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Basic Science



Preclinical Assessment of the Proliferation Capacity of Gingival and Periodontal Ligament Stem Cells from Diabetic Patients

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Abstract

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BACKGROUND: Stem cells have recently received great interest as potential therapeutics alternative for a variety of diseases. The oral and maxillofacial region, in particular, encompasses a variety of distinctive mesenchymal (MSC) populations and is characterized by a potent multilineage differentiation capacity.

AIM: In this report, we aimed to investigate the effect of diabetes on the proliferation potential of stem cells isolated from controlled diabetic patients (type 2) and healthy individuals.

SUBJECTS & METHODS: The proliferation rate of gingival and periodontal derived stem cells isolated from diabetic & healthy individuals were compared using MTT Assay. Expression levels of Survivin in isolated stem cells from all groups were measured by qRt - PCR.

RESULTS: There was a significantly positive correlation between proliferation rate and expression of Survivin in all groups which sheds light on the importance of Survivin as a reliable indicator of proliferation. The expression of Survivin further confirmed the proliferation results from MTT Assay where the expression of stem cells from non-diabetic individuals was higher than diabetic patients. Conclusion: Taking together all the results, it could be concluded that PDLSC and GSC are promising candidates for autologous regenerative therapy due to their ease of accessibility in addition to their high proliferative rates.

Introduction

The capacity of mesenchymal stem cells to self-renew and to differentiate into multiple cells strongly suggests their role as a promising candidate for future cell-based therapies [1][2][3]. The oral and maxillofacial region encompasses a variety of distinctive mesenchymal (MSC) populations, isolated from the tooth and its supporting structures, and is characterized by a potent multilineage differentiation capacity and immunomodulatory properties [1][2][3][4][5]. Of those are the gingival mesenchymal stem cells (GMSCs) which offer a more appealing alternative to other dental originated MSCs regarding their ease of extraction and isolation from the clinically respected gingival tissues with minimal harm to the donor. GMSCs are considered an excellent source of MSCs for cell-based regenerative therapies with maximum stability for the longest period in addition to its uniformly homogenous property and stable phenotype and telomerase activity during prolonged

culture time [3][6][7][8]. Periodontal ligament stem cells (PDLSCs) also represent typical properties of mesenchymal stem cells (MSCs) regarding self-renewal and expression of mesenchymal stem cells surface markers. PDLSCs also possess multilineage differentiation potential into various types of cells such as osteoblasts, adipocytes, chondrocytes and neurocytes in - vitro. Additionally, it is suggested that PDLSCs might belong to a unique population of somatic stem cells due to its distinctive potential to form cementum and PDL-like tissues in - vivo. 9 It is believed that PDLSCs are a suitable candidate cellular source for PDL regeneration as they play an endogenous role in maintaining PDL cell numbers due to their periodontal ligament derivation and their vast differentiation capacity [10]. In the current study, GMSCs and PDLSCs were recruited since both are currently considered ideal cell source for tissue engineering and repair [11]. Previous studies confirmed that GMSCs transplanted into the periodontal defects had been demonstrated to contribute to the periodontal repair and regeneration [12]. Novel studies had demonstrated the great

potential of bone healing when GMSCs were implemented into the mandibular defects in animal models [13]. In recent years, several research groups have attempted to use MSCs as a treatment option for diabetes and its complications [1][2][3][4][5][6][7][8]. However, autologous cell-based therapeutics may be unlikely to succeed if the true impact of diseases like diabetes on stem cells and progenitor cell population is not investigated thoroughly. It is thought that diabetes negatively affects the stem cell niches thus altering their dynamics and disrupting the repair and homeostasis. Predominantly, changes in reactive oxygen species (ROS) and hypoxia from neighbouring cells in case of hyperglycemia (high blood glucose level) is the reason for the alteration of the signalling to the stem cells [14][15]. Diabetes produces a surge in ROS which results in generating a prolonged inflammatory and oxidative environment that leads to the inhibition of stem cell proliferation which in turn causes stem cell senescence impairing innate tissue repair mechanisms and regeneration [16][17].

Diabetes also causes stem cell aberrations resulting in direct implications on tissue function that seem to persist even after return to normoglycemia [18]. These impairments might include changes in migration, recruitment, survival, self - renewal and differentiation capacity. Diabetes-induced in mice was found to alter the intrinsic properties of stem cells and impair their function and their regenerative ability [19][20]. Stem cells investigated in these reports were isolated from tissues other than dental tissues like adipose and bone marrow and has provided accumulating evidence that implies differences in proliferation and differentiation abilities between different sources of stem cells [18][20][21][22][23]. However, most of these reports have either investigated stem cells from healthy individuals without comparing the results to diabetic individuals or has used diabetic induced models.

In this report, we investigate the effect of diabetes on the proliferation potential of stem cells isolated from controlled diabetic patients (type II) and healthy individuals.

Subjects and Methods

Periodontal ligament stem cells were isolated from (PDLSCs) of healthy subjects and controlled diabetic patients (DM type II). Additionally, gingival mesenchymal stem cells (GMSCs) were isolated from the gingiva of healthy subjects and controlled diabetic patients (DM type II). Procedures were performed at the National Research Centre, Cairo, Egypt according to the recommendations and approval of its ethics committee. Informed consent was obtained from all subjects and patients before undergoing teeth extractions.

Sample Size Calculation

Based on previous studies, the expected average of cell viability was 26.2% with variance percentage 1.86% [24][25]. A total sample size of 116 (29 in each of the four groups) was calculated to be sufficient to detect a significant difference between the groups with a power of 80% and a significant level of 5%. The number was then increased to a total sample size of 144 (36 in each group) to allow for losses of around 25%. The sample size was calculated using nQuery Advisor.

Inclusion and Exclusion Criteria

The sample represents a population of Egyptian patients with type 2 diabetes of both sexes, ≥ 18 years of age. Participants represent a consecutive series of patients fulfilling the exclusion/inclusion criteria. Patients were recruited among those referred to the hospital of the faculty of dentistry and oral medicine, Cairo University for dental treatment. Inclusion Criteria included; clinical diagnosis of diabetes mellitus according to American Diabetes Association criteria (ADA) criteria [26], whose age ranged from 18 to 69 years, with duration of diabetes diagnosis over 12 months, and requiring oral antidiabetic drugs for optimal glycemic control in a dose of ≥ 0.7 U/kg/day at least for 1 year in addition to willingness to participate in the study.

Control diabetes was described as proven normal glucose tolerance according to ADA criteria where uncontrolled diabetes was deemed present in diabetics who had fasting blood glucose (FBG) level ≥ 7.0 mmol/L and controlled diabetes in diabetics who had fasting blood glucose (FBG) level ≤ 7.0 mmol/L [26][27]. At the time of entry into the study, all patients had a fasting blood glucose (FBG) level ≤ 7.0 mmol/L and HbA1c $\leq 7\%$. The exclusion criteria included the following: acute or chronic infections; any malignancies; haematological diseases; known immunosuppressive disease (for example, acquired immunodeficiency); acute or chronic pancreatitis; and a history of thoracic or abdominal aorta diseases. After recruitment, all follow - up visits were performed at the same hospital. All patients gave written informed consent.

Cell Isolation and Culture

Human third molars, which were removed for impact reason from both systemically healthy and diabetic patients, were used for tissue biopsy and PDL cell isolation. Meanwhile, gingival tissues surrounding the tooth sockets were collected immediately after tooth extraction for gingival cell isolation and subsequent investigations. Periodontal ligament stem cells (PDLSCs) were acquired from the periodontal ligament tissues by scraping the middle third of the root of extracted impacted third molars. Teeth

surfaces were rinsed with phosphate buffered saline (PBS) under aseptic conditions. The collected tissues were then digested with 3 mg/ml collagenase at 37°C for 15 min. The cell suspension was then transferred to dishes and cultured in RPMI culture media supplemented with 10% Fetal Bovine Serum (FBS) and streptomycin at 37°C in a humidified atmosphere with 5% CO₂. The culture medium was changed every three days.

In parallel, a small of biopsy of gingiva comprised of epithelium and connective tissues was harvested from the extracted third molar's cervical ridge, and the tissue was washed with PBS. The excised gingival tissues were and treated aseptically and incubated overnight at 4°C with 2 mg/ml Dispase to separate the epithelial and the spinous layers. The tissues were then minced into fragments and digested with 4 mg/ml collagenase at 37°C for two h. The dissociated cell suspension were filtered through a 70 µm cell strainer, plated on Petri dishes RPMI culture media supplemented with 10% Fetal Bovine Serum (FBS) and streptomycin at 37°C in a humidified atmosphere with CO₂. The culture medium was changed every three days. Cells from all groups were passaged at a ratio of 1:2 when reaching 70 - 80% confluence. The collected stem cells were grouped as follows; periodontal ligament stem cells from healthy individuals, periodontal ligament from controlled diabetic patients, gingival stem cells from healthy individuals and gingival stem cells from controlled diabetic patients.

Flow Cytometric Analysis

The identity of isolated stem cells was confirmed by analysing surface antigen expression. Isolated stem cells from all groups at passage three were detached by 0.05 % trypsin - EDTA, centrifuged for 5 min at 2000 rpm and resuspended in PBS containing 2 % FBS. Next, they were incubated with antibodies against CD45, CD90 and CD105 for 30 min at 4°C. Isotypes were used as negative control. After washing with PBS, the cells were resuspended in 500 µl PBS containing 2 % FBS and subjected to flow cytometric analyses using CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, FL, USA) and analysed using CXP Software version 2.2.

Assessment of Cell Proliferation by MTT Assay

The proliferation of isolated stem cells was evaluated using MTT assay. For MTT assay, the cells from passage three were seeded in 96 - well plates (2 x 10³ cells per well) and cultured for 72h in DMEM supplemented with antibiotic and 10% FBS. Then, MTT solution (at the final concentration of 0.05 %) was added to each well, and the cells were incubated under the atmosphere of 5 % CO₂ at 37°C. After 3h,

the supernatant was discarded, and the formazan precipitate was dissolved in dimethyl sulfoxide. The MTT Reagent and Detergent Solution were obtained from TACSTM TREVIGEN1 8405 Hegerman Ct. Gaithersburg, supplied ready for use. The optical density (O.D) values were measured at a range from 490 to 630 nm using an ELISA reader (Dynatech MRX 5000; Dynex, Chantilly, VA).

Real-time Quantitative PCR for Survivin Gene Expression

Total RNA of cells of all studied groups was isolated with RNAeasy Mini Kit (Qiagen) and further analysed for quantity and quality with Beckman dual spectrophotometer (USA). The mRNA expression level was quantified by qRT - PCR (Real-time PCR). 1000 mg of the total RNA from each sample was used for cDNA synthesis by reverse transcription using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48 - well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of beta actin housekeeping gene by the $\Delta\Delta Ct$ method. We used 1 mM of both primers specific for each target gene. Survivin primer sequence was 5' - ACCCACACTGTGCCCATCTAC - 3' and antisense 5' - TCGGTGAGGATCTTCATGAGGTA - 3' (gene bank accession number: NG_026370.2) and β - beta actin primer sequence was 5' - GGC GGCACCACCATGTACCCT - 3' and antisense 5' - AGG GGCCGGACTCGTCATACT - 3' (gene bank accession number: NM_001101.3).

Statistical Analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data was summarized using median and interquartile range in quantitative data. Comparisons between quantitative variables were done using the non - parametric Kruskal - Wallis and Mann - Whitney tests. Correlations between quantitative variables were done using Spearman correlation coefficient. P - values less than 0.05 were considered as statistically significant.

Results

MSCs morphology

MSCs were identified in culture by adopting a

fusiform adherent fibroblast like cells in both types PDLSCs and GMSCs for healthy subjects and diabetic patients (Figure 1).

Flow Cytometric Analysis

Surface antigens of all groups were analysed by flow cytometry. GSCs & PDLSCs isolated from healthy & diabetic patients demonstrated relatively high positivity for mesenchymal stem cell-associated markers CD 90 and CD 105, and negative for hematopoietic markers CD45 (Figure1).

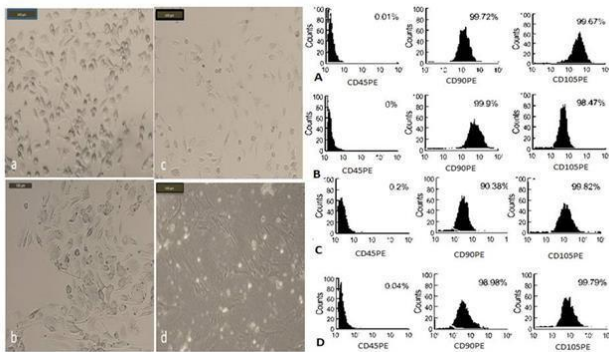


Figure 1: MSCs isolated from all groups in culture; A: PDLSCs isolated from healthy subjects, B: GMSCs isolated from healthy subjects, C: PDLSCs isolated from diabetic patients and D: GMSCs isolated from diabetic patients (Left). FACS analysis for characterisation of isolated MSCs; A: PDLSCs isolated from healthy subjects, B: GMSCs isolated from healthy subjects, C: PDLSCs isolated from diabetic patients and D: GMSCs isolated from diabetic patients. Analysis for all groups revealed high positivity of CD 90 and 105 and negativity of CD 45 for all types of isolated MSCs (Right)

Cell Proliferation by MTT Assay

Figure 2 illustrates the proliferation of PDLSCs and GSCs from normal & diabetic patients as evaluated by MTT assay.

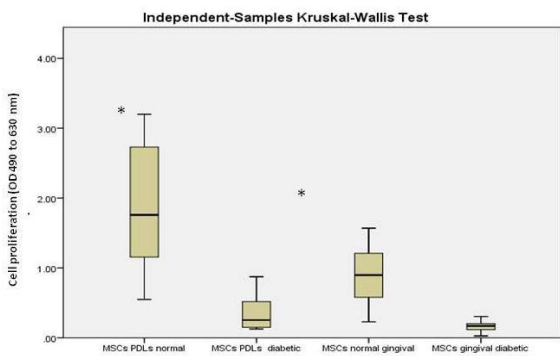


Figure 2: Proliferation of periodontal ligament stem cells (PDLs) and gingival stem cells (GSCs) from normal and diabetic individuals by MTT assay (* indicates statistically significant differences between normal and diabetic individuals in each cell type P < 0.001)

Both in normal and diabetic individuals PDLSCs exhibited greater proliferation rate than GSCs nearly two-fold higher. When comparing proliferation of each cell type about diabetes, it was revealed that the proliferation rate of PDLSC derived from normal individuals was significantly higher than those derived from diabetic individuals. The same

pattern was noticed in GSCs where the proliferation rate was significantly higher in normal cells. The increase in normal PDLSCs was seven folds higher than that in diabetics and 5.29 folds higher in case of GSCs (P < 0.001).

Real-time Quantitative PCR

The expression of Survivin in PDLSCs & GSCs from normal and diabetic patients by Rt - PCR is illustrated in Table 1. Statistical analysis revealed that Survivin expression in PDLSCs isolated from normal individuals was significantly higher than those from diabetic patients (P < 0.05). Similarly, GSCs isolated from normal individuals exhibited expression that is significantly higher than GSCsc from diabetic individuals (P < 0.001).

Table 1: Survivin expression when comparing PDLSCs normal group with diabetic group, and GSCs normal group with diabetic group using multiple comparison post hoc tests (* P - value when comparing PDLSCs group versus PDLSCs diabetic group, ** P - value when comparing GSCs group versus GSCs diabetic group)

	MSCs PDLs Normal	MSCs PDLs Diabetic	MSCs Normal Gingival	MSCs Normal Diabetic	P - value
Survivin Gene Expression	1.24*	0.54	0.84**	0.26	< 0.001
SD	0.74	0.15	0.32	0.07	

Assessment of the relation between proliferation rate and Survivin Gene expression

Next, we analysed the correlation between Proliferation rate & Survivin Gene Expression. There was a significantly positive correlation between proliferation rate and expression of Survivin in all groups illustrating the importance of Survivin as a reliable indicator of proliferation (Figure 3 and Table 2).

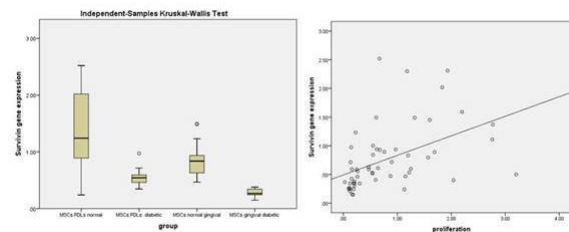


Figure 3: A plot of Survivin gene expression in all groups by RT - PCR (Left) and the relation of its expression with the proliferation potential of studied stem cells groups

Table 2: Correlation between Proliferation and Survivin expression

	Proliferation
Survivin Gene Expression	.621
Correlation Coefficient	
P value	<0.001
N	56

Discussion

Stem cells have recently received great interest as a potential therapeutic alternative for a variety of diseases. This is especially true for a disease like diabetes. The majority of mesenchymal stem cells previously studied for therapeutic purposes have been from sources like bone marrow and adipose tissue [23][28]. Both, PDLSCs and GSCs demonstrated promising potential for use in tissue regeneration [29][30][31]. In the present study, we investigated stem cells isolated from two different oral tissues. Moreover, to address whether oral stem cells derived from different oral tissues have different proliferation ability and whether controlled type II diabetes influences these abilities, we compared the growth rate of gingival and periodontal derived stem cells isolated from diabetic & healthy individuals.

Our results showed that both PDLMSCs and GMSCs demonstrate satisfactory proliferation rates. We observed a significant difference in the proliferation pattern of both tissues. Stem cells isolated from PDL exhibited higher proliferation than those isolated from gingival tissues both in normal and diabetic patients. This was in contrast to multiple reports that showed GMSCs to have a higher proliferation compared to PDLSCs [29][32][33][34]. This pattern of proliferation described in the diabetic patient was in contrast to previous reports that demonstrated that early after the induction of diabetes with streptozotocin, the proliferative capacity of bone marrow-derived stem cells increased [23]. The inclusion of a greater number of diabetics enrolled in our study in contrast to using diabetes-induced models may help explain this result. Our results were in consistence with Stolzing et al who attributed the diminished proliferative capacity of bone marrow cells to the long-term incidence of diabetes [35].

It is worthy to note that in the present study both PDLMSCs and GMSCs were phenotypically similar in diabetic and non - diabetic groups as demonstrated by FACS analysis. The homogeneity of the results in all groups suggests that diabetes has not altered the stemness of either population confirming that the changes associated with diabetes mainly affect the proliferation capacity of the cells.

To further assess the effect of diabetes on PDLSCs and GSCs, we measured the expression levels of Survivin in isolated stem cells from all groups and correlated its expression with the proliferation patterns studied. Survivin is a critical inhibitor of apoptosis-inducing proteins that are up-regulated in many types of malignant diseases. Additionally, it has been demonstrated previously that Survivin expression is correlated with the expression of cell proliferation and survival markers [36]. In the present study, there was a significantly positive correlation

between proliferation rate and expression of Survivin in all groups which sheds light on the importance of Survivin as a reliable indicator of proliferation. The expression on Survivin further confirmed the proliferation results from MTT Assay where the expression of stem cells from non - diabetic individuals was higher than diabetic patients and PDL stem cells demonstrated significantly higher expression than GSCs. Taking together all the results, it could be concluded that PDLSC and GSC are promising candidates for autologous regenerative therapy due to their ease of accessibility in addition to their high proliferative rates. However, it could also be concluded that stem cells from diabetic donors exhibit decreased proliferative potential while maintaining their stemness properties. Thus it is essential to improve methods and factors that would increase the proliferation capacity of stem cells isolated from diabetic patients to ensure the success of regenerative medicine options especially when autologous cells are intended for use.

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