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Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an *in vitro* trial

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Abstract (J Korean Assoc Oral Maxillofac Surg 2024;50:206-215)

Objectives: The current *in vitro* study aimed to assess the effects of ascorbic acid augmented albumin platelet-rich fibrin (AA Alb-PRF) on the wound healing activity of human gingival fibroblasts (HGFs) purported to be a regenerative biomaterial in surgical procedures.

Materials and Methods: All assays were performed on three HGF groups, group I: complete media; group II: Alb-PRF, and group III: AA Alb-PRF. Alb-PRF was prepared following the protocol by Fujioka-Kobayashi et al. (2021). For preparation of AA Alb-PRF, 2,500 μg AA was added to the blood pre-centrifugation. All groups were subjected to 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to estimate cell viability and proliferation, scratch assay for migration (0, 4, 12, and 24 hours) and transwell migration assay for chemotactic migration assessment (24 hours). Outcome variables were optical density (OD) for MTT assay, percentage of wound closure in scratch assay, and number of migrated cells in transwell migration assay. One-way ANOVA for MTT and transwell migration assays and two-way ANOVA for scratch assay with Bonferroni correction were performed with significance set at $P<0.05$.

Results: Cell viability and proliferation (OD: 0.684±0.003 and proliferation: 28%) and wound closure (49.92%±1.62% at 4 hours and 61.39%±0.88% at 12 hours) were significantly higher in group III, while group II demonstrated the maximum number of HGFs migrating across the transwell membrane (9.25±2.49) with $P<0.05$.

Conclusion: HGFs demonstrated a significant increase in viability and proliferation along with rapid wound closure in the presence AA Alb-PRF compared to Alb-PRF alone, indicating additional beneficial effects of AA. Thus, AA Alb-PRF potentiates the wound healing activity of HGFs and could be employed in oral, maxillofacial, and periodontal surgeries as a regenerative biomaterial.

Key words: Ascorbic acid, Platelet rich fibrin, Albumin, Regeneration, Fibroblasts

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I. Introduction

Tissue regeneration in wound healing encompasses a coordinated and interdependent set of events involving various cells, a proper blood supply, an apt scaffold, and signaling biomolecules[1]. Among them, fibroblasts and platelets play an active role in orchestrating these crucial processes[1]. Fibroblasts contribute to wound contraction and remodeling via the production of extracellular matrix (ECM), and platelets play a vital role in tissue repair by releasing abundant cytokines and growth factors, which are also involved in the regulation of fibroblastic activity[2].

Platelet concentrates have been used extensively in dentistry for many years, proving valuable in diverse regenerative applications[3]. Platelet-rich fibrin (PRF), an autologous platelet concentrate, aids in tissue regeneration by serving as a three-dimensional scaffold of fibrin, constituting leukocytes, macrophages, neutrophils, and platelets. In addition, PRF is a natural reservoir of factors responsible for adhesion, coagulation, and angiogenesis[4,5]. PRF has been employed as a therapeutic agent in oral and maxillofacial surgeries such as exodontia, implantology, management of oro-antral communications, and cleft reconstructions and in surgical periodontics such as guided tissue/bone regeneration (GTR/GBR) and recession coverage to promote bone and soft tissue regeneration during wound healing[6-9].

Over the years, several protocols of PRF were advocated

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which have shown distinction in the biological properties of PRF such as stability, cellularity, cell distribution and release of growth factors. A recently introduced protocol of PRF, albumin PRF (Alb-PRF)[3], or extended PRF[10] has gained popularity due to its prolonged degradation time. In this protocol, after centrifugation of blood, the plasma devoid of platelets is collected and heated to produce denatured albumin, which is mixed with liquid PRF to form Alb-PRF. The resultant PRF membrane has a denser protein structure organization with prolonged resorption properties (4-6 months) due to the denatured albumin, while re-addition of the platelet-rich layer offers cellular and growth factor contents[3,11]. To date, Alb-PRF has been employed as a barrier membrane in management of extraction sites, explanted sites, and zygomatic implant surgeries and also as a bio filler for lateral sinus augmentation, gingival recession coverage, and facial scar reduction[10].

Several studies have evaluated the effects of various biomolecules in combination with PRF to enhance its efficacy either by improving fibrin cross-linking or utilizing it as a local delivery system for biomolecules[12]. Ascorbic acid (AA), i.e., vitamin C, is a powerful antioxidant that plays a role in many important processes in the body including collagen synthesis, ECM formation, immune function, cell differentiation, promotion of the growth and regeneration of stem cells, and inhibition of cellular senescence[2,13]. AA is particularly important for healing of both soft and hard tissue wounds as it promotes the growth of fibroblasts, which are crucial for tissue repair. Research has demonstrated that oral vitamin C accelerates the healing process after surgery or tooth extraction[2]. Combination of AA and PRF in the management of intra-bony defects has also shown significant improvement in periodontal parameters[1].

With this background, we hypothesized that the combination of Alb-PRF and AA would prove beneficial in promoting regeneration and would healing. Therefore, the aim of this *in vitro* trial was to evaluate the effect of AA augmented Alb-PRF (AA Alb-PRF) on wound healing activity of human gingival fibroblasts (HGFs) and to determine if it can be utilized as a biomaterial for oral and maxillofacial and periodontal surgeries.

II. Materials and Methods

1. Study design

This *in vitro* trial on HGFs aimed to assess the effect of AA Alb-PRF on their wound healing activity and, was carried out at Bapuji Dental College and Hospital, Davangere and Maratha Mandal's NGH Institute of Dental Sciences, Belagavi in Karnataka, India between December 2021 to January 2022. Ethical approval for the study was provided by the Institutional Review Board of Bapuji Dental College and Hospital, Davangere, Karnataka, India (Ref. No. BDC Exam/548/2021-22), and the study protocol was carried out in accordance with the Declaration of Helsinki (2013). The written informed consent was obtained from all participants.

2. Sample

1) Inclusion and exclusion criteria

Systemically healthy male volunteers, aged between 18 and 25 years, were randomly selected from the outpatient department for harvest of gingival tissue to isolate HGFs and obtain blood samples for the PRF preparations. The volunteers were given detailed verbal and written descriptions of the study. Chronic smokers; subjects on antibiotics 3-6 months prior to the study; individuals on anticoagulants, corticosteroids, or immunosuppressants; and those with history of any active systemic disease/infection related to reduced wound healing such as uncontrolled diabetes, HIV, and leukopenia were excluded.

2) Cell culture

The HGFs were stored at the Central Research Laboratory of Maratha Mandal's NGH Institute of Dental Sciences, Belagavi, Karnataka, India. After obtaining written consent, unerupted mandibular third molar gingival tissue was harvested from a single volunteer (22 years, male) and immediately transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; HiMedia Laboratories Pvt. Ltd.). The tissue was rinsed twice in Dulbecco's phosphate-buffered saline (PBS; HiMedia Laboratories Pvt. Ltd.) and cut into fragments of 1 mm×2 mm with a surgical blade and seeded into a 24-well microtiter plate with complete media, i.e., DMEM enhanced with 10% heat inactivated fetal bovine serum (FBS; Gibco), and 5% antibiotic mixture (Gibco) of gentamicin (10 μg), penicillin (100 Units/mL), and streptomycin (100 μg/mL) for HGF culture. The cells were maintained in an incubator (New Brunswick) at optimum conditions (5% CO_2 at 37ºC) in a humidified atmosphere, and the media was replaced every two days. Fibroblasts were harvested at the fourth passage for use in the assays.

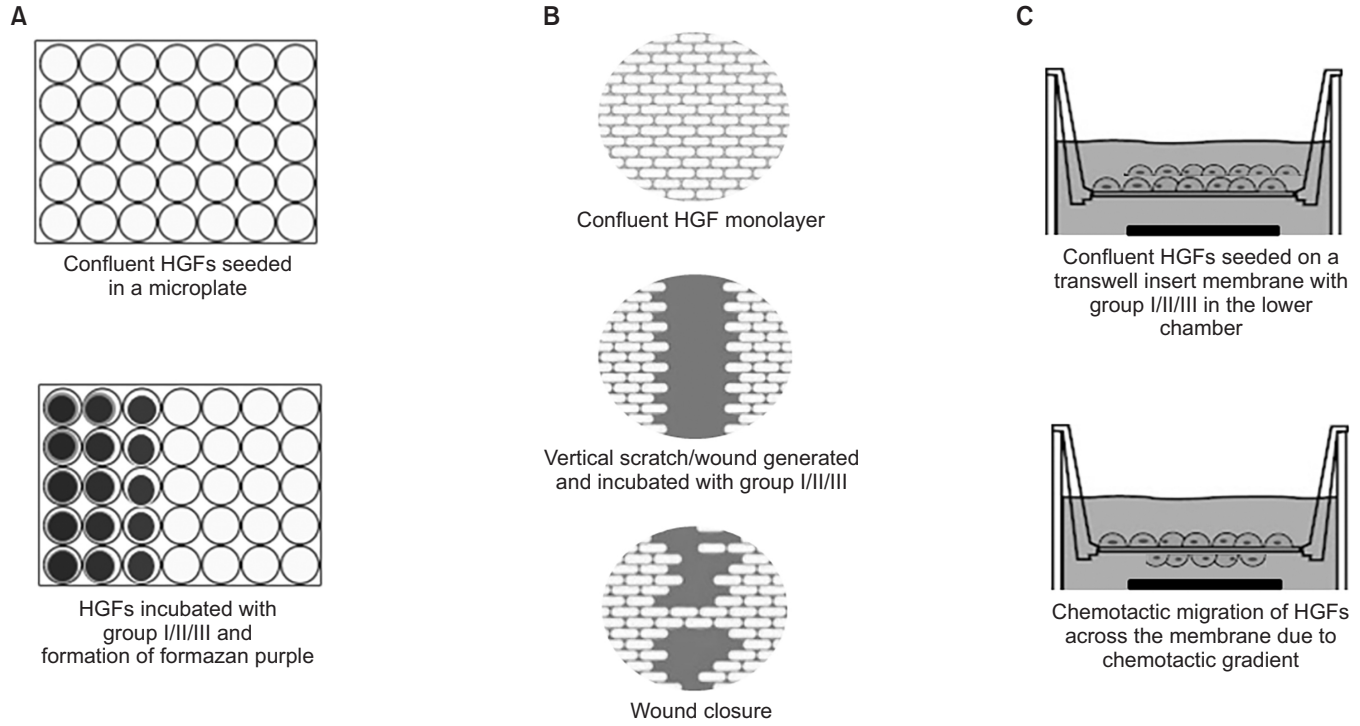

Fig. 1. Schematic representation of the study protocol. A. MTT assay. B. Scratch assay. C. Transwell migration assay. Group I: complete media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin. (MTT: 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, HGFs: human gingival fibroblasts)
Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

3) Preparation of Alb-PRF

Under aseptic conditions, two samples of 10 mL blood were withdrawn from the antecubital vein into S-PRF glass tubes (S-P; Choukroun) and centrifuged (PRF DUO Quattro; Choukroun) at 700g for 8 minutes. After centrifugation, using a 2 mL syringe (Dispovan and Unolok; HMD Ltd.), the upper layer of plasma devoid of platelets was collected and heated for 10 minutes at 75°C to procure denatured albumin (albumin gel), which was allowed to cool to room temperature. Liquid PRF was collected in a second syringe and mixed with the cooled albumin gel using a female-female luer lock device to form Alb-PRF. The injectable Alb-PRF was allowed to polymerize into a gelled membrane in a sterile glass petri dish[3].

4) Preparation of AA Alb-PRF

The AA Alb-PRF was prepared with a similar protocol, where 2,500 μg AA (SCORNIX; Rhythm Biotech Pvt. Ltd.) was mixed with blood before centrifugation to attain a concentration of 250 μg/mL of AA in AA Alb-PRF membrane[2,3,13].

5) Intervention

This *in vitro* study subjected the HGFs to complete media (group I: negative control), Alb-PRF (group II: positive control), and AA Alb-PRF (group III). All the samples were analyzed with the cell viability and proliferation assay, scratch assay, and transwell migration assay for assessment of the wound healing activity of HGFs.(Fig. 1) Four wells were used per group for each assay. Alb-PRF and AA Alb-PRF samples from three donors were utilized to confirm the results in triplicate. The data from a single, representative donor was presented for ease.

6) Cell viability and proliferation assay (MTT assay)

In vitro viability and proliferation effects were assessed by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For that, 2×10^4 cells/well were cultured in a microplate (Nunc MicroWell 96-Well Microplates; Thermo Fisher Scientific) to 100% confluence in 24 hours. The supernatant was removed from the microplate and replaced with fresh DMEM solution, to which complete media, Alb-PRF, and AA Alb-PRF were subsequently added to respec-

tive wells. The cell cultures were cut uniformly to a 1.5 mm diameter using a biopsy punch (Acumen Schimatle). After 24 hours of incubation under optimum conditions, 20 μL (5 mg/mL) stock solution of MTT (HiMedia Laboratories Pvt. Ltd.) was added to every microplate well and further incubated for 4 hours to allow the formation of the formazan product by the cells. Post-incubation, the supernatant was slowly aspirated, and precipitated purple formazan crystals were dissolved by adding 100 μL dimethyl sulfoxide (DMSO; Thermo Fisher Scientific).

7) Scratch assay

Approximately 2×10^4 HGFs were cultured in a micro plate (Nunc MicroWell 96-Well Microplates; Thermo Fisher Scientific) to 100% confluence; treated with complete media, Alb-PRF, and AA Alb-PRF; trimmed to 1.5 mm diameter samples; and incubated at optimum conditions for 24 hours. Sterile 200 μL pipette tips (Accumax) were used to generate a vertical scratch, i.e., a wound, through the confluent layer of cells without excessive force. The older media and cell debris were carefully aspirated and replaced by enough complete media to cover the entire well.

8) Transwell migration assay

For this assay, 1,000 μL (approximately 106 cells/mL) HGF solution was plated onto the membranes in a transwell insert plate (8 μm pore size; HiMedia Laboratories Pvt. Ltd.) and incubated for 10 minutes at optimum conditions. In addition, 3 mL of serum-free media was added as a negative control, and Alb-PRF and AA Alb-PRF were added to respective lower chambers and incubated for 24 hours at optimum conditions. The non-migrated HGFs above the membrane surface were cleared with a cotton swab post-incubation. The cells that had migrated across the membrane to the opposite side were fixed in 70% ethanol (Thermo Fisher Scientific) for 10 minutes, stained with 0.5% crystal violet (cell culture tested, HiMedia Laboratories Pvt. Ltd.), and washed thrice with distilled water.

3. Variables

The predictor variable was treatment of HGFs with AA Alb-PRF. The outcome variables were optical densities in the MTT assay, percentage of wound closure in the scratch assay, and migration of cells across the membrane in the transwell migration assay.

4. Data collection

- MTT assay: The optical density (OD) was recorded at 595 nm using a spectrophotometer (LISA plus; Aspen Diagnostics Pvt. Ltd.)
  a) Cell viability (%)=(mean OD of group I/II/III÷mean OD of group I)×100
  b) Cell proliferation (%)=cell viability (group I/II/III–group I)
- Scratch assay: Wound closure was inspected under an inverted microscope (Olympus) at 0, 4, 12, and 24 hours (×100), imaged, and analyzed using GIMP 2.10 software to measure the reduction in distance (in μm) between the wound edges at the mentioned time points. The values were calculated as percentage of wound closure compared to the wound measurement at 0 hours for each of the groups.
- Transwell migration assay: Cell migration was assessed under an inverted microscope (Olympus) in three random fields (×100), imaged, and analyzed using GIMP 2.10 software (https://www.gimp.org/) to obtain the number of HGFs that had migrated through the membrane.

5. Statistical analysis

The IBM SPSS Statistics program (ver. 20.0; IBM Corp.)

Table 1. Comparison of optical density, cell viability, and proliferation percentages at 24 hours

Group	Optical density	95% CI	*P*-value	Cell viability (%)	Cell proliferation (%)
I	0.535±0.002	0.530-0.539		100	-
II	0.600±0.004	0.600-0.615		114	14
III	0.684±0.003	0.679-0.690	<0.05*	128	28

(CI: confidence interval)
*P<0.05; one-way ANOVA with Bonferroni test.
Values are presented as mean±standard deviation.
Group I: complete media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.
Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

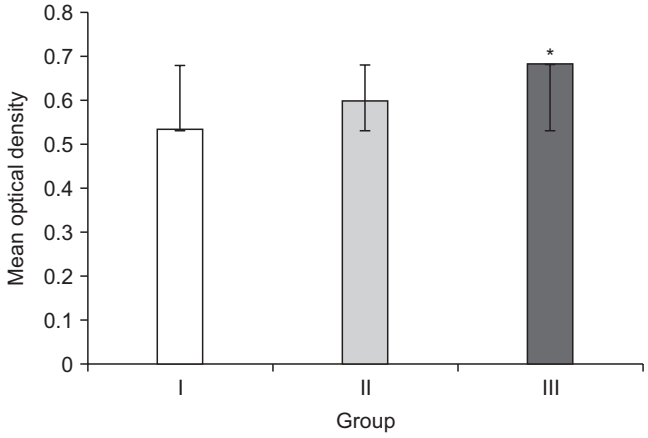

Fig. 2. Mean optical density values at 24 hours. *P<0.05, one-way ANOVA with Bonferroni test. Group I: complete media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.

Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

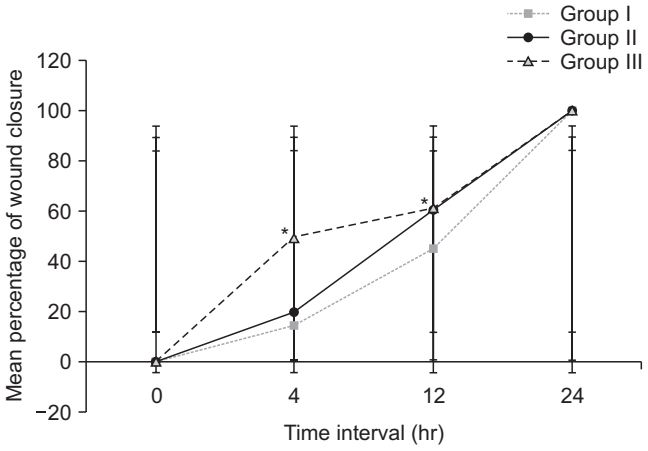

Fig. 3. Mean percentage of wound closure observed at various time intervals. *P<0.05, two-way ANOVA with Bonferroni test. Group I: complete media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.

Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

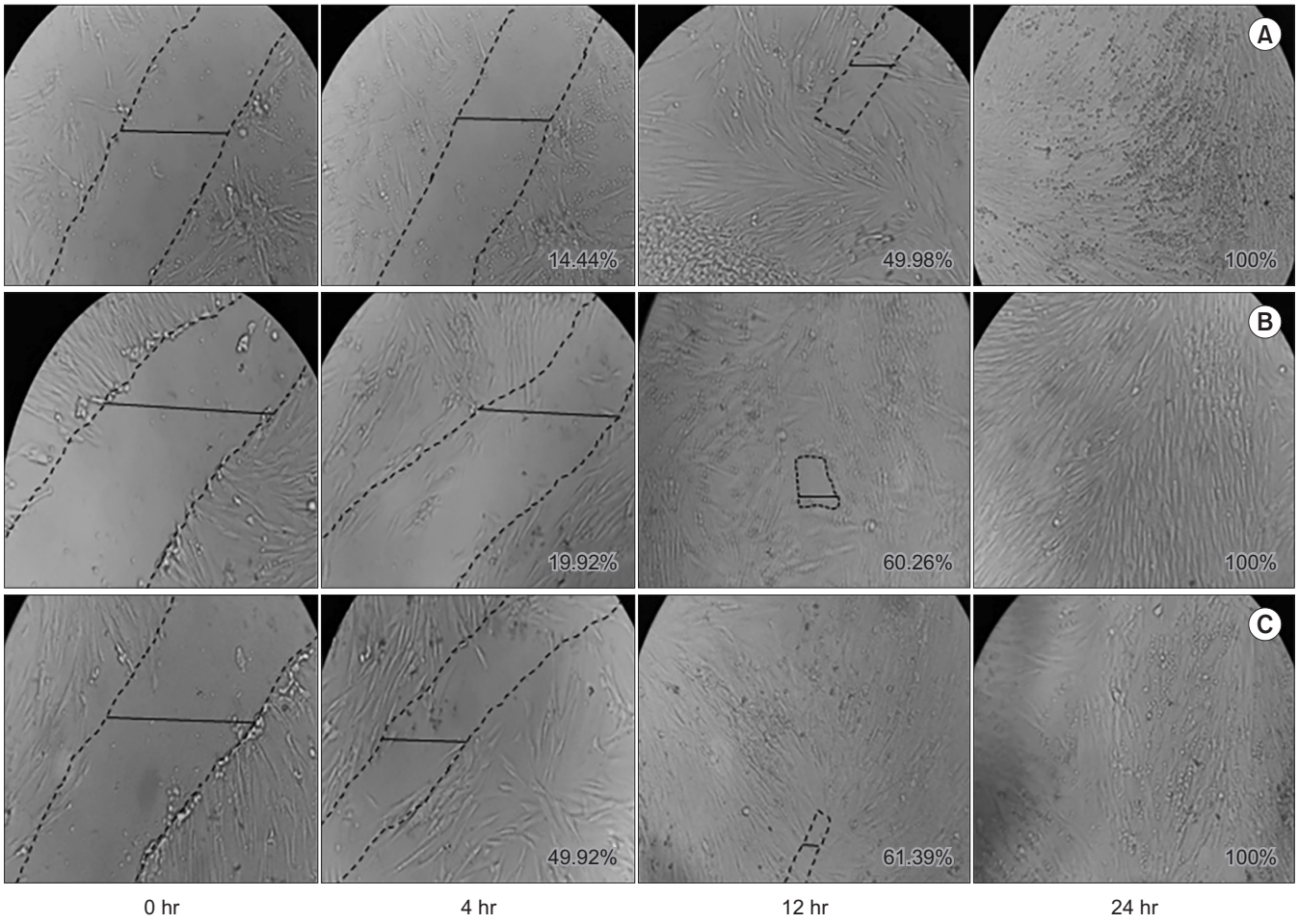

Fig. 4. Cell migration-percentage wound closure at 0, 4, 12, and 24 hours in group I (A), group II (B), and group III (C) (×100). Group I: complete media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.

Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

was employed for statistical analysis. Results were presented as mean and standard deviation or percentage. One-way ANOVA for MTT and transwell migration assays and two-way ANOVA for scratch assay with post-hoc Bonferroni corrections were used for analysis. $P<0.05$ was the cutoff for statistical significance.

III. Results

1. MTT assay

The mean OD values recorded at 24 hours for groups I, II, and III were 0.535±0.002, 0.600±0.004, and 0.684±0.003, respectively. The cell viability in groups I, II, and III was 100%, 114%, and 128%, respectively. In comparison to group I, the cell proliferation for groups II and III was 14% and 28%, respectively. Cell toxicity was not evident in any of the groups. The results showed a significant difference in both cell viability and proliferation between the groups at the 24-hour time point ($P<0.05$). Group III exhibited the highest values, followed by group II and then group I.(Table 1, Fig. 2)

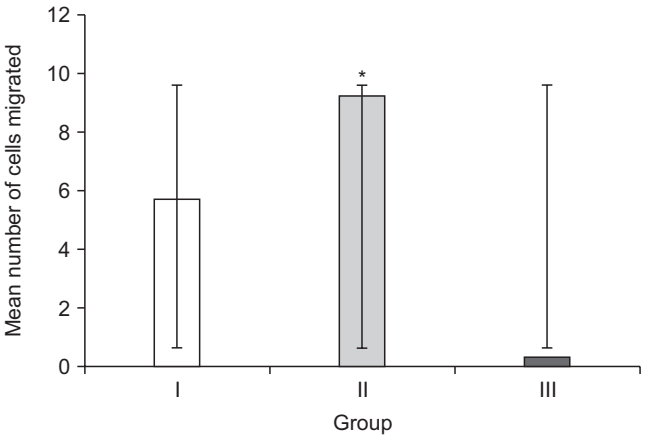

Fig. 5. Mean number of migrated cells across the membrane at 24 hours. *$P<0.05$, one-way ANOVA with Bonferroni test. Group I: serum free media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.
Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

2. Scratch assay

The mean percentages of wound closure at 4 hours were 14.44%±2.37%, 19.92%±3.33%, and 49.92%±1.62% in groups I, II, and III, respectively, and those at 12 hours were 44.98%±2.02%, 60.26%±1.00%, and 61.39%±0.88%. All three groups showed complete wound closure by 24 hours. A significant difference in the percentage of wound closure was seen at 4 and 12 hours, with the fastest closure demonstrated by group III at both time intervals ($P<0.05$).(Fig. 3, 4)

3. Transwell migration assay

The mean numbers of cells migrated after 24 hours in groups I, II, and III were 5.75±4.07, 9.25±2.49, and 0.33±0.77, respectively. The analysis indicated a significant difference among the groups, with the highest cell migration in group II, followed by group I and group III ($P<0.05$).(Fig. 5, 6)

IV. Discussion

The present *in vitro* trial investigated the effects of AA Alb-PRF on wound healing activity of HGFs by assessing cell vi-

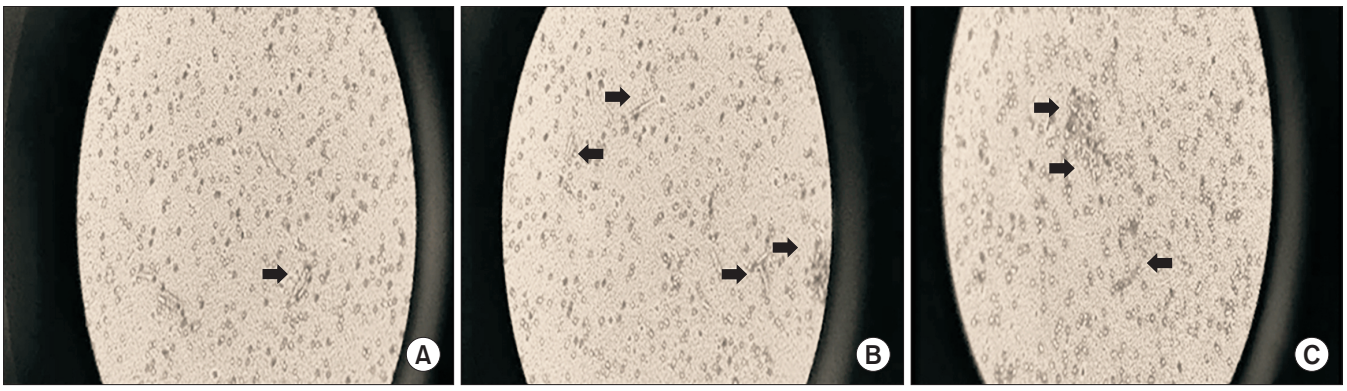

Fig. 6. Transwell migration of fibroblasts (arrows) in group I (A), group II (B), and group III (C) (0.5% crystal violet staining, ×100). Group I: serum free media, Group II: albumin-platelet rich fibrin, Group III: ascorbic acid augmented albumin-platelet rich fibrin.
Manjiri Kulkarni et al: Effects of ascorbic acid augmented albumin platelet-rich fibrin on the wound healing activity of human gingival fibroblasts: an in vitro trial. J Korean Assoc Oral Maxillofac Surg 2024

ability through MTT assay and cell migration through scratch assay and transwell migration assay.

Wound healing encompasses a complex and coordinated set of cellular and biochemical events. First is activation of platelets and fibrin clot formation, resulting in hemostasis. This is followed by infiltration of neutrophils and macrophages, leading to an inflammatory phase[14]. Fibroblasts then migrate and proliferate to establish a new ECM that subsequently matures[14]. These processes are regulated by various chemokines and cytokines, such as platelet-derived growth factor, transforming growth factor-beta 1, vascular endothelial growth factor, interleukin-6, interleukin-1β, and tumor necrosis factor-alpha, as well as the necessary nutrients for uneventful healing[14,15].

PRF as an autologous surgical adjuvant is known to promote and accelerate soft and hard tissue wound healing and regeneration due to its potential to facilitate optimal concentrations of platelets, fibrin, growth factors, leukocytes, and macrophages[7,16]. The degranulation of platelets from PRF releases supraphysiological levels of the aforementioned growth factors into the wound site to promote healing[7]. The renewal of periodontal cells is also promoted by these growth factors, leading to tissue regeneration[5].

Several techniques have been noted to enhance the durability and efficacy of PRF either by additional physical (ultraviolet light, heat) or chemical methods (glucose, glutaraldehyde, etc.)[17]. Association of biomaterials with albumin has demonstrated favorable modulation in the fibrin network ultrastructure and permeability, resulting in formation of fibers with increased thickness and a coarse nodular appearance[18]. Earlier studies have established that heating and denaturing albumin modifies its three-dimensional structure through the creation of new hydrogen and disulfide bonds. These modifications lead to drastic changes in resorption properties and improved stability over time[11]. The combination of denatured albumin and liquid PRF produces Alb-PRF, reportedly having a denser and more stable organization of the protein structure, extended resorption properties, enhanced volume stability (21 days), and an increased duration of release of cells and growth factors (up to 10 days)[3,10,11,19]. The combination has demonstrated improved cellular viability and proliferation of gingival fibroblasts[11] and can be considered as an alternative for autologous PRF membranes, with longer stability and resorption time.

During wound healing, especially in the inflammatory phase, the higher level of catabolism leads to increased uptake of various micronutrients, including AA (vitamin C)[14]. AA is an essential micronutrient for healing but is not synthesized naturally in the human body. Studies have shown that AA levels decrease significantly (up to 70%) at the site of injury and do not fully recover even after two weeks[20]. The micronutrient helps eliminate free radicals, aids in collagen production, modulates immune cell functions, and is necessary for fibroblast proliferation and angiogenesis. Through these processes, AA is rapidly depleted and therefore needs to be supplemented[14,21].

In a clinical trial, Elbehwashy et al.[1] incorporated AA in the blood to prepare PRF for treatment of intra-osseous periodontal defects and reported a substantial improvement in periodontal parameters. The improvement was attributed to the sustained release of AA during surgical wound healing and its subsequent regenerative effects on the resident periodontal cells, in addition to PRF growth factors[1]. AA at a concentration of 250 μM was employed for the preparation of AA Alb-PRF in the present study based on an *in vitro* trial that reported maximum proliferation of the gingival stem cells at that concentration[13].

We hypothesized that AA Alb-PRF would increase the wound healing activity of HGFs and can be employed as a potential regenerative biomaterial in maxillofacial and periodontal surgeries. To the best of our knowledge, this is the first *in vitro* study examining the wound healing activity of HGFs under the influence of AA Alb-PRF.

In the present *in vitro* trial, cell viability and proliferation were assessed with MTT assay. That assay spectrophotometrically measures the reduction of yellow MTT to purple-blue formazan by mitochondrial succinate dehydrogenase produced by metabolically active cells. The greater is the activity of the cells, the greater is the intensity of purple color, and the higher is the absorbance, indicating higher cell viability[22]. The scratch assay inspects the ability of a particular cell line to migrate to and close a wound in a confluent monolayer of cells, simulating *in vivo* cell migration, while the transwell migration assay offers the distinct advantage to analyze migration in response to a chemotactic gradient across a filter membrane[23,24].

Results of previous research have shown that the size of the PRF membranes obtained from male and female patients varies significantly (17%) due to differences in hematocrit values[25]. To reduce this variability and maintain consistency in the average size of the PRF obtained, only male participants were included in the present trial.

Maximum viability (128%, OD: 0.684±0.003) and fastest wound closure demonstrated by HGFs in group III could be

substantiated by confluence of the stimulatory effects of AA along with growth factors released from Alb-PRF. Studies have shown that AA boosts the proliferative and regenerative potentials of gingival fibroblasts along with their ability to produce ECM and remodeling via increased expression of urokinase-type plasminogen activator, hyaluronan-mediated motility receptor, and IL-6[13]. AA also redirects quiescent fibroblasts into the cell cycle and promotes migration[13]. At 4 hours, 49.92% closure is suggestive of the aforementioned stimulatory effects of AA that could have resulted in accelerated initial wound closure. Our results are comparable with those of an *in vitro* study by Chaitrakoonthong et al.[2], where local rinsing with 20 μg/mL AA stimulated gingival fibroblastic viability and migration. The growth factors released at the wound site can act as mitogens and chemoattractants for dermal fibroblasts and gingival and periodontal cells, increasing their proliferation and migration[26]. Group II also showed increased fibroblastic viability (114%), migration, and wound closure, accredited to the mitogenic activity of growth factors released by Alb-PRF. This is comparable to an *in vitro* trial by Fujioka-Kobayashi et al.[3].

Migration of HGFs across the transwell membrane was the lowest in group III compared to the other two groups, and the difference was statistically significant. Chaitrakoonthong et al.[2] reported that while rinsing the fibroblasts with higher concentration of AA (50 μg/mL) reduced their viability and wound closure ability, it promoted their ECM protein expression, thus depicting a dose dependent switch in the fibroblastic activity. Another *in vitro* study, by Baranyi et al.[27], reported that supplementation of AA (100 μM) to the culture medium was found to reduce the migration capability of fibroblasts and therefore to reduce wound closure. From the findings of these various studies it can be postulated that the fibroblastic activity varies depending on the concentration and mode of administration of AA. It is noteworthy to consider this factor while interpreting the outcome of our study. As mentioned previously, the addition of AA in group III improved viability of HGFs, and they demonstrated rapid initial wound closure (49.92% at 4 hours). However, it resulted in decreased transwell migration, which was possibly attributable to enhanced collagen and ECM production at the employed concentration of 250 μM.

Apart from the chemotactic and mitogenic actions of TGF-β1, it is a vital factor involved in the transformation of fibroblasts to myofibroblasts, particularly during remodeling of the granulation tissue[28,29]. Myofibroblasts are pivotal in orchestrating the production of granulation tissue, and they exhibit an amplified capacity for granulation tissue contraction and remodeling, eventually resulting in wound closure and/or scar formation[30]. A scientific investigation documented that co-culture of human dermal fibroblasts with TGF-β1 coupled with AA initiated a switch to a myofibroblast phenotype[27,31]. Also, assessment of growth factor release from Alb-PRF by Fujioka-Kobayashi et al.[3] revealed a significant upsurge in the concentration of TGF-β1 released for up to 10 days. In line with previously listed research studies, it can be hypothesized that AA along with TGF-β1 released from AA Alb-PRF could have accelerated the myofibroblastic differentiation, which probably increased the ECM production and ultimately reduced migration of HGFs across the transwell membrane in group III. However, it is important to note that their viability was not negatively impacted.

Thus, our *in vitro* trial strongly suggests that AA Alb-PRF is a potential biocompatible material for gingival fibroblast-mediated healing and regeneration in the wound microenvironment. As an autologous and stable local delivery agent for AA, enriched with cells and growth factors, Alb-PRF may facilitate wound regeneration[1,3]. Augmentation with AA would synergistically promote the wound healing activity of gingival fibroblasts due the antioxidant and mitogenic properties, while aiding in collagen synthesis. Although the utilization of AA Alb-PRF would add to the cost compared to classic periodontal surgery methods, its benefits outweigh and justify the added expense.

When interpreting the results and deriving conclusions in the current *in vitro* trial, it is crucial to consider a few perspectives. The harvested HGFs and blood samples used for cell culture and in the Alb-PRF preparations were sourced from non-autologous donors. Earlier investigations have highlighted that blood with physiological differences in the number and distribution of platelets, leukocytes, and erythrocytes among blood samples and associated variables like stress, nutrition, sex hormones, and compromised immunity might influence the quality and quantity of PRF and have an impact on study outcomes[32]. Similarly, *in vitro* cell culture conditions, particularly the media supplements, also can phenotypically alter the primary cells, although it remains uncertain whether a comparable alteration occurs in an *in vivo* environment[27].

V. Conclusion

Thus, the current *in vitro* trial demonstrated an improvement in the wound healing activity of HGFs in the presence

of AA Alb-PRF as depicted by their enhanced proliferation and migration. Further studies need to be conducted to evaluate the durability, growth factors, and AA release of AA Alb-PRF and to determine its effect on other cells (osteoblasts, periodontal ligament fibroblasts, and epithelial cells). A suitable concentration of AA should be optimized. Animal trials and later clinical trials assessing the cost-benefit ratio could open a wide range of applications for AA Alb-PRF in the management of oral, maxillofacial, and periodontal wounds including extraction sockets, intrabony defects, ridge and sinus augmentations, and GTR/GBR among many others.

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Authors' Contributions

M.K. and S.N.K. conceptualized, investigated, and administered the project and carried out the methodology, writing, reviewing, and editing of the original draft. M.K. formally collected and organized the research data. G.G.V. and T.M.G. reviewed and edited the original draft. S.N.K., G.G.V., and T.M.G. supervised the research. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Ethics Approval and Consent to Participate

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

References

1. Elbehwashy MT, Hosny MM, Elfana A, Nawar A, Fawzy El-Sayed K. Clinical and radiographic effects of ascorbic acid-augmented platelet-rich fibrin versus platelet-rich fibrin alone in intra-osseous defects of stage-III periodontitis patients: a randomized controlled clinical trial. Clin Oral Investig 2021;25:6309-19. https://doi.org/10.1007/s00784-021-03929-1
2. Chaitrakoonthong T, Ampornaramveth R, Kamolratanakul P. Rinsing with L-ascorbic acid exhibits concentration-dependent effects on human gingival fibroblast in vitro wound healing behavior. Int J Dent 2020;2020:4706418. https://doi.org/10.1155/2020/4706418
3. Fujioka-Kobayashi M, Schaller B, Mourão CFAB, Zhang Y, Sculean A, Miron RJ. Biological characterization of an injectable platelet-rich fibrin mixture consisting of autologous albumin gel and liquid platelet-rich fibrin (Alb-PRF). Platelets 2021;32:74-81. https://doi.org/10.1080/09537104.2020.1717455
4. Dohan Ehrenfest DM, Del Corso M, Diss A, Mouhyi J, Charrier JB. Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane. J Periodontol 2010;81:546-55. https://doi.org/10.1902/jop.2009.090531
5. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part IV: clinical effects on tissue healing. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:e56-60. https://doi.org/10.1016/j.tripleo.2005.07.011
6. Miron RJ, Fujioka-Kobayashi M, Bishara M, Zhang Y, Hernandez M, Choukroun J. Platelet-rich fibrin and soft tissue wound healing: a systematic review. Tissue Eng Part B Rev 2017;23:83-99. https://doi.org/10.1089/ten.teb.2016.0233
7. Fan Y, Perez K, Dym H. Clinical uses of platelet-rich fibrin in oral and maxillofacial surgery. Dent Clin North Am 2020;64:291-303. https://doi.org/10.1016/j.cden.2019.12.012
8. Njokanma AR, Fatusi OA, Ogundipe OK, Arije OO, Akomolafe AG, Kuye OF. Does platelet-rich fibrin increase bone regeneration in mandibular third molar extraction sockets? J Korean Assoc Oral Maxillofac Surg 2022;48:371-81. https://doi.org/10.5125/jkaoms.2022.48.6.371
9. Jung JW, Hong SO, Lee EJ, Kim RY, Jee YJ. The double-barrier technique using platelet-rich fibrin for closure of oroantral fistulas. J Korean Assoc Oral Maxillofac Surg 2023;49:163-8. https://doi.org/10.5125/jkaoms.2023.49.3.163
10. Miron RJ, Pikos MA, Estrin NE, Kobayashi-Fujioka M, Espinoza AR, Basma H, et al. Extended platelet-rich fibrin. Periodontol 2000 2024;94:114-30. https://doi.org/10.1111/prd.12537
11. Gheno E, Mourão CFAB, Mello-Machado RC, Stellet Lourenço E, Miron RJ, Catarino KFF, et al. In vivo evaluation of the biocom-

patibility and biodegradation of a new denatured plasma membrane combined with liquid PRF (Alb-PRF). Platelets 2021;32:542-54. https://doi.org/10.1080/09537104.2020.1775188
12. Miron RJ, Moraschini V, Fujioka-Kobayashi M, Zhang Y, Kawase T, Cosgarea R, et al. Use of platelet-rich fibrin for the treatment of periodontal intrabony defects: a systematic review and meta-analysis. Clin Oral Investig 2021;25:2461-78. https://doi.org/10.1007/s00784-021-03825-8
13. Van Pham P, Tran NY, Phan NL, Vu NB, Phan NK. Vitamin C stimulates human gingival stem cell proliferation and expression of pluripotent markers. In Vitro Cell Dev Biol Anim 2016;52:218-27. https://doi.org/10.1007/s11626-015-9963-2
14. Mohammed BM, Fisher BJ, Kraskauskas D, Ward S, Wayne JS, Brophy DF, et al. Vitamin C promotes wound healing through novel pleiotropic mechanisms. Int Wound J 2016;13:572-84. https://doi.org/10.1111/iwj.12484
15. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010;89:219-29. https://doi.org/10.1177/0022034509359125
16. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:e37-44. https://doi.org/10.1016/j.tripleo.2005.07.008
17. Rahman S, Gayathri GV, Mehta DS. Comparative evaluation of advanced platelet rich fibrin membrane with and without glutaraldehyde crosslinking- a de novo in vitro trial. EC Dent Sci 2019;18:2328-37.
18. Mourão CFAB, Gheno E, Lourenço ES, Barbosa RL, Kurtzman GM, Javid K, et al. Characterization of a new membrane from concentrated growth factors associated with denaturized albumin (Alb-CGF) for clinical applications: a preliminary study. Int J Growth Factors Stem Cells Dent 2018;1:64-9.
19. Horváthy DB, Simon M, Schwarz CM, Masteling M, Vácz G, Hornyák I, et al. Serum albumin as a local therapeutic agent in cell therapy and tissue engineering. Biofactors 2017;43:315-30. https://doi.org/10.1002/biof.1337
20. Kim M, Otsuka M, Yu R, Kurata T, Arakawa N. The distribution of ascorbic acid and dehydroascorbic acid during tissue regeneration in wounded dorsal skin of guinea pigs. Int J Vitam Nutr Res 1994;64:56-9.
21. Yussif NM, Abdul Aziz MA, Abdel Rahman AR. Evaluation of the anti-inflammatory effect of locally delivered vitamin C in the treatment of persistent gingival inflammation: clinical and histopathological study. J Nutr Metab 2016;2016:2978741. https://doi.org/10.1155/2016/2978741
22. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell viability assays. In: Markossian S, Grossman A, Arkin M, Auld D, Austin C, Baell J, et al., eds. Assay guidance manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.
23. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, et al. In vitro cell migration and invasion assays. Mutat Res 2013;752:10-24. https://doi.org/10.1016/j.mrrev.2012.08.001
24. Hulkower KI, Herber RL. Cell migration and invasion assays as tools for drug discovery. Pharmaceutics 2011;3:107-24. https://doi.org/10.3390/pharmaceutics3010107
25. Miron RJ, Dham A, Dham U, Zhang Y, Pikos MA, Sculean A. The effect of age, gender, and time between blood draw and start of centrifugation on the size outcomes of platelet-rich fibrin (PRF) membranes. Clin Oral Investig 2019;23:2179-85. https://doi.org/10.1007/s00784-018-2673-x
26. McClain SA, Simon M, Jones E, Nandi A, Gailit JO, Tonnesen MG, et al. Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. Am J Pathol 1996;149:1257-70.
27. Baranyi U, Winter B, Gugerell A, Hegedus B, Brostjan C, Laufer G, et al. Primary Human fibroblasts in culture switch to a myofibroblast-like phenotype independently of TGF beta. Cells 2019;8:721. https://doi.org/10.3390/cells8070721
28. Marconi GD, Fonticoli L, Rajan TS, Lanuti P, Della Rocca Y, Pierdomenico SD, et al. Transforming growth factor-beta1 and human gingival fibroblast-to-myofibroblast differentiation: molecular and morphological modifications. Front Physiol 2021;12:676512. https://doi.org/10.3389/fphys.2021.676512
29. Smith PC. Role of myofibroblasts in normal and pathological periodontal wound healing. Oral Dis 2018;24:26-9. https://doi.org/10.1111/odi.12773
30. Smith PC, Martínez C, Martínez J, McCulloch CA. Role of fibroblast populations in periodontal wound healing and tissue remodeling. Front Physiol 2019;10:270. https://doi.org/10.3389/fphys.2019.00270
31. Piersma B, Wouters OY, de Rond S, Boersema M, Gjaltema RAF, Bank RA. Ascorbic acid promotes a TGFβ1-induced myofibroblast phenotype switch. Physiol Rep 2017;5:e13324. https://doi.org/10.14814/phy2.13324
32. Bi J, Intriago MFB, Koivisto L, Jiang G, Häkkinen L, Larjava H. Leucocyte- and platelet-rich fibrin regulates expression of genes related to early wound healing in human gingival fibroblasts. J Clin Periodontol 2020;47:851-62. https://doi.org/10.1111/jcpe.13293

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