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Effects of Nano-Hydroxyapatite-Coated Platelet-Rich Fibrin on In Vitro Wound Healing of Gingiva-Derived Mesenchymal Stem Cells

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Abstract

Objectives: Platelet-rich fibrin (PRF) contains bioactive mediators that may support cellular events involved in wound healing. This study compared the effects of conditioned media derived from leukocyte- and platelet-rich fibrin (L-PRF), nano-hydroxyapatite-modified PRF (HA-PRF), and titanium-prepared PRF (T-PRF) on gingiva-derived mesenchymal stem cell (GMSC) wound closure using an in vitro scratch assay.

Materials and Methods: GMSCs were isolated from gingival tissue obtained from a healthy donor and characterized before experimental use. L-PRF, HA-PRF, and T-PRF membranes were prepared under identical centrifugation conditions, and conditioned media were collected after 1 or 3 days of incubation. GMSCs were seeded in 24-well plates, serum-starved, and subjected to a linear scratch. Following exposure to the respective conditioned media, residual wound areas were evaluated at 0, 6, 12, 24, 30, and 36 h. Comparisons among the control, L-PRF, HA-PRF, and T-PRF groups were performed at each observation time using one-way ANOVA, followed by Tukey's post hoc test where appropriate.

Results: For 1-day conditioned media, no significant differences among groups were observed at 6, 12, or 24 h (all $p > 0.05$), whereas a significant between-group difference was detected at 30 h ($p = 0.005$), when complete wound closure had occurred in the L-PRF and HA-PRF groups. For 3-day conditioned media, no significant between-group difference was observed at 6 h ($p = 0.209$), whereas a significant difference emerged at 12 h ($p < 0.001$). At 12 h, HA-PRF showed the smallest residual wound area (33.93 ± 7.96%), compared with L-PRF (57.71 ± 9.46%), T-PRF (69.44 ± 9.25%), and control (80.91 ± 5.33%). Complete wound closure was achieved in the HA-PRF group by 24 h and in the L-PRF group by 30 h, while all groups reached complete closure by 36 h. Direct comparison of the 1- and 3-day conditioned media showed significantly smaller residual wound areas with 3-day HA-PRF-conditioned medium at 6 h ($p = 0.024$) and 12 h ($p = 0.011$).

Conclusions: PRF-conditioned media supported GMSC wound closure, with HA-PRF showing the most favorable early response, particularly with conditioned medium collected after 3 days. These findings suggest that nano-hydroxyapatite modification may enhance the early biological activity of PRF; however, further mechanistic and in vivo studies are required.

Clinical Relevance: Nano-hydroxyapatite modification of PRF may enhance early GMSC-mediated wound closure and may represent a promising approach for improving the regenerative potential of PRF in oral and periodontal wound healing.

1. Introduction

Oral wound healing is a dynamic and highly coordinated biological process that restores the structural integrity and function of damaged tissues through the sequential phases of hemostasis, inflammation, proliferation, and tissue remodeling literature [1]. Successful healing depends on the precise regulation of multiple cellular and molecular events, including inflammatory cell recruitment, angiogenesis,

extracellular matrix deposition, epithelialization, and tissue maturation literature [1, 2]. Among these phases, the proliferative stage plays a pivotal role in determining the quality and rate of tissue regeneration because it is characterized by active cell migration, proliferation, and matrix synthesis literature [3]. Accordingly, considerable efforts have been directed toward developing regenerative strategies capable of enhancing these biological events to improve oral soft tissue repair and periodontal regeneration literature [3].

Gingiva-derived mesenchymal stem cells (GMSCs) have attracted considerable attention in regenerative medicine because of their high proliferative capacity, multilineage differentiation potential, and potent immunomodulatory properties literature [4, 5]. Unlike many other mesenchymal stem cell sources, GMSCs can be obtained through minimally invasive procedures while maintaining phenotypic stability and regenerative potential during in vitro expansion [4, 6]. Beyond their differentiation ability, GMSCs actively contribute to oral wound healing by modulating inflammation, promoting angiogenesis, and secreting paracrine mediators that enhance the migration and proliferation of surrounding cells [5, 7]. These biological characteristics make GMSCs an attractive cellular model for evaluating novel biomaterials and regenerative strategies aimed at improving oral soft tissue healing [6].

Platelet-rich fibrin (PRF) is a second-generation autologous platelet concentrate that has been widely applied in oral and periodontal regenerative procedures because of its favorable biological properties and ease of preparation [8, 9]. Unlike platelet-rich plasma (PRP), PRF forms a dense three-dimensional fibrin matrix that entraps platelets and leukocytes, enabling the sustained release of numerous bioactive molecules, including platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF).[9,10] These growth factors promote cell migration, proliferation, angiogenesis, extracellular matrix synthesis, and tissue remodeling, thereby creating a favorable microenvironment for wound healing and regeneration.[9,11] Consequently, PRF has emerged as an attractive biomaterial for enhancing oral soft tissue healing while modulating the regenerative behavior of mesenchymal stem cells.[9,11]

The biological properties of PRF are influenced not only by centrifugation protocols but also by the physicochemical characteristics of the blood collection tubes used during its preparation.[12,13] Previous studies have demonstrated that modifications of the collection tube surface can alter fibrin architecture, cellular entrapment, and the release kinetics of growth factors, thereby affecting the regenerative potential of the resulting PRF membrane.[12,14] In this context, nano-hydroxyapatite (NanoHAp) has emerged as a promising bioactive coating material because of its excellent biocompatibility, hemocompatibility, and favorable interactions with biological tissues.[15,16] Our previous study demonstrated that PRF prepared using NanoHAp-coated tubes enhanced the viability, proliferation, and biological activity of gingiva-derived mesenchymal stem cells compared with conventionally prepared PRF membranes.[17] However, whether conditioned media released from NanoHAp-coated PRF can further enhance the wound-healing capacity of GMSCs by promoting cell migration has not yet been investigated.

Therefore, the aim of the present study was to compare the effects of conditioned media obtained from leukocyte platelet-rich fibrin (L-PRF), nano-hydroxyapatite-coated platelet-rich fibrin (HA-PRF), and titanium-prepared platelet-rich fibrin (T-PRF) on the wound-healing capacity of gingiva-derived mesenchymal stem cells using an in vitro scratch wound assay.

2. Materials and Methods

2.1. GMSC Culture and Characterization

Gingiva-derived mesenchymal stem cells (GMSCs) used in the present study were obtained from gingival tissue collected from a systemically healthy, nonsmoking male volunteer with no history of medication use. Gingival tissue was obtained during the surgical extraction of an impacted third molar. The study protocol was approved by the Clinical Research Ethics Committee of Eskişehir Osmangazi University (Decision No. 46; 21 September 2023), and written informed consent was obtained from the donor before tissue and blood collection. All methods were performed in accordance with the relevant guidelines and regulations.

GMSCs were isolated and characterized as described in our previous study.[17] Briefly, gingival tissue was washed with Dulbecco's phosphate-buffered saline (DPBS; Capricorn Scientific, Ebsdorfergrund, Germany) and enzymatically digested using 2 mg/mL collagenase type II (Sigma-Aldrich, St. Louis, MO). The isolated cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit HaEmek, Israel), 1% penicillin/streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), and 1% stable glutamine (Capricorn Scientific, Ebsdorfergrund, Germany). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 using a CO_2 incubator (MCO-170M-PE; Panasonic, Kadoma, Japan), and the culture medium was renewed every 3–4 days.[17]

Immunophenotypic characterization was performed by flow cytometry using antibodies against CD90, CD73, CD105, CD44, CD29, CD69, CD34, CD45, CD19, CD25, CD14, and HLA-DR (BioLegend, San Diego, CA, USA). Flow cytometric analysis was performed using a NovoCyte D3005 flow cytometer (Agilent Technologies, Santa Clara, CA, USA), and the data were analyzed using NovoExpress software (version 1.5.0; Agilent Technologies). The mesenchymal phenotype was confirmed by positive expression of mesenchymal stem cell markers and negative expression of hematopoietic and immunologic markers. Multilineage differentiation toward adipogenic, osteogenic, and chondrogenic lineages was also confirmed as previously described.[17]

For the wound-healing experiments, cryopreserved passage-3 GMSCs were rapidly thawed in a 37 °C water bath, washed twice with complete culture medium, and expanded under the culture conditions described above before experimental procedures.

2.2. Production of Nano-Hydroxyapatite-Coated Tubes

Nano-hydroxyapatite (NanoHAp)-coated glass tubes were prepared using an ultrasonic spray coating technique, as described in our previous study.[17] Briefly, conventional glass tubes were cleaned with ethanol and dried with hot air for 4 min before coating. Calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$] (AFG BioScience) and ammonium dihydrogen phosphate [$NH_4H_2PO_4$] (Sigma-Aldrich, St. Louis, MO) were used as calcium and phosphate precursors, respectively, to prepare a 0.5 M precursor solution. The coating procedure was performed using an ultrasonic spray system, with the nozzle positioned coaxially within the glass tube and gradually withdrawn during spraying to obtain a homogeneous coating on the inner surface. The coating process lasted approximately 5–10 min, while the substrate temperature was maintained at 300–350 °C. Nozzle movement and spraying were synchronized using a programmable logic controller system. The 0.5 M precursor concentration and a substrate temperature of 300–350 °C were used to obtain optimal coating integrity and homogeneity.[17]

Conventional glass tubes (Borosil, Mumbai, India), NanoHAp-coated glass tubes, and titanium tubes (Firdevs Dental Medikal Ltd., Konya, Türkiye) were subsequently used for the preparation of L-PRF, HA-PRF, and T-PRF, respectively. Representative images of the three tube types are shown in Figure 1.

2.3. Preparation of PRF Membranes and Conditioned Media

For PRF preparation, venous blood was collected from the same healthy volunteer and immediately transferred, without anticoagulant, into three different types of 10-mL tubes: conventional glass tubes for leukocyte- and platelet-rich fibrin (L-PRF), NanoHAp-coated glass tubes for hydroxyapatite-modified PRF (HA-PRF), and titanium tubes for titanium-prepared PRF (T-PRF). A total of 120 mL of venous blood was collected and distributed into four tubes for each PRF type (10 mL per tube). All three groups were centrifuged at 2700 rpm for 12 min using a tabletop centrifuge (Elektro-Mag, Istanbul, Türkiye). The use of identical centrifugation parameters across the three groups was intended to minimize protocol-related variability and allow tube material and surface characteristics to constitute the principal experimental differences among the PRF preparations [12,13,17]

Immediately after centrifugation, PRF clots were carefully removed using sterile forceps, and the erythrocyte-rich portions were trimmed with sterile scissors. The clots were then placed in a standardized metal PRF box and compressed for 10 min to obtain membranes of uniform thickness. The resulting membranes were cut into 4-mm-diameter discs using a biopsy punch (Plasti-Med, Istanbul, Türkiye). All PRF membranes were prepared by the same investigator immediately after blood collection under standardized laboratory conditions.

For the preparation of PRF-conditioned media, two standardized 4-mm discs obtained from each L-PRF, HA-PRF, and T-PRF membrane were randomly allocated to the designated incubation conditions. The PRF discs were incubated in culture medium for either 1 or 3 days to allow the release of soluble components from the PRF matrices. At the end of each incubation period, the corresponding conditioned media were collected and used for the subsequent in vitro scratch wound healing experiments. Thus, separate 1-day and 3-day conditioned media were obtained for each PRF preparation (L-PRF, HA-PRF, and T-PRF). The overall experimental workflow is illustrated in Figure 2.

2.4. In Vitro Scratch Wound Healing Assay

GMSC migration and wound closure were evaluated using an in vitro scratch wound healing assay, as previously described,[18] with modifications according to the present experimental design. GMSCs were seeded into 24-well culture plates at a density of 2×10^5 cells/well and maintained in complete growth medium for 24 h. The culture medium was subsequently replaced with serum-free medium, and the cells were incubated for an additional 24 h. A linear scratch was then created in the confluent cell monolayer using a sterile pipette tip.

Following scratch formation, the cells were exposed to conditioned media obtained from L-PRF, HA-PRF, or T-PRF membranes after 1 or 3 days of incubation. Cells cultured without PRF-conditioned medium in culture medium supplemented with 1% FBS served as the control group. Images of the scratched areas were acquired immediately after scratch formation (0 h) and at 6, 12, 24, 30, and 36 h using an inverted phase-contrast microscope (Axio Observer D1; Carl Zeiss, Oberkochen, Germany). Images were obtained from the same field at each time point, and at least three images were evaluated for each experimental condition.

Wound areas were quantified using Zen 2 Pro software (Carl Zeiss, Oberkochen, Germany). The wound area at 0 h was considered 100%, and the residual wound area at each subsequent time point was expressed as a percentage of the initial wound area. Lower residual wound area values therefore indicated greater wound closure.

2.5. Statistical Analysis

Statistical analyses were performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). Residual wound area values were expressed as mean ± standard deviation (SD). The effects of PRF preparation (Control, L-PRF, HA-PRF, and T-PRF), conditioned-medium collection period (1 day and 3 days), and observation time (0, 6, 12, 24, 30, and 36 h) were initially evaluated using a three-way factorial ANOVA, including interaction effects. When significant effects were detected, comparisons among groups at each observation time were performed using one-way ANOVA followed by Tukey's post hoc test. Comparisons between the 1-day and 3-day conditioned media within each PRF preparation at corresponding observation times were also performed using one-way ANOVA. Statistical significance was set at $p < 0.05$.

3. Results

3.1. GMSC Characterization

Flow cytometric analysis confirmed that the passage-3 GMSCs retained a mesenchymal immunophenotype. High expression was observed for CD90 (99.40%), CD105 (95.72%), CD73 (99.99%), CD44 (99.97%), and CD29 (99.96%). In contrast, CD69 (13.67%), CD34 (0.19%), CD45 (0.16%), CD19

(0.02%), CD25 (0.00%), CD14 (4.72%), and HLA-DR (0.58%) showed low or negligible expression (Figure 3).

3.2. Effects of PRF-Conditioned Media on GMSC Wound Closure

3.2.1. Wound Closure Following 1-Day PRF Release

For conditioned media collected on day 1, residual wound area progressively decreased over the observation period in all experimental groups. No significant differences among the control, L-PRF, HA-PRF, and T-PRF groups were detected at 6 h ($p = 0.939$), 12 h ($p = 0.309$), or 24 h ($p = 0.149$). At 12 h, the mean residual wound areas were 71.56 ± 6.47% in the control group, 66.80 ± 7.56% in the L-PRF group, 58.74 ± 5.56% in the HA-PRF group, and 80.32 ± 23.74% in the T-PRF group. Although HA-PRF showed the lowest residual wound area among the PRF groups at this time point, the between-group difference was not statistically significant.

At 24 h, complete wound closure was observed only in the HA-PRF group, whereas residual wound areas were 34.96 ± 17.17% in the control group, 25.92 ± 33.58% in the L-PRF group, and 44.86 ± 21.81% in the T-PRF group. At 30 h, complete wound closure was observed in both the L-PRF and HA-PRF groups, whereas residual wound areas of 16.33 ± 3.52% and 20.79 ± 11.57% remained in the control and T-PRF groups, respectively. The overall between-group difference at 30 h was statistically significant ($p = 0.005$). Tukey's post hoc analysis showed significantly smaller residual wound areas in both the L-PRF and HA-PRF groups compared with the control group (both $p = 0.043$) and the T-PRF group (both $p = 0.013$), whereas no significant difference was observed between the control and T-PRF groups. By 36 h, complete wound closure was observed in all groups (Table 1; Figure 4).

3.2.2. Wound Closure Following 3-Day PRF Release

For conditioned media collected on day 3, differences in wound closure among the experimental groups became evident during the early observation period. At 6 h, the mean residual wound areas were 92.30 ± 12.40% in the control group, 89.55 ± 7.05% in the L-PRF group, 79.89 ± 5.50% in the HA-PRF group, and 94.15 ± 4.65% in the T-PRF group. Although HA-PRF exhibited the smallest residual wound area, the overall between-group difference was not statistically significant ($p = 0.209$).

At 12 h, a significant between-group difference was observed ($p < 0.001$). The residual wound area was 80.91 ± 5.33% in the control group, 57.71 ± 9.46% in the L-PRF group, 33.93 ± 7.96% in the HA-PRF group, and 69.43 ± 9.25% in the T-PRF group. HA-PRF exhibited the smallest residual wound area and showed significantly greater wound closure than the control, L-PRF, and T-PRF groups.

At 24 h, complete wound closure was observed in the HA-PRF group, whereas residual wound areas were 26.37 ± 14.86% in the control group, 13.40 ± 1.09% in the L-PRF group, and 22.14 ± 17.19% in the T-PRF group. The overall between-group difference at 24 h was not statistically significant ($p = 0.087$). At 30 h, complete wound closure was observed in both the L-PRF and HA-PRF groups, whereas residual wound areas of 19.63 ± 7.38% and 4.38 ± 4.22% remained in the control and T-PRF groups, respectively.

The overall between-group difference at 30 h was statistically significant (p = 0.001). By 36 h, complete wound closure was observed in all groups (Table 2; Figure 5).

3.3 Comparison of 1- and 3-Day PRF-Conditioned Media

Direct comparisons between the 1- and 3-day conditioned media revealed a time-dependent difference specifically in the HA-PRF group. At 6 h, the residual wound area was significantly smaller following exposure to 3-day HA-PRF-conditioned medium than following exposure to 1-day HA-PRF-conditioned medium (79.89 ± 5.50% vs. 91.66 ± 1.76%, respectively; p = 0.024). A similar difference was observed at 12 h, with residual wound areas of 33.93 ± 7.96% and 58.74 ± 5.56% for the 3-day and 1-day HA-PRF-conditioned media, respectively (p = 0.011). In contrast, no significant differences between the 1- and 3-day conditioned media were observed for L-PRF at 6, 12, or 24 h (all p > 0.05) or for T-PRF at 6, 12, 24, or 30 h (all p > 0.05) (Table 3).

4. Discussion

The present study investigated the effects of conditioned media derived from L-PRF, HA-PRF, and T-PRF on GMSC wound closure using an in vitro scratch wound healing model. The findings demonstrated that the wound-closure response varied according to PRF preparation, conditioned-medium collection period, and observation time. Among the tested preparations, HA-PRF showed the most favorable early response, particularly when conditioned medium collected after 3 days of incubation was used. With 3-day conditioned medium, HA-PRF produced the smallest residual wound area at 12 h and achieved complete wound closure by 24 h. Moreover, direct comparison of the two collection periods showed significantly smaller residual wound areas with 3-day than with 1-day HA-PRF-conditioned medium at both 6 and 12 h. In contrast, comparable collection-period-dependent differences were not observed for L-PRF or T-PRF. These findings suggest that nano-hydroxyapatite modification may influence the early biological activity of soluble factors released from PRF, with this effect appearing to depend on the duration of conditioned-medium collection.

Cell migration is a critical component of wound healing and contributes to the restoration of tissue integrity during the proliferative phase.[1,2] GMSCs are particularly relevant to oral tissue regeneration because of their high proliferative capacity, multilineage differentiation potential, migratory ability, and immunomodulatory properties.[4-7] The in vitro scratch wound healing assay provides a well-established approach for evaluating cellular wound closure by monitoring the progressive reduction of a defined cell-free area.[18] In the present study, all experimental groups showed a progressive reduction in residual wound area over time, indicating ongoing GMSC-mediated wound closure. However, differences in the magnitude and timing of this response among L-PRF, HA-PRF, and T-PRF suggest that the characteristics of the PRF preparation and its released soluble components may influence early cellular events associated with wound repair.

PRF acts as a fibrin-based reservoir for platelet- and leukocyte-derived bioactive mediators that contribute to wound healing and tissue regeneration.[9,10] Growth factors released from PRF, including PDGF, TGF-β1, VEGF, and IGF, contribute to key regenerative processes such as cell migration, proliferation, angiogenesis, and extracellular matrix formation.[9-11] Importantly, the release of these mediators varies over time, suggesting that the biological activity of PRF may depend partly on its release kinetics.[10] In our previous study, HA-PRF demonstrated favorable effects on GMSC viability and proliferation and was associated with increased levels of regenerative mediators, including VEGF and TGF-β1.[17] The present findings extend these observations by demonstrating a favorable early wound-closure response to HA-PRF-conditioned medium, particularly after the 3-day release period. The significantly smaller residual wound area observed with 3-day HA-PRF-conditioned medium at 6 and 12 h compared with the corresponding 1-day conditioned medium suggests that the temporal release profile of HA-PRF may be relevant to its biological effects on GMSCs. Nevertheless, because individual growth factors were not quantified in the conditioned media in the present study, the enhanced early wound-closure response cannot be attributed to a specific mediator or release mechanism.

The biological characteristics of PRF may be influenced not only by the centrifugation protocol but also by the material and surface properties of the collection tube.[11-14] Surface characteristics at the blood–material interface can affect the initial interactions of platelets and other blood components and may consequently influence the properties of the resulting fibrin matrix and its biological activity.[12-14] In this context, nano-hydroxyapatite represents a potentially relevant surface modification because hydroxyapatite nanoparticles can interact with blood components and influence coagulation-related responses, with these effects depending partly on their geometry and physicochemical characteristics.[15,16] In our previous study, NanoHAp-coated tubes were successfully produced and characterized, and HA-PRF prepared using these tubes showed favorable biological interactions with GMSCs.[17] Together with the present finding of enhanced early wound closure with HA-PRF-conditioned medium, these observations suggest that modification of the blood-contacting tube surface may contribute to differences in the biological performance of PRF preparations. However, the present study did not directly evaluate fibrin architecture, platelet activation, or the mechanisms by which the NanoHAp-coated surface influenced PRF formation and subsequent release of soluble factors. Therefore, a direct mechanistic relationship between NanoHAp coating and the enhanced early wound-closure response cannot be established.

The incubation period used to obtain PRF-conditioned media appeared to influence the wound-closure response, particularly in the HA-PRF group. Previous studies have demonstrated that PRF releases bioactive mediators over time and that the concentrations and profiles of these factors may vary during the release period.[10] In the present study, no significant differences among the experimental groups were observed during the early observation period with 1-day conditioned media, whereas with 3-day conditioned media a pronounced between-group difference emerged at 12 h. At this time point, HA-PRF showed a residual wound area of 33.93 ± 7.96%, compared with 57.71 ± 9.46% for L-PRF, 69.43 ± 9.25% for T-PRF, and 80.91 ± 5.33% for the control group. More importantly, direct comparison of the two

collection periods demonstrated significantly smaller residual wound areas with 3-day than with 1-day HA-PRF-conditioned medium at both 6 and 12 h, whereas no significant collection-period-dependent differences were observed for L-PRF or T-PRF at the evaluated time points. These findings suggest that extending the conditioned-medium collection period from 1 to 3 days may alter the biological activity of soluble factors released from HA-PRF and enhance their effects on early GMSC-mediated wound closure. However, because the composition and temporal release profiles of the conditioned media were not directly characterized, the mechanisms underlying this time-dependent response remain to be determined.

The comparative responses of L-PRF and T-PRF further indicate that the effects of PRF-conditioned media were not uniform across the different preparations. Although all PRF groups showed progressive wound closure over time, neither L-PRF nor T-PRF consistently reproduced the favorable early response observed with HA-PRF. This difference was most evident with the 3-day conditioned media at 12 h, when the residual wound area was 33.93 ± 7.96% for HA-PRF, compared with 57.71 ± 9.46% for L-PRF and 69.43 ± 9.25% for T-PRF. At this time point, HA-PRF showed significantly greater wound closure than both L-PRF and T-PRF. However, this advantage was predominantly observed during the early phase of wound closure and did not represent a sustained superiority throughout the entire observation period. By 24 h, complete wound closure had occurred in the HA-PRF group, whereas residual wound areas remained in the other groups; nevertheless, the overall between-group difference was not statistically significant ($p = 0.087$). By 30 h, both HA-PRF and L-PRF had achieved complete wound closure, and all groups reached complete closure by 36 h. Thus, the present findings support an acceleration of early wound closure with HA-PRF rather than a persistent advantage across the entire healing period.

The present findings are consistent with previous in vitro evidence demonstrating that platelet concentrates can enhance cellular behaviors associated with soft-tissue wound healing, while also indicating that the magnitude of these effects may depend on the preparation method. Bi et al. reported that different platelet-derived preparations promoted artificial wound closure in human gingival fibroblasts, with P-PRF producing greater wound filling than L-PRF.[19] Similarly, Pitzurra et al. demonstrated that conditioned media derived from both L-PRF and A-PRF+ promoted proliferation and migration of gingival and periodontal ligament fibroblasts and accelerated artificial wound closure compared with controls.[20] Notably, A-PRF+ maintained the wound-closure response more effectively than L-PRF, further supporting the concept that differences in PRF preparation can influence cellular behavior.[20] Although these studies used fibroblast-based models and PRF preparations that differed from those evaluated in the present study, their findings are consistent with our observation that PRF-conditioned media produced preparation-dependent effects on wound closure. The particularly favorable early response observed with HA-PRF therefore extends this concept to NanoHAp-modified PRF and GMSCs.

An important aspect of the present study is the direct comparison of conditioned media derived from three distinct PRF preparations using GMSCs as the cellular model. Previous studies have demonstrated that PRF-derived conditioned media can promote fibroblast migration and artificial wound closure;[19,20]

however, comparatively limited information is available regarding the response of GMSCs to soluble factors released from differently prepared PRF matrices. To our knowledge, the effect of NanoHAp-modified PRF-conditioned medium on GMSC wound closure has not previously been investigated using this experimental approach. Moreover, evaluating conditioned media collected after both 1 and 3 days allowed the influence of the PRF release period on the cellular response to be examined. The use of conditioned media also eliminated direct contact between the PRF membranes and GMSCs, thereby enabling the observed responses to be associated primarily with soluble components released from the different fibrin matrices. The favorable early response observed with HA-PRF, particularly with the 3-day conditioned medium, therefore provides additional evidence that modification of PRF preparation may influence its biological interactions with cells relevant to oral and periodontal soft-tissue regeneration.

Several limitations should be considered when interpreting the present findings. First, the study was conducted using an in vitro scratch wound healing model, which cannot fully reproduce the complex cellular, vascular, inflammatory, and extracellular matrix interactions involved in wound healing in vivo. Second, GMSCs were obtained from a single healthy donor; therefore, interindividual biological variability and the potential influence of donor-related factors could not be assessed. Third, although the scratch assay is widely used to evaluate cellular wound closure, reduction of the wound area may result from both cell migration and proliferation; therefore, the observed response cannot be attributed exclusively to enhanced GMSC migration.[18] Fourth, conditioned media were evaluated at only two collection periods (1 and 3 days), and longer-term release patterns were not investigated. Finally, the composition and release kinetics of the conditioned media were not directly characterized. Consequently, although the findings indicate a favorable early wound-closure response with HA-PRF, the specific soluble mediators and mechanisms responsible for this effect remain unknown. Future studies combining quantitative profiling of PRF-derived mediators with migration-specific assays, multiple donors, longer release periods, and in vivo wound-healing models are needed to clarify the mechanisms and biological relevance of these findings.

From a regenerative perspective, enhancement of early cellular events associated with wound closure may be particularly relevant in oral and periodontal environments, where healing tissues are continuously exposed to microbial and mechanical challenges. The present findings suggest that NanoHAp modification of PRF may enhance its capacity to support early GMSC-mediated wound closure, particularly through soluble factors released during the first days after preparation. However, the observed advantage of HA-PRF was primarily evident during the early phase of wound closure and should not be interpreted as evidence of superior long-term regenerative efficacy. Accordingly, these findings should be regarded as preliminary in vitro evidence supporting the biological potential of HA-PRF rather than direct evidence of improved clinical wound healing.

5. Conclusions

Within the limitations of this in vitro study, PRF-conditioned media supported progressive GMSC wound closure, with the response varying according to PRF preparation and conditioned-medium collection

period. HA-PRF demonstrated the most favorable early wound-closure response, particularly with conditioned medium collected after 3 days. These findings suggest that NanoHAp modification may enhance the early biological activity of PRF-derived soluble factors on GMSCs. Further mechanistic and in vivo studies are required to determine whether this early cellular response translates into improved oral and periodontal tissue regeneration.

Declarations

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Residual wound area (%) following exposure to 1-day PRF-conditioned media at different observation times.

Observation time	Control	L-PRF	HA-PRF	T-PRF	p-value
0 h	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	—
6 h	90.85 ± 7.98	94.79 ± 6.72	91.65 ± 1.76	95.89 ± 20.66	0.939
12 h	71.56 ± 6.47	66.80 ± 7.56	58.74 ± 5.56	80.32 ± 23.74	0.309
24 h	34.96 ± 17.17	25.92 ± 33.58	0.00 ± 0.00	44.86 ± 21.81	0.149
30 h	16.33 ± 3.52	0.00 ± 0.00	0.00 ± 0.00	20.79 ± 11.58	**0.005**
36 h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	—

Data are presented as mean ± standard deviation (SD) of residual wound area (%). Between-group comparisons at each observation time were performed using one-way analysis of variance (ANOVA). When a statistically significant overall difference was detected, Tukey's post hoc test was used for multiple comparisons. Different superscript letters within the same row indicate statistically significant differences between groups ($p < 0.05$). Significant p-values are shown in bold. A value of 0 indicates complete wound closure. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; SD, standard deviation

Table 2. Residual wound area (%) following exposure to 3-day PRF-conditioned media at different observation times

Observation time	Control	L-PRF	HA-PRF	T-PRF	p-value
0 h	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	—
6 h	92.30 ± 12.40	89.55 ± 7.05	79.89 ± 5.50	94.15 ± 4.65	0.209
12 h	80.91 ± 5.33	57.71 ± 9.46	33.93 ± 7.96	69.44 ± 9.25	**<0.001**
24 h	26.37 ± 14.86	13.40 ± 1.09	0.00 ± 0.00	22.14 ± 17.19	0.087
30 h	19.63 ± 7.38	0.00 ± 0.00	0.00 ± 0.00	4.38 ± 4.22	**0.001**
36 h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	—

Data are presented as mean ± standard deviation (SD) of residual wound area (%). Between-group comparisons at each observation time were performed using one-way analysis of variance (ANOVA). When a statistically significant overall difference was detected, Tukey's post hoc test was used for multiple comparisons. Different superscript letters within the same row indicate statistically significant differences between groups ($p < 0.05$). Significant p-values are shown in bold. A value of 0 indicates

complete wound closure. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; SD, standard deviation

Table 3. Comparison of residual wound area (%) between 1-day and 3-day PRF-conditioned media within each PRF group.

PRF group	Observation time	1-day CM	3-day CM	p-value
L-PRF	6 h	94.79 ± 6.72	89.55 ± 7.05	0.404
	12 h	66.80 ± 7.56	57.71 ± 9.46	0.263
	24 h	25.92 ± 33.58	13.40 ± 1.09	0.554
HA-PRF	6 h	91.65 ± 1.76	79.89 ± 5.50	**0.024**
	12 h	58.74 ± 5.56	33.93 ± 7.96	**0.011**
T-PRF	6 h	95.89 ± 20.66	94.15 ± 4.65	0.894
	12 h	80.32 ± 23.74	69.44 ± 9.25	0.500
	24 h	44.86 ± 21.81	22.14 ± 17.19	0.230
	30 h	20.79 ± 11.58	4.38 ± 4.22	0.082

Data are presented as mean ± standard deviation (SD) of residual wound area (%). Comparisons between 1-day and 3-day conditioned media within each PRF group at corresponding observation times were performed using one-way analysis of variance (ANOVA). Significant p-values are shown in bold. CM, conditioned medium; L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; SD, standard deviation

Figures

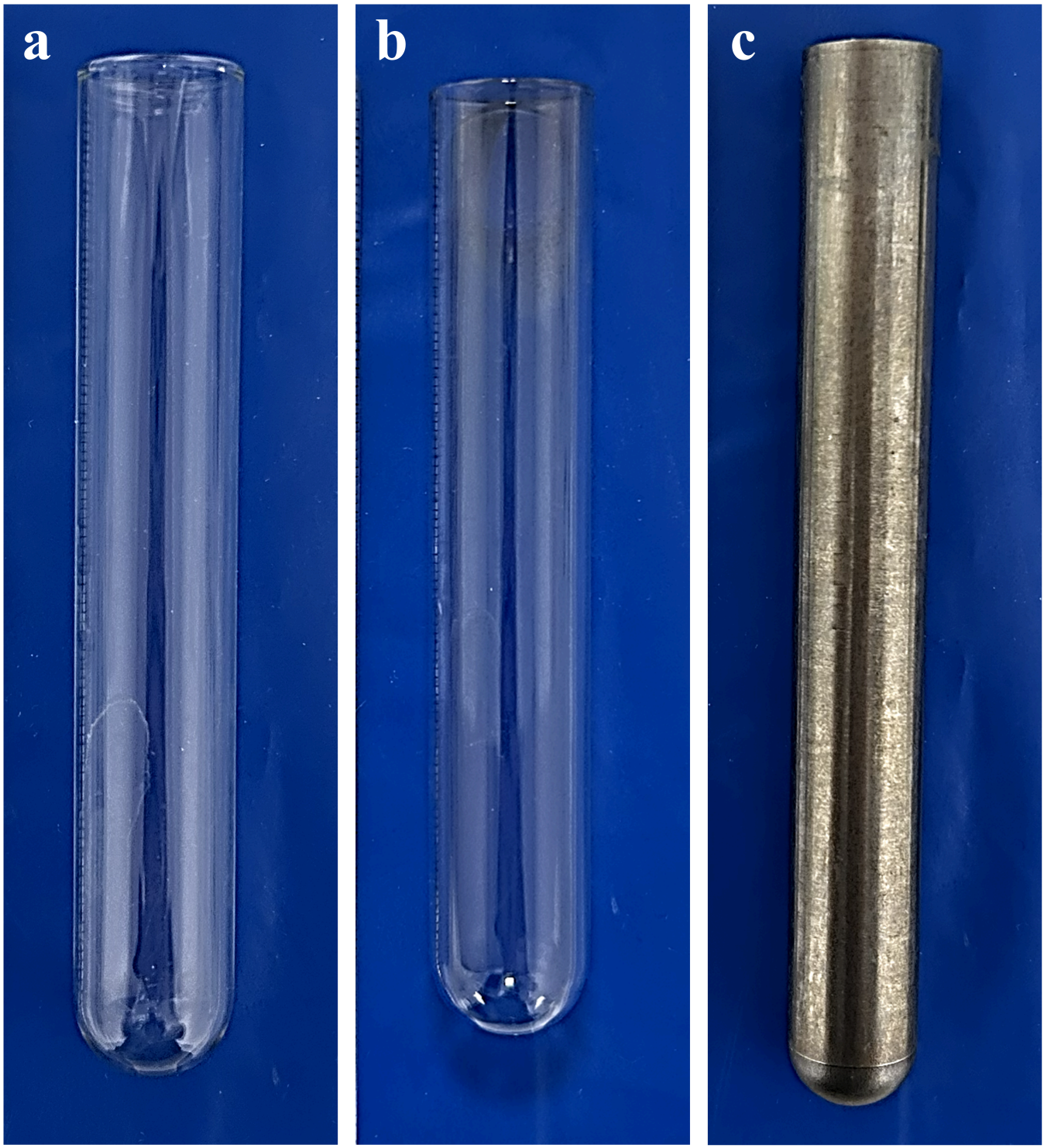


Figure 1

Blood collection tubes used for the preparation of different platelet-rich fibrin (PRF) formulations. (a) Conventional glass tube used for L-PRF preparation, (b) nano-hydroxyapatite-coated glass tube used for HA-PRF preparation, and (c) titanium tube used for T-PRF preparation. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin.

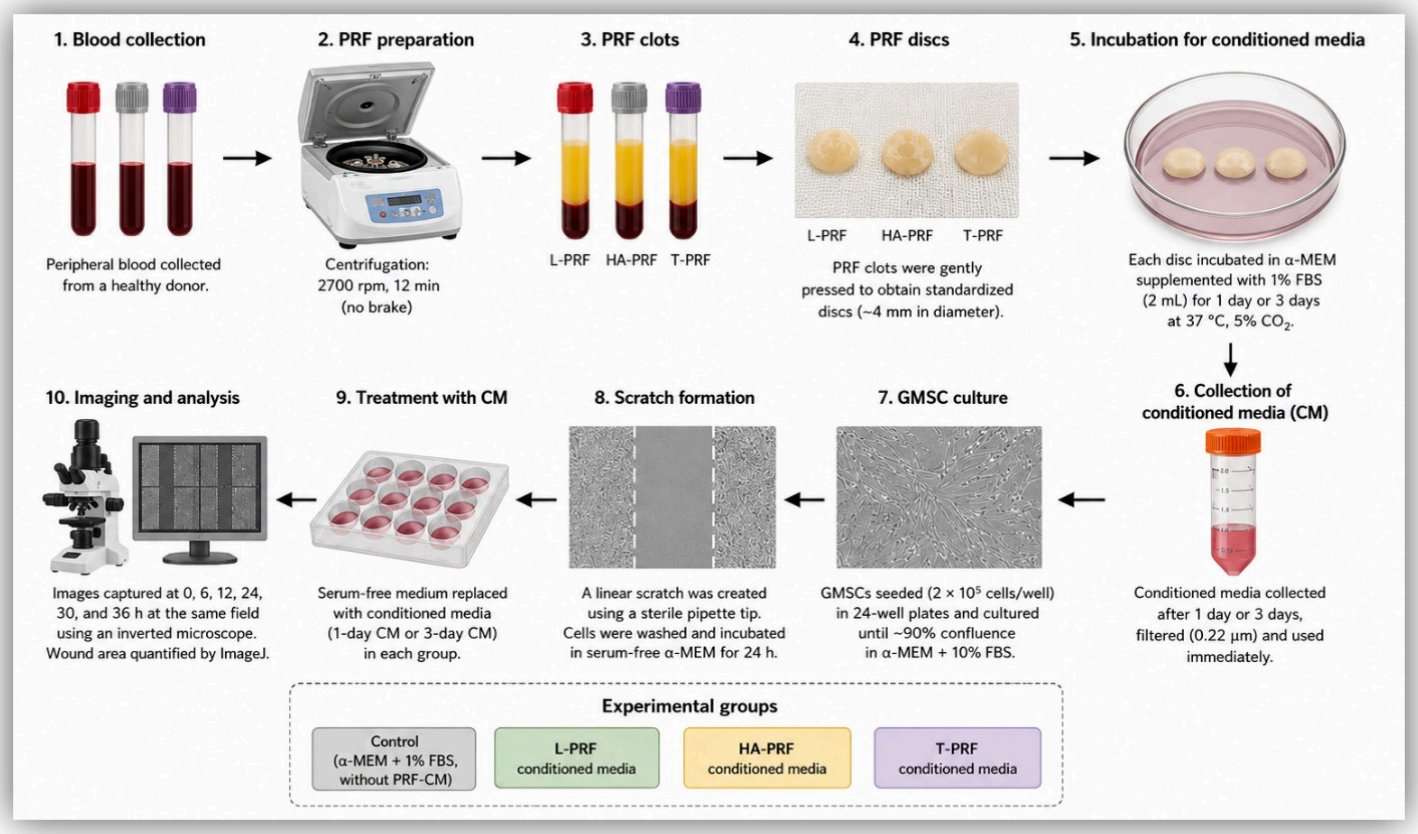


Figure 2

Schematic overview of the experimental workflow. L-PRF, HA-PRF, and T-PRF were prepared using the corresponding blood collection tubes and processed into standardized PRF discs. The PRF samples were incubated for 1 or 3 days to obtain conditioned media (CM), which were subsequently applied to gingiva-derived mesenchymal stem cells (GMSCs) following scratch formation. Wound closure was evaluated at 0, 6, 12, 24, 30, and 36 h. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; CM, conditioned medium; GMSC, gingiva-derived mesenchymal stem cell.

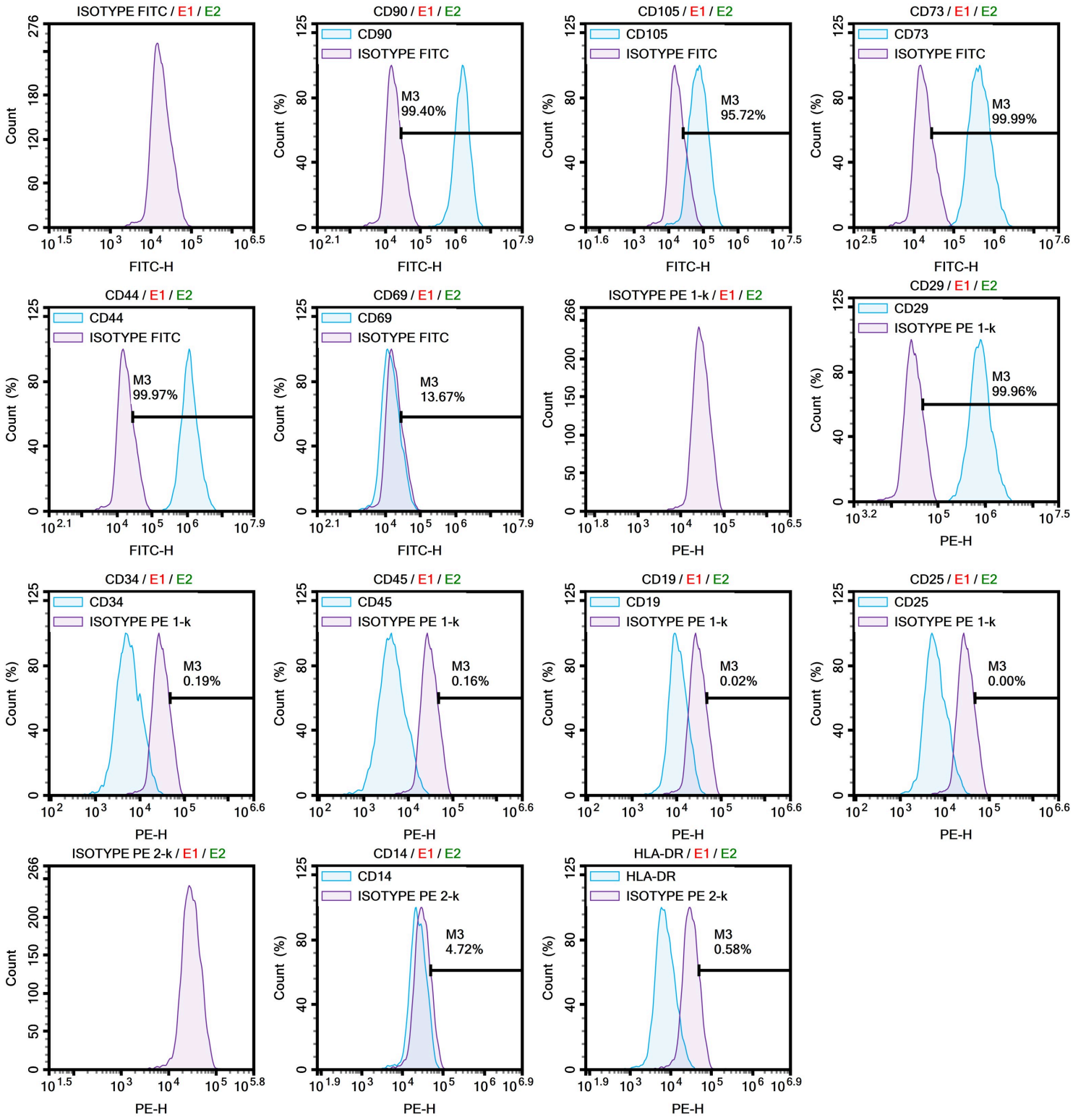


Figure 3

Flow cytometric characterization of gingiva-derived mesenchymal stem cells (GMSCs) at the third passage. Representative flow cytometry histograms demonstrate high expression of the mesenchymal stem cell-associated surface markers CD90 (99.40%), CD105 (95.72%), CD73 (99.99%), CD44 (99.97%), and CD29 (99.96%), whereas CD69 (13.67%), CD34 (0.19%), CD45 (0.16%), CD19 (0.02%), CD25 (0.00%),

CD14 (4.72%), and HLA-DR (0.58%) showed low or negligible expression. Target antibody staining is shown in blue and the corresponding isotype controls in purple.

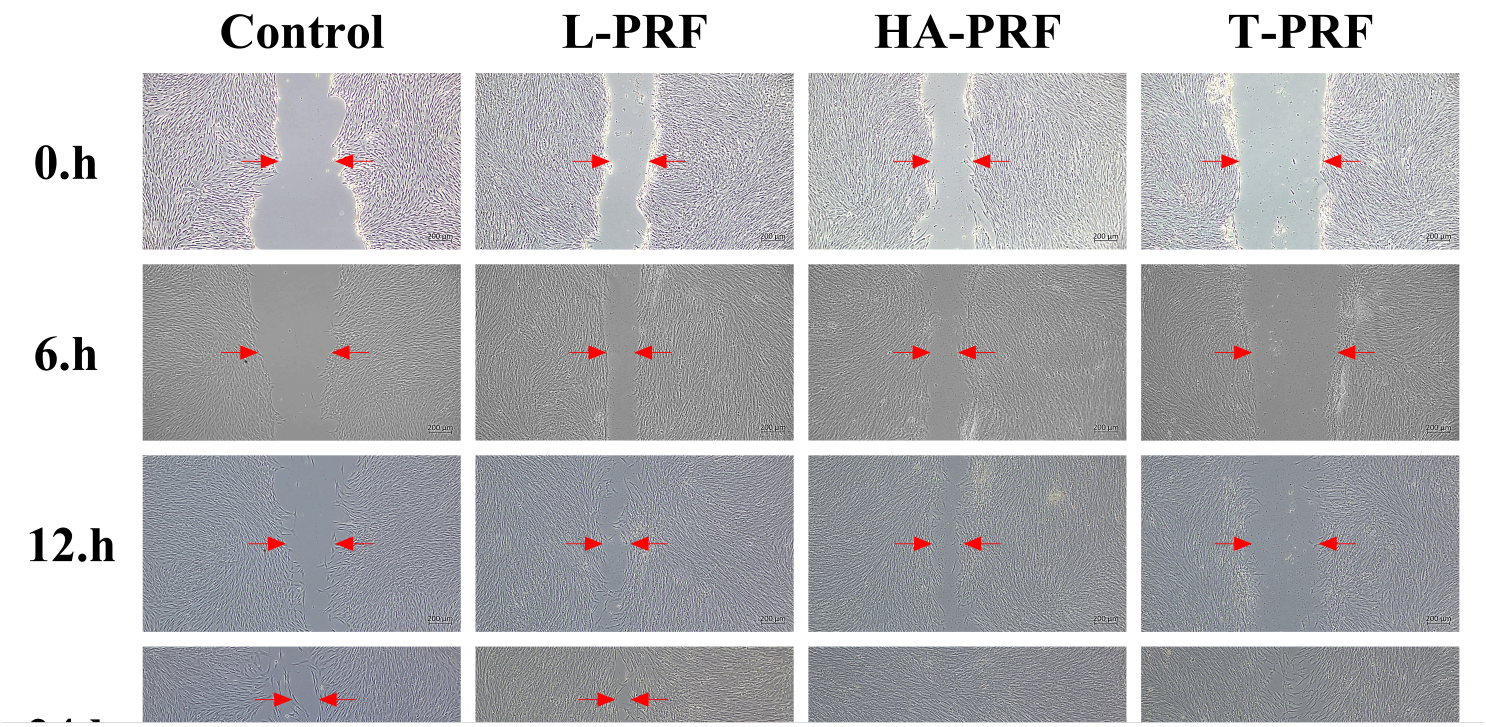


Figure 4

Representative phase-contrast microscopic images of GMSC wound closure following exposure to 1-day PRF-conditioned media. Images show the progressive closure of the scratch wound in the Control, L-PRF, HA-PRF, and T-PRF groups at 0, 12, 24, 30, and 36 h. Red arrows indicate the margins of the residual wound area. Complete wound closure was observed in all groups by 36 h. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; GMSC, gingiva-derived mesenchymal stem cell.

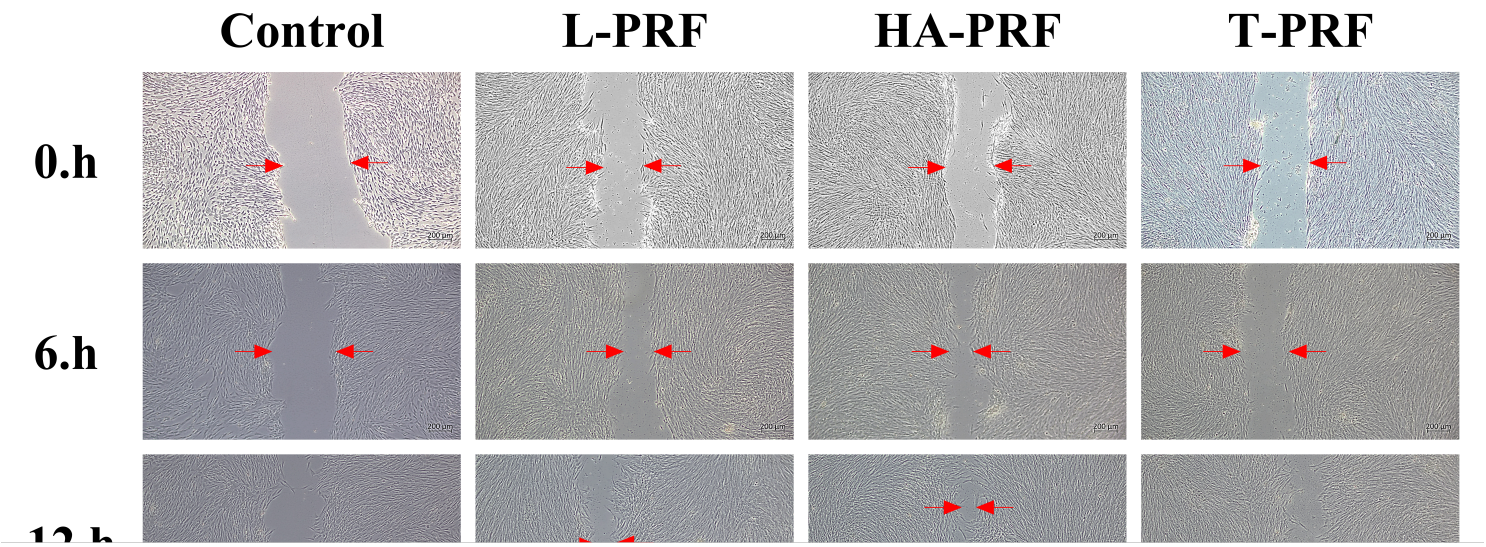


Figure 5

Representative phase-contrast microscopic images of GMSC wound closure following exposure to 3-day PRF-conditioned media. Images show the progressive closure of the scratch wound in the Control, L-PRF, HA-PRF, and T-PRF groups at 0, 12, 24, 30, and 36 h. Red arrows indicate the margins of the residual wound area. Complete wound closure was observed in the HA-PRF group by 24 h and in the L-PRF group by 30 h, whereas complete closure in the Control and T-PRF groups was observed by 36 h. L-PRF, leukocyte- and platelet-rich fibrin; HA-PRF, nano-hydroxyapatite-coated platelet-rich fibrin; T-PRF, titanium-prepared platelet-rich fibrin; GMSC, gingiva-derived mesenchymal stem cell.

Supplementary Files

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