

Gene and cellular assessment of wound healing with a novel natural cocktail gel dressing: a new method for quantitative wound closure time assessment

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Abstract

Introduction: This study aimed to enhance wound healing using a novel natural cocktail gel dressing composed of purslane, human amniotic membrane (hAM), and platelet-rich plasma (PRP). In addition, a new ratio-based analytical approach was applied to evaluate the healing dynamics in each treatment group, revealing correlations between the healing rate and the respective treatment compound.

Methods: Under aseptic conditions, specific amounts of freeze-dried decellularized hAM, PRP, and hydroalcoholic extract of purslane (HAEP) powder were prepared. The study groups included hAM gel, PRP gel, HAEP gel, a cocktail gel (HAEP + PRP + hAM), and phenytoin gel (positive control). Cytotoxicity was evaluated using the MTT assay. *In vivo*, seven groups were assessed on days 7, 14, and 21. Wound closure rate was analyzed via photographic imaging, and tissue samples were collected for H&E staining. Wound healing dynamics were further evaluated using 14/7-day and 21/14-day ratios.

Results: The cocktail gel significantly enhanced wound healing compared with other groups ($p < 0.05$), improving cell migration, M2 macrophage polarization, and angiogenesis. The ratio-based analysis indicated that the cocktail group exhibited the fastest healing between days 7 and 14, while purslane and hAM groups showed superior healing between days 14 and 21.

Conclusions: This study introduces the use of 14/7 and 21/14-day ratios for the first time as a quantitative measure of healing progression, bridging macroscopic closure rates with underlying cellular and molecular changes. The combination of purslane, PRP, and hAM significantly accelerated healing and reduced closure time, suggesting a synergistic effect. The proposed ratio-based approach provides a more accurate evaluation of wound healing phases.

Key words: Wound healing, human amniotic membrane, platelet-rich plasma, purslane

Introduction

Injuries represent a major global public health concern, accounting for approximately 10% of all deaths worldwide, disability and a substantial share of disability-adjusted life years (DALYs). According to recent reports, the economic burden of trauma and wound related morbidity remains enormous, with road traffic accidents, occupational hazards, burns, and falls among the leading causes (Abdulrazaq and Fan, 2025; Abdulrazaq and Fan, 2024; Duarte et al., 2021). These injuries often result in acute and chronic wounds that challenge healthcare systems globally. Therefore, the development of cost-effective and biologically robust strategies for wound healing has become a priority in regenerative medicine. Wound healing is a complex, multi-stage process in which alterations at any stage can significantly impact overall recovery (Enderami et al., 2024). It typically progresses through four overlapping but distinct phases: 1. Hemostasis (coagulation): Occurs within minutes after injury. Vasoconstriction reduces blood flow, while platelets aggregate to form a clot and release clotting factors, resulting in a stable fibrin mesh. 2. Inflammatory phase: Begins shortly after injury and generally lasts 3–7 days. This stage is characterized by an immune response to prevent infection and remove cellular debris. Neutrophils and macrophages migrate to the wound site to clear pathogens and necrotic tissue. While essential, prolonged inflammation can hinder healing and result in chronic wounds. Conversely, shortening this phase excessively may also be detrimental; therefore, regulating its duration is critical in wound care management. 3. Proliferative phase: Initiates around days 2–3 and may last up to two weeks. Fibroblasts produce collagen, forming the structural framework of new tissue (Singh et al., 2023). Angiogenesis ensures adequate oxygen and nutrient delivery, while epithelial cells migrate to cover the wound surface. (Raina et al., 2021). Granulation tissue comprising fibroblasts, collagen, and new capillaries fill the wound bed. This phase is crucial for effective wound healing management, as most cell migration occurs during this period (Li et al., 2024a). 4. Maturation (remodeling) phase: Commences approximately three weeks after injury and may persist for months or years. During this stage, collagen fibers are reorganized, tensile strength increases, and scar tissue matures. Given these mechanisms, the use of agents that modulate both the inflammatory and proliferative phases can significantly improve wound healing outcomes (Bartold and Ivanovski, 2025; Huang et al., 2024a). One such agent is *Portulaca oleracea* L. (purslane), a plant with documented nutritional, medicinal, and antimicrobial properties. (Li et al., 2024b) . Purslane extract promotes wound

healing by increasing collagen synthesis and angiogenesis, while reducing inflammation through the inhibition of prostaglandin and thromboxane production from arachidonic acid (Ghorani et al., 2023). Another widely used material in regenerative medicine is the human amniotic membrane (hAM). First applied clinically in the early 20th century, (Elkhenany et al., 2022) hAM has been employed for treating burns, chronic ulcers, ocular surface disorders, and surgical wound dehiscence (Abazari et al., 2020; Ingraldi et al., 2023). Its extracellular matrix components elastin, collagen types I, III, IV, V, and VI, and hyaluronic acid create a biocompatible scaffold for epithelial cell growth (Abazari et al., 2022a; Hashemi et al., 2018; Ingraldi et al., 2023; Jahanafrooz et al., 2023). Furthermore, the absence of HLA-A, -B, and -D antigens reduces immunogenicity, and its intrinsic anti-inflammatory properties support tissue regeneration (Mankuzhy et al., 2021). Platelet-rich plasma (PRP), derived from autologous blood, is another potent wound healing agent. Platelets adhere to injured endothelium, activating coagulation and releasing alpha granules containing over 300 bioactive molecules, including growth factors, cytokines, and extracellular matrix regulators (Park and Park, 2024). PRP stimulates angiogenesis, fibroblast proliferation, extracellular matrix remodeling, and mesenchymal stem cell differentiation (Hojjat et al., 2023; Jin et al., 2024).

While each of these agents purslane, hAM, and PRP has been individually shown to enhance wound healing, no study to date has evaluated their combined effect. The present study aimed to develop topical formulations containing these three components and investigate their synergistic potential in promoting wound repair. Additionally, a novel ratio-based approach was introduced to assess phase-specific healing dynamics.

Material and methods

Plant extraction

Fresh leaves of *Portulaca oleracea* were collected and authenticated by a plant taxonomist. A voucher specimen was deposited in the institutional herbarium. Leaves were shade-dried, ground into small particles using an electric grinder, and extracted multiple times with 80% methanol. For each extraction, 100 g of dried plant material was placed in a decanter funnel and covered with hydroalcoholic solvent (2 cm above the plant material). The mixture was left at room temperature for 48 h, after which the extract was collected. This process was repeated twice.

Combined extracts were concentrated under reduced pressure at 40 °C using a rotary evaporator, lyophilized, and stored at 4 °C in the dark until use.

PRP and amniotic membrane powder preparation

Platelet-Rich Plasma (PRP): Human venous blood was collected into tubes containing anticoagulant citrate dextrose. PRP was prepared using a double-spin method: First spin: 250 g for 10 min to separate red blood cells (bottom), buffy coat (middle), and platelet-rich plasma (top). Second spin: The plasma and buffy coat layers were centrifuged at 1000 g for 10 min to pellet platelets. The pellet was resuspended in plasma (3-5 mL) and activated with 10 µL of 10% CaCl₂. After 5 min, platelet clots formed and were lyophilized into PRP powder.

Human Amniotic Membrane (hAM): Placentae from cesarean deliveries were collected immediately post-operation, placed in sterile phosphate-buffered saline (PBS) with penicillin-streptomycin, and transported on ice. The amniotic membrane was mechanically separated from the chorion, washed repeatedly with cold PBS, and decellularized using sodium dodecyl sulfate (SDS) and Triton X-100. The tissue was then digested with pepsin in acetic acid at 37 °C under constant shaking, lyophilized, and stored as hAM powder.

Gel preparation and evaluation

Six gel formulations were prepared: 1. hAM gel: 1% (w/v) hAM powder. 2. PRP gel: 1% (w/v) PRP powder. 3. HAEP gel: 1% (w/v) HAEP powder. 4. Cocktail gel: 1% (w/v) each of hAM, PRP, and HAEP powders. 5. Carrier gel – no active ingredients. 6. Phenytoin gel: 1% (w/w) commercial formulation (positive control).

Each powder was dispersed in distilled water containing 2% (w/v) Tween 80. Methylparaben (50 mg) and propylparaben (50 mg) were added as preservatives. The mixtures were homogenized at 5000 rpm for 10 min. Carbopol 941 (1% w/v) was used as the gelling agent. After complete hydration, pH was adjusted to 6.0–6.5 with triethanolamine. Homogeneity was confirmed by visual inspection. For pH measurement, 2.5 g of gel was dispersed in 25 mL distilled water, incubated for 2 h, and measured in triplicate. pH values ranged from 6.3 to 6.8.

Animals

Sixty-three adult male Wistar rats (200–253 g, 115–130 days old) were obtained from the Animal Research Center of Mazandaran University of Medical Sciences. Animals were housed in ventilated cages at 24 ± 2 °C with a 12/12 h light–dark cycle and ad libitum access to food and water. All procedures complied with international animal care guidelines and were approved by the Ethics Committee (IR.MAZUMS.REC.1236).

Study design

Full-thickness dorsal skin wounds were created under aseptic conditions. Rats were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg), and dorsal fur was shaved. Using a scalpel, 1.5×1.5 cm wounds were made down to the panniculus carnosus muscle.

Animals were randomly assigned to seven groups (n = 9 each): 1. Negative control. 2. Positive control (phenytoin gel). 3. HAEP gel (purslane). 4. PRP gel. 5. hAM gel. 6. Cocktail gel. 7. Carrier gel. The outcome assessments were performed by an investigator blinded to treatment allocation. Treatments were applied daily. On days 7, 14, and 21, three rats per group were euthanized for wound assessment. Phenytoin gel (1%) was selected as the positive control based on its proven stimulatory effects on granulation tissue formation and epithelialization, as well as its widespread use and cost-effectiveness in preclinical wound models. Future studies may include more contemporary comparators such as silver-based dressings.

Wound closure rate calculation

Wound areas were photographed and analyzed using ImageJ v1.54j. Closure rate was calculated as:

$$D_c = \lim_{\Delta t \rightarrow 0} D = \lim_{\Delta t \rightarrow 0} -\frac{\Delta A}{P_{avg} \Delta t} = -\frac{dA}{P dt} = -\frac{dr}{dt}$$

Formula 1: wound healing rate

Where A: area, d: delta, lim: limit, P: perimeter, P_{avg} : average wound perimeter measured at two visits, r: length perpendicular to the wound contour, DA: change in superficial wound area between two visits, dt: time between two visits (7, 14 and 21). $dA = \lim_{\Delta t \rightarrow 0} \Delta A$ when ΔA approach 0 = wound area at the first day (Vidal et al., 2015).

Healing ratios between specific time points of 14/7 and 21/14 days

In this study, a novel approach has been adopted to analyze wound healing. The 14/7-day ratio represents the transition between proliferative and early remodeling phases, whereas the 21/14-day ratio corresponds to late remodeling. These intervals were selected because they reflect the major biological turning points of acute wound healing, enabling phase-specific comparison of treatment effects (Mansour et al., 2023a; Mansour et al., 2023b).

Histological analysis:

Wound tissues and margins were excised, fixed in 10% formalin for 48 h, processed, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin (4 min) and eosin (10 min), dehydrated, and mounted with Entellan®. Images were captured using an Olympus BX41 microscope (Hamburg, Germany).

RNA extraction and quantitative real-time PCR

Fibroblasts were cultured for 7 days in standard medium with either hAM or cocktail treatments. Total RNA was extracted using RNX-Plus (Sinaclon, Iran) and quantified by UV–Vis spectrophotometry. cDNA synthesis was performed using PrimeScript™ RT (TaKaRa, Japan). Relative expression of VEGFa, bFGF, and IL-1 β was measured with SYBR Premix Ex Taq™ (TaKaRa) using β -actin as the reference gene. PCR reactions were run on a StepOne™ system (Applied Biosystems, USA).

The procedures were carried out as in previous studies (Hashemi et al., 2024).

Cell viability assay

Fibroblasts (5×10^3 cells/cm²) were seeded in 24-well plates and treated with hAM or cocktail for 1, 3, or 5 days at 37 °C in 5% CO₂. MTT solution (5 mg/mL) was added for 3 h, formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm (Abazari et al., 2022b).

Histopathological examination

On days 7, 14, and 21, tissue samples including part of the wound area and adjacent normal skin were collected for histopathology. Samples were fixed in 10% neutral buffered formalin for at

least 48 h, embedded in paraffin, and serially sectioned at 5 μm thickness with 15 μm spacing between sets. Sections were stained with hematoxylin and eosin and examined for: re-epithelialization, granulation tissue deposition, collagen density and organization, inflammatory cell infiltration, and angiogenesis. A board-certified pathologist, blinded to group assignments, evaluated all samples using light microscopy. Scoring followed established histological grading methods.

Statistical analysis

Since each measurement was done three times, data normality was assessed using the Shapiro Wilk test, and homogeneity of variance was evaluated using Levene's test. One-way ANOVA followed by Tukey's post-hoc test was used for multiple group comparisons. Effect sizes (η^2) and 95% confidence intervals were calculated to estimate the magnitude of differences. Statistical analyses were conducted using SPSS v23.0, and $p < 0.05$ was considered statistically significant.

Results

Gene Expression

In vitro experiments were conducted to assess the angiogenic potential of fibroblasts treated with either hAM or cocktail formulations. The results (Figure 1) showed that VEGFa and bFGF expression levels were significantly higher in both the hAM-treated and cocktail-treated fibroblasts compared to the control group ($p < 0.05$). Although IL-1 β expression was also elevated in these groups, the increase was not statistically significant.

Cell viability assay

The cell viability (MTT) assay evaluated fibroblast proliferation in the presence of hAM or cocktail formulations. As shown in Figure 2, fibroblasts treated with hAM exhibited increased proliferation on days 3 and 5, with a statistically significant difference observed on day 5 compared to controls ($p < 0.05$). In the cocktail-treated fibroblasts, proliferation increased on days 1, 3, and 5 compared to controls. The increases on days 3 and 5 were significant ($p < 0.05$), whereas the day 1 increase was not.

Wound Closure

Wound healing rates were calculated according to the formula described in the Materials and Methods section (Formula 1). Figure 3 shows wound healing rates on days 7, 14, and 21, with quantitative data presented in Table 1 and representative images in Figure 4. Day 7: The purslane group showed the highest healing rate among all groups ($p < 0.02$), followed by the negative control, cocktail, positive control, hAM, carrier, and PRP groups. The last two groups had nearly identical rates ($p > 0.05$). Day 14: The purslane and cocktail groups exhibited almost identical healing rates, both higher than those of the other groups. The remaining order was positive control, hAM, PRP, negative control, and carrier groups. Day 21: All groups except the negative control and carrier groups achieved complete wound closure.

Analysis of the 14/7 and 21/14-day ratios (Figure 5) indicated that the cocktail group had the fastest healing rate between days 7 and 14, while the purslane and hAM groups outperformed others between days 14 and 21. Complete closure was observed in all groups except the negative control.

Histological Studies

Day 7: In the cocktail group, neutrophil numbers were lower than in other groups, and most macrophages had polarized to the M2 phenotype (large, oval-shaped nuclei). The hAM group showed more M2 macrophages than neutrophils, while the PRP group contained both M1 and M2 macrophages alongside neutrophils. The purslane group displayed blood pools, suggesting active angiogenesis. The negative control and carrier groups exhibited higher neutrophil and macrophage counts compared to all treatment groups. In the hAM group, migrated cells appeared to differentiate into primary fibroblasts, a phenomenon also observed in the cocktail group but to a lesser degree.

Day 14: Keratinocyte migration from the wound edges was evident in all groups, with the most extensive migration seen in the purslane group, followed by the cocktail group. The PRP and hAM groups showed less migration but better cellular organization, with a more developed stratum corneum. The negative control and carrier groups had minimal keratinocyte migration and keratinization. Overall, dermal layer differentiation was more pronounced in the hAM and cocktail groups.

Day 21: A complete epidermal layer was present in all groups. Epidermal appendages were better developed in the hAM and cocktail groups compared to others. Type I collagen appeared more abundant in the carrier, negative control, and to a lesser extent PRP and purslane groups. These findings suggest that PRP, purslane, and even the carbomethylcellulose vehicle accelerated wound closure, but inclusion of hAM promoted superior skin remodeling.

Analysis of Healing Ratios and Overall Findings

The analysis of healing dynamics using the 14/7-day and 21/14-day ratios provided additional insight into the temporal effects of each treatment. As illustrated in Figure 5, the cocktail group exhibited the highest 14/7-day ratio, indicating a significantly faster healing rate during the first week compared to all other groups. This suggests that the combination of hAM, HAEP, and PRP was most effective during the proliferative phase. In contrast, the 21/14-day ratio was highest in the purslane and hAM groups, indicating these treatments contributed more substantially to wound remodeling in the later stages. This aligns with histological findings that showed more organized dermal and epidermal layers in these groups by day 21. Complete wound closure was achieved in all groups except the negative control by the end of the study. Notably, while the positive control (phenytoin) group had a relatively high 14/7-day ratio, full closure was not achieved within 21 days, suggesting that its effect plateaued during the remodeling phase. These results underscore that different treatments may exert their greatest impact at distinct stages of the wound healing process. The ratio-based analysis thus provides a more nuanced understanding of healing progression than endpoint measurements alone.

Discussion

In this study, we developed a gel formulation composed of freeze-dried hAM powder, lyophilized PRP powder, and freeze-dried HAEP powder. The cocktail formulation demonstrated superior wound healing effects compared to any of the components used individually. This enhanced efficacy appears to result from the synergistic actions of its constituents at different stages of the wound healing process. Previous studies have documented the wound healing properties of each component individually. For purslane, Jinglin et al. (2022) investigated *Portulaca oleracea* extracts in a mouse model of deep tissue pressure injury (DTPI). They

reported improved wound healing through increased neovascularization, upregulation of VEGF-A, and reduced iron accumulation in damaged tissue (Guo et al., 2022). Similarly, Liu et al. demonstrated that *P. oleracea* extract enhanced healing in skin wounds infected with methicillin-resistant *Staphylococcus aureus* (Liu et al., 2023). In our study, histological findings confirmed that purslane promoted angiogenesis, particularly during the first week post-injury.

Regarding hAM, Heydari et al. found that hAM promoted wound healing by modulating inflammation, reducing infection, and delivering essential growth factors, making it especially effective during inflammatory and proliferative phases (Heydari et al., 2024). Murphy et al. reported that both hydrogel and lyophilized powder forms of hAM accelerated wound closure in a porcine model, with the powder form offering practical advantages for storage and application (Murphy et al., 2020). Our histological results showing diverse patterns of cell migration at the wound site, highlight the variety of growth factors present in hAM (Bernabé-García et al., 2017). PRP's role in wound healing has also been widely studied. Yuan et al. compared leukocyte-rich PRP (L-PRP) and leukocyte-poor PRP (P-PRP) in rat pressure ulcers. Both accelerated healing, but L-PRP elicited a stronger early inflammatory response and more angiogenesis (Yuan et al., 2023). Huang et al. developed a zwitterionic hydrogel containing PRP for diabetic rat wounds, achieving >90% closure by day 12 (Huang et al., 2024b). Our findings align with this evidence, demonstrating PRP's ability to enhance both early inflammation and tissue repair.

Importantly, our results show that although angiogenesis is critical, excessive angiogenesis similar to prolonged inflammation can be detrimental, leading to excessive type I collagen deposition and scar formation (Figure 6c). This was most notable in the negative control group, where increased fibroblastic activity and dense type I collagen were associated with more prominent scar tissue. In contrast, the cocktail group showed more balanced neovascularization and better epidermal remodeling, including the formation of hair follicles (Figure 6i).

Current approaches to measuring wound healing rate usually focus on one factor at a specific moment (days 7, 14 and 21) (Mansour et al., 2023a). But by creating an index that allows time-point comparisons, the impact of treatments can be understood more clearly and precisely.

A major innovation in this study was the use of healing rate ratios (14/7-day and 21/14-day) to assess phase-specific effects of treatments. Most acute wounds are in the proliferative phase by day 7, characterized by re-epithelialization and granulation tissue formation (Cañedo-Dorantes and Cañedo-Ayala, 2019; Enderami et al., 2024). By day 14, the process transitions toward the

remodeling phase, with collagen maturation and wound contraction (Cañedo-Dorantes and Cañedo-Ayala, 2019; Landén et al., 2016).

Comparing healing rates across these intervals allows for the detection of treatments that sustain momentum versus those whose effects taper off (El Ayadi et al., 2020; Singer, 2022; Thiruvoth et al., 2015). We can conclude that if the day 14/ 7 healing rate is high, it suggests sustained healing momentum. If it drops significantly, it may indicate slow healing in the later phase. Day 21/14 ratio, a higher ratio might indicate continued active healing, while a low ratio may suggest plateauing or delayed remodeling. By the 21st day of wound healing, the remodeling (maturation) phase is dominant. Key cellular events include; Fibroblast activity and ECM remodeling, myofibroblast contraction and angiogenesis regression (Moretti et al., 2022; Singer, 2022). Our results demonstrated that although the purslane group exhibited a faster initial wound healing rate compared to other groups, the calculated healing rate ratios (day 14/7 and day 21/14) indicated that the cocktail group demonstrated a more sustained and effective healing process overall. This observation suggests a potential synergistic interaction between the components of the cocktail namely, the amniotic membrane, PRP, and purslane extract each of which has individually been shown to enhance various stages of wound repair. The combination appears to support both early and late phases of healing, possibly by providing a wider range of growth factors and bioactive molecules that modulate inflammation, promote angiogenesis, and accelerate tissue regeneration. These findings are in line with previous studies emphasizing the importance of combinatorial therapies in achieving optimal wound healing outcomes. The obtained results indicate that wound healing rate at the ratio of 14/7 in mixture composes, cocktail group, was more than other groups (figure 5). Therefore, however, the time of wound healing stages has overlapped with each other, but it has a main duration for each stage. Approximately, hemostasis and inflammation phase are in the first week and the proliferation phase is in the second week. According to other studies, prolonged inflammation phase causes acute wound to become chronic. Therefore, it seems that an appropriate wound healing composition makes proliferation phase longer than inflammatory phase. Also, it is less likely to cause scar tissue. In our study this result was also observed, too. Amnion, cocktail and positive control groups had bigger ratio of day 14/7 (table 2) than other groups. These three groups had no scar formation (figure 6). However, wound closure in positive control group was not complete within 21 days (figure 3). Moreover, the result of hematoxylin and eosin staining demonstrate;

cell immigration in the cocktail group is more than other groups, too. Therefore, by establishing a criterion for comparing the wound condition at these ratios, it can be understood that: evaluating treatment effects of wound coverage ingredients materials, because some wound healing agents may have a greater impact during a specific phase of wound healing but exhibit different effects at another time interval. So, it seems that using these ratios can provide more precise information about the healing properties of a substance. And ultimately, it seems that too, these ratios (day 14/7 and day 21/14 ratio) can be used as an indicator of the effectiveness of a specific substance in the wound healing process. The use of these ratios in the present study can serve as an example of their application. With this method, the wound closure rate can be calculated more accurately and correlated with tissue changes. We propose that using these ratios as analytical tools could provide more nuanced insights into treatment efficacy, especially for agents with phase-specific actions. They may serve as valuable adjuncts to conventional healing assessments in both preclinical and clinical wound healing research. A limitation of this study is the relatively small sample size ($n = 9$ per group), which may affect the statistical power of some comparisons. Nevertheless, the consistency of findings across histological, molecular, and morphometric analyses supports the robustness of the observed effects.

Although the cocktail gel exhibited strong preclinical efficacy, translation to clinical practice may face challenges, including donor-to-donor variability in hAM and PRP, regulatory considerations, and formulation scalability. Addressing these aspects will be essential before human application.

Conclusion

The combination of purslane extract, PRP, and hAM demonstrated a synergistic effect in accelerating wound healing, reducing inflammation, and enhancing tissue regeneration. This tri-modal approach significantly shortened wound closure time and improved the quality of regenerated tissue compared to individual treatments. The novel ratio-based method for assessing wound closure dynamics provided a more precise understanding of healing progression and treatment-specific effects in different wound healing phases. Given its promising outcomes, this combination therapy could serve as a cost-effective and biologically potent strategy for wound care, meriting further clinical investigation. While the findings are promising, further

optimization and clinical validation are required to address variability and regulatory challenges inherent to biological materials.

CRedit authorship contribution statement

Reza Eshaghi-gorji and ReyhanehNassiri Mansour: Writing – original draft, Validation, Investigation, Methodology. Maryam Soleimanifar, Emran Habibi, Majid Saeedi, Fereshteh Talebpour Amiri, Seyyed Mobin Rahimnia, Keyvan Mehdipour-chari and Mansoureh Mirzaei: Investigation, Methodology. Behnaz Bageshlooyafshar and Amir Melati: Investigation. Seyed Ehsan Enderami and ReyhanehNassiri Mansour: Writing – review & editing, Supervision, Conceptualization, Investigation, Methodology.

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.

Data availability

Data will be made available on request.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT AI in order to improve the readability and language of the manuscript.

References:

Abazari, M.F., Soleimanifar, F., Enderami, S.E., Nasiri, N., Nejati, F., Mousavi, S.A., Soleimani, M., Kiani, J., Ghoraieian, P., Kehtari, M., 2020. Decellularized amniotic membrane scaffolds improve differentiation of iPSCs to functional hepatocyte-like cells. *Journal of cellular biochemistry* 121, 1169-1181.

Abazari, M.F., Torabinejad, S., Karizi, S.Z., Enderami, S.E., Samadian, H., Hajati-Birgani, N., Norouzi, S., Nejati, F., Mansouri, V., 2022a. Promoted osteogenic differentiation of human induced pluripotent stem cells using composited polycaprolactone/polyvinyl alcohol/carbopol nanofibrous scaffold. *Journal of Drug Delivery Science and Technology* 71, 103318.

Abazari, M.F., Torabinejad, S., Karizi, S.Z., Samadian, H., Jalili-ghelichi, S., Norouzi, M., Askari, H., Poortahmasebi, V., Enderami, S.E., Soleimanifar, F., 2022b. Biologically modified electrospun polycaprolactone nanofibrous scaffold promotes osteogenic differentiation. *Journal of Drug Delivery Science and Technology* 68, 103050.

Abdulrazaq, M.A., Fan, W., 2025. A priority based multi-level heterogeneity modelling framework for vulnerable road users. *Transportmetrica A: Transport Science*, 1-34.

Abdulrazaq, M.A., Fan, W.D., 2024. Temporal dynamics of pedestrian injury severity: A seasonally constrained random parameters approach. *International Journal of Transportation Science and Technology*.

Bartold, M., Ivanovski, S., 2025. Biological processes and factors involved in soft and hard tissue healing. *Periodontology* 2000 97, 16-42.

Bernabé-García, Á., Liarte, S., Moraleda, J.M., Castellanos, G., Nicolás, F.J., 2017. Amniotic membrane promotes focal adhesion remodeling to stimulate cell migration. *Scientific reports* 7, 15262.

Cañedo-Dorantes, L., Cañedo-Ayala, M., 2019. Skin acute wound healing: a comprehensive review. *International journal of inflammation* 2019, 3706315.

Duarte, J., Marques, A.T., Santos Baptista, J., 2021. Occupational accidents related to heavy machinery: a systematic review. *Safety* 7, 21.

El Ayadi, A., Jay, J.W., Prasai, A., 2020. Current approaches targeting the wound healing phases to attenuate fibrosis and scarring. *International Journal of Molecular Sciences* 21, 1105.

Elkhenany, H., El-Derby, A., Abd Elkodous, M., Salah, R.A., Lotfy, A., El-Badri, N., 2022. Applications of the amniotic membrane in tissue engineering and regeneration: the hundred-year challenge. *Stem cell research & therapy* 13, 1-19.

Enderami, S.E., Mansour, R.N., Hasanzadeh, E., Mahabadi, J.A., Abazari, M., Asadi, P., Hojjat, A., 2024. Applications of blood plasma derivatives for cutaneous wound healing: A mini-review of clinical studies. *Regenerative Therapy* 27, 251-258.

Ghorani, V., Saadat, S., Khazdair, M.R., Gholamnezhad, Z., El-Seedi, H., Boskabady, M.H., 2023. Phytochemical characteristics and anti-inflammatory, Immunoregulatory, and antioxidant effects of *Portulaca oleracea* L.: a comprehensive review. *Evidence-Based Complementary and Alternative Medicine* 2023, 2075444.

Guo, J., Peng, J., Han, J., Wang, K., Si, R., Shan, H., Wang, X., Zhang, J., 2022. Extracts of *Portulaca oleracea* promote wound healing by enhancing angiology regeneration and inhibiting iron accumulation in mice. *Chinese Herbal Medicines* 14, 263-272.

Hashemi, J., Pasalar, P., Soleimani, M., Khorramirouz, R., Fendereski, K., Enderami, S.E., Kajbafzadeh, A.M., 2018. Application of a novel bioreactor for in vivo engineering of pancreas tissue. *Journal of cellular physiology* 233, 3805-3816.

Hashemi, S.M.J., Enderami, S.E., Barzegar, A., Mansour, R.N., 2024. Differentiation of Wharton's Jelly-derived mesenchymal stem cells into insulin-producing beta cells with the enhanced functional level on electrospun PRP-PVP-PCL/PCL fiber scaffold. *Tissue and Cell* 87, 102318.

Heydari, P., Mojahedi, M., Javaherchi, P., Sharifi, M., Kharazi, A.Z., 2024. Advances and impact of human amniotic membrane and human amniotic-based materials in wound healing application. *International Journal of Biological Macromolecules*, 136596.

Hojjat, A., Mansour, R.N., Enderami, S.E., Hassannia, H., Mahdavi, M., Mellati, A., Salarinia, R., Saburi, E., 2023. The differentiation and generation of glucose-sensitive beta like-cells from menstrual blood-derived stem cells using an optimized differentiation medium with platelet-rich plasma (PRP). *Acta Histochemica* 125, 152025.

Huang, B., Yang, M., Kou, Y., Jiang, B., 2024a. Absorbable implants in sport medicine and arthroscopic surgery: A narrative review of recent development. *Bioactive Materials* 31, 272-283.

Huang, N.-C., Huang, N.-C., Kang, L.-Y., Hsieh, P.-S., Dai, L.-G., Dai, N.-T., Huang, C.-J., 2024b. Enhanced diabetic rat wound healing by platelet-rich plasma adhesion zwitterionic hydrogel. *Annals of Plastic Surgery* 92, S2-S11.

Ingraldi, A.L., Audet, R.G., Tabor, A.J., 2023. The preparation and clinical efficacy of amnion-derived membranes: a review. *Journal of functional biomaterials* 14, 531.

Jahanafrooz, Z., Bakhshandeh, B., Behnam Abdollahi, S., Seyedjafari, E., 2023. Human amniotic membrane as a multifunctional biomaterial: recent advances and applications. *Journal of biomaterials applications* 37, 1341-1354.

Jin, F., Li, X., Chen, J., Liu, J., Wang, Y., 2024. Clinical study on the role of platelet-rich plasma in human acellular dermal matrix with razor autologous skin graft repair of giant congenital pigmented nevus in children. *Journal of Plastic, Reconstructive & Aesthetic Surgery* 90, 305-314.

Landén, N.X., Li, D., Ståhle, M., 2016. Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences* 73, 3861-3885.

Li, A., Ma, B., Hua, S., Ping, R., Ding, L., Tian, B., Zhang, X., 2024a. Chitosan-based injectable hydrogel with multifunction for wound healing: A critical review. *Carbohydrate Polymers*, 121952.

Li, K., Xia, T., Jiang, Y., Wang, N., Lai, L., Xu, S., Yue, X., Xin, H., 2024b. A review on ethnopharmacology, phytochemistry, pharmacology and potential uses of *Portulaca oleracea* L. *Journal of Ethnopharmacology* 319, 117211.

Liu, G., Liu, A., Yang, C., Zhou, C., Zhou, Q., Li, H., Yang, H., Mo, J., Zhang, Z., Li, G., 2023. *Portulaca oleracea* L. organic acid extract inhibits persistent methicillin-resistant *Staphylococcus aureus* in vitro and in vivo. *Frontiers in Microbiology* 13, 1076154.

Mankuzhy, P.D., Ramesh, S.T., Thirupathi, Y., Mohandas, P.S., Chandra, V., Sharma, T.G., 2021. The preclinical and clinical implications of fetal adnexa derived mesenchymal stromal cells in wound healing therapy. *Wound repair and regeneration* 29, 347-369.

Mansour, R.N., Hasanzadeh, E., Abasi, M., Gholipourmalekabadi, M., Mellati, A., Enderami, S.E., 2023a. The effect of fetal bovine acellular dermal matrix seeded with Wharton's jelly mesenchymal stem cells for healing full-thickness skin wounds. *Genes* 14, 909.

Mansour, R.N., Karimizade, A., Enderami, S.E., Abasi, M., Talebpour Amiri, F., Jafarirad, A., Mellati, A., 2023b. The effect of source animal age, decellularization protocol, and sterilization method on bovine acellular dermal matrix as a scaffold for wound healing and skin regeneration. *Artificial organs* 47, 302-316.

Moretti, L., Stalfort, J., Barker, T.H., Abeyayehu, D., 2022. The interplay of fibroblasts, the extracellular matrix, and inflammation in scar formation. *Journal of Biological Chemistry* 298, 101530.

Murphy, S.V., Skardal, A., Nelson Jr, R.A., Sunnon, K., Reid, T., Clouse, C., Kock, N.D., Jackson, J., Soker, S., Atala, A., 2020. Amnion membrane hydrogel and amnion membrane powder accelerate wound healing in a full thickness porcine skin wound model. *Stem cells translational medicine* 9, 80-92.

Park, S., Park, J.K., 2024. Back to basics: the coagulation pathway. *Blood research* 59, 35.

Raina, N., Rani, R., Gupta, M., 2021. Angiogenesis: aspects in wound healing, *Endothelial Signaling in Vascular Dysfunction and Disease*. Elsevier, pp. 77-90.

Singer, A.J., 2022. Healing mechanisms in cutaneous wounds: tipping the balance. *Tissue Engineering Part B: Reviews* 28, 1151-1167.

Singh, D., Rai, V., Agrawal, D.K., 2023. Regulation of collagen I and collagen III in tissue injury and regeneration. *Cardiology and cardiovascular medicine* 7, 5.

Thiruvoth, F.M., Mohapatra, D.P., Kumar, D., Chittoria, S.R.K., Nandhagopal, V., 2015. Current concepts in the physiology of adult wound healing. *Plastic and Aesthetic Research* 2, 250-256.

Vidal, A., Zerón, H.M., Giacaman, I., Romero, M.d.S.C., López, S.P., Trillo, L.E.M., Pérez, D.A.P., Concha, M., Torres-Gallegos, C., Orellana, S.L., 2015. A simple mathematical model for wound closure evaluation. *Journal of the American College of Clinical Wound Specialists* 7, 40-49.

Yuan, Z., Wang, Y., Li, Y., Lin, C., Wang, S., Wang, J., Ma, C., Wu, S., 2023. Comparison of leukocyte-rich and leukocyte-poor platelet-rich plasma on pressure ulcer in a rat model. *Journal of Burn Care & Research* 44, 860-868.

Legends:

Figure 1. In vitro test for angiogenesis. VEGF, bFGF were identified to be expressed more highly in both fibroblasts treated with amnion and cocktail. (n=3) (**P<0.0001)

Figure 2. The MTT test result demonstrates that, in day 5 of culture, cell proliferation is significantly higher in the fibroblast with amnion group and fibroblast with cocktail group than the control group. (n=3) (*P<0.05)

Figure 3. wound closure rate: full-thickness skin defects were made in each group and the process of healing was followed since 3, 14 and 21 days after defecting. The rate of wound closure was calculated and they were compared with the first day of defecting. This comparison was done between 7 groups (negative control, positive control, carrier, PRP, purslane, amnion and cocktail). (n=3)

Figure 4: illustration of Wound healing rate calculation between studied groups on days 7, 14 and 21. (n=3)

Figure 5. $\frac{14}{7}$ and $\frac{21}{14}$ day ratio of wound healing rate for each group. (n=3)

Figure 6: Hematoxylin and eosin staining of the study groups on days 7, 14, and 21 post-wounding, showing tissue changes over time. (n=3)

Table 1: Wound healing rate calculation between study groups on days 7, 14 and 21

Table 2: day 14/ day 7 and day 21/ day14 wound healing rate ratio between study groups