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Assessment of angiogenic potential of mesenchymal stem cells derived conditioned medium from various oral sources

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Abstract

Background: Abnormal angiogenesis hamper blood vessel proliferation implicated in various biological processes. The current method available to clinically treat patients to enhance angiogenesis is administering the angiogenic growth factors. However, due to a lack of spatiotemporal control over the substantial release of these factors, numerous drawbacks are faced such as leaky vasculature. Hence, stem-cell-based therapeutic applications are running their race to evolve as potential targets for deranged angiogenesis. In clinical dentistry, adequate tissue vascularization is essential for successful endodontic therapies such as apexogenesis and apexification. Furthermore, wound healing of the extraction socket and tissue regeneration post-surgical phase of treatment including implant placement require angiogenesis as a foundation for the ultimate success of treatment. Mesenchymal stem cells (MSCs) secrete certain growth factors and cytokines in the culture medium during the proliferation. These factors and cytokines are responsible for various biological activities inside human body. Oral cavity-derived stem cells can secrete growth factors that enhance angiogenesis.

Aim: The aim of the study was to investigate the angiogenic potential of conditioned medium (CM) of MSCs derived from different oral sources.

Methods: Oral tissues such as dental pulp of adult and deciduous teeth, gingiva, and buccal fat were used to isolate dental pulp MSCs (DPSCs), exfoliated deciduous teeth, gingival MSCs, and buccal fat derived MSCs. MSCs conditioned medium (CM) from passage four cells from all the sources were obtained at 48 h interval and growth factor analysis was performed using flow cytometry. To assess the functionality of the CM, Chick Yolk Sac Membrane (YSM) assay was performed.

Results: CM obtained from DPSCs showed higher levels of vascular endothelial growth factor, fibroblast growth factor, and hepatocyte growth factor as evidenced by flow cytometry. Furthermore, DPSC-CM exhibited significantly higher pro-angiogenic potential when assessed in in-ovo YSM assay.

Conclusion: DPSCs so far seems to be the best source as compare to the rest of oral sources in promoting angiogenesis. A novel source of CM derived from buccal fat stem cells was used to assess angiogenic potential. Thus, the present study shows that CM derived from oral cavity-derived-MSCs has a dynamic and influential role in angiogenesis.

Relevance for Patients: CM derived from various oral sources of MSCs could be used along with existing therapies in medical practice where patients have compromised blood supply like in diabetes and in patients with debilitating disorders. In clinical dentistry, adequate tissue vascularization is essential for successful wound healing, grafting procedures, and endodontic therapies. DPSCs-CM shows better angiogenic potential in comparison with other oral sources of MSCs-CM. Our findings could be a turning point in the management of all surgical and regenerative procedures requiring increased angiogenesis.

1. Introduction

Angiogenesis, also termed neovascularization, is a dynamic process that holds paramount importance in numerous biological life processes. It forms an essential part of various physiological processes right from the embryonic development of the fetus in utero and continues throughout life in healthy and diseased states. Abnormal angiogenesis hampers blood vessel proliferation implicated in various biological processes [1]. The entire procedure of angiogenesis is a well-synchronized pathway made up of sequential events. In clinical dentistry, adequate tissue vascularization is essential for successful endodontic therapies such as apexogenesis and apexification. Furthermore, wound healing of the extraction socket and tissue regeneration post-surgical phase of treatment, including implant placement, require angiogenesis as a foundation for the ultimate success of treatment [2].

Various regulatory factors and enzymes form a part of this pathway. These include: Angiopoietin (ANG), vascular endothelial growth factor (VEGF), basic Fibroblast Growth Factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and many more. Each factor has a different story to build during induction, initiation, amplification of cells, migration, cell stabilization, wound healing process, inflammatory response, and suppressing angiogenesis [3,4]. The method currently available to clinically treat patients to enhance angiogenesis is administering the angiogenic growth factors. However, due to a lack of spatiotemporal control over the substantial release of these factors, numerous drawbacks are faced like leaky vasculature. Hence, stem-cell-based therapeutic applications are running their race to evolve as potential targets for deranged angiogenesis [5].

Mesenchyme is a Greek word for middle infusion, which refers to the capability of the cells to proliferate and migrate during early embryogenesis in between ectodermal and endodermal layers [6]. Mesenchymal stem cells (MSCs) are fibroblast like cells that can proliferate and differentiate into multiple lineages such as osteocytes, chondrocytes, adipocytes, and neural cells. To prove the stemness of the MSCs, the International Society of Cellular Therapy has determined specific guidelines [7]. MSCs adhere to plastic surface under normal conditions of cell culture. Apart from this, particular cell surface-specific markers (cluster of differentiation [CD]) such as CD90, CD73, and CD105, are present and CD34, CD45, and HLA-DR are absent.

MSCs are derived from pre-natal tissues such as the umbilical cord, wharton's jelly, cord blood, placenta, and amniotic fluid and membrane. It is also found in post-natal tissues such as bone marrow (BM), adipose tissue, dermal tissues, and visceral organ cells. Over the past decade, MSCs have been isolated from adult oral cavity tissues. These include dental pulp, gingiva, periodontal membrane, apical papilla, and buccal fat pad [8]. MSCs are known to possess immunomodulatory and anti-inflammatory properties. Furthermore, MSCs have been proven to be essential in tissue regeneration. They also can enhance vascularization and accelerate healing potential following certain pathological conditions [9].

MSCs exhibit their therapeutic effects through paracrine mechanisms. MSCs secrete certain growth factors and cytokines which are responsible for various biological activities. This unique property of MSCs has led to development of a novel by-product of the stem cells, which is termed the conditioned medium (CM) or the secretome [9]. This by-product of CM is helpful in various therapeutic applications [10]. MSCs-CM is also known to secrete growth factors which help in promoting angiogenesis and aid in tissue healing mechanisms through their potent paracrine signaling [11].

Oral tissues are derived from ectomesenchymal components and are further derived from neural crest cells. This unique property of oral derived MSCs makes them an ideal candidate for isolation, culture, and application in various pre-clinical studies to acknowledge their therapeutic potency [12]. Extensive research has happened on various sources of oral cavity derived MSCs (OC-MSCs). However, none of the studies could portray a better candidate for selection in terms of angiogenic potential. Hence, this study was focused on the assessment of the growth factors profile and pro-angiogenic potential of the CM obtained from MSCs isolated from various tissue oral sources.

2. Materials and Methods

2.1. Ethical approval and patient consent

The Institutional Ethics Committee of Dr. D. Y. Patil Dental College and Hospital, Pune, India approved the study protocols involved in the present study (DYPV/EC/566/2020). The patients from whom specimens were collected were informed about the research and use of their biological sample, and informed consent was taken from them at the commencement of the study.

2.2. Sample collection

In this study, four different sources of the tissues were used for isolation of MSCs. Permanent teeth: Sound teeth extracted from impacted third molars to isolate dental pulp MSCs (DPSCs). Deciduous teeth: Exfoliated sound teeth extracted during routine dental procedures to isolate stem cells from exfoliated deciduous teeth (SHED). Gingiva: Healthy gingiva surgically excised during crown lengthening to isolate gingival MSCs (GMSCs). Buccal pad of fat: Healthy buccal adipose fat tissue excised during flap surgeries to isolate buccal fat MSCs (BFMSCs). For isolation of DPSCs, sound third molars were obtained from routine dental extractions from otherwise healthy patients aged 18–25 years. For isolation of SHED, sound deciduous teeth samples were obtained from healthy young patients with age ranging from 6 years to 15 years during routine dental procedures. For isolation of GMSCs, healthy gingiva samples were obtained from patients aged 18–25 years, subjected to crown lengthening procedures. For isolation of BFMSCs, healthy AT derived from the buccal area from patients aged 18–25 years subjected to flap surgeries was obtained. The selected donors had no relevant medical history and were otherwise healthy. The details regarding the donors have been included in [Supplementary Table 1](#). Following the surgical extraction or tissue removal from the patient, the

specimen was first dipped in povidone-iodine solution, then dipped in sterile saline solution and later placed in a falcon tube containing phosphate buffered saline (PBS) (Gibco, pH 7.4) with 1% antibiotic-antimycotic solution (CELL clone). The falcon-containing sample was then stored at 4°C until processing was carried out. The collected specimen was usually processed within 24 h of collection.

2.3. Tissue processing

For processing permanent and deciduous teeth (pulp extirpation), the tooth was drilled using an air-rotor hand piece and straight fissure burs to extract the pulp tissue from the tooth. After the initial drill, the tooth was opened using an elevator. The pulp specimen was placed in a sterile petri dish containing PBS for further experimentation. For, processing gingiva and buccal pad of fat tissues were disinfected and directly used for explant culture.

2.3.1. Explant culture for MSCs isolation

MSCs from OC-MSCs were isolated by explant method as per the methodology established in our laboratory [13]. The tissue samples were washed thoroughly with PBS 3–4 times. A T25 flask was incubated with fetal bovine serum (FBS) (Gibco) on the adherent surface at 37°C in a CO₂ incubator for 15 min. Once the tissue is disinfected, it was cut into small pieces, approximately 1–2 mm, using a sterile surgical blade. These sections were rewashed with PBS and transferred to a sterile petri plate. The tissue sections were soaked in FBS containing a 1% antibiotic-antimycotic solution. Further, these sections were transferred to the incubated T25 flask using 200 µl pipette tips. The flask was then incubated for 24 h. Furthermore, after the first 24-h incubation was complete, 4–5 ml of Minimum Essential Medium Alpha (MEM-α) (Gibco), supplemented with 10% FBS and 1% antibiotic-antimycotic solution, was added. The flask was then incubated for 4–6 days.

2.4. Isolation of stem cells

Post 6–7 days, all the flasks were observed under an inverted phase-contrast microscope (OLYMPUS) for cell out growth. Freshly made complete medium was changed in all the flasks and further incubated.

2.5. Cell passaging

Cell outgrowth surrounding the tissue was observed and trypsinized when cells are 70–80% confluent. For trypsinization, the medium in the flask was discarded, and PBS wash was given to remove remnants of FBS. 4 ml of trypsin (Gibco [0.25%]) was added into the flask. After 1 min of incubation, the flask was checked under a microscope. To stop trypsin action, an equal quantity of complete medium was added to the flask. Further, this cell suspension was added in a falcon tube and centrifuged at 1800 rpm for 5 min. Once a pellet of cells was formed, the supernatant was removed, and it was suspended in a complete medium. The suspension was transferred to a new sterile T25 flask and was labeled passage zero (P0). The medium of the flask was changed every 2 days. Once the flask was 80–90% confluent with

cells, passaging was carried out again. The same procedure was repeated till cells reached passage 4.

2.6. Cell characterization

MSCs isolated from each source were characterized using specific cell surface markers by flow cytometry. The panel of antibodies consisted of CD90, CD73, CD105 (PE Tagged), and CD34, CD45, and HLADR (FITC Tagged) (BD Biosciences). Passage 4 cells were used for FACS analysis. Approximately 100 µl of the cell suspension (1×10^6 cells/ml) was transferred in an eppendorf tube. Then, cells were fixed with 4% paraformaldehyde for up to 30 min at room temperature. Post-fixation, cells were washed with PBS containing 0.5% bovine serum albumin (Gibco). Cells were incubated with specific antibodies for an hour. After incubation, cells were washed and suspended in 500 µl PBS. Samples were then acquired on the FACS analyzer. The data analysis was performed by using Cell Quest Pro software (Supplementary Figure 2).

2.7. Tri-lineage differentiation

2.7.1. Osteogenic differentiation

MSCs from all four sources were seeded in 6-well plates at the density of 1×10^4 cells/well in triplicates for control and osteogenic differentiation. Cells after reaching 80% confluency were used for induction. For osteogenic induction, medium consisted of MEM-α supplemented with 1% FBS and 1% antibiotic-antimycotic solution, 10 nM dexamethasone, 0.1 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate solution. The culture medium was replaced every 3 days for the next 21 days post-induction. Later, osteogenic differentiation was confirmed using alizarin red staining.

2.7.1.1. Alizarin red staining

After 21 days, the culture medium from the 6-well plate was removed, and the cells were rinsed gently with PBS. Further, the cells were fixed using 4% paraformaldehyde for 30 min at room temperature, followed by gently rinsing with distilled water. For alizarin red staining, 2% solution of alizarin red (Ph 4.1, Sigma, Cat A5533) were prepared and filtered using a 0.22 µm membrane filter. Further, 1 ml of freshly prepared stain was added into the 6-well plate that was incubated at room temperature for an hour. After incubation was complete, the stain was removed and the cells were gently washed. The cells were observed under the microscope (OLYMPUS Phase Contrast Microscope) [13].

2.7.2. Chondrogenic differentiation

Once cells are 80–90% confluent, they were induced for chondrogenesis. Cells cultured in complete MEM-α were used as control. Chondrogenic differentiation medium was comprised of, sodium pyruvate (1 mM), dexamethasone (100 nM), ascorbate-2-phosphate (50 mg/ml), TGF-β (310 ng/ml), and L-proline (40 mg/ml). The culture medium was replaced every 3 days for the next 21 days. Alcian blue staining was performed to confirm the chondrogenic differentiation.

2.7.2.1. Alcian blue staining

The culture medium was discarded and cells were washed gently using PBS. Then, the cells were fixed using 4% paraformaldehyde for 30 min at room temperature. Alcian blue solution (1%) was prepared in 0.1N HCL and cells were stained cells for an hour. After staining, cells were washed with distilled water and visualized under a phase contrast microscope [14].

2.7.3. Adipogenic differentiation

MSCs from all four sources were differentiated into adipocytes using induction medium which consisted of dexamethasone (100 nM), indomethacin (0.25 μM), 3-isobutyl-1-methylxanthine (0.1 mM), and insulin (0.1 M). The induction medium was replaced every 3 days for the next 15 days. The adipogenesis was confirmed using oil red o staining.

2.7.3.1. Oil red O staining

Oil red o stain (30 mg) was dissolved in 100 ml of isopropanol to prepare stock solution. Working solution of oil o red was prepared by mixing three parts of stock of oil o red with two parts of distilled water and filtered. After 15 days, cells were washed with PBS and fixed with 4% paraformaldehyde. Further, 500 μl/ml working solution of oil red o stain was added to each well and incubated for 1 h at room temperature. After incubation, the cells were rinsed with distilled water and observed under an OLYMPUS phase contrast microscope [13].

2.8. Conditioned medium (CM) preparation

To collect CM, DPSC, SHED, GMSC, and BFMSCs were separately seeded into the T75 cell culture flask at the density of 1×10^4 cells. Further, cells were allowed to achieve 80–90% confluency. Then, the culture medium was removed, and cells were washed with sterile PBS to remove the serum content inside the flask. Furthermore, fresh MEM-α supplemented with 1% antibiotic-antimycotic solution without serum was added to the respective culture flask and incubated for 48 h. Post-incubation and culture medium from all the flasks were filtered through 0.22 μm syringe filters and stored at –80°C until further analysis.

2.9. Growth factor analysis

Growth factors in the CM collected from four sources were analyzed using LEGEND plex Multi-analyte flow assay kit (Human Growth Factor Panel (13-plex); Biolegend, San Diego, CA, USA; Cat. No. 740180), as per the manufacturer's instruction. The growth factors assessed included Angiopoietin (ANG), FGF, Hepatocyte Growth Factor (HGF), PDGF-AA, PDGF-BB, Granulocyte-Colony Stimulating Factor (G-CSF), TGF-α (TGF-α), and VEGF. The LEGEND plex multi-analyte flow assay is a bead-based immunoassay. At first, 25 μl of CM from all four sources were incubated for 2 h with the micro beads. Further, the detection antibodies were added and incubated for 30 min. Then, samples were rinsed with a buffer solution after incubation. The samples were then centrifuged at 2000 rpm for 5 min to recover the pellet. The pellet was then reconstituted in

200 μl of sheath fluid. The samples were acquired using a flow cytometer (Attune NxT, Thermo Fisher Scientific, Waltham, MA, USA). The data were analyzed using LEGEND plex Data Analysis Software (BioLegend, San Diego, CA, USA) [15].

2.10. Yolk sac membrane (YSM) assay for functional assessment of conditioned medium

To assess the functionality of the conditioned medium from various sources, YSM assay was employed as described previously [16]. Day 0 chick eggs were obtained from the Venkateshwara Hatcheries Pvt. Ltd., Pune (India) and incubated in the humidified incubator for 48 h at 37°C. After incubation, a small hole was created at the eggs' blunt end, and approximately 3–4 ml of albumin was removed. Further, 200 μl of conditioned medium obtained from the DPSC, SHED, GMSC, and BFMSCs were added at the top of the YSM. Culture medium without preconditioning with MSCs was kept as control. The opening was sealed with transparent tape and eggs were incubated in the humidified incubator for 24h. Pictures of the blood vessel sprouting were taken and quantitatively analyzed using the Wim CAM online tool (Wimasis) for the measurement of total vessel network length, vessel density, and total segments.

2.11. Statistical analysis

The comparative evaluation of the expression of growth factors in various MSCs-CM was done using the one-way ANOVA- *post hoc*-Tukey HSD test. The analysis was performed in IMB SPSS statistics version 20 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation of MSCs

The isolated MSCs from all four sources showed spindle-shaped morphology under the microscope (Figure 1).

3.2. Surface marker characterization

Immunophenotypic characteristics were assessed to analyze the expression of CD markers using flow cytometry. The MSCs derived from all oral cavity sources showed positive expression of CD90, CD105, and CD73 whereas negative expression of CD34, CD45, and HLA-DR (Figures 2-5).

3.3. In vitro tri-lineage differentiation

MSCs derived from all sources were characterized for their tri-lineage differentiation potential. Alizarin red staining exhibited accumulation of matrix mineralization and calcium deposits. Whereas, undifferentiated cells did not show matrix mineralization and calcium deposits. MSCs that differentiated into chondrocytes were evidenced by blue color deposition, revealing extracellular matrix proteoglycan staining. In adipogenic differentiation of MSCs, intracellular deposition of lipid droplets was seen as evidenced by oil red o staining (Figure 6A). Tri-lineage differentiation of MSCs isolated from oral sources and

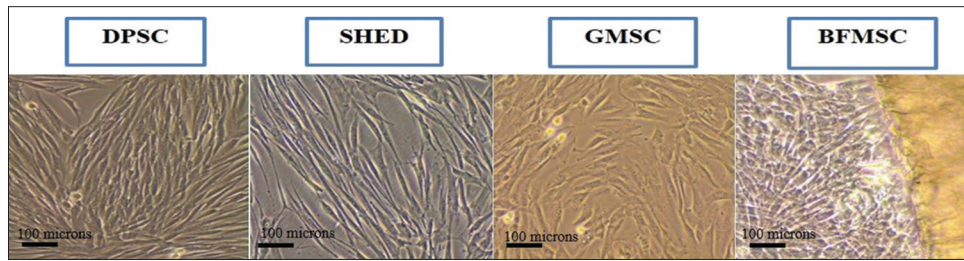


Figure 1. Isolated MSCs from dental pulp (DPSCs), tissue of sound permanent and deciduous teeth (SHED), healthy gingival (GMSCs), and buccal fat pad tissue (BFMSCs), respectively, showing spindle-shaped morphology under a phase-contrast microscope (magnification $\times 200$).

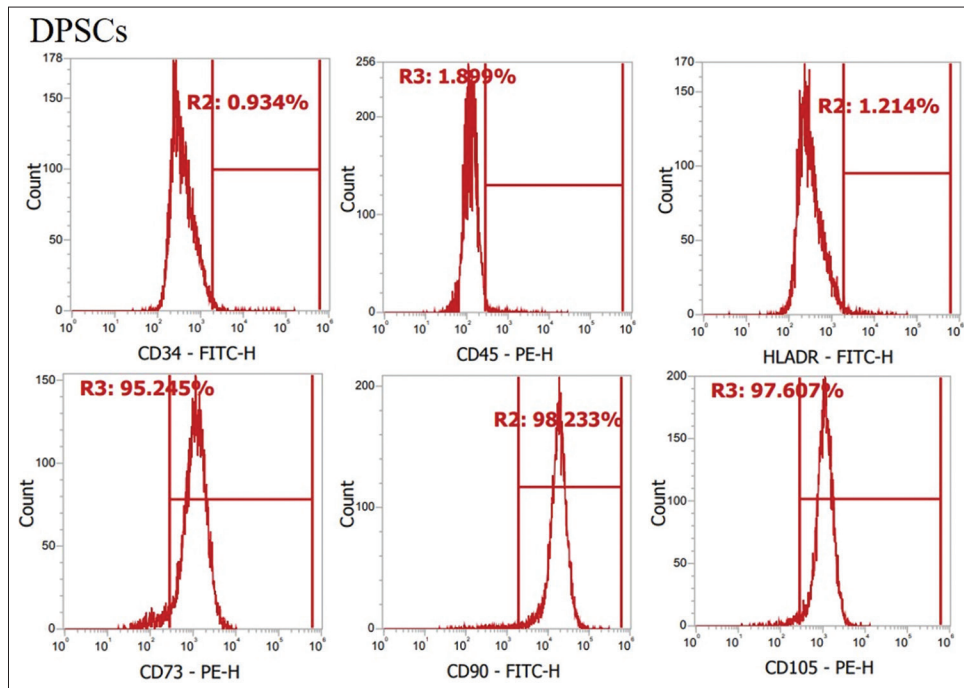


Figure 2. Immunophenotypic expression of cluster of differentiation (CD) by flow cytometry. MSCs derived from DPSCs showed positive expression of CD90 (98.23%), CD105 (98.8%), and CD73 (95.245%) and negative expression of CD34 (0.934%), CD45 (1.89%), and HLA-DR (1.21%).

stained with alizarin red, alcian blue, and oil red o, respectively, were quantitatively analyzed using Image J analysis software (Figure 6B) (Supplementary Figure 1).

3.4. Growth factor analysis of CM

Flow cytometry was used to compare the expression of pro-angiogenic growth factor levels to compare expression in each oral source of MSCs-CM. An intra-group comparison was performed between GMSCs-CM, SHED-CM, DPSCs-CM, and BFMSCs-CM. The concentration of expressed growth factors was compared to the published literature [17]. DPSC-CM showed a significantly higher expression of FGF and HGF as compare to other sources. The highest expression of HGF was found in DPSC-CM ($P < 0.01$), followed by SHED-CM, BFMSC-CM, and GMSC-CM. Growth factors such as VEGF, TGF- α , PDGF, and angiopoietin were also expressed in significant concentration in DPSC-CM. VEGF and G-CSF were significantly higher in SHED-CM ($P < 0.01$). Levels of PDGF-AA and angiopoietin did not show a statistically significant difference in intra-group comparison. GMSC-CM and

BFMSC-CM showed a relatively higher expression of TGF- α compared to DPSC-CM ($P < 0.01$). Intra-group comparison is depicted in Figure 7.

3.5. Functional assessment of the conditioned medium using YSM assay

Followed by the analysis of the growth factors in the conditioned medium obtained from GMSCs-CM, SHED-CM, DPSCs-CM, and BFMSCs-CM, we investigated the pro-angiogenic potential of the conditioned medium in the YSM assay. Our results clearly showed that DPSCs-CM enhances blood vessel formation as compared to the GMSCs-CM, SHED-CM, and BFMSCs-CM. A quantitative assessment of angiogenesis revealed that DPSCs-CM significantly enhanced the total vessel network length, vessel density, and total segments. Although GMSCs-CM, SHED-CM, and BFMSCs-CM could enhance angiogenesis, the observed pro-angiogenic effect was higher in the DPSCs-CM, as revealed by the quantitative assessment of the YSM assay (Figure 8).

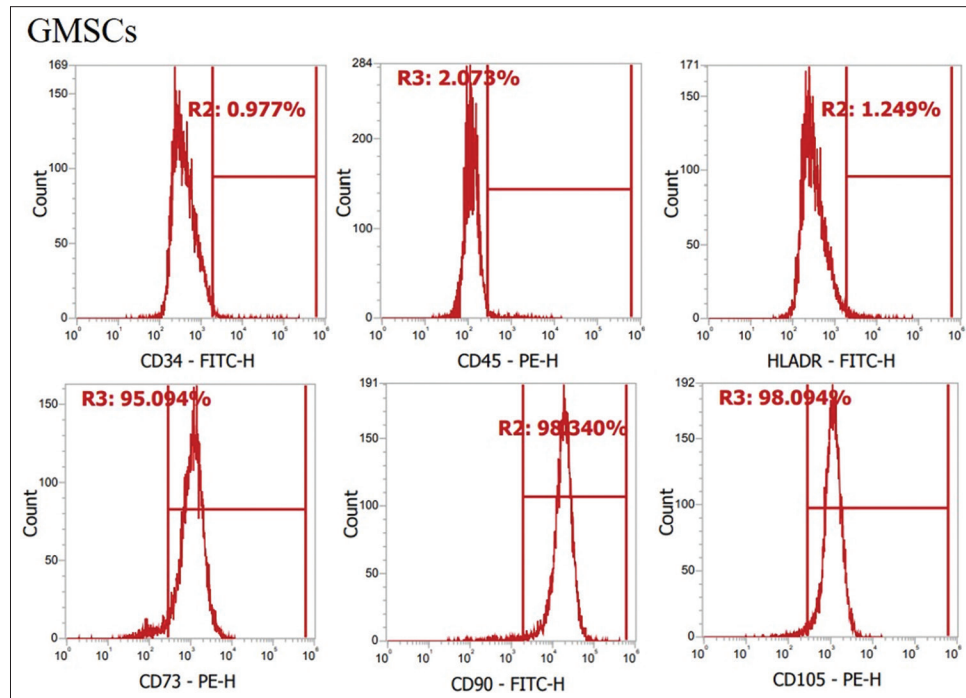


Figure 3. Immunophenotypic expression of cluster of differentiation (CD) by flow cytometry. GMSCs showed positive expression of CD90 (98.34%), CD105 (98.09%), and CD73 (95.09%) and negative expression of CD34 (0.977%), CD45 (2.073%), and HLA-DR (1.24%).

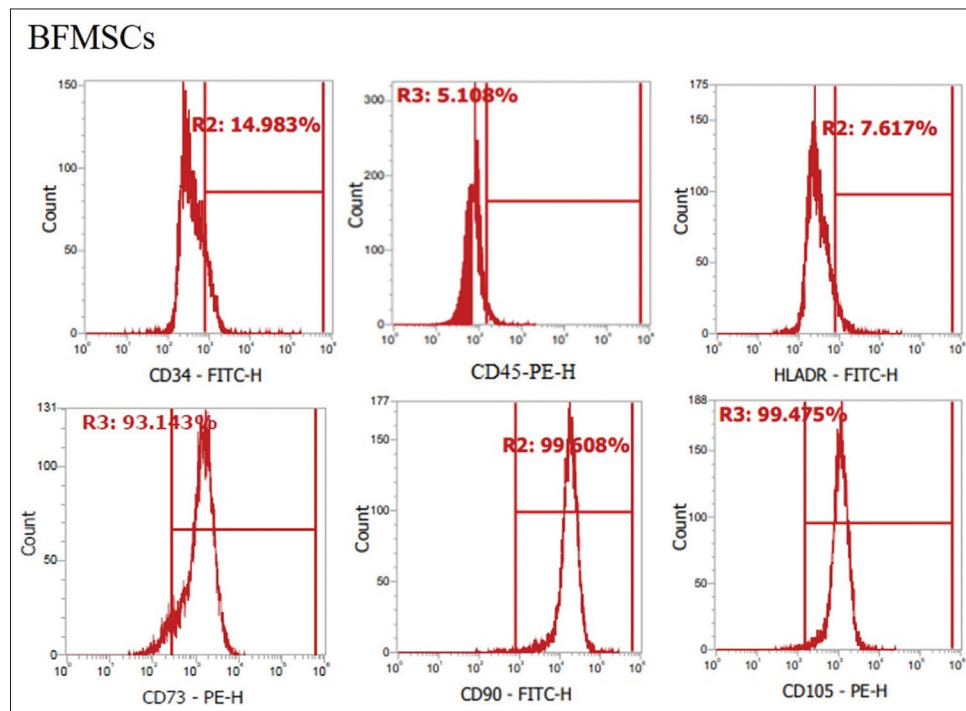


Figure 4. Immunophenotypic expression of cluster of differentiation (CD) by flow cytometry. BFMSCs showed positive expression of CD90 (99.5%), CD105 (99.47%), and CD73 (93.143%) and negative expression of CD34 (14.9%), CD45 (5.1%), and HLA-DR (7.6%).

4. Discussion

For over a decade, numerous studies have attempted to identify the actual role players of angiogenesis. At present, several studies

are aimed at establishing therapeutic angiogenesis. These include the administration of chemical components known as growth factors which have a central role in angiogenesis [18,19]. Angiogenesis is thus considered a fine balance between the inducers and the

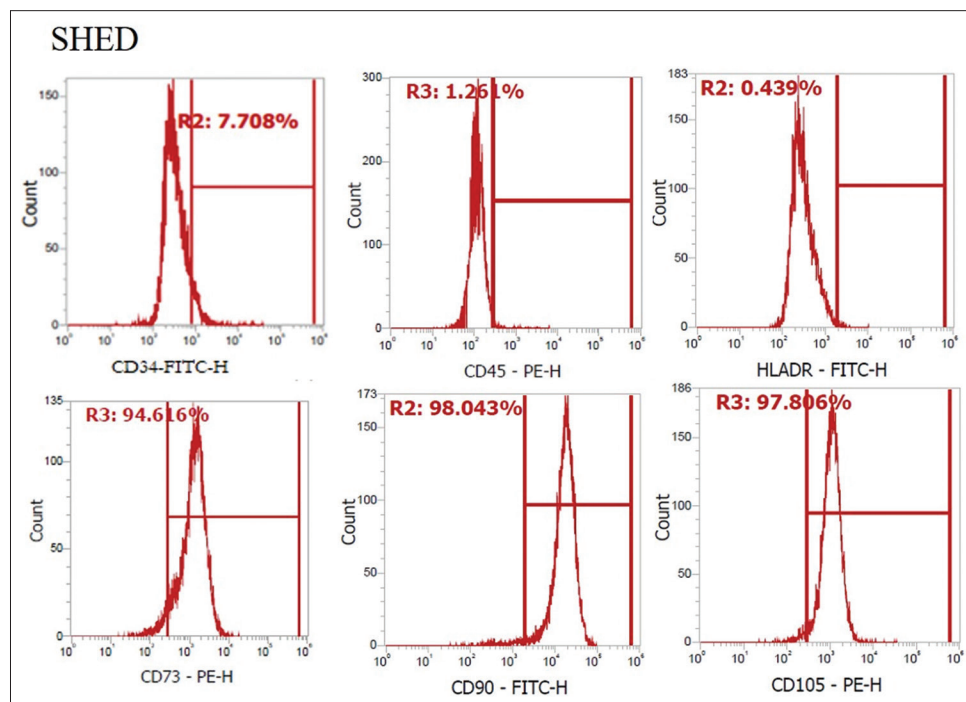


Figure 5. Immunophenotypic expression of cluster of differentiation 9CD by flow cytometry. SHED showed positive expression of CD90 (98.043%), CD105 (97.806%), and CD73 (94.616%) and negative expression of CD34 (7.7%), CD45 (1.26%), and HLA-DR (0.439%).

inhibitors of the angiogenesis [20]. The growth factors assessed in the present study are essential for angiogenesis and are considered significant targets to manipulate angiogenesis [21].

OC-MSCs have captured attention of researchers over the past decade [22]. The group of OC-MSCs includes (a) stem cells in the dental pulp, which are procured from both adult pulp tissue (DPSC) as well as stem cells derived from SHED, (b) GMSC derived in human gingival tissue, (c) BFMSC, (d) periodontal ligament derived MSCs, (e) apical papilla stem cells of incompletely developed tooth bud (SCAP), (f) dental follicle derived stem cells which are procured from third molars, and (g) MSCs can be procured from the BM of alveolar bone in the maxilla and mandible (BM-MSC) [23]. Altogether, OC-MSCs, when combined, could be fabricated into a powerful weapon to battle several diseases [24].

The field of regenerative medicine has accepted MSCs as promising potential candidate for therapeutic applications. MSC-CM can be used as a replacement tool instead of a cell-based therapeutic approach due to their paracrine potential [25]. The use of cell free therapy in regenerative medicine could help us overcome the possible limitations of cell-based therapy, of which graft rejection is the most prominent so far [26]. Numerous studies have investigated MSC-CM-derived growth factors and their potential in various physiologic and pathologic processes [24, 25, 27]. The present study focuses on pro-angiogenic counterparts in these growth factors. Levels of pro-angiogenic factors such as VEGF, FGF, and PDGF in the CM derived from OC-MSCs and their pro-angiogenic potential is studied. To date, no study have compared more than two sources of OC-MSCs for angiogenic

potential in the MSC-CM. The present study compared the quantitative expression level of different angiogenic growth factors in four different sources of OC-MSC-derived CM. Furthermore, functional assessment of angiogenic potential was tested using YSM model.

Growth factor analysis of CM derived from DPSC (DPSC-CM) was been carried out previously by Martínez *et al.* [26] who showed that DPSCs secreted pro-angiogenic growth factors such as VEGF, PDGF-BB, and HGF. They also studied the endothelial proliferation of DPSC *in vitro* and thus found an up regulation of expression of endothelial markers like von Willebrand Factor, thus concluding the angiogenic inclination of DPSC. Shen *et al.* [28] concluded in their study that CM derived from DPSCs that higher levels of expression of VEGF and PDGF-BB were evidenced compared to CM derived from other sources of MSCs. In the present study, VEGF, FGF, and HGF were expressed at significantly higher levels in DPSC-CM as compared to sources of OCMSCs. All these three factors share a significant contributory role in promoting angiogenesis right from endothelial cell recruitment to the maturation of blood vessels. In addition to the growth factor analysis, several studies have assessed the angiogenic potential of DPSC. In an *in vitro* assay carried out by Dissanayaka *et al.*, [29] DPSCs were found effective in forming vessel-like structures confirming the pro-angiogenic role of DPSCs.

DPSCs-CM has proven their potential in *in vivo* studies as well. It has been shown to establish marked neovascularization in an ischemic mice model [30-32]. Knowing the angiogenic potential of DPSC-CM due to their dynamic content of growth factors, *in vivo* models of pulp regeneration have been established

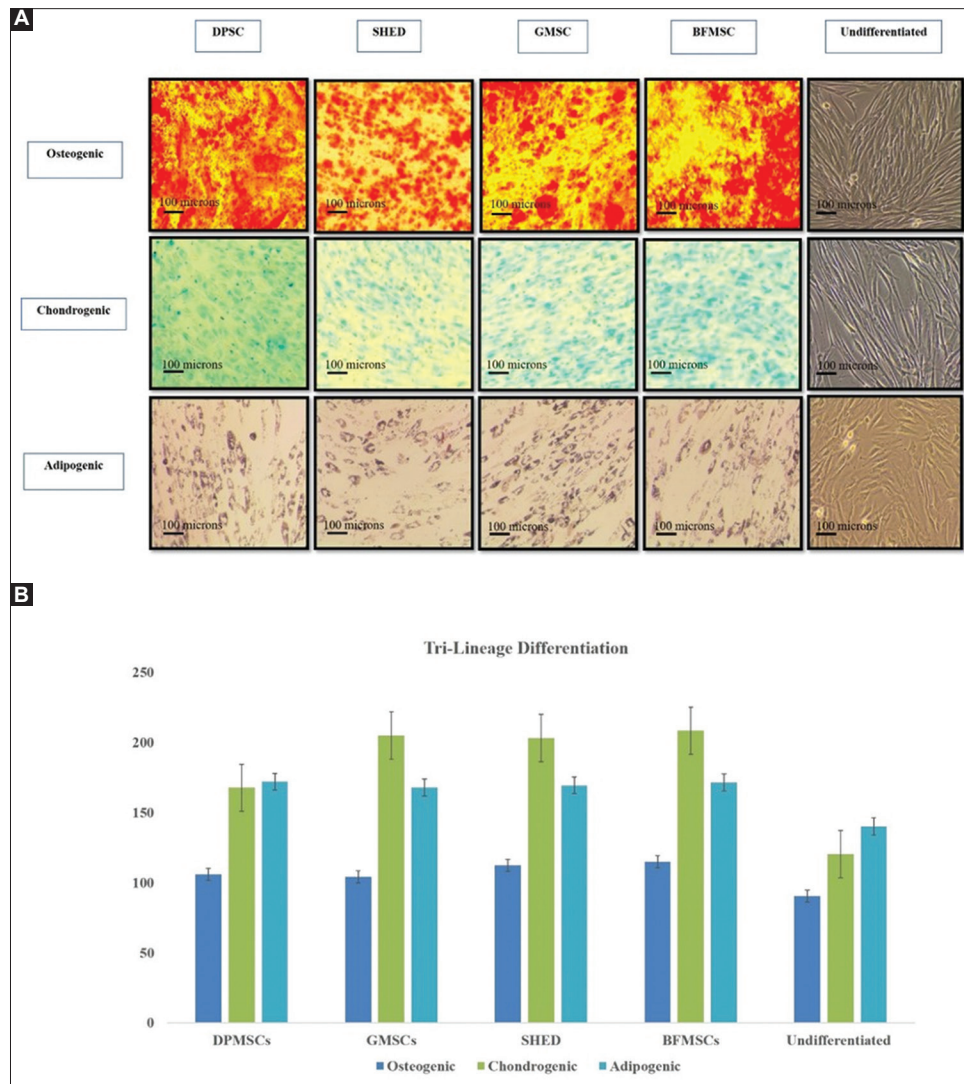


Figure 6. (A) *In-vitro* osteogenic, chondrogenic, and adipogenic differentiation of DPSCs, SHED, GMSCs, and BFMSCs. MSCs without induction were kept as control. MSCs derived from these oral sources were cultured *in vitro* under osteogenic, chondrogenic, and adipogenic induction conditions for 21, 18, and 15 days, respectively. Matrix mineralization is shown by alizarin red staining depicting osteogenic differentiation, and cartilage matrix is shown by alcian blue staining depicting chondrogenic differentiation. Further, lipid-containing adipocytes are shown by oil red o staining depicting adipogenic differentiation (magnification 200). (B) Quantitative assessment of *in-vitro* differentiation of DPSCs, SHED, GMSCs, and BFMSCs in osteogenic, chondrogenic, and adipogenic lineages when stained with alizarin red, alcian blue, and oil red o, respectively. Data analysis was performed using Image J analyzer. Data are presented as mean \pm SD. Error bars indicate SD.

where ectopic tooth transplantation or scaffold implantation was carried out [27,33,34]. With DPSC-CM having a greater levels of VEGF, FGF, and HGF, it drives the recruitment of endothelial cells, their proliferation, migration and maturation, and vessel-like morphogenesis, and ultimately its maturation to blood vessels be taken care of with just one source of MSCs.

SHED is a dynamic source of MSCs due to their higher proliferation potential, plasticity, and unique secretory profile. In this study, CM derived from SHED showed enhanced expression of VEGF, HGF, and G-CSF. This can be correlated to their function of cell proliferation, migration, and tube formation with the enhanced release of pro-angiogenic mediators. Our findings support the claim by Xu *et al.* [35], where expression

of pro-angiogenic growth factors such as VEGF that were highly expressed in SHED compared to the control group. Yet another study by Hiraki *et al.* [36] assessed various angiogenic growth factors in CM derived from SHED which concluded that CM is a better alternative in promoting angiogenesis than the stem cells. Interestingly, hypoxic environment is known to augment the angiogenic potential of SHED. It is also used as a perivascular source to form functional vessel-like structures when transplanted *in vivo* [37]. The overall features exhibited by CM derived from SHED can form immature vessel-like structures from the pre-existing vasculature. Thus, it can be a potent source for inducing angiogenes that is in the desired field [38].

Human gingival cells are blessed by a unique contribution from

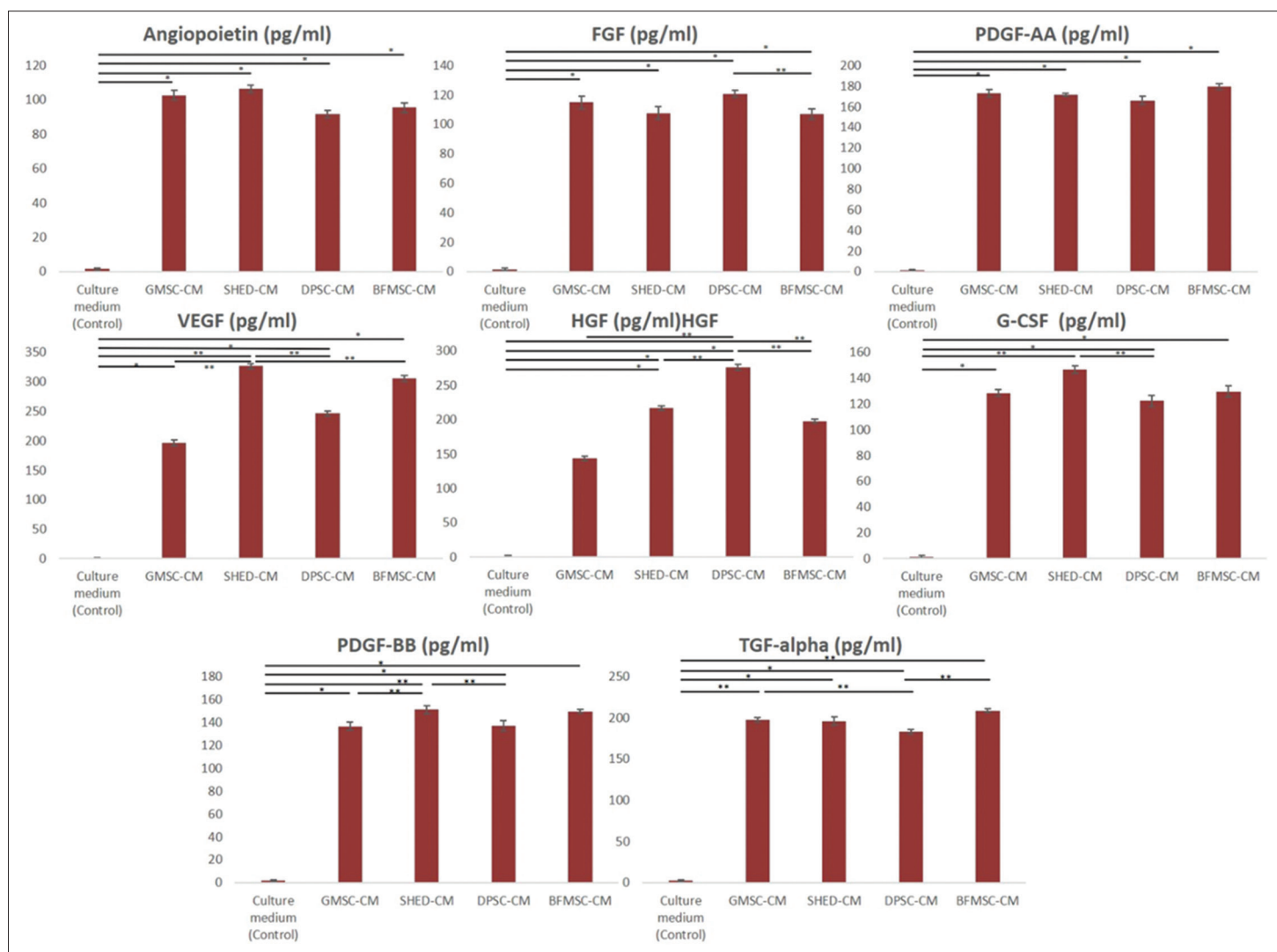


Figure 7. Assessment of levels of pro-angiogenic growth factors such as Angiopoietin (ANG), Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF), Platelet-derived Growth Factor (PDGF-AA, PDGF-BB), Granulocyte-Colony Stimulating Factor (G-CSF), Transforming Growth Factor-Alpha (TGF- α), and Vascular Endothelial Growth Factor (VEGF) in CM obtained from MSCs derived from GMSCs, SHED, DPSCs, and BFMSCs. Culture medium without preconditioning with MSCs was kept as control. Data shown are Mean \pm SD. Error bars show SD; * $P < 0.05$; ** $P < 0.01$.

the neural crest cells, the perifollicular mesenchyme and partly by the dental follicular cells. This unique origin of gingiva and its close approximation with the tooth has made GMSCs an exclusive candidate for stem cells [39]. Jin *et al.* [40] and Angelopoulos *et al.* [41] quantified the expression of angiogenic growth factors such as VEGF and HGF and found that their expression is significantly greater than the control group which is in agreement with our findings. In the present study, GMSC-CM showed significantly higher expression of HGF. HGF is known to enhance the morphogenesis of the capillary tube. Thus, GMSC-CM could assist in the morphogenesis of vessel formation. There is limited literature on angiogenic potential of GMSCs and, therefore, further studies are required to confirm its potential.

A novel route to isolate adipose-derived stem cells was introduced to the oral cavity in recent years. The fatty tissue mass in the vicinity of the oral cavity thus derived its name from the

native location as buccal fat pad-derived MSCs. It is also known as Bichat's fat pad [42]. MSCs derived from this source were later recognized as BFMSCs [43]. There is much evidence of studies conducted on the osteogenic potential of BFMSCs [44,45]. However, studies related to its angiogenic potential are rare. Furthermore, pro-angiogenic potential of BFMSCs have not been explored. The present study is the first to report the angiogenic potential of CM derived from BFMSCs. BFMSC-CM showed presence of VEGF, HGF, and PDGF, thus, suggesting its potential use in cell proliferation, migration, and tube formation. In addition, it recruits smooth muscle cells for the maturation of the formed vessels. There is no supporting literature regarding the angiogenic potential of BFMSC-CM, but this aspect can be further explored to showcase their angiogenic potential.

To further verify results, we performed a functional assessment assay on the YSM of a chick embryo. In ovo YSM model has

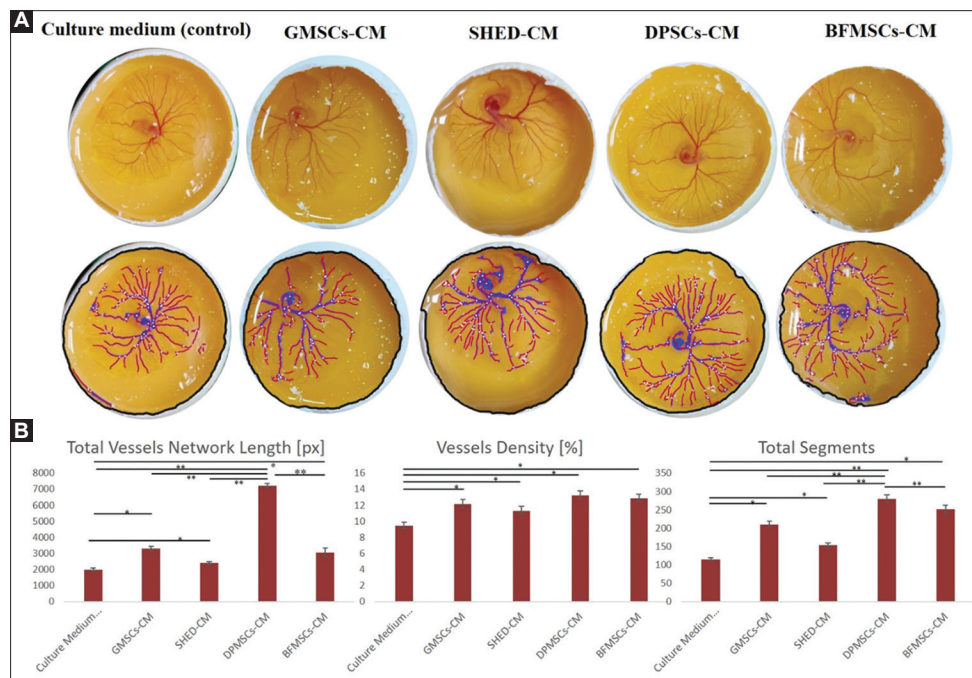


Figure 8. (A) Assessment of the angiogenic potential of GMSCs-CM, SHED-CM, DPSCs-CM, and BFMSCs; culture medium without preconditioning with MSCs was kept as control; (B) Original and analyzed images of the yolk sac membrane assay with Wim CAM online tool (Wimasis) for quantitative assessment of the angiogenic potential in terms of total vessels network length, vessels density, and total segments; Data shown are Mean \pm SD (* $P < 0.05$, ** $P < 0.001$).

been used to screen angiogenic, anti-angiogenic agents, and various toxicological studies [46]. In the present study, we used the YSM model to screen the best MSC-CM source from four different oral sources. We measured three different parameters to evaluate the most potential source for augmenting angiogenesis. Our data reveal an increase in total vessel network length, vessel density, and total segments on YSM assay when supplemented with DPSC-CM, which was significantly higher than rest of the OC-MSCs. With enough literature supporting the more substantial angiogenic potential of DPSCs and DPSC-CM, our study indicates that DPSC serves as a significant source of stem cells augmenting angiogenesis both *in vitro* and *in ovo*.

Conditioned medium has been reported for its therapeutic potential in clinical trials as well. Reports have been found where scaffolds soaked in conditioned medium derived from BM MSCs when transplanted during maxillary sinus lift procedures augmented bone regeneration. There have been no safety concerns and there were no local or systemic problems following the engraftment [47]. Another clinical study focused on bone regeneration and angiogenesis during maxillary sinus lift procedures. Promising results were encountered when CM derived from BM MSCs was used [48]. The use of CM derived from umbilical cord MSCs provided therapeutic effects on patients with atopic dermatitis. Furthermore, CM influences cutaneous immune response by regulating Th2 and B cells [49]. A split-face clinical assessment of embryonic cell-derived CM was conducted on 42 participants who had ablative and non-ablative laser operations. The findings of this clinical trial support the use of soluble

embryonic cell-derived CM to speed up the healing of wounds following laser resurfacing operations. CM encouraged faster, scarless wound healing, and more typical skin recovery [50]. Thus, the present study emphasized the use of CM as a source of cell free cultured medium with excellent clinical and translational value.

5. Conclusion

The critical drivers of angiogenesis are VEGF, FGF, and HGF, which help in overall angiogenesis, beginning from endothelial cell induction to blood vessel maturation. Among all the four sources assessed in this study, DPSC-CM shows the maximum presence of the critical factors for angiogenesis, thus proving itself as one of the best sources for angiogenic potential. YSM assay performed in the present study further proves that findings from growth factor analysis hold translational value. SHED-CM consists of HGF, VEGF, and G-CSF in equally more significant amounts; thus, it can assist in angiogenesis, but there is a lack of factors responsible for vessel maturation. GMSC-CM showed presence of higher levels of HGF. Although, further studies are required to explore the benefits of GMSC-CM. A novel source of CM derived from BFMSCs was used to assess its potential for angiogenesis, which exhibited significant quantities of VEGF, HGF, and PDGF; these factors can enhance angiogenesis at the desired site. Thus, the present study shows that CM derived from OC-MSCs has a dynamic and influential role in angiogenesis. Thus, CM can be further used alone or in conjunction with other graft materials or combinations, to promote the angiogenesis at the desired site of action.

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Conflict of Interest

All authors declare no conflict of interest.

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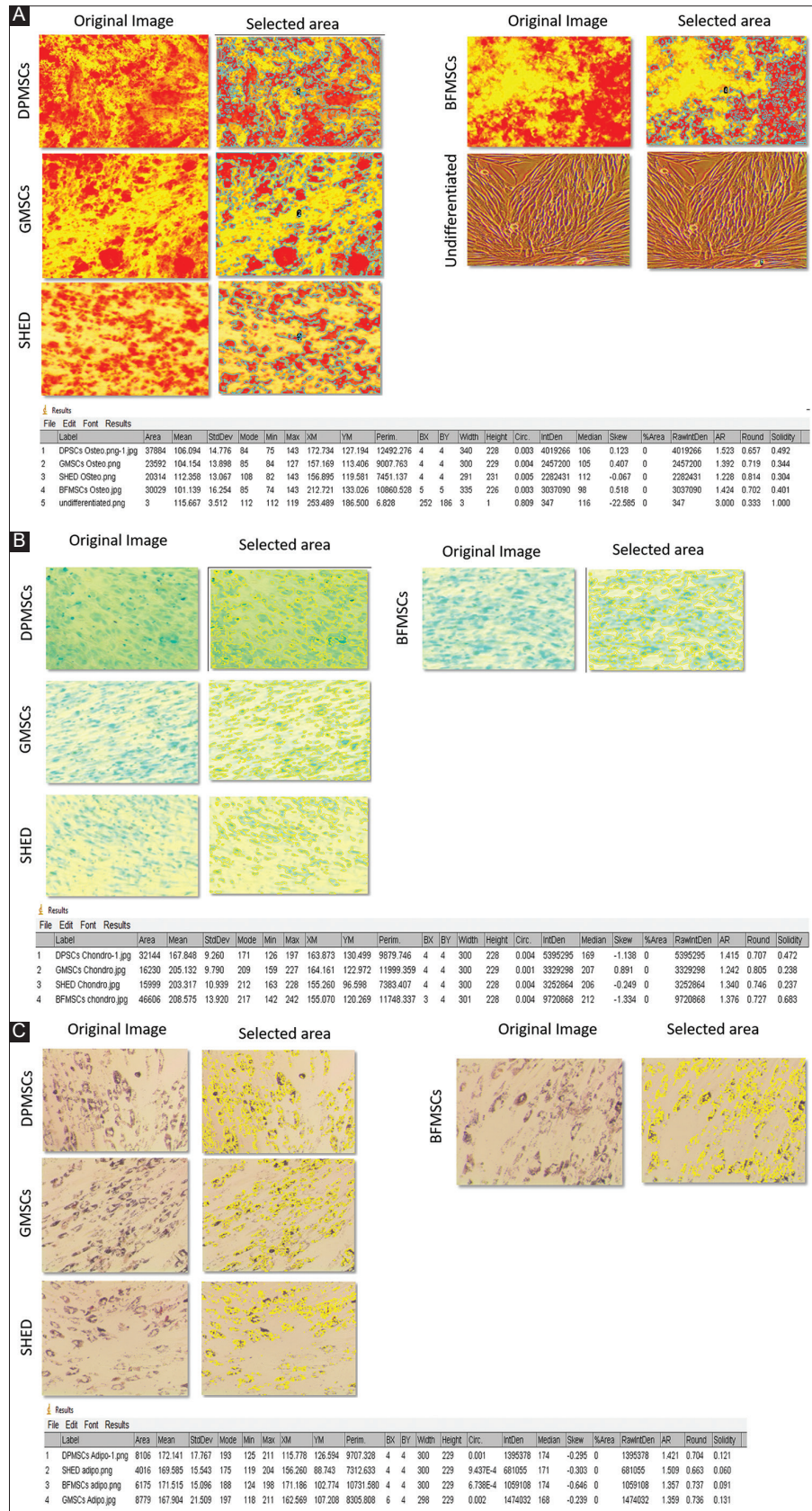
Assessment of angiogenic potential of mesenchymal stem cells derived conditioned medium from various oral sources

Supplementary File

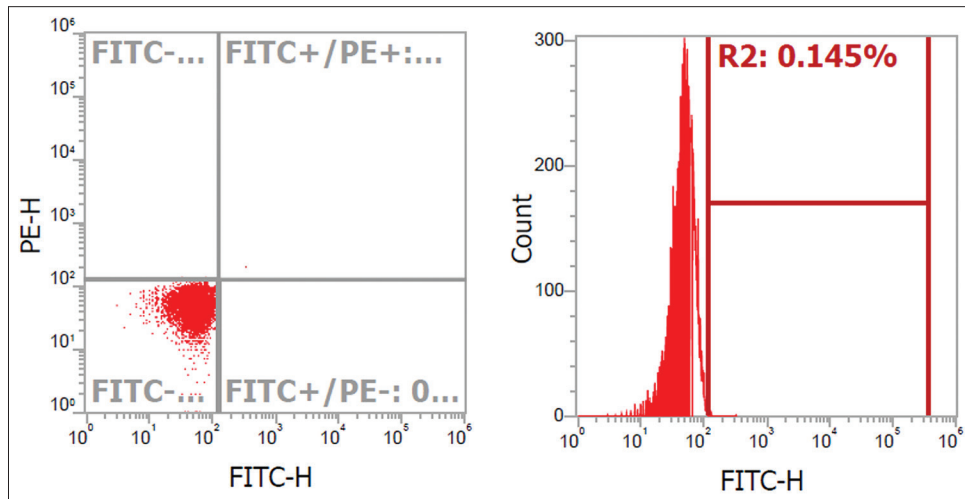
Supplementary Table 1. Demographic details of the donors.

Source of MSCs	Number of samples	Age of the donor Mean±S.D.	Gender of the donor	Tooth number
DPMSC	N=6	22±2 years	Males=3 Females=3	Maxillary/Mandibular third molars
SHED	N=6	10±2 years	Females=3 Males=3	Maxillary/Mandibular deciduous molars
GMSC	N=6	20±2 years	Males=3 Females=3	Not applicable
BFMSC	N=6	23±2 years	Males=3 Females=3	Not applicable

DPMSCs: Dental pulp mesenchymal stem cells, GMSCs: Gingival mesenchymal stem cells, BFMSC: Buccal fat mesenchymal stem cells, SHED: Exfoliated deciduous teeth



Supplementary Figure 1. Image J analysis of Tri-lineage differentiation of dental pulp mesenchymal stem cells, exfoliated deciduous teeth, gingival mesenchymal stem cells, and buccal fat mesenchymal stem cells. (A) Osteogenic differentiation. (B) Chondrogenic differentiation. (C) Adipogenic differentiation.



Supplementary Figure 2. Mouse IgG-PE isotype control was used for the characterization of mesenchymal stem cells from various sources.