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ORIGINAL ARTICLE

PHENOTYPIC CHARACTERISTICS AND DIFFERENTIATION POTENTIAL OF GINGIVAL MESENCHYMAL STEM CELLS IN HYPERGLYCEMIA — AN *EX VIVO* EXPLORATORY STUDY

Características fenotípicas y potencial de diferenciación de las células madre mesenquimales gingivales en la hiperglucemia: un estudio exploratorio *ex vivo*

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ABSTRACT

Background: The therapeutic use of gingival mesenchymal stem cells (GMSCs) as autologous cells may pose the challenge of alterations inflicted by the hyperglycemic environment.

Objective: This study aims to assess the effects of hyperglycemia on the characteristics of GMSCs in diabetics.

Materials and Methods: 10 patients who consented and fulfilled the criteria for inclusion and exclusion were recruited and categorized as test (HbA1c > 6.5) and control (HbA1c < 6.0). Gingival explants were obtained from gingival collar of teeth, washed, digested and cultured. The cells were subjected to microscopic observation to assess phenotype characteristics, and flow cytometry and qRT-PCR to assess differentiation potential. Stem cell markers CD90, CD73, CD105, CD34, CD45, HLA DR & HLA ABC, osteogenic differentiation markers RUNX2 & OCN, adipogenic differentiation markers PPARG2 & FABP4 and chondrogenic differentiation markers SOX9 & AGCN were evaluated.

Results: Microscopic appearance of spindle shaped cells was found to be comparable in both groups. Flow cytometry results demonstrated comparable expressions with both groups, samples being positive for CD90, CD73, CD105, HLA ABC and negative for CD34, CD45 & HLA DR. There were variations in the expression of markers when assessed for differentiation potentials.

Conclusions: The hyperglycemic environment did not manifest any changes in the phenotypic characteristics of GMSCs among diabetics. However, the expression of certain differentiation markers was significantly altered in the diabetic test population included. Further research is being conducted to understand the GMSCs in a hyperglycemic environment with an aim to develop strategies to optimize its clinical implications.

Keywords: Gingiva; Mesenchymal stem cells; Diabetes mellitus; Cell Differentiation; Hyperglycemia; Flow cytometry.

RESUMEN

Antedededentes: El uso terapéutico de células madre mesenquimales gingivales (GMSC) como células autólogas puede plantear el desafío de las alteraciones infligidas por el entorno hiperglucémico.

Objetivo: Este estudio tiene como objetivo evaluar los efectos de la hiperglucemia sobre las características de las GMSC en diabéticos.

Materiales y Métodos: Se reclutaron y categorizaron 10 pacientes que dieron su consentimiento y cumplieron los criterios de inclusión y exclusión como prueba (HbA1c > 6,5) y control (HbA1c < 6,0). Los explantes gingivales se obtuvieron del cuello gingival de los dientes, se lavaron, digirieron y cultivaron. Las células se sometieron a observación microscópica para evaluar las características fenotípicas y a citometría de flujo y qRT-PCR para evaluar el potencial de diferenciación. Se evaluaron los marcadores de células madre CD90, CD73, CD105, CD34, CD45, HLA DR y HLA ABC, marcadores de diferenciación osteogénica RUNX2 y OCN, marcadores de diferenciación adipogénica PPARG2 y FABP4 y marcadores de diferenciación condrogénica SOX9 y AGCN.

Resultados: Se encontró que la apariencia microscópica de las células fusiformes era comparable en ambos grupos. Los resultados de la citometría de flujo demostraron expresiones comparables en ambos grupos, siendo las muestras positivas para CD90, CD73, CD105, HLA ABC y negativas para CD34, CD45 y HLA DR. Hubo variaciones en la expresión de los marcadores cuando se evaluaron los potenciales de diferenciación.

Conclusiones: El entorno hiperglucémico no manifestó ningún cambio en las características fenotípicas de las GMSC entre los diabéticos. Sin embargo, la expresión de ciertos marcadores de diferenciación se alteró significativamente en la población de prueba de diabetes incluida. Se están realizando más investigaciones para comprender las GMSC en un entorno hiperglucémico con el objetivo de desarrollar estrategias para optimizar sus implicaciones clínicas.

Palabras Clave: Encía; Células madre mesenquimatosas; Diabetes mellitus; Diferenciación celular; Hiperglucemia; Citometría de flujo.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that affects major organs like the kidneys, heart and eyes. Various treatment options such as diet control, use of oral hypoglycemic agents, and insulin have been incapable of achieving sustained glucose homeostasis. Whole pancreas or islet transplantation seemed effective but pose surgery-associated risks and complications of long term immunosuppression, and deficiency of organ donors.

The therapeutic potential of mesenchymal stem cells (MSCs) in medical conditions is being tested through various clinical trials.[1] In response to changes in microenvironment, trophic factors are released by MSCs to repair and regenerate injured tissues.[2]

In the recent years, MSCs have been tried in experiments and clinical trials, as a therapeutic approach for DM with the hope of correcting metabolic dysregulation and restoring immune homeostasis.[3] MSCs have shown to reverse hyperglycemia by adopting to an insulin secreting phenotype, homing and engrafting into the injured tissues, inducing endogenous progenitor cells to regenerate insulin producing β-cells.[2-8]

Self-replication and survival of pre-existing β-cells have been enhanced by MSCs.[6] Hyperglycemia causes withdrawal of cell cycle, reduced proliferation, alternate differentiation, cell phenotype changes, increased migration, lowered angiogenesis, lowered immunomodulation and senescence associated phenotypic changes in MSCs.[9]

Gingival mesenchymal stem cells (GMSCs) have been the most sought after MSCs for cellular therapy in the recent times for the following reasons.[10]

- They are readily accessible and demonstrate expandable nature.
- They can be procured with minimally invasive surgical procedures.
- They have demonstrated higher proliferation capacity than bone marrow derived MSCs and umbilical cord-derived MSCs.
- They have expressed a lesser mean population doubling time than of bone marrow derived MSCs. (39.6±3.2h; 80.4±1.2h respectively).
- They exhibit stable morphology and do not lose telomerase activity during long-term cultures.

GMSCs demonstrate potent differentiation and immunomodulatory/anti-inflammatory capacities and hence have been extensively investigated for regenerative therapy. During the innate immune response, GMSCs modulate the activation of immune cells, such as macrophages, mast cells, dendritic cells, and natural killer cells.[11]

They have shown promise in the management of skin, autoimmune, inflammatory, nerve and oro-maxillofacial disorders.[12-17] GMSCs have been tried in treating DM as well.

Zhang *et al.,*[11] reported that GMSCs could be promising in treating DM type 1. They noted sustained control of blood glucose and lag in onset of diabetes with diminished levels of IL-17, IFNý, CD4 and CD8 T cells in the spleen and pancreatic lymph node.[11] Junaid *et al.,*[13] demonstrated that gingival cells exposed to glucose mediated oxidative stress showed obliterated cellular proliferation and apoptotic gene expression mediated by TNF family in GMSCs.

The use of GMSCs as autologous cells may pose the challenge of alterations inflicted by the hyperglycemic environment. This study is the first of its kind to assess the effects of hyperglycemia on phenotype characteristics and differentiation potential of GMSCs from diabetics through *in vitro* experiments with the objective of utilizing GMSCs to treat DM.

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MATERIALS AND METHODS

Periodontally healthy patients aged between 18 - 55 years who were advised gingivectomy/gingivoplasty/crown lengthening/tooth extraction in the outpatient department of Periodontology of a tertiary Dental hospital in South India, were recruited into the study in accordance with the Helsinki Declaration of 1975 (as revised in 2013), after obtaining a written informed consent from them following approval from the Institutional Ethical Committee of JSS Dental College and Hospital (JSSDCH/PGS/ETHICAL/2015-16).

The test and controls were age and gender matched in pairs. Their HbA1c was evaluated and were grouped to test (HbA1c > 6.5) and control (HbA1c < 6.0). Smokers, lactating and pregnant women and individuals with systemic diseases other than DM were excluded from the study. 10 subjects assigned to 5 pairs were initially included in the study as per the inclusion and exclusion criteria. 3 subjects dropped out of the study. 1 sample got contaminated in the laboratory. Finally, 3 pairs of samples were available to be evaluated for the study.

Gingival explants were obtained from gingival collar of teeth advised for gingivectomy/gingivoplasty/crown lengthening/extraction procedures using No. 15 Bard Parker blade.

The excised gingival tissue was washed in PBS (Phosphate buffered solution, Gibco), later teased and digested overnight with 0.5 mg/mL collagenase Blend type H (Sigma-Aldrich) at 37°C in an incubator. The digested tissue was washed with PBS and plated in Knockout Dulbecco's Modified Eagle Medium (KO-DMEM, Gibco, 4.28 catalog No.10829-18) supplemented with 10% FBS (Australian FBS, hi Media, catalog No.RM9951), Glutamax (Gibco, catalog No.35050-061) and antibiotics namely, 100 U. mL^{-1} penicillin, 100 µg. mL^{-1} streptomycin and 1% amphotericin.
After 24 hours, the floating debris was removed and the adherent cells were allowed to grow till confluence.
They were passaged further with 0.25% trypsin-EDTA and plated in complete medium containing KO-DMEM, FBS 10%, Glutamax-1% and antibiotic - antimycotic -1%. The flasks were incubated at 37°C in 5% CO_2. The cells were allowed to grow for 3-4 days. They reached confluence; they were checked using phase contrast inverted microscopy. The basic medium was changed on a regular basis 3 times a week.
Flow cytometry was used to determine the immunophenotypic characteristics of GMSCs.
The primary antibodies used were:

- CD73 - catalog No.550257
- CD105 - catalog No.561443
- CD90 - catalog No.555596
- CD45 - catalog No.555482
- CD34 - catalog No.550761
- HLA D - catalog No.347363
- HLA ABC - catalog No.555552

from BD Pharmingen.

Fluorescein conjugated FACS buffer was added to centrifuged cells and analyzed with flow cytometer using cell quest pro-software.

Assessing the Trilineage differentiation potential of GMSCs

Trilineage differentiation potential of cells was assessed by expression of differentiation markers after inducing the cells for differentiation.

1. Osteogenic differentiation.

GMSCs were plated with a density of 50,000 cells/well in 24-well culture plates and culture in a-MEM medium. On reaching confluence, the cells were transferred to a culture media consisting of a-MEM, 10% FBS, 1% penicillin, streptomycin, and β-glycerophosphate (10 mM), dexamethasone (0.1 mM, Sigma, catalog No.D4902) and ascorbic

acid (50 mg/mL, Sigma, catalog No.A4403) and cultured again. Care was taken to renew the media once in 2 days.

2. Adipogenic differentiation.

GMSCs were cultured on a 24-well culture plate with a density of 30,000 cells/well in a medium containing a-MEM. On reaching confluence, they were cultured. The medium contained a-MEM, antibiotics-1% penicillin, streptomycin, and dexamethasone (1 mM) along with 10% FBS, insulin (10 mg/mL), indomethacin (100 mM) and 3-isobutyl-1-methylxanthine (100 mg/mL, Sigma, catalog I 7018).

3. Chondrogenic differentiation.

GMSCs were cultured on 24-well culture plates with micro-masses and later incubated for 1 hour. They were plated with a density of 30,000 cells/well. Differentiation medium consisting of a-MEM, 1% penicillin, streptomycin, and dexamethasone (0.1 mM) and 10% FBS, insulin (10 mg/mL), transforming growth factor-b (10 ng/mL), ascorbic acid (50 mg/mL) along with bone morphogenetic protein-6 (0.025 mg/mL) and insulin-transferrin-selenium-A (Sigma, catalog No.1884) were added. Furthermore, ascorbic acid (5 µL) was added once a day.

Trilineage differentiation potential of cells was assessed by expression of differentiation markers after inducing the cells for differentiation. GMSCs were inducted to osteogenic, adipogenic and chondrogenic differentiation by culturing in the respective media and assessed for expression of osteogenic genes (RUNX2, OCN), adipogenic genes (PPARG2, FABP4) and chondrogenic genes (SOX9, AGCN).

RNA was isolated using TRIzol™ (Invitrogen) based phase separation method following im-proved single step RNA isolation using mono-phasic solution. Quantity and purity of RNA was assessed by UV-Spectrophotometry following the Nanodrop-direct RNA quantification method based on Beer's-

Figure 1. Cultured primary cells derived from human gingival tissue exhibited typical fibroblast-like morphology in both (a) test and (b) control samples of the 3 pairs. (Images at 10x magnification, Nikon Eclipse TE 2000S Inverted Microscope used.)

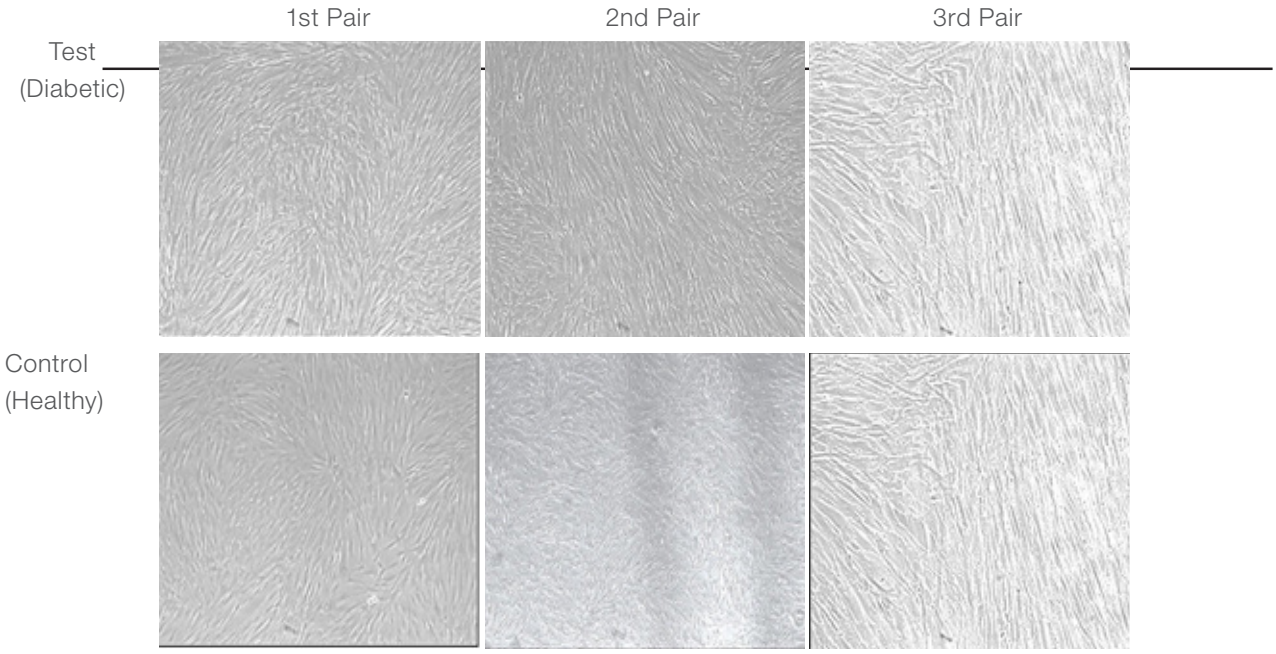

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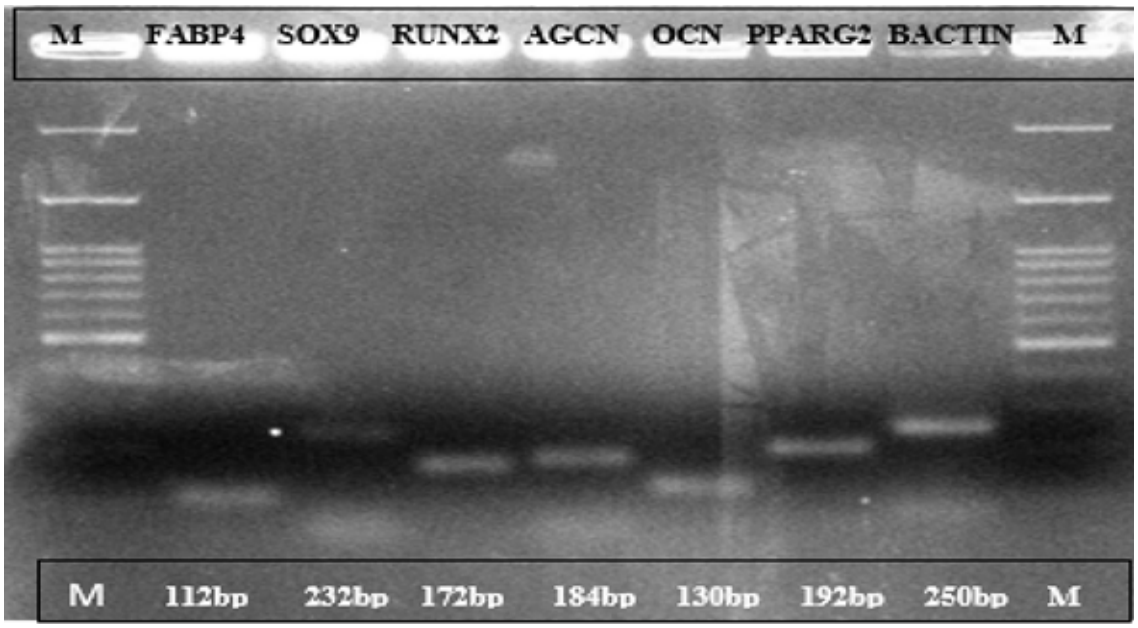
Figure 2. PCR products of all genes- 2.5% agarose gel representative image.



Lambert's law of absorbance using monochromatic source of light. Bleach gel electrophoresis was done using Et-Br (ethidium – bromide) dye based denaturing bleach gel electrophoresis method. cDNA was synthesized by following the Verso cDNA 1st strand synthesis by reverse transcription method.

Real time quantitative PCR (RT-qPCR) with relative quantification was carried out following three steps SYBR green fluorescence dye-based qPCR analysis method. Primer sequences are displayed in Table 1.

Target genes with primer sequence and product size were used under controlled qPCR reaction conditions. Separate qPCR- master mixes were prepared for each set of genes. A single batch of qPCR analysis included 2 subsets which included gene of interest (GOI) and reference (house-keeping gene) beta actin gene.

Gradient PCR was done to establish optimum annealing conditions for each GOI using master cycler gradient-PCR. The method was employed for relative gene quantification normalized with the house-keeping gene. The real time was done with the Rotor-Gene Q-5PLEX HRM-QIAgen machine.

Data analysis

1. Statistical analysis of percentage expression of surface markers of GMSCs by Flow cytometer.

Data were expressed as mean ± standard deviation and analyzed by a two-tailed nonparametric Mann-Whitney U test using SPSS 23 software with *p*-value significance set at <0.05.

2. RT-qPCR Data Analysis.

RT-qPCR data acquisition and analysis was done using relative quantification using the 'delta delta Ct' (ΔΔCt) method. The expressions of target genes were normalized to the reference gene and relative to the calibrator which was finally expressed as fold change (FC). The FC was calculated by the formula:

$$\text{Fold100 change} = 2^{-\Delta\Delta Ct}$$

Expression of each targeted gene involved in different stages of differentiation of GMSCs was compared among healthy and diabetic individuals based on FC. Upregulation or downregulation

of GOI was determined in terms of FC and interpreted to study variations among samples from different groups.

RESULTS

The microscopic appearance of the cultured gingival samples obtained from the test and control, which were gender and age matched, from the 3rd-5th passage were comparable showing small round and spindle shaped cells after 48 hours. Plastic adherent spindle shaped cells were visible by Day 5 and 80% confluent plates were visible from Day 12. Homogenous spindle shaped cells were seen from the 1st passage, (Figure 1).

Both the test and control displayed positive expression of CD73, CD90, CD105, HLA ABC with a mean percentage of expression of 94.95 & 95.93; 95.90 & 88.53; 73.97 & 73.89; 98.83 & 98.16 respectively and negative or weakly positive plastic adherent expression of CD34, CD45 and HLA DR with a mean percentage of expression of 0.03 & 0.010; 0.57 & 1.72; 0.65 & 2.58 respectively.

There was no statistically significant variation in the percentage of expression of markers between the test and control when assessed by Mann-Whitney U test with *p*-value significance set at <0.05, (Table 2). However, there were variations in the expression of CD105 showing comparatively higher expre-ssion in diabetic GMSCs (96.00%, 77.37%) than the healthy GMSCs in the first pair of samples but lower (49.88%, 54.85%) in second and third pair (76.02%, 89.42%). Variation in percentage of expression was also noticed with CD90 showing higher percentage in diabetic GMSCs in comparison to healthy GMSCs (70.19%) though not statistically significant, (Table 3).

RT-qPCR analysis demonstrated trilineage potential of GMSCs from both the diabetic and healthy groups with the expression of its respective markers, (Figure 2). However, few variations in the expression of differentiation markers were noticed between and within the diabetic and healthy groups, (Table 4). With respect to osteogenic differentiation, RUNX2 showed a 2.5-fold upregulation in expression in the

Table 1. Primer sequences used in the RT-qPCR experiment.

No.	Gene Targets	Codes	Primer Sequence (5´•3´)	Product Size (bp)
1	Runt-Related Transcription Factor-2	RUNX2	GCCCGTGGCCTTCAAGGTGG TCGTCCACTCCGGCCCACAA	172
2	Osteocalcin	OCN	ATGAGAGCCCSTCACACTCCTC CGTAGAAGCGCCGATAGGC	130
3	Peroxisome Proliferator Activated Receptor y-2	PPARG2	CCATGCTGTTATGGGTGAAA TCAAAGGAGTGGGAGTGGTC	192
4	Fatty Acid Binding Protein-4	FABP4	ACCTTAGATGGGGGTGTCCTGGT CGCCTTTCATGACGCATTCCACC	112
5	SRY-Box Transcription Factor-9	SOX9	CGGACACCGAGAACACGCGG GCCTGCGCCCACACCATGAA	232
6	Aggrecan	AGCN	CACTTCTGAGTTCGTGGAGG ACTGGACTCAAAAAGCTGGG	100
7	Beta Actin (House-Keeping Gene)	BACTIN	CATGTACGTTGCTATCCAGGC CTCCTTAAAGTCACGCACGAT	250

first diabetic sample with no difference in expression in the other diabetic samples when compared with healthy samples. Expression of osteocalcin was increased in the first diabetic sample while the other 2 samples showed a down modulation. The results are suggestive of the alteration induced by hyperglycemia on the differentiation potential of GMSCs in diabetics, (Table 5).

Both control and diabetic GMSCs when subjected to adipogenic differentiation show a reduced expression in PPARG and increased expression of FABP4. The expression of FABP4 was significantly higher in diabetic GMSCs derived from all the 3 patients compared to control ones, (Table 5).

With respect to the expression of chondrogenic differentiation markers, SOX9 showed a 4-fold upregulation in the second sample and a down-regulation in the third diabetic sample compared to the sample from the healthy group. Aggrecan showed a 4-fold upregulation in the first pair and downregulation in the third compared to the control, (Table 5).

Table 2. Flow cytometry analysis of surface markers expressed by test and control.

Yes No	Surface Markers	Test Percentage Mean	Test SD	Control Percentage Mean	Control Standard Deviation	*p*-value Mann-Whitney U test
1	CD73	94.9467	5.13574	95.9267	3.81513	0.827
2	CD90	98.0467	2.55142	88.5267	15.98227	0.513
3	CD105	73.9667	23.12846	73.8800	17.54726	0.827
4	CD34	0.0267	0.03055	0.1000	0.15620	0.822
5	CD45	0.5733	0.74675	1.7200	1.97228	0.513
6	HLA DR	0.6467	0.89030	2.5767	3.25371	0.275
7	HLA ABC	98.8300	0.85750	98.1567	2.39953	0.827

Table 3. Percentage expression of stem cell markers CD73, CD90, CD105, HLA ABC, CD34, CD45, HLA DR of GMSCs from test and control groups of the 1st paired sample, 2nd paired sample and 3rd paired sample.

	GROUP	CD73	CD90	CD105	HLA ABC	CD34	CD45	HLA DR
1st paired sample	Test	89.63	98.98	96.00	99.52	0.02	1.43	1.67
	Control	92.13	99.50	77.37	99.41	0.02	3.94	6.30
2nd paired sample	Test	95.33	95.16	49.88	97.87	0.00	0.06	0.05
	Control	95.89	95.87	54.85	95.39	0.00	0.17	0.28
3rd paired sample	Test	99.88	100	76.02	99.10	0.06	0.23	0.22
	Control	99.76	70.19	89.42	99.67	0.28	1.05	1.15

Table 4. Ct values of gene of interest (RUNX2, OCN, PPARG2, FABP4, SOX9, AGCN) from the isolated gingival stem cells from both the Control and Test groups and house-keeping gene (hkg), beta actin from RT- PCR.

CT VALUES OF OSTEOGENIC, CHONDROGENIC AND ADIPOGENIC GENES FROM RT PCR

	TEST		CONTROL		TEST		CONTROL	
OSTEOGENIC MARKERS								
	Run x 2 Differentiated		Run x 2 Non Differentiated		Run x 2 Differentiated		Run x 2 Non Differentiated	
	AVG	S.D	AVG	S.D	AVG	S.D	AVG	S.D
1	28.27	0.28	27.40	0.32	28.61	0.81	27.44	0.19
2	30.62	0.41	32.03	0.34	31.50	0.04	30.24	0.28
3	30.8	0.14	28.70	0.13	28.96	0.55	28.29	0.09
	OCN Differentiated		OCN Non Differentiated		OCN Differentiated		OCN Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	25.27	0.09	33.88	0.71	33.39	0.81	33.27	0.27
2	36.02667	0.34	36.96	0.30	34.78	0.37	33.84	0.57
3	33.56	0.30	32.27	0.17	34.95	0.34	34.8	1.42
	B Actin Hkg Differentiated		B Actin Hkg Non Differentiated		B Actin Hkg Differentiated		B Actin Hkg Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	17.67	0.14	17.07	0.16	17.64	0.07	18.55	0.36
2	17.81	0.10	18.98	0.18	19.85	0.29	18.1	0.25
3	18.13	0.07	18.24	0.08	25.48	0.19	25.47	0.25
ADIPOGENIC MARKERS								
	Pparg2 Differentiated		Pparg2 Non Differentiated		Pparg2 Differentiated		Pparg2 Non Differentiated	
	AVG	S.D	AVG	S.D	AVG	S.D	AVG	S.D
1	39.07	0.39	38.87	0.25	39.04	0.18	37.99	0.53
2	39.10	0.32	38.86	0.42	38.97	0.66	37.96	0.44
3	36.98	0.83	38.80	0.39	38.93	0.58	38.36	0.36
	FABP4 Differentiated		FABP4 Non Differentiated		FABP4 Differenrentiated		FABP4 Non Differenrentiated	
	AVG	S.D	AVG	S.D	AVG	S.D	AVG	S.D
1	29.43	0.48	33.84	0.36	35.76	0.48	36.08	0.82
2	30.19	1.13	34.77	0.37	31.2	0.30	34.15	0.56
3	29.32	0.04	36.73	0.59	32.15	0.71	34.10	0.63
	B actin Hkg Differentiated		B actin Hkg Non Differentiated		B actin Hkg Differentiated		B actin Hkg Non Differentiated	
	AVG	S.D	AVG	S.D	AVG	S.D	AVG	S.D
1	24.01	0.12	24.37	0.66	24.75	0.97	24.59	0.50
2	22.36	0.64	26.40	0.53	22.93	0.23	23.92	0.42
3	22.21	0.59	26.51	0.35	23.09	0.71	24.29	0.50
	B actin Hkg Differenrentiated		B actin Hkg Non Differentiated		B actin Hkg Differenrentiated		B actin Hkg Non differenrentiated	
	AVG	S.D	AVG	S.D	AVG	S.D	AVG	S.D
1	23.86	0.19	24.44	0.49	25.75	0.39	24.27	0.08
2	22.69	0.72	24.16	0.83	23.83	0.49	24.26	0.37
3	22.13	0.63	26.54	0.32	22.9	0.50	24.25	0.49
CHONDROGENIC MARKERS								
	Sox 9 Differentiated		Sox 9 Non Differentiated		Sox 9 Differentiated		Sox 9 Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	31.36	0.16	30.8	0.13	30.80	0.13	30.25	0.46
2	31.62	0.17	32.47	0.40	32.47	0.40	30.81	0.64
3	31.15	0.07	29.22	0.33	29.22	0.33	29.46	0.92
	AGCN Differentiated		AGCN Non Differentiated		AGCN Differentiated		AGCN Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	23.93	0.78	32.92	0.19	32.91	0.19	30.66	0.27
2	35.16	0.03	38.25	0.28	38.25	0.28	31.26	0.62
3	34.16	0.61	32.88	0.72	32.88	0.72	30.58	0.28
	B Actin Hkg Differentiated		B Actin Hkg Non Differentiated		B Actin Hkg Differentiated		B Actin Hkg Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	22.69	0.34	20.77	0.41	22.97	0.47	22.51	0.13
2	17.72	0.16	20.16	0.35	17.87	0.19	18.15	0.11
3	18.19	0.23	17.74	0.40	18.65	0.44	18.18	0.15
	B Actin Hkg Differentiated		B Actin Hkg Non Differentiated		B Actin Hkg Differentiated		B Actin Hkg Non Differentiated	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1	19.49	0.20	17.59	0.34	19.35	0.03	18.79	0.23
2	21.09	0.07	26.30	0.56	18.81	0.25	19.14	0.38
3	20.29	0.03	18.39	0.16	20.55	0.10	18.99	0.26

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Table 5. Fold change values of genes/markers (RUNX2, OCN, PPARG2, FABP4, SOX9 and AGCN) for GMSCs isolated from Test and Control groups.

DIFFERENTIATION	MARKERS/ GENES	PAIRS	TEST	CONTROL
Osteogenic	RUNX2	1st	2.177905	0.648591
		2nd	0.524969	0.344245
		3rd	0.323721	0.877238
	OCN	1st	2.319315	0.534819
		2nd	0.810288	1.837265
		3rd	0.389101	1.082784
Adipogenic	PPARG2	1st	0.731396	0.550923
		2nd	0.052031	0.251033
		3rd	0.183373	0.468105
	FABP4	1st	15.08924	4.069964
		2nd	6.63799	5.854481
		3rd	8.085149	1.658995
Chondrogenic	SOX9	1st	1.462343	0.648481
		2nd	4.347108	1.182724
		3rd	5.552757	3.61768
	AGCN	1st	9.673671	3.250515
		2nd	0.235768	0.527265
		3rd	1.642243	3.055837

DISCUSSION

With the forethought of harnessing the potential of GMSCs for cell therapy in diabetics, the characteristics of GMSCs in diabetics was evaluated to assess for any alterations that may be induced by hyperglycemia in diabetic milieu. The phenotypic and trilineage differentiation potential of GMSCs from diabetic and healthy subjects have shown comparable results in this study. The results have demonstrated that GMSCs isolated and cultured from diabetics abide by the criteria put forth by International Society for Cell and Gene Therapy (ISCT).[18]

The microscopic appearance of homogeneous spindle shaped fibroblast like cells seen by the 2nd-3rd passages in our study are in accordance with the studies of Zhang *et al.,*[19] and Mitrano *et al.,*[20] who reported a homogeneous spindle shaped appearance of GMSCs. Studies with bone marrow derived MSCs have confirmed comparable surface phenotype, MSC expansion and self-renewability between diabetic and healthy samples.[21] However, Junaid *et al.,*[13] has reported obliterated proliferation and apoptotic gene expression of GMSCs on exposure to glucose mediated oxidative stress.

GMSCs isolated from diabetic and healthy individuals have shown positive expression of CD90, CD73, CD105, and HLA ABC and negative expression of hematopoietic markers CD34, CD45, and HLA DR, which is in accordance with studies highlighting the stem cell marker profile of GMSCs.[20] Apart from a few statistically

non-significant variations in the percentage of expression of stem cell markers (CD73, CD90, CD105, CD34, CD45, HLA ABC, HLA DR), flow cytometry analysis revealed a comparable stem cell marker profile of both diabetic and healthy.

Comparable stem cell marker profile of diabetic and healthy bone marrow derived MSCs has been reported by Phadnis *et al.,*[21] Dermal cells and bone marrow derived MSCs have also shown comparable immunophenotypic characteristics with their diabetic counterparts.[22] These were not affected by number of passages or time in culture or minor differences between donors.[23]

Tan *et al.,*[24] demonstrated that isolated murine pericardial adipose tissue with high CD73 displayed anti-inflammatory activity by upregulating anti-inflammatory genes in comparison to populations with low CD73. The high expression of CD73 in diabetic GMSCs in this study suggests their reparative and anti-inflammatory functions in the hyperglycemic environment. CD105+ cells have shown trilineage differentiation potential.[25] Our results showing positive expression of CD105 in both groups suggest GMSCs trilineage differentiation potential.

Although expression of HLA ABC did not vary in diabetic GMSCs, there was a reduction in the expression of HLA DR. It could be speculated that hyperglycemia altered the expression of HLA DR on diabetic GMSCs which could alter their ability to suppress the persistent inflammation observed in periodontitis. The difference in expression was explained to be the response to certain inflammatory cytokines.[26]

Overall, the flow cytometry analysis in our study presents a comparable stem cell marker profile of GMSCs between the two groups barring the variations in percentage of expression of few markers which are statistically non-significant.

The enhanced osteogenic potential of diabetic GMSCs in our study as evaluated by RUNX2 and ONC is in accordance with reports by Li *et al.,*[27] of stimulated proliferation and osteogenic differentiation of bone marrow MSCs with high glucose concentrations *in vitro.* The increased expression of osteogenic markers in diabetic group in our study may be indicative of the altered effect of hyperglycemia on GMSCs which are basically not osteogenic.[28]

The increased expression of FABP4 in diabetic GMSCs may be indicative of the proinflammatory microenvironment induced by hyperglycemia. Our results are suggestive of the alteration in adipogenesis induced by hyperglycemic state in diabetic GMSCs.[15,29-31]
We could speculate the increased SOX9 expression in our study in diabetic sample could be due to the increased upregulation of TGF-β signaling in hyperglycemic environment leading to enhanced chondrogenesis in GMSCs.[32] Persistent hyperglycemia has shown to reduce the osteogenic and chondrogenic differentiation potential of adipose stem cells while enhancing its adipogenic potential.[29]

These effects of hyperglycemia may also be suggestive of the role of other additional factors like age of the patient, the chronicity and the severity of the glucose impairment.

Challenges and Limitations

The processes followed during research were technique sensitive demanding high standards of disinfection. Diligent mana-gement of working time with minimal traffic in the laboratory helped to overcome contamination hurdles.

The expensive reagents posed a challenge to financial management. The smaller sample size limited the profoundness of the results of the study. Though the stem cell markers used

suffices for ISCT criteria, newer marker profiles could be added to enhance the identification of additional potential characteristics of these cells.

CONCLUSION

The hyperglycemic environment in the diabetics did not manifest any changes in the phenotypic characteristics of GMSCs that abided to the criteria put forth by ISCT. Although, alterations in expression of differentiation markers were observed. The results do support the potential of utilizing GMSCs from diabetics in stem cell Therapy. However further research into additional factors with a larger sample need to be validated to mandate use of GMSCs clinically among diabetics.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

Suman Basavaraju: Research concept and design, Collection and/or assembly of data, Data analysis and interpretation, Writing the article, Critical revision of the article, Final approval of the article.

MR Dhakshaini: Research concept and design, Collection and/or assembly of data, Data analysis and interpretation, Final approval of the article.

Anshukumar Yadav: Research concept and design, Collection and/or assembly of data, Data analysis and interpretation, Final approval of the article.

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