Article published online: 2024-06-20

Compromised Differentiation Potential of Diabetic Dental Pulp Stem Cells

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J Health Allied Sci[NU]

Abstract

Background Dental pulp-derived mesenchymal stem cells (DPSCs) are documented to be a promising source for the treatment of a diverse spectrum of diseases including type 2 diabetes mellitus (T2DM). However, alterations in the characteristics of DPSCs from the T2DM patients are still unclear.

Objective The purpose of this study was to compare the characteristics of dental pulp stem cells obtained from diabetic and nondiabetic healthy individuals.

Methods Dental pulp stem cells from nondiabetic (ND-DPSCs) and diabetic (D-DPSCs) were isolated by the explant culture method. Both cells were expanded in identical culture conditions and subsequently differentiated into osteogenic, chondrogenic, and adipogenic conditions. D-DPSCs and ND-DPSCs were characterized for a panel of MSCs-specific surface markers. Senescence associated with β-galactosidase was performed. In addition, we also performed an in vivo chick embryo yolk sac membrane assay for angiogenesis.

Results Findings of this study showed that diabetes mellitus affected the osteogenic and chondrogenic differentiation, while adipogenic differentiation was significantly higher in D-DPSCs as compared to ND-DPSCs. Clonogenic ability and angiogenic potential of ND-DPSCs is higher than D-DPSCs despite similar surface marker expressions.

Conclusion Diabetes affects the stemness of D-DPSCs in terms of clonogenic, osteogenic, and chondrogenic differentiation and angiogenic potential, reflecting the adverse effects of hyperglycemia even on dental pulp stem cells.

Keywords
- angiogenesis
- dental pulp stem cells
- diabetes mellitus
- differentiation
- stemness

Introduction

Type 2 diabetes mellitus (T2DM) is approaching epidemic proportions globally.[1] T2DM is a heterogeneous metabolic disease characterized by higher blood glucose levels with dysregulation of carbohydrate, protein, and lipid metabolism.[2] The gradual progression and insidious nature of the disease affects the physiological functioning of the body systems posing a challenge in maintaining normal levels of blood glucose during treatment to delay systemic complications. The persistent hyperglycemia in T2DM is implicated in vascular complications of the heart, blood vessels, eyes, kidneys, and nerves, and wound healing in diabetic patients.[3]

The field of stem cells holds immense potential and is rapidly becoming a treatment option for many diseases.[4]

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DOI https://doi.org/10.1055/s-0044-1787848.
ISSN 2582-4287.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Stem cell therapy is a new ray of hope to treat T2DM and related disorders. Mesenchymal stem cells (MSCs) are one of the most extensively investigated stem cell sources which possess unique immunosuppressive and regenerative properties.[5] The dental pulp is one of the most commonly used sources of adult tissue MSCs due to their ease of isolation, ectodermal origin, unique proliferation, differentiation, and secretory properties with minimal ethical concerns. However, there is limited data on the affects of metabolic syndromes such as T2DM on cellular and functional properties. Although it is known that diabetes affects several organs, it remains to be seen whether it affects the stem cell properties of dental pulp and its mechanism. Hence, the purpose of this study was to investigate whether there is a difference in the stem cell properties obtained from the dental pulp of diabetic and healthy individuals in the context of surface markers, trilineage differentiation, angiogenesis, and clonogenicity.

Materials and Methods

Sample Collection

This project was approved by the Institutional Committee for Stem Cells Research (IC-SCR) no. IC-SCR/001/2019. Dental pulp was obtained from extracted teeth from the patients reporting to the department of oral and maxillofacial Surgery. For the diabetic dental pulp, patients were selected with 10 to 20 years of diabetic history within the age group of 40 to 60 years, hemoglobin A1C between >7/%, blood serum fasting 100 to 120 U/mL, and postprandial above 180 U/mL. Premolar teeth ($N = 10$) from the T2DM ($n = 5$) and nondiabetic patients ($n = 5$) of age 40 to 45 years were collected after extraction. The extraction was by oral and maxillofacial surgeons after a written informed consent was obtained as per the institutional guidelines (►**Table 1**).

Table 1 Demographic chart

Sr. no.	Age	Gender	HbA1C	Tooth number
Nondiabetic group				
1	44	M	5.5	45
2	46	M	6.2	35
3	55	F	5.8	14
4	53	M	6.0	34
5	45	F	5.5	24
Diabetic group				
1	43	F	7	14
2	60	F	7.5	25
3	52	M	8	34
4	57	M	8.7	45
5	69	F	7.2	44

Abbreviations: F, female; HbA1C, hemoglobin A1C; m, male.

Isolation of Diabetic Dental Pulp Stem Cells and Nondiabetic Dental Pulp Stem Cells

Dental pulp was removed and separated from the teeth by vertical section using an air rotor and was immediately transferred to phosphate buffer saline (PBS) (Gibco) containing 1% antimycotic-antibiotic (AA) solution (Gibco). The dental pulp was then cut into 1- to 2-mm fragments and placed in a T25 culture flask. Dental pulp fragments were completely covered with fetal bovine serum (FBS) (Gibco), and incubated for 24 hours at 37°C with 5% CO_2. After incubation, explants were cultured in Dulbecco-modified Eagle medium (DMEM) (Gibco) supplemented with 10% FBS and 1% AA solution at 37°C in a humidified atmosphere containing 5% CO_2 until the outgrowth of cells was observed. Once the cells achieved 70 to 80% confluency, they were subcultured up to passage 4 for further studies. The cells obtained from all samples were pooled together.

Flow Cytometry Analysis

Diabetic dental pulp stem cells (D-DPSCs) and nondiabetic DPSCs (ND-DPSCs) were characterized for a panel of MSC-specific surface markers, that is, positive for CD90, CD105, and CD73 (phycoerythrin [PE] tagged), and negative for hematopoietic lineage markers such as CD34, CD45, and human leukocyte antigen (HLA)-DR (fluorescein isothiocyanate tagged) by flow cytometry as previously described.[6]

Colony-Forming Unit-Fibroblast and Cell Proliferation Assay

The colony-forming unit-fibroblast (CFU-F) assay was performed in triplicate to assess the ability of the cells to form colonies. D-DPSCs and ND-DPSCs were seeded (5×10^2 cells/well) in a 6-well culture plate along with DMEM containing 10% FBS and 1% AA solution. After 8 to 9 days of incubation, cells were washed with PBS and stained with 0.3% crystal violet solution. Colonies were observed under the microscope (Olympus). The analysis of CFU-F was performed by using the open-CFU software. Cell proliferation assay was performed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Trilineage Differentiation Potential

Adipogenic Differentiation

For adipogenic differentiation, the D-DPSCs and ND-DPSCs were seeded in a 24-well plate at a density of 2.5×10^4 cells/well containing a complete growth medium. After 70 to 80% confluence was achieved, cells were treated with the adipogenic induction medium (DMEM supplemented with, 1% AA solution, 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin, and 0.5 mM isobutyl-methylxanthine) twice a week for 18 days. Adipogenic differentiation was confirmed by the accumulation of the lipid droplets with a 0.3% oil red O stain as previously described.[7]

Osteogenic Differentiation

D-DPSCs and ND-DPSCs were seeded at a density of 2.5×10^4cells/well in a 24-well plate within a complete growth medium. Once the cells achieved 70 to 80% confluency, they were treated with osteogenic induction medium (DMEM along with 1% FBS, 0.1 µM dexamethasone, 50 µM

ascorbate-2-phosphate, and 10mM β-glycerophosphate). The induction medium was replaced twice a week for up to 21 days. Cells without any treatment of induction medium were kept as control. After 21 days, cells were fixed with 4% formaldehyde and stained with 2% Alizarin red S solution (pH 4.1–4.3) to assess the matrix mineralization and calcium deposition.

Chondrogenic Differentiation

To induce chondrogenic differentiation, 2.5×10^4 cells/well of D-DPSCs and ND-DPSCs were seeded in a 24-well plate within a complete growth medium and allowed to become 70 to 80% confluent. Further, cells were cultured in the DMEM supplemented with 1 × -ITS, 1 mM sodium pyruvate, 100 nM dexamethasone, 2 mM ascorbate-2-phosphate, 40 µg/mL L-proline, and 10 ng/mL transforming growth factor-β3. Cells were differentiated for 28 days at 37°C in an atmosphere containing 5% CO_2. The induction medium was replaced twice a week. After 28 days, cells were stained for glycosaminoglycans by using 0.1% safranin O to confirm the chondrogenic differentiation of D-DPSCs and ND-DPSCs.

Quantitative analysis for trilineage differentiation was performed using an enzyme-linked immunosorbent assay plate reader. Differentiated cells were fixed with 4% formalin for at least 1 hour and rinsed with PBS before incubation in freshly diluted respective stains for 10 minutes. Stains were solubilized using 0.1% acetic acid and the resulting absorbance was measured at 420 nm using a spectrophotometer.

Cell Senescence Assay

Cell senescence assay was performed using β-galactosidase (β-gal) test. β-gal is a classic maker of senescence in cellular study. Cellular senescence of D-DPSCs and ND-DPSCs was determined at passages 15 and 18. The cells were washed with PBS, fixed, and stained for β-gal by using the X-gal staining solution and incubated overnight at 37°C as per the manufacturer's instructions (Cell Signaling Technology, Cat-9860).

Cells were then examined under a microscope at 10× magnification (Olympus, Japan) for the development of blue coloration. Cells senescence analysis was performed by using the Image J software.

Yolk Sac Membrane Assay for Angiogenesis

The ex vivo yolk sac membrane (YSM) assay was performed in triplicate to investigate the comparative angiogenic potential of D-DPSCs and ND-DPSCs condition media as previously described.[8] Condition medium from D-DPSCs and ND-DPSCs were obtained by culturing cells in a T75 culture flask for 48 hours without serum. Freshly collected fertilized eggs were obtained from Venkateshwara Hatcheries, Pune, Maharashtra, India and then incubated for 48 hours in a humidified incubator. Small windows were created at the air cell position of eggs. Fertilized eggs were then treated with a 100-µL conditioned medium. DMEM without preconditioning was used as control. The windows were closed using transparent sealing tape and the eggs were incubated at 37.0°C for 48 hours in a humidified incubator. After incubation, the eggshells were cut open to expose complete sprouting of YSM blood vessels. The images for control and experimental YSMs were captured using a digital camera (Nikon). Captured images were further processed for the angiogenic parameters such as total tube length, branching points, and number of tubes using online WimTube software as described previously.[8]

Statistical Analysis

All the experiments were performed thrice and the samples were run in triplicate ($n = 3$). All the data values were represented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance tests using the SPSS (IBM) software ($^{*}p < 0.05$ and $^{**}p < 0.001$).

Results

D-DPSCs and ND-DPSCs Exhibited Similar Surface Marker Profiles

After 7 days of explant culture, cell growth was seen around the tissue (►**Fig. 1A** and **B**). Upon subsequent passaging up to passage 4, D-DPSCs and ND-DPSCs exhibited fibroblast-like morphology (►**Fig. 1C** and **D**). The flow cytometric analysis revealed that D-DPSCs showed positive expression of CD105 (99.52%), CD73 (98.8%), and CD90 (93.2%), and negative expression for HLA-DR (3.6%), CD45 (0.64%), and CD34 (8.5%) (►**Fig. 2A**). ND-DPSCs showed positive expression of CD105 (98.3%), CD73 (98.54%), and CD90 (93.1%), and negative expression for HLA-DR (3.6%), CD45 (0.62%), and CD34 (10.7%) (►**Fig. 2B**). There was no significant difference in the expression of CD markers in the DPSCs obtained from diabetic and nondiabetic individuals.

Clonogenicity of D-DPSCs is hampered: The present study found that ND-DPSCs (►**Fig. 3A**) exhibited higher CFUs as compared to D-DPSCs (►**Fig. 3B**). Significance difference is

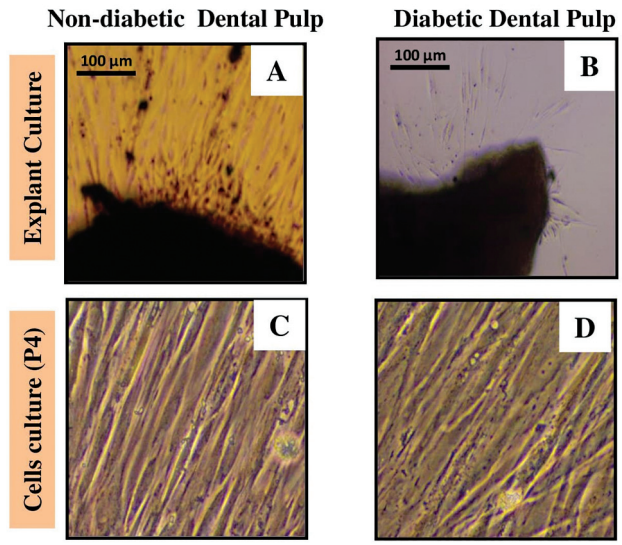

Fig. 1 Isolation and culture of nondiabetic (**A**) and diabetic dental pulp (**B**) by explant culture showing cell outgrowth after 7 days of culture. Fibroblast-like morphology of nondiabetic (**C**) and diabetic dental pulp (**D**) at passage 4 (magnification 10×).

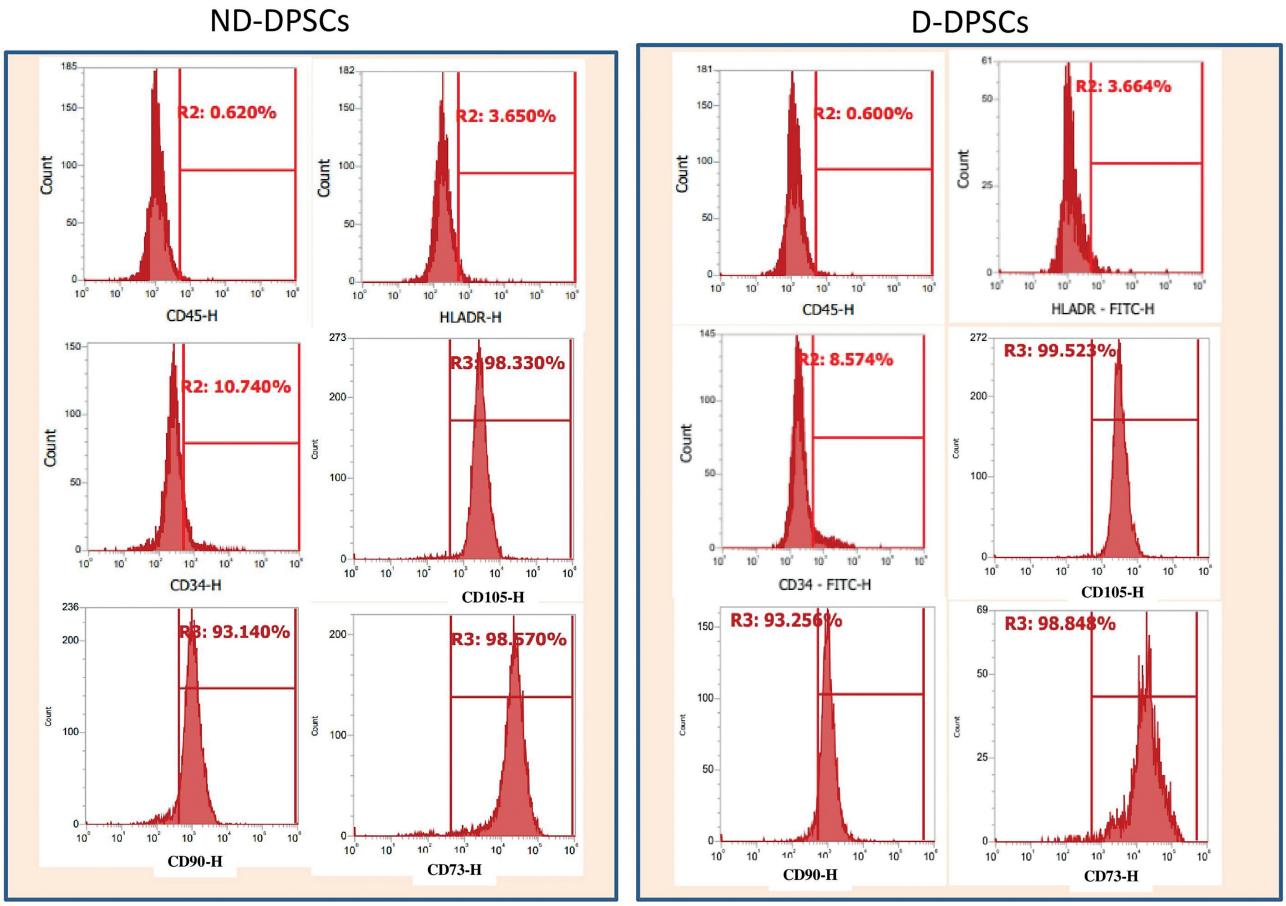

Fig. 2 Mesenchymal stem cell (MSC)-specific surface markers analysis performed by flow cytometry for diabetic dental pulp stem cells (D-DPSCs) and nondiabetic dental pulp derived stem cells (ND-DPSCs). D-DPSCs and ND-DPSCs both are positive for CD90, CD105, and CD73 and negative for CD34 and human leukocyte antigen (HLA)-DR.

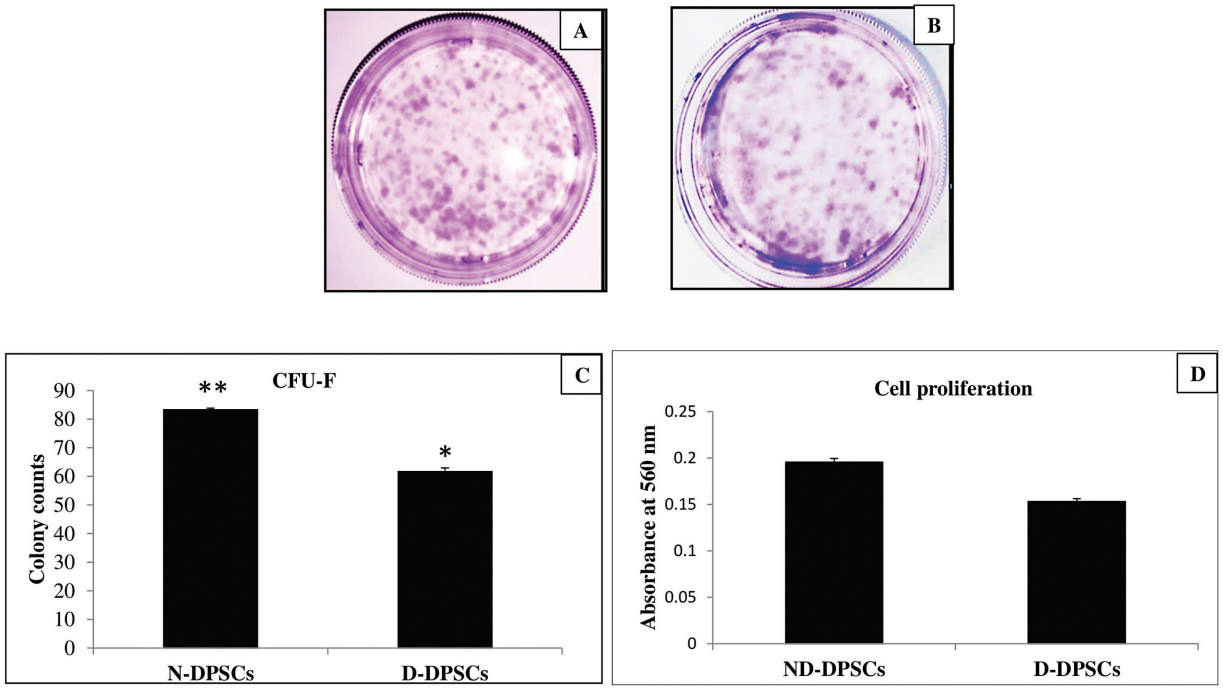

Fig. 3 Colony-forming unit (CFU) like fibroblast (**A**). Nondiabetic dental pulp stem cells (ND-DPSCs), (**B**) diabetic dental pulp stem cells (D-DPSCs), (**C**) colony count analysis of ND-DPSCs and D-DPSCs by open-CFU software. (**D**) Cell proliferation by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

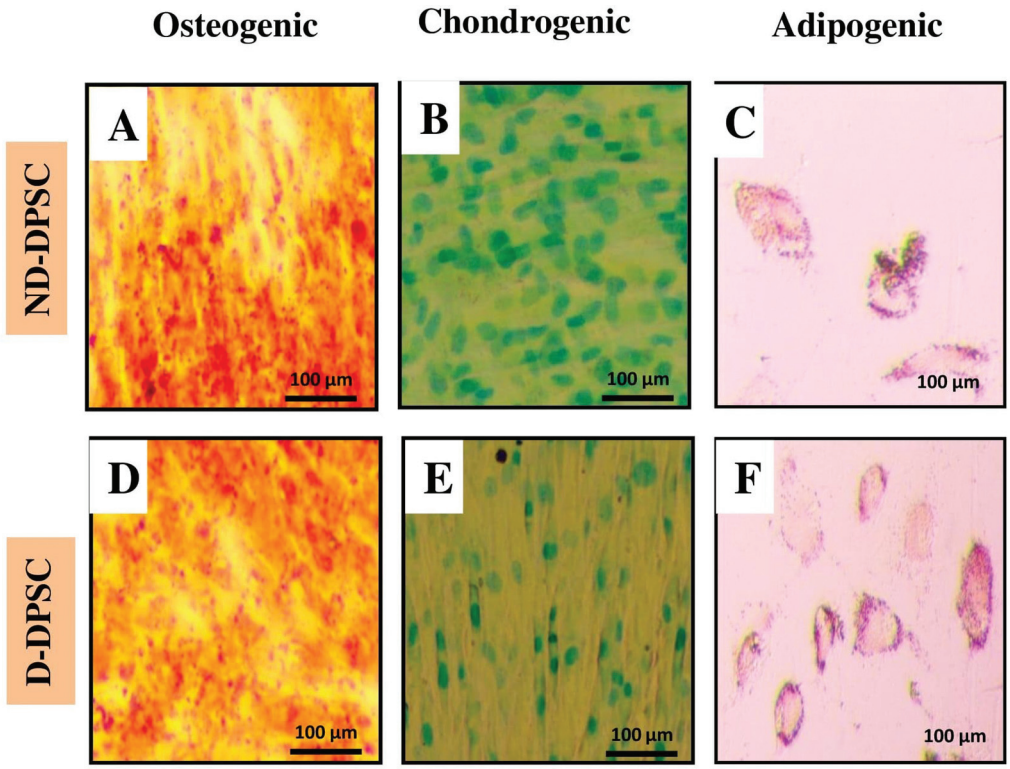

Fig. 4 Trilineage differentiations of nondiabetic dental pulp stem cells (ND-DPSCs) and diabetic dental pulp stem cells (D-DPSCs). Osteogenic (**A**, **D**) Alizarin red shows calcium deposition, chondrogenic (**B**, **E**) Alcian blue shows glycosaminoglycans, and adipogenic (**C**, **F**) oil red O stain shows lipid droplets.

shown in ►**Fig. 3C**. ND-DPSCs also showed higher proliferation rate as compared to D-DPSCs (►**Fig. 3D**).

Trilineage Differentiation Potential of ND-DPSCs is Compromised

The ND-DPSCs and D-DPSCs were able to differentiate into osteocytes, adipocytes, and chondrocytes as evidenced by the Alizarin red, oil red O, and Alcian blue staining, respectively. Interestingly, osteogenic and chondrogenic differentiation in D-DPSCs were relatively lower when compared to ND-DPSCs as evidenced by Alizarin red for calcium deposition for osteocyte and Alcian blue staining for glycosoaminoglycan for chondrocyte differentiation (►**Fig. 4**). Whereas adipogenic differentiation was significantly higher in the D-DPSCs as compared to the ND-DPSCs as shown by oil O red staining (►**Fig. 4**). Spectrophotometric measurement was performed to crosscheck the microscopic results of trilineage differentiation of ND-DPSCs and D-DPSCs and revealed a similar outcome (►**Fig. 5**).

Evaluation of Cell Senescence Analysis

Cell senescence was performed using β-gal senescence assay on D-DPSC and ND-DPSCs at passages 15 and 18 (►**Fig. 6A**). These observations relate cell senescence with the process of aging. High levels of senescence-associated β-gal indicate high cell aging. Our findings showed that the amount of blue, β-gal positive cells was higher (25–35%±) in D-DPSCs than in the ND-DPSCs (20–25%±) at passages 15 and 18 (►**Fig. 6B**).

Angiogenic Potential of D-DPSCs Conditioned Medium is Defective

We were interested in finding out if the diabetic microenvironment compromised the angiogenic potential of D-DPSCs. The results showed that ND-DPSCs exhibited better angiogenic potential as compared to the D-DPSCs as revealed by the YSM assay (►**Fig. 7**). Furthermore, analysis showed that treatment of conditioned medium obtained from ND-DPSCs significantly enhances the total tube length, branching points, and number of tubes as compared to the D-DPSCs.

Discussion

Stem cell therapy harnesses the therapeutic potential of embryonic and adult stem cells and has long been exploited for clinical applications.[9–11] The DPSCs are a readily available adult source that can be easily derived from teeth indicated for extraction.[12,13] Studies have shown that dental pulp exhibits a significant reduction in blood flow and collateral circulation in long-standing diabetic conditions.[14] The present study endeavored to determine whether diabetes affected the stem cell properties of dental pulp from diabetic individuals. In diabetes, the bone marrow is unable to release quality stem cells which are important for damage repair. Normally, MSCs and mesenchymal progenitor cells can aggregate in injured areas and differentiate into new vascular endothelial cells to restore homeostasis. However, in the case of diabetes, their angiogenic differentiation ability is

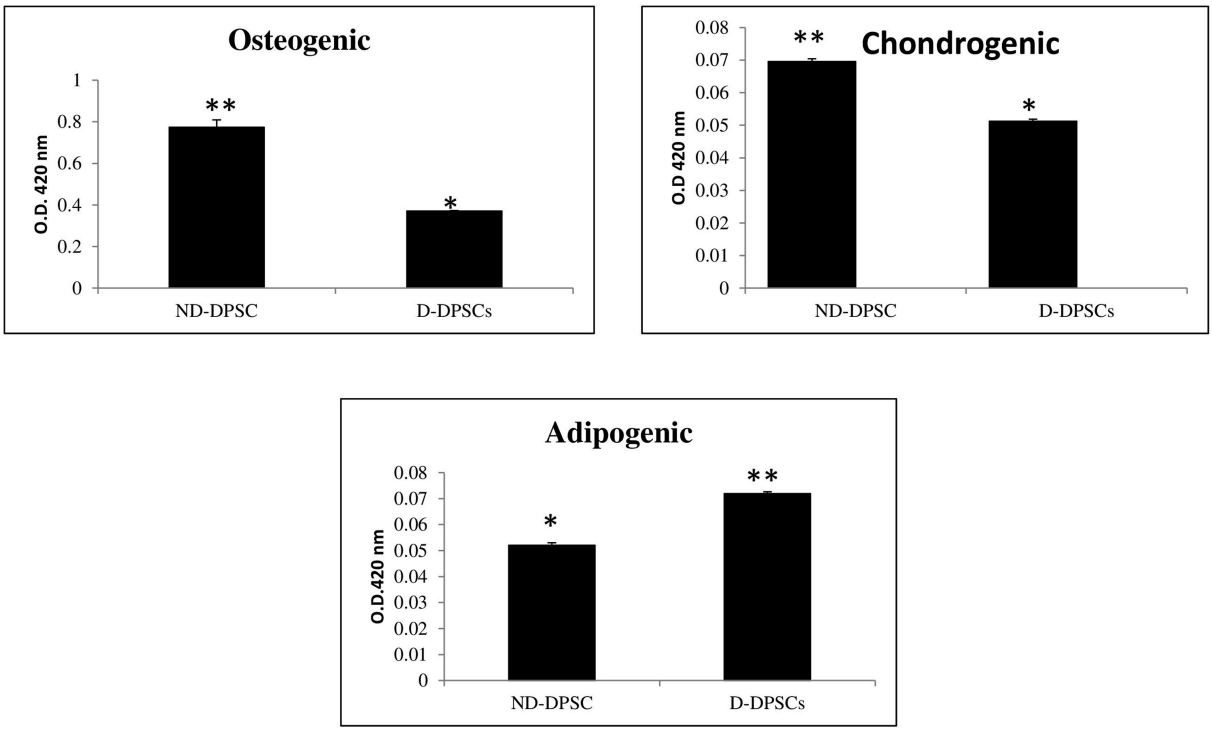

Fig. 5 Quantitative analysis. (**A**) Osteogenic, (**B**) chondrogenic, and (**C**) adipogenic differentiation of nondiabetic dental pulp stem cells (ND-DPSCs) and diabetic dental pulp stem cells (D-DPSCs). Quantitative data are presented as mean ± standard error of the mean (SEM); $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *t*-test.

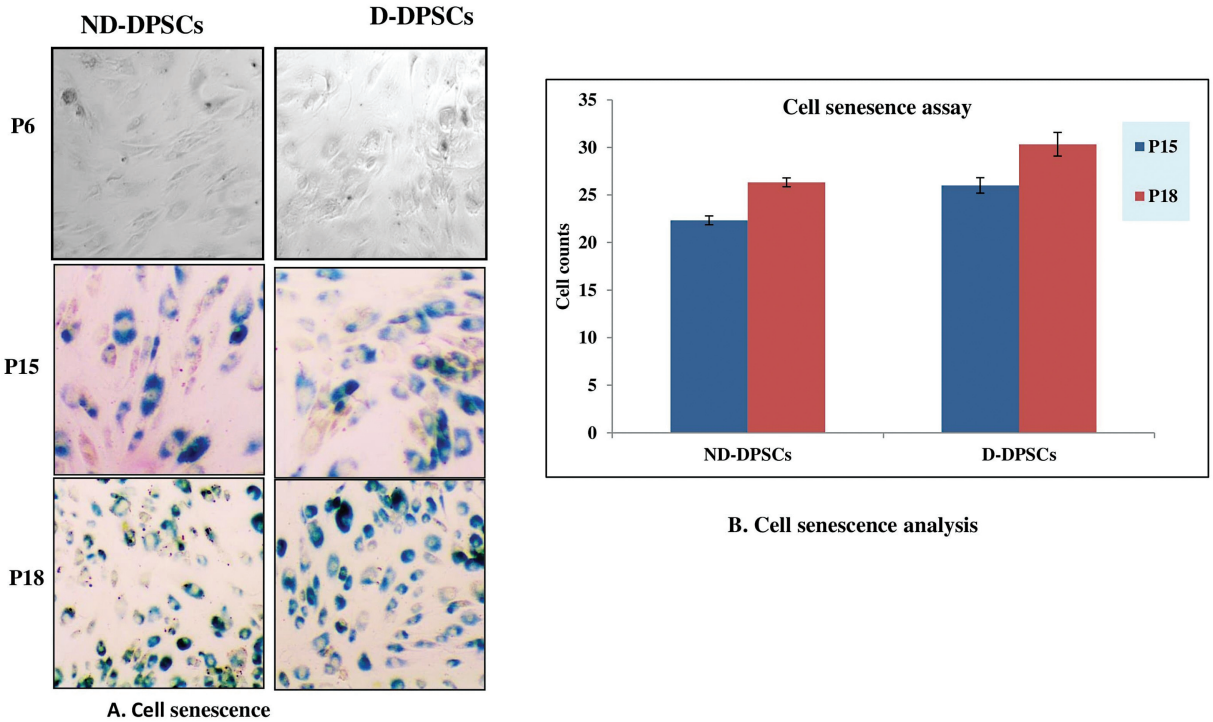

Fig. 6 (A) Cell senescence (10×). (B) Cell senescence analysis using the Image J software for nondiabetic dental pulp stem cells (ND-DPSCs) and diabetic dental pulp stem cells (D-DPSCs).

aberrant, and as a result, are incapable of repairing damaged blood vessels. In diabetic conditions, stem cells and progenitor cells suffer from "mobilopathy" and are unable to activate for tissue repair, inducing the progression of diabetic complications. Previous studies have reported the isolation, characterization, and differentiation potential of DPSCs from healthy individuals.[15,16] However, detailed characterization such as surface marker analysis, CFU-F, and trilineage

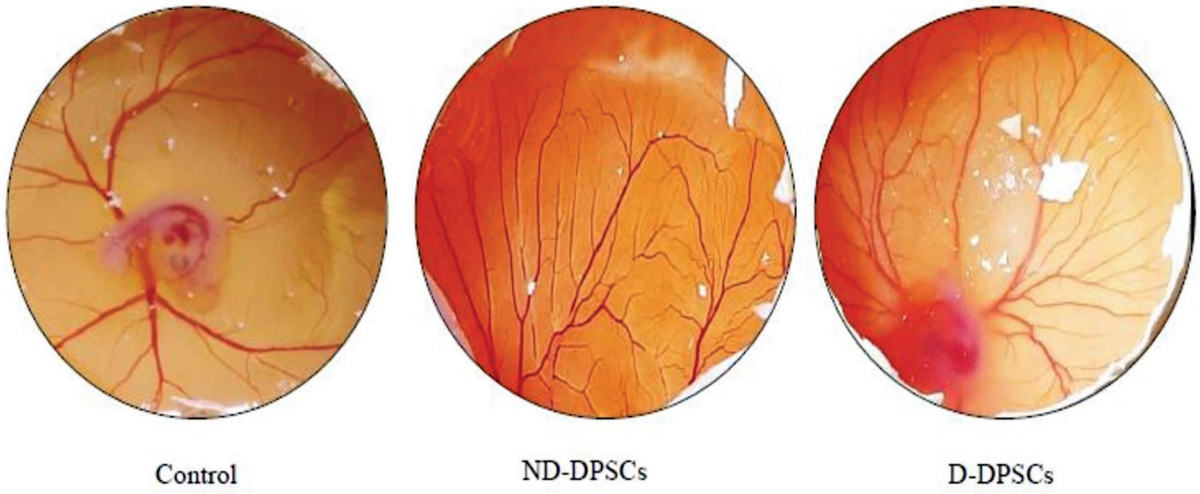

Fig. 7 Angiogenic effect of condition media in yolk sac membrane (YSM) model in control, nondiabetic dental pulp stem cells (ND-DPSC), and diabetic dental pulp stem cells (D-DPSCs) show primary, secondary, tertiary, and quaternary blood vessels after 24 hours of treatment.

differentiation of DPSCs obtained from diabetic patients was not documented.[6,17] The MSCs successfully differentiating into osteoblasts, chondroblasts, and adipocytes through induction medium has been previously demonstrated.[6]

It has been reported by several investigators that dental pulp tissue contains a significant number of stem cells.[6,18] In this investigation, we compared the stem cell properties of DPSCs collected from nondiabetic and diabetic individuals.

According to our results, it was evident that there was no significant difference in the surface marker expression of cells CD90, CD105, CD73, CD34, and CD45-PE. Therefore, it can be implied that the diabetic microenvironment did not affect the cell surface marker expression of the DPSCs in T2DM individuals. Our results also showed that the D-DPSCs exhibited lower CFU ability when compared to the ND-DPSCs. The diabetic microenvironment may affect the colony-forming ability of D-DPSCs, emphasizing the existence of a potential biological mechanism by which diabetes may affect stemness.

MSCs also can secrete growth factors, cytokine, and extracellular nanoparticles containing functional messenger ribonucleic acid (mRNAs), microRNAs, long noncoding RNAs, proteins, and lipids that aids in angiogenesis or new blood vessel formation during wound healing. In the yolk sac model, D-DPSCs show fewer angiogenic effects as compared with ND-DPSCs. Evidence on the ability of endogenous MSCs to secrete angiogenic factors under DM is limited.

In addition, disease state of the donors could impair proliferation and differentiation capabilities.[19] In similarity, ND-PSCCs significantly showed higher numbers than from D-DPSCs.

The present study is mostly the first of its kind to address the direct impact of diabetes on DPSCs. D-DPSCs showed diminished osteogenic and chondrogenic differentiation potential suggesting that diabetic microenvironment may play a role in the determination of stem cells. Moreover, D-DPSCs exhibited a higher propensity toward adipogenic differentiation highlighting the potential impact of diabetic microenvironment on the stem cell properties.

In a previous study, it was shown that the diabetic microenvironment influences the behavior of MSCs derived from the bone marrow of diabetic patients.[20] Another 2019 study also reported evidence on the effects of diabetes mellitus on MSC function.[20,21] T2DM-associated osteopathy is therefore potentially linked to the reduced osteoprogenitor population in the bone marrow of people with T2DM.[22] T2DM impairs bone health including delay in bone regeneration and a decrease in bone biomechanics. Also, impairment of vascularization and angiogenesis linked with T2DM affected the bone healing process.[23]

Findings of this study demonstrated that T2DM impaired osteogenic and chondrogenic differentiation. However, it did not alter the ability of D-DPSCs to differentiate into adipocytes. It was demonstrated that the diabetic status of the DPSC impacted the overall presence and concentration of angiogenic factors within the conditioned medium.

Senescence is a normal feature seen at higher passages. DPSCs are constantly subjected to various kinds of stress. Based on cell type, intensity of stress, and nature of cell, the cell may opt renewal, death, or senescence. However, the data indicated that senescence is severe in D-DPSCs. Diabetes affects stem cell functions, leading to differentiation and secretory dysfunctions through different molecular pathways. T2DM also resulted in abnormal changes in the number of stem cells, which may be due to abnormal proliferating rate and increased apoptosis. The present results supported the earlier findings indicating the importance diabetic microenvironment in altering the stemness of MSCs in dental pulp. Further studies on this may corroborate the findings of this study.

Conclusion

Taken together, our data demonstrates the influence of the diabetic microenvironment on the performance of D-DPSCs concerning their differentiation potential, angiogenic ability, and stemness. The D-DPSCs showed increased adipogenic differentiation.

Funding
This study was funded by Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India [DPU927(22)2018].

Conflict of Interest
None declared.

Acknowledgment
The authors would like to thank Regenerative Medicine Laboratory, Department of Oral and Maxillofacial Surgery, and Department of Periodontology for support and help.

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