



Proliferation Capacity and Expression of Cell Cycle Genes in Normal and Gestational Diabetes Affected WJMSCs

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ABSTRACT

Wharton's jelly derived mesenchymal stem cells (WJMSCs) are a well-known source for the regenerative approach. Current investigation shows that the metabolic disorders like gestational diabetes mellitus (GDM) are not only responsible for several disease factors in adult life but also have adverse effects on perinatal environment as well as on the cellular properties of WJMSCs. This study was designed to determine the effects of gestational diabetes mellitus (GDM) on proliferation of WJMSCs (GDM-WJMSCs). Mesenchymal stem cells were isolated from Wharton's jelly tissue of normal(healthy) samples and from the GDM affected sample. The isolated cells were characterized for the presence of stem cells markers by Immunocytochemistry. After characterization, the analysis of GDM effect on WJMSCs proliferation rate was analyzed by calculating the population doubling time of the cells at different passages (P0-P3). Moreover, the expression of the important cell cycle genes (*CDC42*, *CDC48*, *CDC20*, and *CCNA2*) was analyzed by quantitative PCR to determine the effect of GDM at gene level. The isolated cells expressed the stem cell specific marker *CD90* which confirmed that the isolated cells are WJMSCs. Population doubling time (PDT) was found to be increased in the early passages (P0-P1) in GDM-WJMSCs as compared to control WJMSCs. However, at later passages (P2, P3 stages) PDT was decreased and an improvement in the proliferation rate was observed. The fold change expression of cell cycle genes was found to be decreased in GDM-WJMSCs as compared to control WJMSCs. This study concluded that the GDM affect the expression of cell cycle genes, hence the cells proliferate slow as compared to the normal cells. The GDM affected cells had a high population doubling time in the early passages but when the cells were grown *in vitro* in controlled environment the cells proliferate efficiently, suggesting that the GDM affected WJMSCs can also be utilized for the regenerative purpose after improving the proliferation rate of the cells.

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Authors' Contribution

NR collected the data, wrote the article, analyzed all the data and did all the experiments with seniors' supervisors. SU designed the study, conducted the experiment and analyzed the data. MS helped out in formulation of data and arranged all the data in sequence and helped in writing. BW supervised the study with her expertise. KH performed the isolation of primary culture planned the study, and helped out to in different experiments. HS, TU and SR helped out in sample collection, procedures and writing.

Key words

Gestational diabetes, Regenerative medicine, Population doubling time, Wound healing, Inverted microscope

INTRODUCTION

Regenerative medicine is a branch of medicine related to evolving treatments for the repair of injured, unhealthy or impaired cells, tissues or organs to maintain the normal function of the cells (Bajada *et al.*, 2008). Invention and exploration in nanotechnologies, tissue engineering, bioimaging and stem cells are vital to the advancement of regenerative medicine, as validated by current case studies and scientific trials (Forraz and McGuckin, 2011).

In the human body, an endogenous system of renewal and renovation are present, where stem cells are present nearly in every tissue. These cells have two important properties, i.e., self-renewal ability, and the capability of multiline age differentiation into diverse cell lineages. High differential potential, easy isolation procedures and good proliferation rate made them a decent choice for regenerative medicine application (Kolli *et al.*, 2010; Azzopardi and Blundell, 2018).

On the basis of sources, we classify the stem cells into two main categories, embryonic and non-embryonic stem cells. The embryonic stem cells (ESCs) are pluripotent, self-recommencing cells, isolated from the blastocyst cell mass and can be distinguished into all three germ layers cells (b) Non-Embryonic Stem cells, typically include stem cells from adult sources, have limited differentiation ability. These cells can be sequestered from several tissues including bone marrow, adipose tissue, dental tissues, skin, umbilical cord and placenta and are the widely used

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stem cells in different clinical conditions (Li *et al.*, 2017).

Large numbers of mesenchymal stem cells (MSCs) are present in Wharton jelly tissue, that grasp countless prospective for usage in cell-based beneficial and clinical approaches because of the inherent capability of self-renewal and differentiation into numerous cells i.e., adipose tissue, bone marrow, skeletal muscle and cartilage (Ross *et al.*, 2006) dependent on the precise micro environment.

Wharton's Jelly derived MSCs are utilized for different clinical conditions. Literature reports that their properties are altered by several metabolic conditions. Recent studies have shown a link between gestational diabetes mellitus (GDM), a communal metabolic condition during pregnancy and development of functional alterations in stem cells properties (An *et al.*, 2017). GDM is a temporary metabolic condition resulting from glucose derangement and intolerance due to insulin insensitivity during pregnancy (Buchanan *et al.*, 2012). Literature reports that gestational diabetes affects the cellular characteristics of WJ-MSCs. GDM affected WJMSCs also showed decreased multilineage differentiation capacities than normal WJ-MSCs. Additionally, GDM affected WJ-MSCs showed diminished mitochondrial activity which causes reduced proliferation capacity of MSCs and premature senescence (Jensen and Mehta, 2016; Lawlor, 2013; Kim *et al.*, 2014). In 2015 a study showed the role of gestational diabetes related endothelial dysfunctioning. Microarray data shows different genes like *CDC20*, *CDC A2*, *CDC A8* participate in different steps of cell division cycle. *CDC A2* gene translates a pointing subunit of the cell-cycle related protein phosphatase which helps in nuclear envelope renovation and regulate the DNA damage reaction. *CDC A8* gene encodes the protein which is essential for chromatin-stimulated microtubule maintenance and formation of spindles and *CDC20*, functions to initiate chromatin separation and cell cycle entrance into anaphase. These genes have been reported to be altered in GDM (Samar *et al.*, 2015).

This study was designed to determine the effects of GDM on the proliferation of the WJMSCs by analyzing the population doubling time of the cells at different passages and on the gene expression of cell cycle genes. This study was different from the previous study (Samar *et al.*, 2015) as Samar *et al.* (2015) identified the decreased function of cell cycle genes from microarray data and worked on endothelial cells. We identified for the very first time about the actual cause of decreased proliferation rate in diabetic WJMSCs at genetic level.

MATERIALS AND METHODS

Study setting

This was an experimental study, performed at

Multi-Disciplinary Research Lab and PCR lab Ziauddin University Karachi, Pakistan after taking ethical approval from the Ethical Research Committee Ziauddin University.

Inclusion criteria

Umbilical cords were obtained from normal and gestational diabetic mothers of full-term cesarean sections, aged between 18 to 30 yrs. (primary gravida), after taking informed consent. The verdict of GDM was founded on the International Association of Diabetes and Pregnancy Study Groups Consensus and Panel recommendation, (OGTT at 20 weeks, controlled GDM patients by diet and exercise).

Exclusion criteria

All Hepatitis B and C positive mothers, a pre-pregnancy diagnosis of diabetes any complicated pregnancies with conditions of fetal anomaly/ IUGR, PIH, preeclampsia, ruptured membranes/ placenta previa were excluded for the study.

Isolation and expansion of WJMSCs

The tissue was washed many times with sterilized PBS solution and cut into 3mm thick portions. After that Wharton's jelly was scratched with the help of the scalper. Trypsin was added for tissue digestion and cells harvesting. Following digestion, the tissue pieces were cultured in Dulbecco's modified eagle medium (DMEM) containing pen/strep, antifungal drug, sodium pyruvate, L glutamine and 10% fetal bovine serum (FBS) in 5% CO₂ incubator at 37 °C for further cell proliferation.

Subculturing

When 80% cells confluency was achieved, cells were transferred into T25 cm flasks. Medium were aspirated prior cells washing with sterile PBS. 2-3 mL of 0.25% trypsin was added to the cells and incubated at 37 °C for 4-5 min. After incubation whole medium was supplemented to inhibit the trypsin action and the detached cells were collected in falcon tubes, followed by centrifugation at 300 X g for about 8 min. The cell pellet was re-suspended in the fresh medium and cultured in two T25 flasks for further cell proliferation.

Characterization of WJMSCs by immunocytochemistry

For the confirmation of isolated WJMSCs, Immunocytochemistry was performed WJMSCs (~10,000 cells) were cultured in 24 well plate. After proper cell attachment, medium was aspirated and the cells were gently rinsed twice with 1X PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 15 min. The cells were rinsed

2-3 times with 1X PBS and incubated in blocking solution overnight at 4 °C with primary antibodies at 1:100 dilution against CD90 and ACTIN. After incubation, the antibody solution was removed and cells were rinsed with PBS and incubated for 1 h at RT with Alexa fluor 546 donkey anti rabbit secondary antibody at a dilution of 1:200. This was followed by washing with 1X PBS. Nuclei were counterstained with 0.5µg/mL 4, 6 Diamidino-2-phenylindole (DAPI) for 5min. Finally, cells were rinsed 5 times with PBS, and observed under inverted fluorescent microscope (Fluoid cell imaging system).

After the confirmed presence of WJMSCs by Immunocytochemistry, experiments for population doubling time and gene expression analysis were performed.

Population doubling time

It is defined as the period unit used by a cell inhabitant to grow and double its number. For population doubling time (PDT), normal-WJMSCs and GDM-WJMSCs were plated on 6-well culture plates at 10,000 cells per well. The experiment was done in triplicates. Time taken by the cells to reach 80-90% confluency was observed. The cells were harvested with trypsin and the PDT was calculated at different passages (P0, P1, P2, P3) by using the following formula.

$PDT = \text{culture time (CT)} / \text{population doubling number (PDN)}$

Population doubling number = $\log N_i / N_f * 3.31$ where, N_i = initiating numbers; N_f = numbers at harvesting stage.

Gene expression analysis of cell cycle genes (CDCA2, CDCA8, CDC20, CCNA2)

RNA isolation and quantification

RNA was isolated from the cultured cells in 30µl reaction volume using TRIZOL method. Medium was removed and cells were washed with 1X PBS twice. After that cell were trypsinized and the pellet was resuspended in Trizol reagent. Chloroform was added for the phase

separation for 15 min followed by centrifugation at 12000 X g. After centrifugation, upper aqueous part was used for the extraction of RNA. Chilled isopropanol was added for RNA precipitation and then centrifuged at 10,000 X g for about 10 min at 4 °C. 75% ethanol was added for rehydration of RNA. Pellet obtained after centrifugation was dried out and resuspended in 30µL sterile, nuclease free water. The isolated total RNA was quantified with Multi Scan Sky Spectrophotometer.

CDNA synthesis

One microgram RNA was reverse transcribed using Revert Aid First Strand cDNA synthesis kit (Invitrogen, USA) according to manufacturer's protocol. For the reaction, 1 µg RNA, 1 µL oligo Di was added in the mixture and the total volume was made to 12 µL with Nuclease free water. Then the mixture was incubated at 70°C for 5 min. After that 4 µL 5X reaction buffer, 2 µL dNTPs and 1µL RNase out (20U/µL) were supplemented within the solution tube and incubated at 25 °C for 5 min. After this incubation process, 1 µL of Reverse Transcriptase enzyme was further added, and the sample was incubated at 42°C for 60 min and at 70 °C for 10 min. After incubation, the cDNA was stored at -20 °C for further usage

Quantitative PCR

Expressions of cell division cycle genes were analyzed by quantitative-PCR. All the experiments were done in triplicates. The primers used for q-PCR are enlisted in Table I. 0.4 µL cDNA was added in 9.6 µL SYBR Green master mix and 10µl primers to create a total of 20 µL volume. The denaturation, annealing and extension steps were repeated for 40 cycles. CT values were acquired and relative fold change was calculated by using the following formula. GAPDH was used as housekeeping gene.

$\Delta Ct = Ct (\text{Gene of interest}) - Ct (\text{Housekeeping gene})$

$\Delta\Delta Ct = \Delta Ct (\text{Sample}) - \Delta Ct (\text{Control})$

$\text{Fold Change} = 2^{(-\Delta\Delta Ct)}$

Table I. List of primers of cell cycle genes.

Marker	Primer sequences 5' → 3'	Product size	Annealing temperature
CDCA2	F: TTGGATGAGGAGTGGTTGGGTGT R: TTGGTTGTGGCTGAGAATGCCTT	181	56°C
CDCA8	F: GCTCGTTCACCAAGGTTTCAGCTTA R: TCTCGATCAGTCCTCCACACCTA	188	57°C
CDCA20	F: TCCATTGTGACCTTCAGCCT GCTA R: CTTGGATGCCTGGAGAGTCAATCA	180	57°C
CCNA2	F: GGGGTCAGCTCGTTACTCAAATTTA R: CACTTCTGGCCACACTTCATACGCA	186	58°C

Statistical analysis

SPSS version 20 was used for investigating the data. All numerical morals were obtained as mean± S.E of mean. The mean and SEM of the treatment group was generated by independent sample t test. P-value < 0.05 was taken as significant.

RESULTS

Characteristics of WJMSCs

The isolated cells had shown positive expression of the stem cell marker (*CD90*) and the internal control (*Actin*) confirming the isolation of WJMSCs from the Wharton's jelly of the cord tissue (Fig. 1a, b).

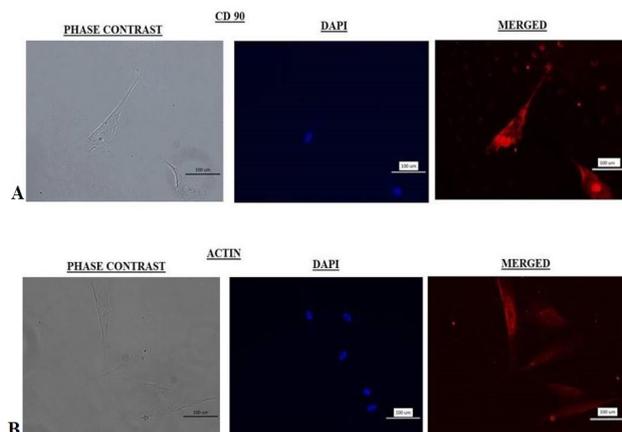


Fig. 1. Immunocytochemistry of WJMSCs CD90 protein (A) ACTIN (B) and protein showed positive expression for the WJMSCs in cord tissue.

PDT

For PDT WJMSCs (control) and GDM-WJMSCs were allowed to grow in the 6 well plate at different passages and the time taken by the cells to reach the confluency was calculated. It was observed that the GDM WJMSCs showed significant decreased proliferation rate with increased PDT especially at P0, P1 stages as compared to WJMSCs (control). We also found that the PDT decreased at later passages i.e., P2, P3 in both groups (Tables II, III).

Expression of cell cycle genes

To determine the effect of GDM on cell proliferation at gene level, the expression of cell cycle genes was analyzed by quantitative PCR and compared with control. GDMWJMSCS showed decreased expression of *CDCA2*, *CDC48*, *CDC420* and *CCNA2* ($p < 0.001$) as compared to the control cells (Fig. 2).

Table II. Effect of GDM on population doubling time (PDT) of WJMSCs. GDM WJMSCs showed a significantly significant decreased proliferation rate with increased population doubling time. We also find out with increased passing stage (P2, P3) within both groups, PDT was decreased and an improvement in the proliferation rate was seen for both groups implicating betterment for GDM WJMSCs group as well.

Passage stage	Control Mean±S.D	GDM WJMSCs Mean±S.D	P value
P0	39.26±0.3	40.12±0.6	0.009
P1	33.7±0.64	35.4±0.40	0.018
P2	22.4±0.30	26.1±0.20	0.0001
P3	15.66±0.35	16.83±0.15	0.015

Independent sample t test was used. Significant at <0.05.

Table III. Multiple comparison of PDT in GDM group.

Stages	GDM MEAN±S.D	Oneway ANOVA P-value	Multiple comparison
P0	40.1±0.6		P0-P1 P0-P2 (significant) P0-P3
P1	35.4±0.40		P1-P0 P1-P2 (significant) P1-P3
P2	26.1±0.20	<0.0001*	P2-P0 P2-P1 (significant) P2-P3
P3	16.8±0.15		P3-P0 P3-P1 (significant) P3-P2

*, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; *, significant; **, highly significant; ***, very highly significant.

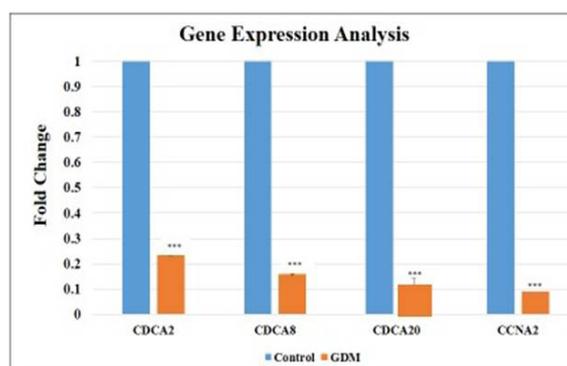


Fig. 2. Relative genes expression of cell division cycle genes (*CDCA2*, *A8*, *A20*, *CCNA2*), in control and GDM WJMSCs. There was a significant change in gene expression of two groups. As compared to control, GDM WJMSCs showed highly significant down regulation of cell cycle genes, which confirms the decreased proliferation rate in GDM WJMSCs.

DISCUSSION

GDM, a communal metabolic condition occurs due to intolerance of glucose during second trimester of gestation (Kanafi *et al.*, 2013). This condition is mostly pronounced due to the abnormalities in the metabolism of carbohydrate in pregnancy period which leads to glucose intolerance. However, majority of these women are reported to have normal carbohydrate tolerance postpartum (Imam, 2012). The main problem is high glucose level in maternal blood which passes through the placenta into the fetal blood. GDM may result in type 2 diabetes in 50% of women who had GDM in later life. It's well known that diabetes is characterized as causing metabolic syndrome and the root cause of many adverse medical conditions. Studies show that Asian women have a greater tendency this glucose intolerance condition than those of other continents. Many adverse effects of GDM have been reported that affects the mother and fetus both, early diagnosis and suitable treatment is imperative for better outcomes of GDM affected pregnancies (Vrachnis *et al.*, 2012).

Different literature reports decreased proliferation rate in stem cells affected by hyperglycemic state during pregnancy. Kim *et al.* (2014), and Baines *et al.* (2014) showed a substantial alteration in the propagation of UCMSCs isolated from GDM patients compared to control. Their study revealed that GDM affected stem cells did not proliferate till passage 6 or 7 compared to N-UCMSC in cultured environment. Another study reported that hyperglycemia during pregnancy leads to reduction in the proliferation rate and increased doubling time of stem cells to divide and proliferate (Leboyer *et al.*, 2016).

In 2012 a study reported that because of increased level of LDH due to diabetes, proliferation rate was reduced in diabetes affected WJMSCs. This condition mostly found in those females stem cells who were prone to GDM at high levels and their glucose level was controlled by insulin (Kolluru *et al.*, 2012), which may be further exert their own effect to increase the LDH levels and cause decrease cell proliferation.

Another study stated that due to hyperglycemic condition, increased activity of senescence associated beta-galactosidase was found and also an augmented manifestation of cell division cycle inhibitors (p16, p21 and p27 expression), in GDM WJMSCs compared to NWJMSCs signifying their effects on premature cellular senescence.

An *et al.* (2017) compared the proliferation rate between GDM affected MSCs and normal MSCs. Their findings suggested that GDM leads to adverse effects on cell harvesting and proliferation rate of MSCs during

early passaging stages. However, they also found that the decelerated proliferative ability of GDM-MSCs during early passages was improved at later passaging stages because of low glucose conditions (An *et al.*, 2017).

In this study, we assessed the proliferation rate of WJMSCs isolated from the normal and GDM patients and found out decreased proliferation rate in GDM WJMSCs as compared to control cells at the early passage stages (P0, P1). Another very important finding we noted was that, at later passages (P2, P3), the population doubling time was decreased in both groups of WJMSCs, and especially in GDM affected WJMSCs (Fig. 2). This positive finding favors our argument that if favorable environment is provided to the GDM WJMSCs, they have the capability to grow and will improve and increase their proliferation rate as well. This brings us to our proposal that we can treat these cells to make them efficient, for different clinical purposes.

We also performed the gene expression analysis of cell cycle genes, which regulate the different stages of cell cycle. Samar *et al.* (2015) discussed the cause of decreased cells proliferation in human umbilical cord derived endothelial cells. They found them mechanism of reduced proliferation, which would be due to alterations in the level of VEGF expression and secretion since proliferation of endothelial cells is mainly controlled by VEGF. They also described the role of gestational diabetes in endothelial dysfunction. From his microarray data that the different cell cycles genes participate at different steps of the cell division cycle, altered due to GDM (Samar *et al.*, 2015).

In this study it was observed that the expression of the cell cycle genes was significantly ($p < 0.001$) decreased in GDMWJMSCs as compared to the control cells (Fig. 3). We conclude that GDM plays an important role in the cell cycle delay which is evident from the expression of these cell cycle genes, leading to increase population double time hence slow proliferation of the cells. However, as the cells were cultured in the controlled conditioned the proliferation rate was found to be improved.

Several preconditioning approaches have been used to improve stem cell functions by treatment with various growth factors, hypoxic shock, and anti-aging compounds (Penno *et al.*, 2011; Petersen *et al.*, 2011). Furthermore, studies on WJSMCs would help to shed light on the molecular mechanisms and processes underlying the developing route to metabolic conditions like diabetes and obesity.

CONCLUSION

This study concluded that the actual cause of decreased proliferation rate in GDM-WJMSCs is the down regulation of cell cycle genes. The GDM affected cells had a high

population doubling time in the early passages but when the cells were grown *in vitro* in controlled environment the cells proliferate efficiently. This shows that in the presence of favorable environment, GDM WJMSCs will propagate and maintain their integrity. Suggesting that the GDM affected WJMSCs can also be utilized for the regenerative purpose after improving the proliferation rate of the cells.

These findings may prove to be beneficial in the utilization of these cells for different clinical applications and in stem cells banking. Further studies are required to evaluate the complete molecular mechanism of functional alterations in GDM WJMSCs and improve their clinical and therapeutic approaches.

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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