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Enhancing angiogenesis through secretomes: Insights from scratch wound assay

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ABSTRACT

Background: This study uses a scratch wound healing assay to investigate the angiogenic potential of secretomes derived from dental mesenchymal stem cells (DMSCs). Angiogenesis, defined as the process of generating new blood vessels, plays a pivotal role in the mechanisms of tissue regeneration and repair.
Methodology: We cultured DMSCs under standard conditions, collected the secretomes, and applied them to human endothelial cells cultured in a scratch-assay setup. Endothelial cell migration into the scratch area was observed and quantified over a 48-h period. Results were compared to controls treated with a standard growth medium.
Results: Preliminary findings indicate that DMSC-derived secretomes of dental pulp significantly enhance the migration of endothelial cells compared to controls, suggesting a strong angiogenic potential.
Conclusion: These findings show that DMSC secretomes from dental pulp can help with angiogenesis. This could have big effects on the development of new treatments for tissue repair and regeneration. This study provides valuable insights into the paracrine mechanisms through which DMSCs may contribute to angiogenesis, highlighting their potential in regenerative medicine applications.

1. Introduction

The physiological process of angiogenesis, which creates new blood vessels from pre-existing ones, is essential for tissue regeneration and repair. This process is especially critical in response to injuries and regenerative medicine applications. Among various sources of stem cells, dental mesenchymal stem cells (DMSCs) have attracted significant attention due to their promising regenerative capabilities and ease of harvest.[1] DMSCs sourced from dental tissues including dental pulp, periodontal ligament, and gingiva, exhibit potential in influencing wound healing processes, in part due to their secretomes. These secretomes are complex mixtures of proteins, lipids, and nucleic acids, including crucial signaling molecules like growth factors and cytokines, which are believed to play pivotal roles in angiogenesis.[2,3]

Dental pulp stem cells (DPSCs) exhibit remarkable proangiogenic potential, making them highly promising for clinical applications in pulp regeneration. They secrete a range of angiogenic factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), and can differentiate into pericyte-like cells that stabilize newly forming blood vessels.[4,5] This capacity to induce and support neovascularization is essential for regenerating functional pulp tissue. Furthermore, their combined angiogenic and neurogenic properties enhance their therapeutic utility not only in regenerative endodontics but also in treating ischemic diseases, offering a novel approach to stem cell-based regenerative medicine.[6]

The scratch wound healing assay is a robust *in vitro* model to mimic the cell migration and proliferation necessary for angiogenesis. It allows researchers to visualize and quantify the effects of DMSC-derived secretomes on endothelial cells, the linchpins of angiogenesis. By creating a "wound" or gap in a confluent cell monolayer, the assay simulates a simplified version of the cell migration and proliferation that

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occur during angiogenesis *in vivo*.[7] In this study, we employ this assay to investigate how DMSC secretomes influence cell behaviors critical to angiogenic processes. We hypothesize that these secretomes can significantly enhance the angiogenic capabilities of endothelial cells, thereby supporting the use of DMSCs in therapeutic angiogenesis. This could have profound implications for developing advanced treatments for various conditions, including chronic wounds, cardiovascular diseases, and tissue engineering applications.[8,9] By delving into the mechanistic aspects of how DMSC-derived secretomes promote endothelial cell function, this study aims to contribute valuable insights into the molecular underpinnings of stem cell-mediated angiogenesis. Moreover, it explores the potential for developing novel DMSC-based therapies that harness the natural regenerative processes of the body to enhance tissue repair and recovery.

Collective cell migration is a defining aspect of various biological processes, including wound healing, cancer metastasis, immune responses, angiogenesis, and embryonic development. The process of wound healing is a complex interplay of cellular and biochemical mechanisms crucial for the restoration of structurally damaged tissue. The process entails dynamic interactions and communication among various cell types, engagement with molecules in the extracellular matrix, and regulated synthesis of soluble mediators and cytokines. In cutaneous wound healing, skin cells migrate from the wound margins into the wound area to facilitate repair and restore skin integrity. *In vitro* analysis of cell migration is essential for quantifying alterations in cell migratory capacity following experimental interventions.[10] Cell migration is the physical movement of single cells, cell sheets, and clusters from one location to another. The word “cell motility" is frequently used interchangeably, but it may properly indicate a less structured and deliberate movement of cells.[11] Investigating the coordinated movement of cells in a two-dimensional confluent monolayer under controlled laboratory settings enables researchers to recreate and examine essential processes involved. An alternative approach, which monitors the movement of individual cells, has been documented in scientific literature.[12] While there is debate on whether the assay can accurately simulate a real, inherently more intricate wound, it enables modeling and examining cell movement under precisely specified circumstances.[13] The procedure involves producing a linear and narrow scratch in a continuous cell layer, resulting in a gap. Photographs of the cells filling the gap are captured at regular intervals and analyzed to measure the extent of movement.[14]

2. Methodology

The current research protocol was approved by the Institutional Ethics Review Committee of Dr. D. Y. Patil Vidyapeeth, Pune, India (Reference number: DYPV/EC/566/2020). Prior to specimen collection, patients were apprised of the study objectives concerning their biological samples, and their informed permission was secured.

2.1. Isolation of stem cells

This investigation utilized four distinct sources of tissues for the isolation of mesenchymal stem cells (MSCs). Permanent dentition: Healthy teeth removed from impacted third molars to obtain dental pulp mesenchymal stem cells (DPSCs). Deciduous teeth: Sound teeth that have been extracted and exfoliated are collected via normal dental operations in order to isolate stem cells from exfoliated deciduous teeth (SHED). Gingiva: Healthy gingival tissue was surgically removed during the crown lengthening surgery to extract gingival mesenchymal stem cells (GMSCs). Buccal fat pad: During flap procedures, healthy buccal adipose tissue is removed in order to collect buccal fat mesenchymal stem cells. To isolate dental pulp stem cells (DPSCs), entire third molars were taken during normal dental extractions from otherwise healthy adults aged 18 to 25.

To isolate SHED, samples of sound deciduous teeth were collected from healthy young patients aged between 6 and 15 years during standard dental procedures. To isolate GMSCs, samples of healthy gingiva were collected from patients within the age range of 18–25 years who underwent crown lengthening procedures. Healthy adipose tissue was taken from the buccal area of individuals aged 18–25 years who had flap operations to isolate BFMSCs. The selected donors had no relevant medical history and were generally in good health. Following the surgical extraction or tissue excision from the patient, the specimen was initially immersed in a povidone-iodine solution. Subsequently, it was immersed in sterile saline solution and then transferred to a falcon tube containing phosphate buffered saline (PBS) (Gibco, pH 7.4) augmented with 1 % antibiotic-antimycotic solution (CELL clone). The falcon carrying the sample was thereafter preserved at 4 °C until processing occurred. The obtained specimen was generally processed within 24 h after its acquisition.[15]

2.2. Tissue processing

In the procedure for processing both permanent and deciduous teeth through pulp extirpation, an air-rotor handpiece equipped with straight fissure burs was employed to meticulously drill into the tooth, facilitating the extraction of the pulp tissue. Subsequent to the initial drilling procedure, the dental architecture was accessed via the utilization of an elevator instrument. The pulp specimen was strategically placed in a sterile petri dish containing phosphate-buffered saline (PBS) to facilitate the forthcoming experimental protocols. The gingival and buccal pad of fat tissue samples also underwent a disinfection process and were subsequently employed for explant culture.[15]

2.3. Explant culture for MSCs isolation

Mesenchymal stem cells (MSCs) derived from oral cavity mesenchymal stem cells (OC-MSCs) were isolated utilizing the explant method, following the established protocols in our laboratory.[15] The tissue samples were subjected to a comprehensive washing procedure using phosphate-buffered saline (PBS) performed 3 to 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. After disinfection, the tissue was sectioned into small fragments, approximately 1–2 mm in size, using a sterile surgical blade. The sections were subjected to a rewash procedure using PBS and then transferred to a sterile petri dish for further analysis. Tissue sections were immersed in fetal bovine serum (FBS) containing a 1 % antibiotic-antimycotic solution. The sections were subsequently transferred to the incubated T25 flask using 200 μl pipette tips. The flask was incubated for 24 h. Following the initial 24-h incubation period, 4–5 ml of Minimum Essential Medium Alpha (MEM-α) (Gibco), supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotic-antimycotic solution, was added. The flask was incubated for 4–6 days.[15]

2.4. Isolation of stem cells

All flasks were inspected using an inverted phase-contrast microscope (OLYMPUS) to measure cell outgrowth after six to seven days. After being refilled with freshly made complete medium, each flask was then put through incubation.[15]

2.5. Cell passaging

Cell outgrowth surrounding the tissue was observed, and trypsinization was performed upon reaching 70–80 % confluence. The trypsinization process involved the removal of the medium from the flask, followed by a wash with PBS to effectively eliminate residual FBS components. Four milliliters of trypsin (Gibco [0.25 %]) were added to the flask. After 1 min of incubation, the flask was examined under a microscope. To inhibit trypsin activity, an equivalent volume of

complete medium was added to the flask. The cell suspension was then transferred to a Falcon tube and centrifuged at 1800 rpm for 5 min. After the pellet containing cells was formed, the supernatant was discarded, and the pellet was resuspended in complete medium. The suspension was then transferred to a new sterile T25 flask and labeled as passage zero (P0). The medium composition of the flask was modified every two days. Once cellular growth reached 80–90 % confluence, the passaging process was performed again. The same methodology was consistently applied until the cells reached passage 4.[15]

2.6. Cell characterization

Mesenchymal stem cells (MSCs) obtained from various sources were subjected to characterization through the application of specific cell surface markers, utilizing flow cytometry as the analytical technique. The antibody panel included CD90, CD73, and CD105, with the latter being PE tagged. CD34, and HLADR, labeled with FITC, sourced from BD Biosciences. Four passages of cells were utilized for fluorescence-activated cell sorting (FACS) analysis. One hundred microliters of the cell suspension, with a concentration of 1 × 10ˆ6 cells/ml, were transferred into an Eppendorf tube. The cells were fixed with a 4 % paraformaldehyde solution for up to 30 min at room temperature. After fixation, the cells were washed with phosphate-buffered saline (PBS) containing 0.5 % bovine serum albumin (Gibco). Cells were incubated with specific antibodies for 1 h. After the incubation period, the cells underwent a washing process and were then resuspended in 500 μl of phosphate-buffered saline (PBS). Samples were collected using the FACS analyzer. Data analysis was performed using Cell Quest Pro software.[15]

2.7. Tri-lineage differentiation

2.7.1. Osteogenic differentiation

In 6-well plates, mesenchymal stem cells from all four sources were injected at a density of 1 × 10^4 cells per well. Triplicate arrangements were made for the evaluation of osteogenic differentiation and control. When cells reached 80 % confluency, they were used for induction. The culture medium used for osteogenic induction was made with MEM-α and supplemented with 1 % fetal bovine serum (FBS) and 1 % antibiotic-antimycotic solution. The medium also included 0.1 mM ascorbic acid-2-phosphate, 10 nM dexamethasone, and a 10 mM β-glycerophosphate solution. Over the course of 21 days after the induction phase, the culture medium was changed every three days. Following this, alizarin red staining was used to confirm the osteogenic differentiation process.[15]

2.7.2. Alizarin red staining

After 21 days, the culture medium from the 6-well plate was removed, and the cells were rinsed with phosphate-buffered saline (PBS). The cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. Temperature monitoring is essential, followed by a gentle rinse with distilled water. A 2 % solution of alizarin red (pH 4.1, Sigma, Cat A5533) was prepared and filtered through a 0.22 μm membrane filter. Furthermore, 1 ml of freshly prepared stain was added to the 6-well plate and incubated at room temperature for 1 h. Following the incubation period, the staining agent was removed, and the cells were subjected to a gentle washing procedure. The cellular structures were analyzed using an OLYMPUS Phase Contrast Microscope.[15,16]

2.7.3. Chondrogenic differentiation

After the cells reached 80–90 % confluence, they were inducted for chondrogenesis; the control group consisted of cells cultured in complete MEM-α. The chondrogenic differentiation medium was composed of sodium pyruvate at 1 mM, dexamethasone at 100 nM, ascorbate-2-phosphate at 50 mg/ml, TGF-β at 310 ng/ml, and L-proline at 40 mg/ml. The culture medium was changed every 3 days for 21 days, and the chondrogenic differentiation process was validated using Alcian blue staining.[15]

2.7.4. Alcian blue staining

The culture media was discarded, and the cells were meticulously washed with PBS. The cells were subsequently fixed with 4 % paraformaldehyde for 30 min at room temperature. Alcian blue solution at a concentration of 1 %. The solution was formulated in 0.1N HCl, and the cells underwent staining for 1 h. Following the staining method, the cells were washed with distilled water and then studied under a phase contrast microscope.[16,17]

2.8. Adipogenic differentiation

Mesenchymal stem cells (MSCs) derived from all four sources underwent differentiation into adipocytes utilizing an induction medium composed of dexamethasone (100 nM), indomethacin (0.25 mM), 3-isobutyl-1-methylxanthine (0.1 mM), and insulin (0.1 μM). This induction medium was systematically replaced every three days over a period of 15 days. The successful adipogenesis was validated through oil red O staining methodology.[15]

2.8.1. Oil red O staining

Oil Red O stain (30 mg) was dissolved in 100 mL of isopropanol to create a stock solution. Three parts of the stock oil-of-red solution were mixed with two parts of distilled water, a suitable solvent, and then filtered to create a functional solution. After 15 days, the cells were cleaned with phosphate-buffered saline (PBS) and then fixed with a 4 % paraformaldehyde solution. Each well was then filled with a 500 μl/ml working solution of Oil Red O stain, and it was left to incubate for an hour at room temperature. An OLYMPUS phase contrast microscope was used to observe the cells after they had been rinsed with distilled water after the incubation time.[15,16]

2.8.2. Conditioned medium (CM) preparation

Dental pulp stem cells (DPSC), human exfoliated deciduous tooth stem cells (SHED), gingival mesenchymal stem cells (GMSC), and buccal fat derived mesenchymal stem cells (BFMSCs) were all separately seeded into T75 cell culture flasks at a density of 1 × 10ˆ4 cells in order to gather conditioned media. To mitigate donor-associated heterogeneity, secretome preparations from three independent donors for each cell source were pooled prior to experimental use. This approach was adopted to ensure a more representative and consistent biological profile, thereby enhancing the reliability and generalizability of the observed effects. The cells were also allowed to achieve an 80–90 % confluence. Confluence. To successfully remove the serum components from the flask, the growth media was then removed and the cells were washed with sterile PBS. Furthermore, a new MEM-α without serum and supplemented with 1 % antibiotic-antimycotic solution was added to the designated culture flask and incubated for 48 h. All flasks' post-incubation and culture media were filtered using 0.22 μm syringe filters before being kept at −80 °C for further examination.[15]

2.8.3. Scratch wound healing assay

The endothelial cells used were HUVECs (Human Umbilical Vein Endothelial Cells), which were placed into a 6-well plate and grown until they reached 80 % confluence. A consistent-diameter strip was created using a 200-μL pipette tip across the middle of the well. Following the scratch, the cells were thoroughly rinsed with PBS to eliminate cell debris. The wells were divided into six groups, each with three wells, and the entire experiment was performed in triplicate.

The six groups were:

1. Negative control: treated with plain MEM-α
2. Positive control: treated with MEM-α and VEGF
3. DPSCs-CM: treated with secretome derived from DPSCs
4. SHED-CM: treated with secretome derived from SHED
5. GMSCs-CM: treated with secretome derived from GMSCs
6. BFMSCs-CM: treated with secretome derived from BFMSCs

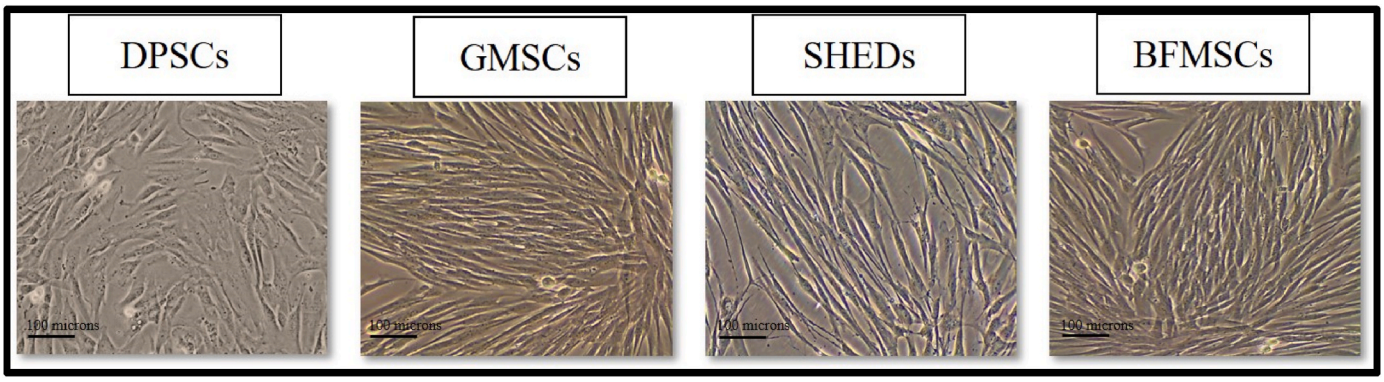

Fig. 1. Mesenchymal stem cells (MSCs) are derived from dental pulp stem cells (DPSCs), sound permanent and deciduous teeth (SHED), healthy gingival stem cells (GMSCs), and buccal fat pad tissue. At a magnification of × 200 using a phase-contrast microscope, the cells exhibited a spindle-shaped morphology.

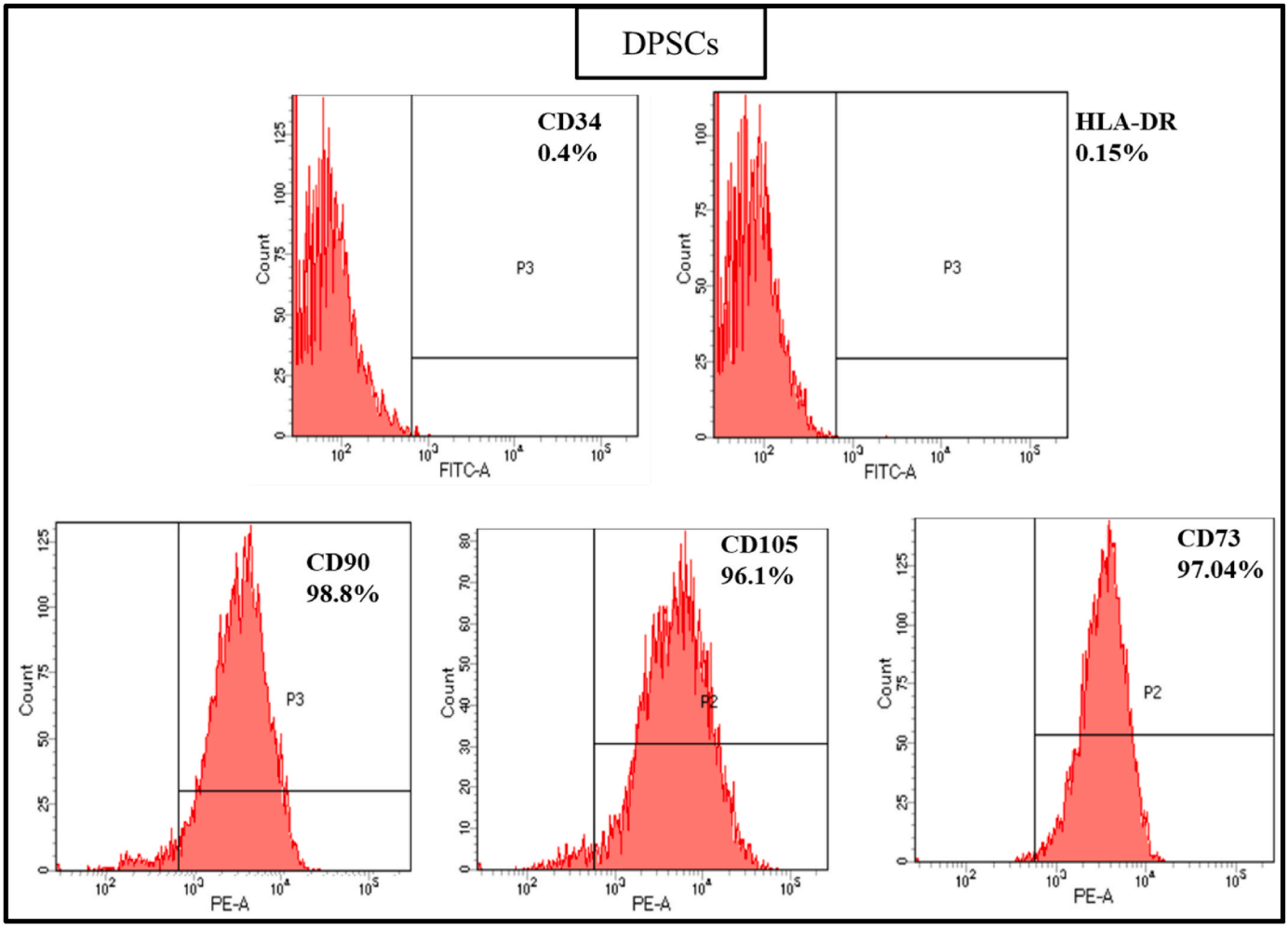

Fig. 2. The analysis of immunophenotypic expression of cluster of differentiation (CD) utilizing flow cytometry techniques. Mesenchymal stem cells (MSCs) obtained from dental pulp stem cells (DPSCs) exhibited a high positive expression of CD90 at 98.8 %, CD105 at 96.1 %, and CD73 at 97.04 %. Conversely, the expression levels of CD34, and HLA-DR were notably low, recorded at 0.4 %, and 0.15 %, respectively.

Two milliliters of each solution were added to the respective wells of each group and cultured for 48 h in a CO2 incubator. Photographs were taken at 0, 12, 24, and 48 h under an OLYMPUS phase contrast microscope. Images were obtained and measured using ImageJ software. Quantitative analysis was performed for cell migration using IBM SPSS Statistics version 20 software.

3. Results

3.1. Isolation of MSCs

The isolated MSCs exhibited a spindle-shaped morphology from all four sources when observed under the microscope (Fig. 1).

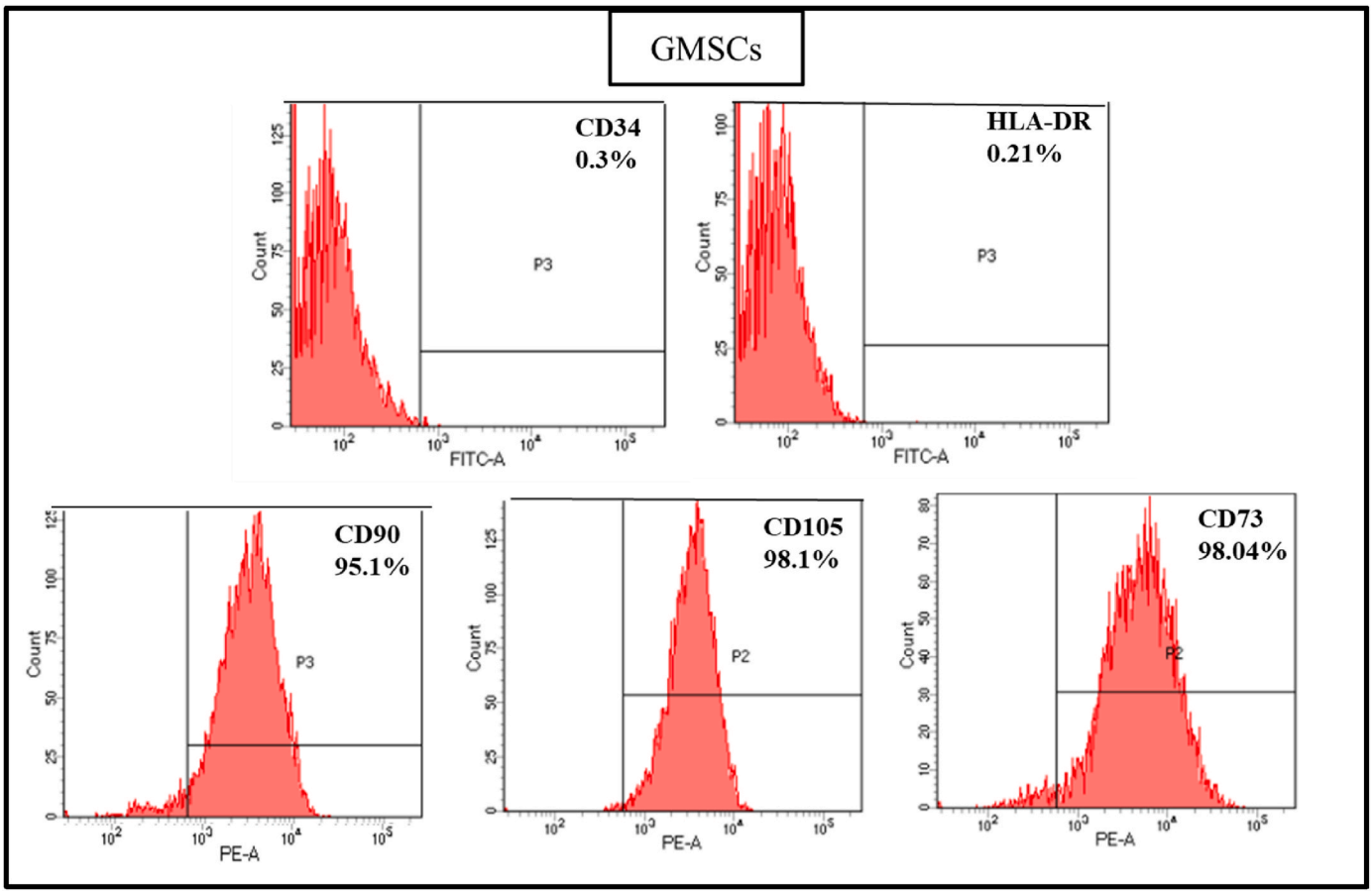

Fig. 3. The analysis of immunophenotypic expression of cluster of differentiation (CD) utilizing flow cytometry techniques. Mesenchymal stem cells (MSCs) obtained from Gingival mesenchymal stem cells (GMSCs) exhibited a high positive expression of CD90 at 95.1 %, CD105 at 98.1 %, and CD73 at 98.04 %. Conversely, the expression levels of CD34, and HLA-DR were notably low, recorded at 0.3 %, and 0.21 %, respectively.

3.2. Surface marker characterization

The immunophenotypic characteristics were evaluated to investigate the expression of CD markers through the application of flow cytometry techniques. The mesenchymal stem cells obtained from various sources within the oral cavity exhibited a positive expression profile for CD90, CD105, and CD73, while demonstrating negative expression for CD34, and HLA-DR (Figs. 2–5).

3.3. In vitro tri-lineage differentiation

Mesenchymal stem cells obtained from various sources were evaluated for their capacity to differentiate into three lineages. Alizarin red staining demonstrated the accumulation of matrix mineralization and calcium deposits. Undifferentiated cells exhibited no matrix mineralization or calcium deposits. The differentiation of MSCs into chondrocytes was indicated by blue color deposition, demonstrating extracellular matrix proteoglycan staining. During the adipogenic differentiation of mesenchymal stem cells, intracellular lipid droplet accumulation was observed, as demonstrated by Oil Red O staining (Fig. 6).

3.4. Scratch wound healing assay

Following the application of the pipette tip to induce wounds on the cellular monolayers, there were variations in the size of the resulting defects among the different groups. The area percentage, area pixel, width pixel, and standard deviation pixel were assessed quantitatively for comparison among various experimental groups for the increased cell migration ability under the effect of secretome derived from various oral sources. The comparison was made at 12 h, 24 h, and 48 h for each group (Fig. 7).

This study findings unequivocally show that conditioned media from dental pulp stem cells (DPMSCs-secretome) significantly promote cell migration compared to conditioned media from gingival mesenchymal stem cells (GMSCs-secretome), stem cells from human exfoliated deciduous teeth (SHED-secretome), and buccal fat derived mesenchymal stem cells (BFMSCs-secretome). Objective evaluation of the images showed that DPMSCs-secretome greatly increased the percentage of area, pixel area, pixel width, and pixel standard deviation. While the secretomes derived from GMSCs, SHED, and BFMSCs promoted cell migration, the wound-healing impact was more pronounced in DPMSCs-secretome, as demonstrated by the quantitative evaluation of the scratch wound healing experiment.

A one-way analysis of variance (ANOVA) was performed to evaluate the impact of secretomes derived from various dental mesenchymal stem cell (DMSC) sources on the migration of endothelial cells in the scratch wound assay. The analysis demonstrated a statistically significant difference among the groups ($p < 0.05$), suggesting that the origin of the secretome exerted a quantifiable influence on the angiogenic response. The post-hoc analysis employing Tukey's test provided additional evidence that the dental pulp stem cell (DPSC) group demonstrated a markedly enhanced degree of endothelial cell migration in comparison to the other groups. This indicates that secretomes derived from dental pulp stem cells exhibit enhanced proangiogenic capabilities, thereby supporting their potential utility in regenerative applications. (Fig. 8).

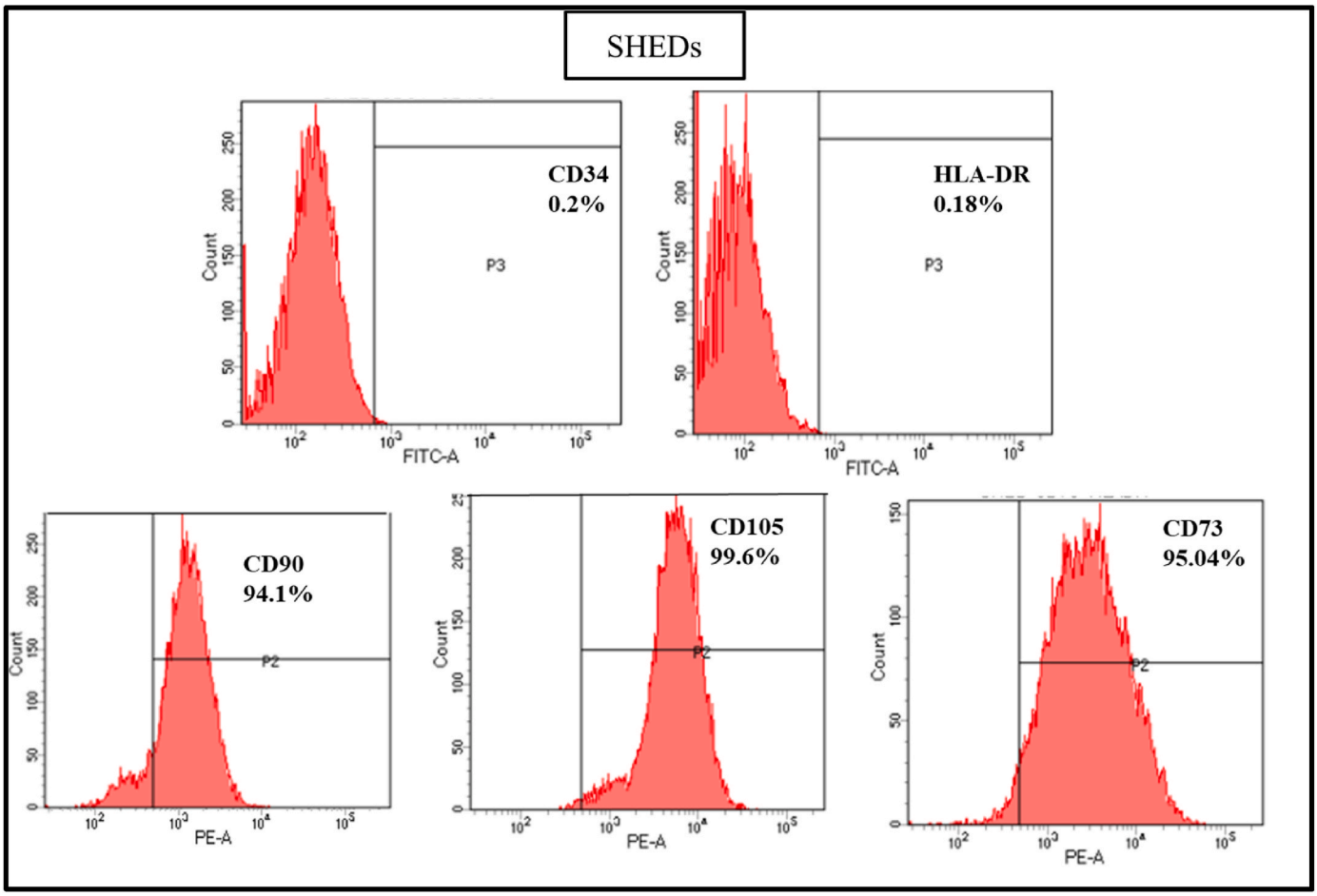

Fig. 4. The analysis of immunophenotypic expression of cluster of differentiation (CD) utilizing flow cytometry techniques. Mesenchymal stem cells (MSCs) obtained from Stem cells from human exfoliated deciduous teeth (SHED) exhibited a high positive expression of CD90 at 94.1 %, CD105 at 99.6 %, and CD73 at 95.04 %. Conversely, the expression levels of CD34, and HLA-DR were notably low, recorded at 0.2 %, and 0.18 %, respectively.

4. Discussion

The present investigation elucidates the angiogenic capabilities of secretomes derived from dental mesenchymal stem cells (DMSCs) through the application of an *in vitro* scratch wound assay, which serves as a recognized model for simulating cellular migration. Dental mesenchymal stem cells (DMSCs), especially those isolated from dental pulp, exhibit significant regenerative capabilities attributed to their capacity for secreting a diverse range of trophic factors. The influence of these secretomes on endothelial behavior is pivotal to the angiogenic cascade.

A substantial collection of research indicates that mesenchymal stem cells (MSCs) primarily promote tissue regeneration via their paracrine mechanisms, as opposed to direct differentiation or cellular integration. The secretomes derived from mesenchymal stem cells, which include soluble cytokines, chemokines, growth factors, and extracellular vesicles, play a pivotal role in the processes of angiogenesis, immunomodulation, and tissue repair. The paracrine hypothesis has gained substantial acceptance within the scientific community, supported by a multitude of studies that illustrate how mesenchymal stem cells (MSCs) facilitate their regenerative effects through the establishment of a bioactive environment that promotes cellular migration, proliferation, and survival.[18]

The findings of the present study indicate that secretomes obtained from dental pulp stem cells markedly improve the migration of endothelial cells across the scratch wound gap, thereby corroborating their proangiogenic properties. This observation supports the hypothesis that factors secreted by DMSCs have the potential to stimulate the migration and possibly the proliferation of endothelial cells, thereby replicating initial processes involved in neovascularization. The observed increase in migration during the scratch assay indicates that the secretome is likely composed of biologically active elements that can influence endothelial function.

Indeed, the composition of the secretome is significantly affected by the origin of mesenchymal stem cells (MSCs), the environmental stimuli present during culture, and various external conditioning factors. Comparative proteomic analyses have demonstrated notable differences in the angiogenic capabilities of MSC secretomes, which are influenced by their tissue of origin. For instance, the secretomes derived from Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) exhibit a more comprehensive and resilient angiogenic protein profile compared to those obtained from adipose or bone marrow sources. This encompasses increased concentrations of critical angiogenic mediators, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietin-1 (ANG-1), and transforming growth factor beta (TGF-β).[19] The current investigation centers on DMSCs; however, these results necessitate subsequent comparative proteomic analyses to determine the positioning of dental pulp-derived secretomes within the angiogenic hierarchy.

A noteworthy element of MSC secretomes is cysteine-rich protein 61 (Cyr61), classified within the CCN protein family. Cyr61 is critically involved in the processes of endothelial adhesion, proliferation, and the formation of tubular structures. A previous study elucidated the role of Cyr61 as a predominant proangiogenic factor within the secretomes of

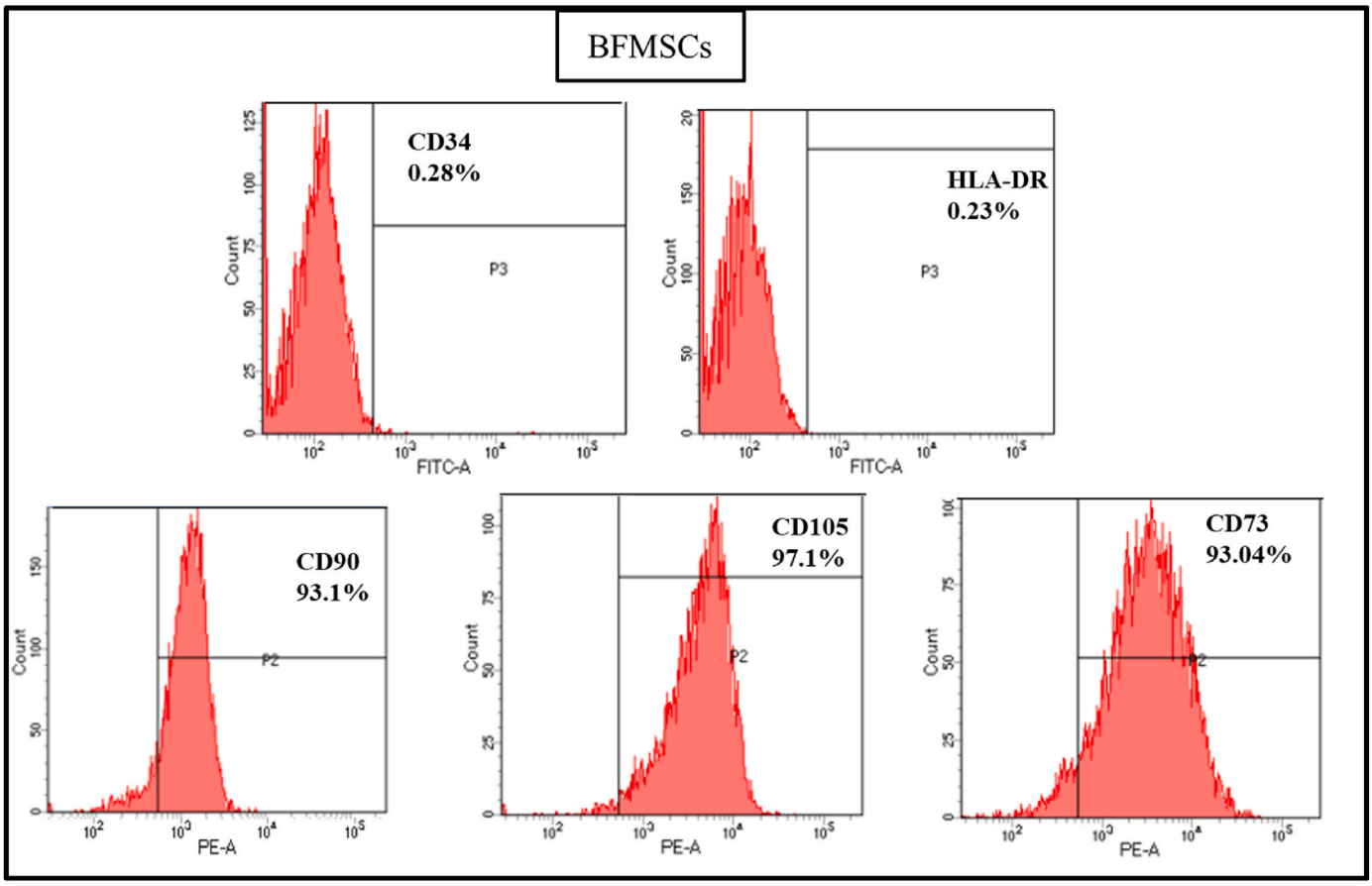

Fig. 5. The analysis of immunophenotypic expression of cluster of differentiation (CD) utilizing flow cytometry techniques. Mesenchymal stem cells (MSCs) obtained from Buccal Fat (BFMSC's) exhibited a high positive expression of CD90 at 93.1 %, CD105 at 97.1 %, and CD73 at 93.04 %. Conversely, the expression levels of CD34, and HLA-DR were notably low, recorded at 0.28 %, and 0.23 %, respectively.

bone marrow-derived mesenchymal stem cells (MSCs).[19] The absence of Cyr61 resulted in a significant reduction of the angiogenic capabilities of the secretome, while the introduction of recombinant Cyr61 reinstated these capabilities, highlighting its crucial function in the process of neovascularization. Although Cyr61 has not been examined in the context of DMSC-derived secretomes, it may serve as a significant molecular factor that warrants further investigation in subsequent research endeavors.

Alongside soluble proteins, extracellular vesicles (EVs)—which encompass exosomes and microvesicles—have been identified as essential mediators of paracrine signaling. Extracellular vesicles serve to encapsulate and safeguard their bioactive components, such as mRNAs, miRNAs, and proteins, from degradation by enzymes. The internalization of these vesicles by target cells, including endothelial cells, results in phenotypic modulation that facilitates angiogenesis. For example, extracellular vesicles originating from hypoxia-preconditioned mesenchymal stem cells exhibit enhanced angiogenic properties by increasing vascular endothelial growth factor expression and stimulating the PI3K/AKT signaling pathway in endothelial cells.[20,21] Their diminutive dimensions and compatibility with biological systems render them promising candidates for therapeutic applications, particularly within the realm of cell-free regenerative medicine.

The implementation of the scratch assay in this investigation facilitates the quantification of endothelial migration, serving as a proxy for assessing angiogenic potential. Although this assay fails to encompass the full spectrum of *in vivo* angiogenesis complexities—such as lumen formation, basement membrane degradation, and pericyte recruitment—it establishes a fundamental platform for evaluating the effects of DMSC-derived secretomes. Subsequent investigations may integrate supplementary assays, including tube formation assays, aortic ring assays, or *in vivo* Matrigel plug models, to corroborate and enhance the existing findings.

Angiogenesis represents a meticulously controlled mechanism characterized by the equilibrium between pro-angiogenic and anti-angiogenic factors. The MSC secretome shifts this equilibrium in favor of vascular regeneration through the delivery of a specific combination of signaling molecules. It is essential to note that the composition of the secretome can be adjusted through a range of preconditioning strategies. Hypoxic culture conditions effectively replicate the physiological niche of mesenchymal stem cells (MSCs) and promote the expression of hypoxia-inducible factor 1-alpha (HIF-1α) along with its downstream effectors. This process subsequently enhances the secretion of vascular endothelial growth factor (VEGF) and angiopoietin-2.[21] In a comparable manner, the process of inflammatory priming utilizing cytokines such as IFN-γ or TNF-α has demonstrated an enhancement in the immunomodulatory and angiogenic capabilities of secretomes derived from mesenchymal stem cells (MSCs). The implementation of these strategies in DMSC cultures may enhance their regenerative efficacy prior to clinical application.

Furthermore, contemporary research underscores the potential therapeutic advantages of manipulating MSC secretomes to achieve improved regenerative results. The process of genetic modification aimed at the overexpression of proangiogenic genes, including VEGF or bFGF, alongside the loading of extracellular vesicles with synthetic miRNAs, has the potential to markedly enhance therapeutic results in the context of ischemic tissue repair. This bioengineering methodology enhances efficacy while simultaneously providing improved reproducibility, precise dose control, and scalability, which are essential for

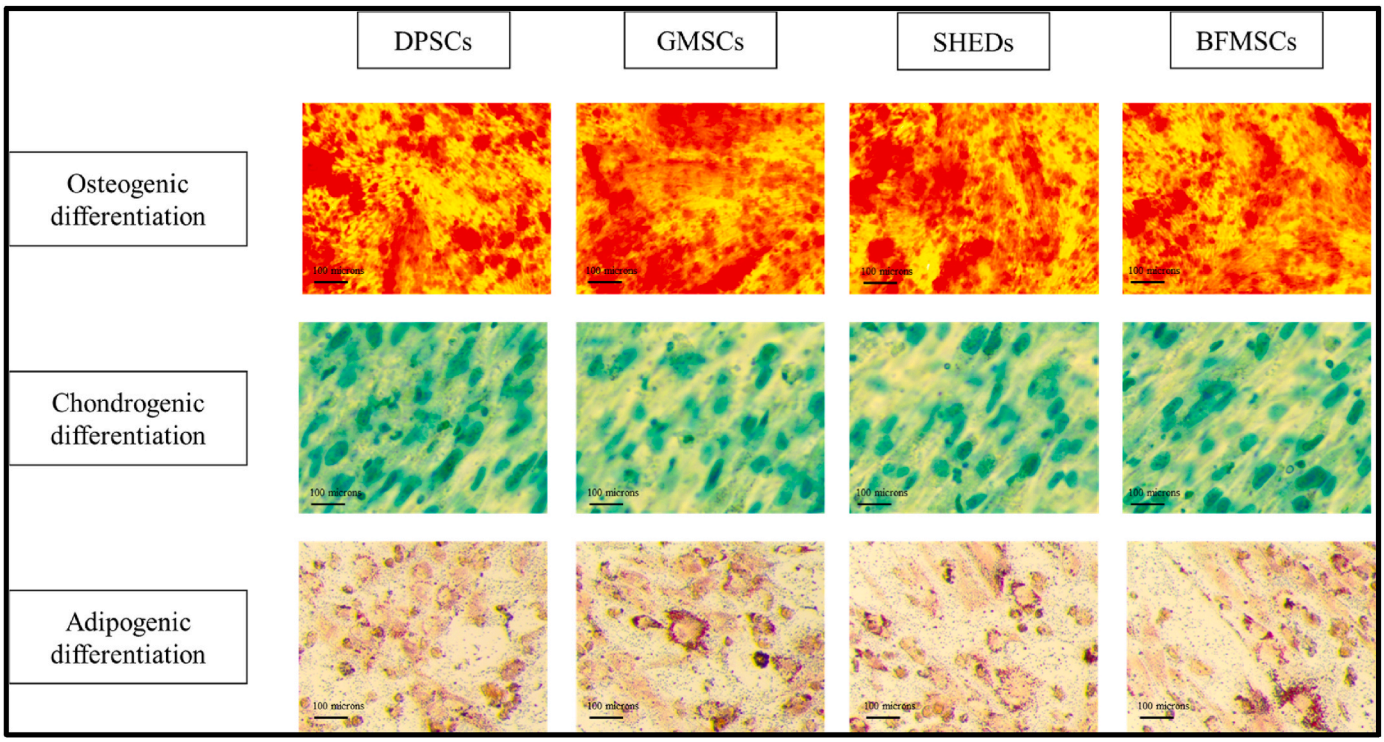

Fig. 6. Representative images showing successful osteogenic (Alizarin Red S), chondrogenic (Alcian Blue), and adipogenic (Oil Red O) differentiation of DPSCs, GMSCs, SHEDs, and BFMSCs, confirming their tri-lineage mesenchymal potential. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

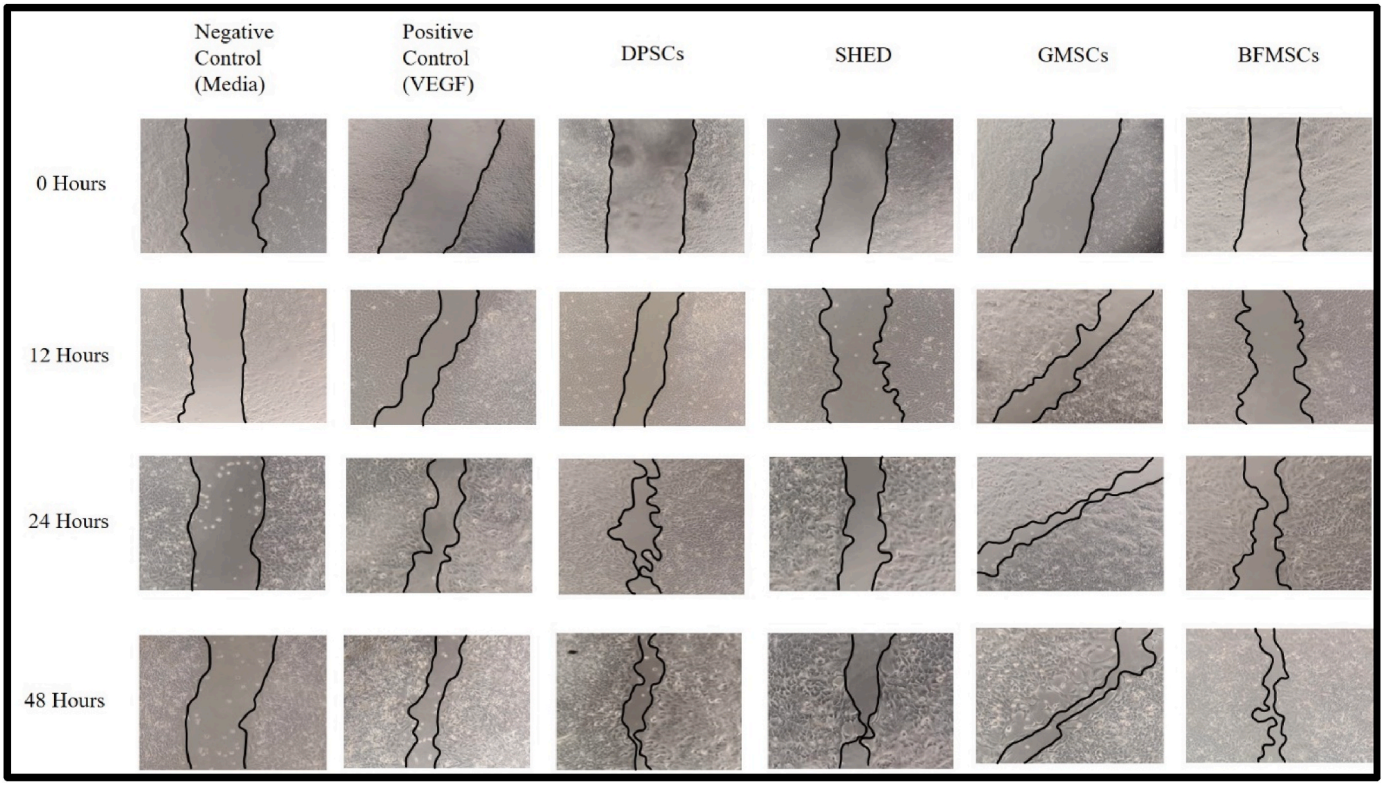

Fig. 7. Assessment of wound healing potential of MSCs derived from GMSCs-secretome, SHED-secretome, DPSCs-secretome, and BFMSCs-secretome; culture medium without preconditioning with MSCs was kept as a negative control. The images are taken at 0 h, 12 h, 24 h, and 48 h. The black outline suggests the migration distance of endothelial cells during the wound-healing process. Closure of scratch was almost completely accomplished by DPSCs-secretome, followed by other DMSCs secretome.

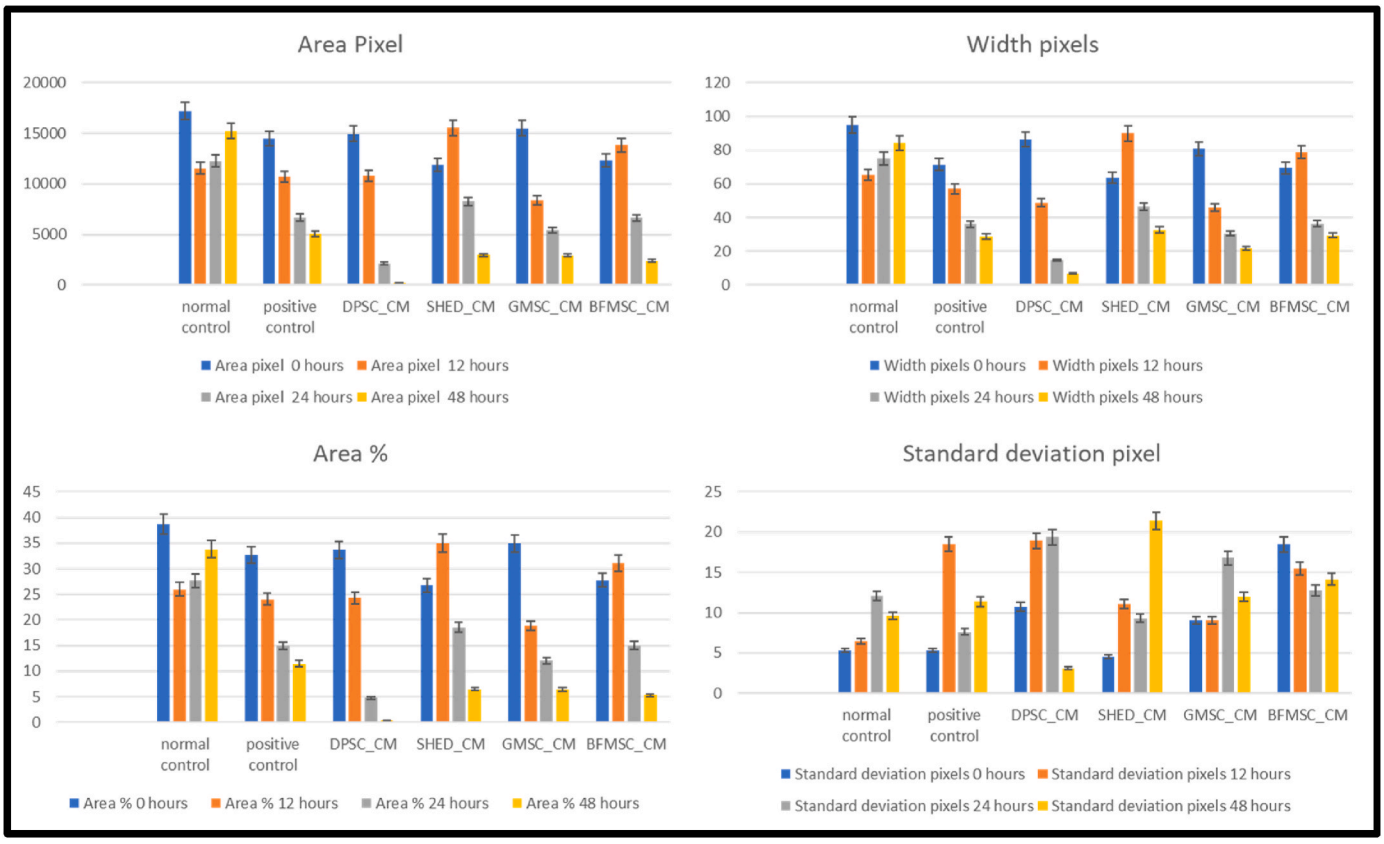

Fig. 8. Quantitative assessment of the scratch wound healing experiment. An objective evaluation revealed that secretomes derived from DPMSCs significantly enhanced the proportion of area, pixel area, pixel width, and pixel standard deviation. Although secretomes from GMSCs, SHED, and BFMSCs were shown to enhance cell migration, the wound-healing effect was more significant in DPMSCs-secretome. Data shown are Mean ± SD.

successful clinical translation. Another important factor is the immunomodulatory potential of MSC secretomes, which indirectly facilitates angiogenesis by establishing a favorable immune environment. The modulation of inflammatory cytokine expression, specifically the downregulation of IL-6 and TNF-α, alongside the enhancement of anti-inflammatory mediator release, such as IL-10 and TSG-6, by MSC secretomes contributes to the stabilization of nascent vasculature and facilitates the process of functional tissue repair.[21] The dual mechanism of action, which involves the direct stimulation of endothelial cells alongside the modulation of the immune microenvironment, positions MSC secretomes as particularly advantageous for applications in wound healing, cardiovascular pathologies, and diabetic ulcer management.

Collectively, our results validate the angiogenic potential of secretomes derived from dental pulp-derived mesenchymal stem cells and situate them within a wider framework of regenerative approaches. Through the facilitation of endothelial migration and the simulation of early angiogenic signaling, these secretomes have the potential to act as a foundational platform for the development of next-generation biologics within the realms of tissue engineering and regenerative medicine.

Nonetheless, the issue of standardization continues to present significant challenges. The variability observed in the composition of the secretome can be attributed to differences among donors, the number of passages, and the specific culture conditions employed, all of which may significantly influence the consistency of therapeutic outcomes. Therefore, thorough characterization employing proteomics, transcriptomics, and functional assays is essential prior to the translation of DMSC secretomes into clinical applications. It is essential to establish regulatory pathways for the assessment of safety, potency, and efficacy of these cell-free products in accordance with Good Manufacturing Practice (GMP) standards.

Future directions encompass the implementation of sophisticated molecular profiling techniques, the integration of preconditioning protocols, and the utilization of *in vivo* functional assays to comprehensively achieve their therapeutic potential. As the discipline advances towards precision medicine, DMSC secretomes present a cell-free, customizable, and biologically potent mechanism for enhancing angiogenesis and facilitating tissue repair across a diverse range of diseases.[22]

An important limitation of our study is the absence of quantitative protein analysis of the secretome. While we acknowledge that protein quantification is essential for ensuring standardization and enhancing the comparability of results across studies, we were constrained by the unavailability of requisite analytical facilities at the time of experimentation. Future work should incorporate robust protein quantification methods to enable more precise characterization of the secretome and facilitate reproducibility across experimental settings. This study was conceived as an initial investigation, with a specific focus on endothelial cell migration—a critical early event in the angiogenic cascade. While our findings provide preliminary insights into the angiogenic potential of the secretome, we recognize the importance of employing more comprehensive and physiologically relevant models. Accordingly, we have acknowledged the need for future studies incorporating advanced *in vitro* assays, such as tube formation and spheroid sprouting, as well as *in vivo* models, to more thoroughly elucidate the pro-angiogenic mechanisms and therapeutic implications of the secretome.

The therapeutic potential of mesenchymal stem cell-derived secretome has garnered considerable interest, particularly in the context of regenerative medicine. In this study, we highlight potential clinical applications of the secretome, notably in wound healing and ischemic tissue repair, where enhanced angiogenesis is critical for tissue regeneration. However, translating secretome-based therapies into clinical

practice presents several challenges. These include the need for scalable and reproducible production protocols, the development of efficient and targeted delivery systems to preserve bioactivity at the site of injury, and the navigation of complex regulatory frameworks governing biologics. Addressing these challenges will be essential for the successful clinical translation of secretome-derived therapeutics.

5. Conclusion

This study has demonstrated the potent angiogenic effects of dental mesenchymal stem cell (DMSC) secretomes. The enhanced proliferation and migration of endothelial cells in our scratch wound assays highlight the significant potential of DPSC secretomes in promoting angiogenesis. This capability is vital for applications in regenerative medicine, where efficient vascularization is crucial for effective tissue repair and integration. DPSC-derived secretomes stand out due to their accessibility and the unique range of angiogenic factors they secrete, positioning them as a valuable tool for developing treatments for conditions that require enhanced vascular growth, such as chronic wounds and diabetic ulcers. In conclusion, DPSC secretomes represent a promising avenue for enhancing natural healing processes and developing minimally invasive regenerative therapies. By leveraging these secretomes, future research can open new therapeutic possibilities, improving outcomes across a spectrum of clinical conditions that depend on rapid and effective angiogenesis.

Patients consent

Not needed since it's an invitro research.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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