSCs have ability to differentiate into islet-like cells and possess immunomodulatory abilities, thanks to which it is possible to decrease the risk of immune rejection.Recently, it was showed that human umbilical cord-derived MSCs are superior to those obtained from subcutaneous adipose tissue in terms of differentiation into ILPCs [15].

Stem cells are thought to mediate repair via five primary mechanisms:

Providing an antiinflammatory effect.

Homing to damaged tissues and recruiting other cells, such as endothelial progenitor cells, that are necessary for tissue growth.

Supporting tissue remodeling over scar formation.

Inhibiting apoptosis.

Differentiating into bone, cartilage, insulin like producing cells, and ligament tissue.

In this study we checked patients files for exclusion criteria which were maternal age (< 16 or > 36 years), fever (> 38°C), ruptured membranes (≥ 18 hr. before delivery), umbilical cord was not in a good condition as too short or thin and serologic positive results (syphilis, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus).

we do extraction of mesenchymal stem cells from Wharton's Jelly of human umbilical cord using collagenase type 1 and Hyaluronidase and these were in accordance with Koliakos et al. [16], then differentiation of Mscs to insulin like producing cells using two step protocol which include culture of cells for three days in serum free DMEM with Trichostatin -A .Then culture for another 7 days in high glucose medium containing 1:1 ratio of DMEM : DMEM F12 with 10% fetal bovine serum and glucagon like peptide -1.

Our study found that functional insulin-like producing cells were generated from WJ-MSCs after in vitro differentiation. The expression of insulin producing cells was confirmed by determination of in vitro insulin release in response to increasing glucose concentration . These results provide evidence that WJ-MSCs have capability to be reprogrammed in vitro to become insulin like producing cells as we found there was stepwise increase in insulin

release in response to increasing glucose concentration.

We choose umbilical cord as a source of mesenchymal stem cell because of the following points:

1-To bypass the ethical and political debates.

2-Significant proliferative and differentiation potential.

3-No tumorigenicity.

4-Karyotype stability.

5-High immunomodulatory activity

6-Unique combination between prenatal and postnatal MSCs properties.

And these points also reported by Takahashi et al [17] ,and Weiss et al [18] as their study illustrate thate there is no immune rejection occurred after xenotransplantation of MSCs in the lack of immunosuppressive therapy. In this study, HUC-MSCs were isolated on the basis of their ability to adhere to plastic. At the end of expansion, these cells assumed a spindle-shaped morphology and were negative for hematopoietic cell markers[19] . And this is the same as our results.

Gabr et al[20] reported that characterization of mesenchymal stem cell by flow cytometric analysis showed that stem cells expressed higher level of CD105 but negligible level of CD14 and these data show that the cells obtained from umbilical cord were MSCs not hematopoietic cells and this same our results.

Zalzman et al and Lee et al [21,22] reported that stem cells can transdifferentiate into insulin like-producing cells in a high-glucose cell culture in vitro . Many protocols have been studied to induce differentiation of stem cells obtained from different sources to form ILPCs [23].

Although, the proportion of generated ILPCs was modest. In an attempt to increase the proportion and optimize insulin production, the current study was designed to compare the efficiency of two different protocols for their differentiation potentials into ILPCs.In the current study, we have utilized TSA in the two-step protocol, and - mercaptoethanol in the three-step protocol to induce differentiation of mesenchymal stem cells to ILPCs.

TSA is a natural product isolated from the metabolites of strains of *Streptomyces hygroscopicus* with antifungal and antibiotic activities [24]. Evidences were provided that TSA has the potential of chromatin remodeling and can allow differentiation of mesenchymal stem cells into ILPCs under appropriated culture conditions in the presence of high glucose concentrations and GLP-1[25].

Tang et al [26] found that two steps in cell culture conditions, high-glucose concentration and β -cell promoting factors, were important for inducing differentiation of MSCs into insulin like-producing cells.

Glucose is well known as a growth factor for β cells and a physiologic regulator for the insulin gene. It up-regulates insulin gene expression in pancreatic β cells and stimulates stem cells to differentiate into insulin like-producing cells [27] .It promotes cells replication in vitro as well as in vivo at concentrations of 20–30 mmol/L and increase insulin content in the cells[28] .GLP-1 is an incretin hormone capable of converting intestinal epithelial cells into functional ILPCs [29] .

It should be noted that the two-step protocol (TSA-based)

had previously used bone marrow-derived cells from mice. In this study, we report, the utilization of such protocols for the differentiation of HUC-MSCs to form ILPCs. Even under the conditions of induction mentioned above, only some of the HUMSCs could differentiate into insulin like-producing cells and this was particularly evident with the two step protocol (TSA based) more than three step protocol.

So in our research we found that TSA based protocol is better because of its simplicity and shorter period needed for differentiation . This was in agreement with Gabr et al [20] .Some cells expressed insulin, which might represent β -cells while others didn't express insulin. The insulin was not detected in the undifferentiated MSCs. These results were also reported by many investigators. The poor insulin release in response to glucose challenge was also reported by many investigators [31] . And this was different from our study as there is good release of insulin which mean good differentiation.

Comparisons between the reported data are difficult because of different units of measurements were used for reference . In our study, data show that, for a similar number of cells, insulin release at a concentration of 25Mm of glucose was ranged between (0.012-0.011 ng/ug/hour).

Kim et al [32] compared insulin release by differentiated human MSCs isolated from different sources. At a concentration of 25mM of glucose, insulin release ranged between (0.004–0.016ng/ug). Wei et al [33] reported that insulin release by differentiated human MSCs at a glucose concentration of 16.7mm ranged between (0.008–0.04ng/ug). Ilie et al [34] . reported that insulin release by human \otimes cells at 15mM glucose concentration was approximately $0.27\text{ng}/1 \times 10^6$ cells/hour.

CONCLUSION:

Wharton's jelly mesenchymal stem cells provide an excellent source for treatment of diabetes. due to its ability to differentiate into insulin like producing cells using the TSA-based differentiation protocol in view of its simplicity and the short duration needed for differentiation. Also WJ-MSCs are readily available in large quantities and have a low risk of immune rejection.

Conflict of interest statement:

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