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# Transforming PRP into a Shelf-Stable therapy: Lyophilization preserves angiogenic and regenerative properties

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ABSTRACT

***Objective(s):*** Platelet-rich plasma (PRP) is widely used in regenerative medicine due to its high concentration of bioactive growth factors; however, its short shelf-life limits its clinical applicability. Lyophilization has been proposed to extend PRP stability, but its impact on biological efficacy remains unclear. This study evaluated whether lyophilization alters the biochemical composition and regenerative function of PRP derived from expired platelet units unsuitable for transfusion.

***Materials and Methods:*** Pooled isogroup PRP was processed as either liquid PRP or lyophilized PRP. Growth factors (FGF-1, PDGF, VEGF) and endotoxin levels were quantified. Biological activity was assessed in human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs) using MTT assays, *in vitro* wound closure, gene expression analysis (*MMP-1, MMP-3, Ang1, PDGFR*), and CD31 immunocytochemistry at 5% and 10% concentrations, with fetal bovine serum (FBS) as a control.

***Results:*** Lyophilization resulted in moderate reductions in FGF-1, PDGF, and VEGF relative to Liquid-PRP ($P<0.05$), while endotoxin levels remained acceptable. In HDFs, liquid-PRP induced higher MMP-1 and MMP-3 expression, whereas lyophilized-PRP promoted lower but sufficient expression, consistent with controlled extracellular matrix remodeling, with comparable wound-closure outcomes between PRP groups. In HUVECs, lyophilized PRP preserved cell viability, migration, and CD31 expression, comparable to liquid PRP and FBS. Angiogenic gene expression showed a concentration-dependent response, with maximal activation at 5% PRP, and no functional impairment after lyophilization ($P<0.05$).

***Conclusion:*** Despite a moderate reduction in growth factors, lyophilized PRP retained regenerative efficacy, supporting lyophilization as a viable strategy for the stable, sustainable reuse of PRP.

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## Introduction

Regenerative medicine aims to enhance tissue repair and overcome the limitations of natural wound-healing processes, particularly in conditions where healing is slow or impaired (1). The skin is the body's largest organ and serves as a critical barrier against environmental, mechanical, and microbial threats. When this barrier is disrupted, the body initiates a highly coordinated wound-healing process that comprises four overlapping phases: hemostasis, inflammation, proliferation, and remodeling (2). Immediately after injury, clot formation and platelet activation limit bleeding and recruit immune cells. This is followed by an inflammatory phase that clears debris and pathogens. During the proliferative phase, fibroblasts migrate into the wound to synthesize extracellular matrix, keratinocytes re-epithelialize the surface, and endothelial cells form new blood vessels to restore tissue perfusion. Finally, the remodeling phase reorganizes collagen and strengthens the repaired tissue. Although efficient in healthy individuals, wound healing can be delayed by aging, chronic diseases, infection, or inadequate vascular supply, underscoring the need for therapies that enhance cellular activity and accelerate repair (1-3).

The clinical translation of regenerative therapies remains limited by challenges related to biomaterial stability, preservation, and controlled biological activity (4). Among

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the biologically based therapies in the field of regenerative medicine developed in recent years, platelet-rich plasma (PRP) has emerged as a widely used treatment due to its high concentration of growth factors that promote angiogenesis, cell proliferation, and extracellular matrix formation (5, 6). These properties make PRP an attractive option for improving wound healing in a variety of clinical settings (1, 5). PRP has emerged as a regenerative therapy capable of supporting multiple stages of the wound-healing process. Upon activation, platelets release a concentrated mixture of growth factors, including Platelet-derived growth factor (PDGF), Transforming growth factor β (TGF-β), Vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF), and Insulin-like growth factor 1 (IGF-1), as well as cytokines and extracellular vesicles that stimulate cell proliferation, migration, angiogenesis, and collagen deposition (7). In wound environments, PRP enhances fibroblast activity, promotes endothelial cell tube formation, modulates inflammation, and improves matrix remodeling. Clinically, PRP has been used in acute and chronic wounds, burns, diabetic ulcers, and surgical sites, often resulting in accelerated healing and improved tissue quality (7, 8).

PRP is autologous, and the entire extraction procedure is often performed just before injection into the injured site (9). Despite its therapeutic potential, conventional PRP is limited by its short shelf life. Because platelets degrade rapidly after preparation, PRP must typically be used immediately, which restricts its practicality, increases preparation costs, and complicates standardized clinical use (6). On the other hand, the clinical use of PRP is limited not only by its short shelf life but also by the burden of on-demand preparation. These limitations underscore the importance of preservation strategies that maintain PRP's regenerative efficacy.

In parallel with these challenges, blood banks routinely discard platelet concentrates that are no longer suitable for transfusion due to expiration or failure to meet transfusion criteria, even though these units may still contain biologically active growth factors (10). Repurposing such non-transfusion-grade platelet products could provide a sustainable, cost-effective source of PRP for regenerative applications, provided that their biological activity is preserved. For these reasons, there is growing interest in developing preservation methods that extend PRP's usability without compromising its biological activity.

Lyophilization (freeze-drying) has recently been proposed as a promising strategy for stabilizing biologically active materials during long-term storage. This technique removes water while preserving structural and biochemical integrity, potentially enabling PRP to retain its growth-factor activity even after reconstitution (11, 12). However, the influence of lyophilization on the functional performance of PRP, particularly its ability to stimulate angiogenesis and support wound healing, remains insufficiently characterized.

The present study investigates the effects of lyophilization on the biological activity of PRP, with particular emphasis on critical processes in wound repair and angiogenesis. Elucidating how lyophilization affects PRP functionality could inform optimized preservation strategies and broaden the clinical applicability of PRP-based regenerative therapies. To our knowledge, for the first time, non-transfusion-grade PRP will be lyophilized without lyoprotectants and then systematically evaluated and compared with Liquid-PRP across multiple healing parameters, including angiogenesis, wound closure, gene expression, and growth factor profiles.

## Materials and Methods

### *PRP preparation and freez-drying procedure*

In this study, PRP units were sourced from the Imam Reza Blood Donation Center in Mashhad, Iran. These units were deemed unsuitable for transfusion due to red blood cell (RBC) contamination. Three platelet concentrate bags of blood group $A^+$, stored for 5 days, were used and transported to the laboratory under standard conditions (22-24 ˚C) within 2-4 hr. Platelet concentrates of the same ABO blood group were selected to enable isogroup pooling and avoid ABO incompatibility, thereby ensuring immunological consistency in PRP preparation.

Under sterile conditions, the PRP bags were pooled and centrifuged at 320g for 5 min to sediment the residual RBCs. After separating the contaminated layer from PRP, platelet counts were measured using a hematology autoanalyzer (Auto Hematology Analyzer BC-6000, Mindray, China). The purified PRP samples were then frozen at −80 °C and subsequently freeze-dried using a laboratory freeze dryer (Christ Alpha 2-4 LD Plus, Germany). The lyophilized PRP was kept in a refrigerator (4 ˚C) until the testing.

Before each test, the PRP was resuspended in sterile normal saline to the same volume as the original plasma before lyophilization. All experiments were conducted to compare liquid PRP before freeze-drying with the lyophilized PRP. The experimental groups are presented in Table 1.

### *Biochemical assays*

To assess the effect of lyophilization, human FGF1, PDGF, and VEGF concentrations in lyophilized PRP samples were quantified using commercial ELISA kits. FGF1 levels were measured with the Quantikine® Human FGF acidic/FGF1 ELISA (Cat. No. DFA00B, R&D Systems, Minneapolis, USA). PDGF and VEGF were assessed using DuoSet® ELISA Development Systems (Cat. Nos. DY220 and DY293B-05, R&D Systems, Minneapolis, USA). Lyophilized PRP samples were reconstituted, diluted as needed, and applied to ELISA plates coated with kit-supplied capture antibodies. After a 2-hour incubation at room temperature with standards and samples, plates were washed and incubated with the appropriate biotinylated detection antibodies and streptavidin-HRP. Color development was achieved using 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and reactions were terminated with 2 N sulfuric acid. Absorbance was measured at 450 nm with wavelength correction at 540/570 nm, and analyte concentrations were calculated using a four-parameter logistic (4-PL) standard curve.

### *Safety assay*

Endotoxin levels were quantified using a competitive ELISA kit (Abbexa Endotoxin ELISA Kit, Cat. No. abx514093, Abbexa Ltd., UK) according to the manufacturer's instructions. Lyophilized PRP samples were reconstituted in endotoxin-free diluent and processed immediately. A serial 1:2 dilution of the supplied endotoxin standard was prepared, and all reagents were equilibrated to room temperature before use. Fifty microliters of standards and samples were added to pre-coated wells, followed by the addition of Detection Reagent A and incubation at 37 ˚C for one h. After washing, Detection Reagent B was added,

**Table 1.** Description and definition of the experimental groups and culture conditions used for HDF and HUVEC cell models in this study

| Name | Definition | Fibroblast cell model | Endothelial cell model |
|---|---|---|---|
| FBS 5% (v/v) | The cultured cell in DMEM.F12 with 5% of FBS | HDF | HUVEC |
| FBS 10% (v/v) | The cultured cell in DMEM.F12 with 10% of FBS | HDF | HUVEC |
| Liquid-PRP 5% (v/v) | The cultured cell in DMEM.F12 with 5% of pooled PRP before freeze-drying | HDF | HUVEC |
| Liquid-PRP 10% (v/v) | The cultured cell in DMEM.F12 with 10% of pooled PRP before freeze-drying | HDF | HUVEC |
| Lyophilized-PRP 5% (v/v) | The cultured cell in DMEM.F12 with 5% of pooled PRP after freeze-drying and resolving in normal saline | HDF | HUVEC |
| Lyophilized-PRP 10% (v/v) | The cultured cell in DMEM.F12 with 10% of pooled PRP after freeze-drying and resolving in normal saline | HDF | HUVEC |

In PRP groups, the PRPs were used instead of fetal bovine serum (FBS), so these groups did not contain FBS
HDF: Human Dermal Fibroblast; HUVEC: Human Umbilical Vein Endothelial Cells; PRP: Platelet-Rich Plasma

and plates were incubated for 45 min at 37 ˚C, followed by TMB substrate development and reaction termination. Absorbance at 450 nm was recorded, and endotoxin concentrations were calculated from the standard curve, accounting for the inverse relationship between OD and concentration in the competitive assay.

***Functional studies***

In functional experiments, cells were exposed to 5% or 10% liquid PRP or lyophilized PRP. Treatment outcomes were compared with parallel groups cultured in 5% or 10% FBS.

*Cytocompatibility evaluation*

For cell proliferation evaluation, HUVECs and HDFs were used (both purchased from the Iran National Cell Bank, Pasteur Institute, Iran). After reaching confluency under standard culture conditions (37 ˚C, 95% humidity, 5% $CO_2$) in DMEM/F12 supplemented with 10% FBS, the cells were trypsinized and seeded into 96-well plates at a density of $5\times10^3$ cells/well. The plates were incubated overnight under standard culture conditions, and treatments were applied the following day. The defined groups then received treatment. After 24 hr of treatment, cell proliferation was assessed using the MTT assay.

To prepare the MTT solution, 100 mg of MTT (Sigma-Aldrich, USA) powder was dissolved in 20 mL of sterile PBS (1x) and filtered through a 0.22 µm syringe filter. The working concentration of MTT for the assay was 0.5 mg/mL. To perform the test, after 24 hr of treatment, the culture medium was removed, and 100 µl of the MTT working solution was added to each well, followed by incubation in a $CO_2$ incubator for 4 hr. After the formation of formazan crystals, the MTT solution was aspirated and replaced with DMSO. The plates were then incubated at room temperature for 30 min to allow complete solubilization of the crystals. Absorbance was measured at 570 nm using an Epoch microplate reader (BioTek Instruments, USA). All steps of the MTT procedure were carried out in the dark.

*Gene expression*

For quantitative real-time PCR analysis, gene-specific primers were designed from mRNA sequences available in the NCBI database. In HUVECs, primers were designed for *PDGFR* and *Ang-1* to assess angiogenesis-related gene expression. In HDFs, primers targeting *MMP1* and MMP3 were intended to evaluate genes associated with wound-healing capacity and extracellular matrix remodeling. All primers were checked for specificity using BLAST and were synthesized by a commercial provider. Primer sequences and product sizes are presented in Table 2. GAPDH was picked as a housekeeping gene.

To evaluate potential changes in gene expression, specific genes associated with healing and angiogenesis were selected. Cells were seeded at $3\times10^5$ cells per well in 6-well plates, and allowed to reach 90% confluence, then the medium was replaced with the pre-defined treatment. After treatment with the designated experimental groups for 24 hr, the culture medium was removed, and TRIzol™ Reagent (Thermo Fisher Scientific, USA) was added to each well. Following cell lysis, the lysates were transferred to microcentrifuge tubes, and cold chloroform (Sigma-Aldrich, USA) was added at a 1:5 (chloroform: TRIzol) ratio, followed by vigorous shaking for 10 seconds. Tubes were incubated on ice for 15 min and then centrifuged at

**Table 2.** Primer sequences and related information used for gene amplification in this study

| Gene name | Sequence | Tm (°) |
|---|---|---|
| Hs *Ang1* | F: AGCGCCGAAGTCCAGAAAAC | 61 |
| | R: TACTCTCACGACAGTTGCCAT | 59 |
| Hs *MMP1* | F: AAAATTACACGCCAGATTTGCC | 59 |
| | R: GGTGTGACATTACTCCAGAGTTG | 60 |
| Hs *MMP3* | F: ACAAAGGATACAACAGGGACCAA | 59 |
| | R: ACCGAGTCAGGTCTGTGAGT | 60 |
| Hs *PDGFR* | F: AGCACCTTCGTTCTGACCTG | 50 |
| | R: TATTCTCCCGTGTCTAGCCCA | 59 |
| Hs *GAPDH* | F: TGTGGGCATCAATGGATTTGG | 60 |
| | R: ACACCATGTATTCCGGGTCAAT | 60 |

HS: Homo sapiens; Ang1: Angiopoietin-1, MMP1: Matrix Metalloproteinase-1; MMP3: Matrix Metalloproteinase-3; PDGFR: Platelet-Derived Growth Factor Receptor; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

12,000 g for 15 min at 4 ˚C using an Eppendorf refrigerated microcentrifuge (Eppendorf AG, Germany).

The upper aqueous phase was carefully transferred to a new microcentrifuge tube, and an equal volume of isopropanol (Sigma-Aldrich, USA) was added. Samples were incubated overnight at -20 ˚C to precipitate RNA. The following day, tubes were centrifuged at 12,000 g for 15 min at 4 ˚C to pellet the RNA. Pellets were washed with 75% ethanol, vortexed, and centrifuged at 10,000 g for 10 min at 4 ˚C. After air-drying, RNA pellets were resuspended in DEPC-treated water. RNA quality and concentration were assessed by measuring the OD260/280 using a NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, USA). All procedures were performed under standard, sterile conditions.

Complementary DNA (cDNA) was synthesized using the SMOBIO cDNA synthesis kit (SMOBIO, Taiwan). Components were added to a nuclease-free microcentrifuge tube on ice, and the mixture was gently mixed by pipetting up and down. Reaction components were used according to the manufacturer's instructions. The mixture was briefly centrifuged to collect all components at the bottom of the tube, then incubated in a thermal cycler using the following protocol: priming for 10 min at 25 ˚C, reverse transcription for 60 min at 46 ˚C, RTase inactivation for 5 min at 85 ˚C, and an optional hold at 4 ˚C.

Real-time PCR was performed using the SYBR Premix Ex Taq II master mix (TaKaRa, Japan) on a Roche real-time PCR system (Roche Diagnostics GmbH, Mannheim, Germany). GAPDH was used as the internal control (housekeeping) gene to normalize target gene expression levels. Amplification followed the protocols described in previous research (13, 14). Cycle threshold (Ct) values were analyzed using the $2^{-}\Delta\Delta Ct$ method, and results are presented as relative fold changes.

*Angiogenesis evaluation*

The HUVECs were seeded at $1\times10^5$ cells per well in 6-well plates. After ensuring cell attachment, the supernatant was removed, and treatments were applied for 24 hr. Immunofluorescence staining for CD31 was performed to assess PECAM-1 expression. HUVECs were fixed with 10% formalin for 2 hr, washed, permeabilized on ice, and then blocked with 1% bovine serum albumin (BSA) in TBS at room temperature. Samples were incubated overnight at 4 ˚C with the primary anti-CD31 antibody (PECAM-1 (H-3), sc-376764; Santa Cruz Biotechnology, USA), washed, and incubated with the secondary antibody (Goat Anti-Mouse IgG H&L (FITC), ab6785; Abcam, UK) for 10 min at room temperature. Slides were then washed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA), dehydrated through graded ethanol and xylene, and mounted with coverslips. Fluorescence images were captured using an Olympus BX50 fluorescence microscope (Olympus Corporation, Japan) equipped with an Olympus DP72 digital camera (Olympus Corporation, Japan).

*In vitro wound closure*

A scratch-wound assay was performed to assess the effects of Liquid-PRP and lyophilized-PRP on wound closure in HDFs and HUVECs. HDFs and HUVECs were seeded at $5\times10^4$ cells/well in 12-well plates and cultured to appropriate confluence. After removing the medium, a linear scratch was generated across the monolayer using a sterile pipette tip. Detached cells were removed by washing the wells twice with sterile solution. Media with the defined composition listed in Table 1 were added to the respective treatment wells. Cells maintained in standard DMEM. F12 supplemented with FBS served as the control group. All experimental conditions were performed in triplicate. Following treatment, plates were incubated under standard conditions, and images of the scratch area were captured at 0 hr and 24 hr using an inverted microscope (Olympus Corporation, Japan). Wound closure was quantified using ImageJ software by measuring the scratch width at each time point, and cell migration/proliferation was expressed as the percentage of closure relative to the initial wound area.

*Sterility evaluation*

To assess potential bacterial contamination, a sterility test was performed using thioglycolate broth. Lyophilized PRP samples were aseptically transferred into sterile tubes containing thioglycolate medium and incubated at 37 ˚C for up to 1 week. Samples were monitored daily for turbidity, which was considered indicative of microbial contamination. Any PRP sample showing turbidity was classified as contaminated, and both the sample and all associated experimental data were excluded from further analysis.

***Statistical analysis***

All experiments were performed in triplicate. One-way ANOVA was used as the primary method for evaluating statistical significance across multiple groups. For ELISA-based assays, paired t-tests were used to compare treatment conditions. A $P$-value<0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism software (version 10). To minimize concentration-dependent effects, comparisons were limited to treatments at the same concentrations. Thus, 5% FBS was evaluated against 5% liquid and lyophilized PRP, and 10% FBS against 10% liquid and lyophilized PRP.

## Results

The study design represents the experimental step of the study. First, the lyophilized PRP was prepared, and then biochemical composition and functional assessments were performed. Figure 1 briefly outlines the study design.

***PRP evaluation***

**Biochemical assays**

To examine the influence of lyophilization on platelet-derived growth factor content, the concentrations of FGF-1, PDGF, and VEGF were measured using an ELISA kit, in Liquid-PRP and in the same PRP after lyophilization and reconstitution. Before processing, the PRP concentrates were pooled, and the platelet count was adjusted to $1\times10^6$ platelets/µl to ensure consistency across samples. The pooled-adjusted PRP was then lyophilized. As shown in Table 3, all three growth factors were significantly lower in the lyophilized PRP than in the liquid preparation. Quantitative analysis demonstrated that FGF-1 was 1.2-fold higher, PDGF was 1.7-fold higher, and VEGF was 1.17-fold higher in Liquid-PRP than in lyophilized PRP. These findings indicate that lyophilization leads to a measurable decrease in the detectable concentrations of platelet-derived growth factors in PRP.

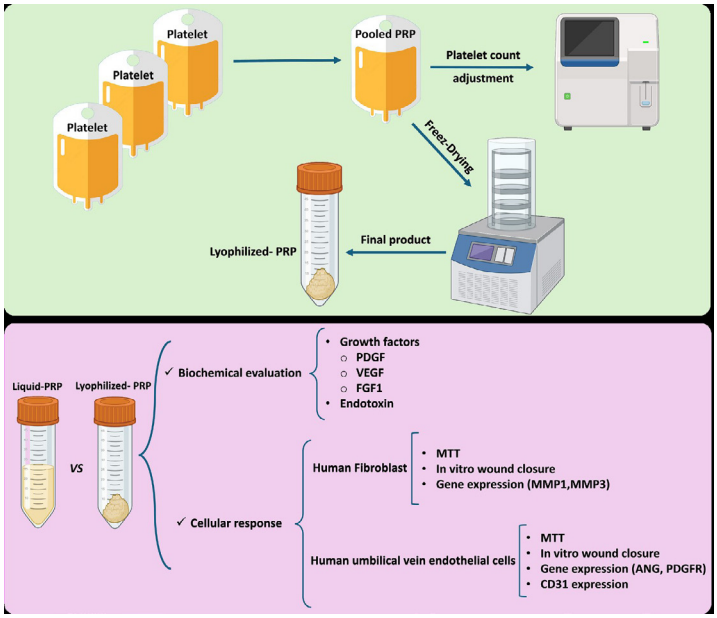


**Figure 1.** Study design. The PRP concentrates were pooled and then freeze-dried. Various tests were performed to assess the biochemical and cellular responses of Liquid-PRP and Lyophilized-PRP
Angiopoietin-1 (Ang1), Cluster of Differentiation 31 (CD31), Fibroblast Growth Factor-1 (FGF1), Human Umbilical Vein Endothelial Cells (HUVEC), Matrix Metalloproteinase-1 (MMP1), Matrix Metalloproteinase-3 (MMP3), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Platelet-Derived Growth Factor (PDGF), Platelet-Derived Growth Factor Receptor (PDGFR), Platelet-Rich Plasma (PRP), and Vascular Endothelial Growth Factor (VEGF)

## Safety assay

Studies of platelet-derived products commonly set an endotoxin limit of ≤0.5 EU/ml, as reported by Schallmoser *et al.*, Oeller *et al.*, and commercial manufacturers (15, 16). Therefore, many research groups use 0.5 EU/ml as the practical acceptance threshold. In our study, endotoxin levels measured by ELISA were <0.01 EU/ml, confirming that both pooled Liquid-PRP and Lyophilized-PRP were free of detectable endotoxin and remained within acceptable limits.

## *Functional studies*

### Cytocompatibility evaluation

The MTT assay was performed to assess cellular responses across the experimental groups, and the results are summarized in Figure 2. Evaluation of HDFs revealed comparable trends across groups; however, notably higher

**Table 3.** Results of growth factor analysis and endotoxin in liquid-PRP and Lyophilized-PRP

| Experimental groups | FGF-1 (ng/ml) | PDGF (ng/ml) | VEGF (ng/ml) | Endotoxin (EU/ml) |
|---|---|---|---|---|
| Liquid-PRP | 705 | 377 | 235.35 | 0 |
| Lyophilized- PRP | 5[illegible]3 $^{\#\#}$ | 221 $^{\#}$ | 200.95 $^{\#}$ | 0.01 |

Significant differences between Liquid-PRP and Lyophilized- PRP ($^{\#}P<0.05$, $^{\#\#}P<0.01$)
FGF-1: Fibroblast Growth Factor-1; PDGF: Platelet-Derived Growth Factor; PRP: Platelet-Rich Plasma; VEGF: Vascular Endothelial Growth Factor

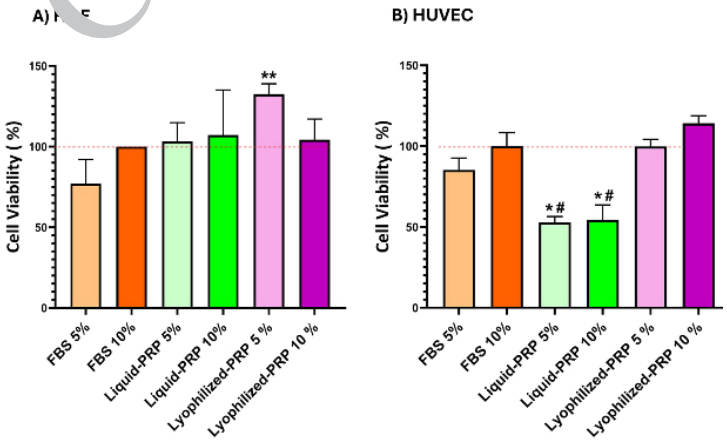


**Figure 2.** MTT test. A) HDF, the best result was related to cells treated with Lyophilized-PRP 5% ($P<0.05$). B) HUVEC, Liquid-PRP did not support cell viability compared to other groups. Comparisons were restricted to treatments at the same concentrations (5% or 10%; *, significant difference compared with FBS; #, considerable difference between Liquid- and Lyophilized-PRP; $P<0.05$)
HDF: Human Dermal Fibroblast; HUVEC: Human Umbilical Vein Endothelial Cells; PRP: Platelet-Rich Plasma

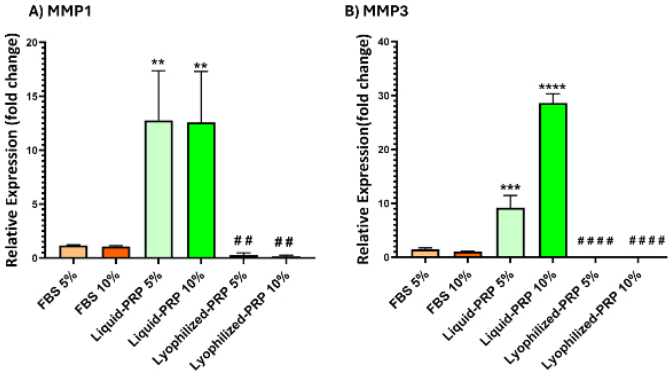


**Figure 3.** HDF gene expression. A) *MMP1*, the highest expression was related to cells treated with Liquid -PRPs ($P$<0.05). B) Liquid-PRP showed higher *MMP3* expression compared to other groups. Comparisons were restricted to treatments at the same concentrations (5% or 10%; *, significant difference compared with FBS; #, considerable difference between Liquid- and Lyophilized-PRP; $P$<0.05)
MMP1: Matrix Metalloproteinase-1; MMP3: Matrix Metalloproteinase-3; PRP: Platelet-Rich Plasma

cell viability was observed in HDFs treated with 5% Lyophilized-PRP, nearly twofold that of HDFs cultured with 5% FBS. In contrast, among the other tested cell types, HUVECs, Lyophilized-PRP preserved endothelial viability and metabolic activity at levels comparable to or exceeding those of FBS controls, whereas Liquid-PRP showed reduced viability. Overall, these findings suggest that Lyophilized-PRP may have a more favorable effect on cell proliferation than other treatments in HDFs. On the other hand, in HUVECs, Liquid-PRP may exert a less supportive effect on HUVEC viability than FBS or Lyophilized-PRP treatment.

## Gene expression

*HDF gene expression*

MMPs, including MMP1 and MMP3, play a critical role in wound healing by remodeling the ECM, facilitating cell migration, and promoting tissue repair. Their expression must be tightly regulated: moderate increases support early ECM remodeling, whereas excessive or prolonged expression can damage newly formed tissue. Here, MMP1 and MMP3 expression was evaluated in HDF cells and is presented in Figure 3. MMP1 expression was markedly upregulated in the Liquid-PRP group (~12-fold) compared with FBS, whereas Lyophilized-PRP treatment resulted in a substantial decrease (~0.14-fold). Similarly, MMP3 expression was substantially higher in Liquid-PRP-treated cells (10%; ~28-fold) and was strongly reduced in Lyophilized-PRP-treated cells (~0.04-fold) relative to FBS. The differences between FBS and Lyophilized-PRP were not statistically significant, whereas both were significantly lower than the Liquid-PRP group.

These findings suggest that Liquid-PRP may strongly promote early ECM remodeling through MMP induction, which could accelerate initial wound repair but risk excessive matrix degradation if prolonged, leading to scar formation. In contrast, lyophilized PRP maintains low MMP levels, potentially preserving ECM integrity but possibly delaying early remodeling during wound healing. Although relative fold-changes in MMP1 and MMP3 expression appeared high, baseline levels in control cells were low, and absolute expression values remained within physiologically relevant ranges reported in previous PRP studies.

*HUVECs gene expression*

To evaluate angiogenesis-related gene expression, changes in Ang1 and PDGFR levels were assessed in HUVECs treated according to the defined experimental groups. The results showed an increase in Ang1 expression in cells treated with Liquid-PRP; however, this change was not statistically significant compared with the FBS-treated group. In contrast, Ang1 expression was significantly lower in cells treated with Lyophilized-PRP than in cells treated with either Liquid-PRP or FBS ($P$<0.05).

Regarding *PDGFR* expression, cells treated with Liquid-PRP showed higher levels than those in the other groups, with a statistically significant difference compared with Lyophilized-PRP. As illustrated in Figure 4, the lowest *PDGFR* expression was observed in cells treated with Lyophilized-PRP, particularly at the 10% concentration. These results suggest that Liquid-PRP may promote angiogenesis-related gene expression, whereas Lyophilized-PRP showed lower *Ang1* and *PDGFR* expression, particularly at higher concentrations.

## Angiogenesis evaluation

Fluorescence analysis revealed that both PRP-treated groups showed higher CD31 expression than the FBS control. Within the PRP groups, Liquid-PRP showed slightly higher CD31 expression than Lyophilized-PRP; however, this difference was not statistically significant. These findings indicate that PRP enhances PECAM-1 expression in HUVECs and that lyophilization does not alter its pro-angiogenic potential. The results are represented in Figure 5.

## *In vitro* wound healing

To evaluate the effects of Liquid-PRP and Lyophilized-PRP on wound closure compared with the FBS control, this assay was performed in both HDF and HUVEC cells. In HDFs, the fastest wound closure was observed in the FBS group; however, there was no significant difference between Liquid-PRP- and Lyophilized-PRP-treated cells. In other words, regarding wound closure in HDFs, both forms of PRP performed similarly, and lyophilization did not appear to affect efficacy. In HUVECs, the highest wound closure was achieved in the Liquid-PRP group, although differences among all groups were not statistically significant. These results suggest that while PRP supports wound closure, lyophilization does not substantially alter its effect, and Liquid-PRP may have a slight advantage in endothelial cells, as shown in Figure 6.

## *Sterility evaluation*

All lyophilized PRP samples remained clear and

transparent throughout the 7-day incubation in thioglycolate broth, with no evidence of turbidity or microbial growth. Accordingly, all samples were confirmed sterile, and no exclusions were required due to contamination.

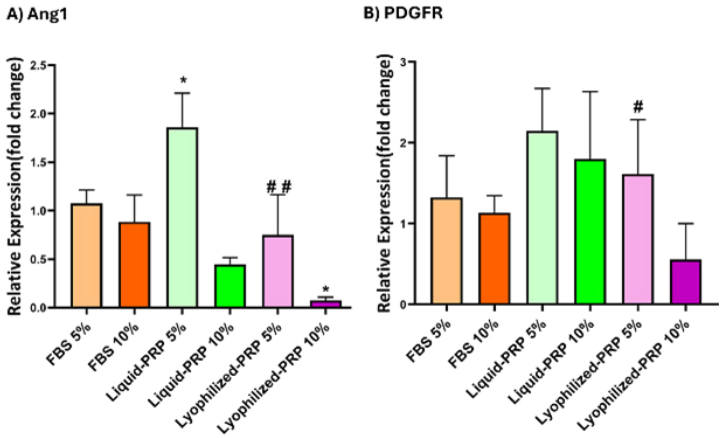


**Figure 4.** HUVEC gene expression. A) *Ang1*, the highest expression was related to cells treated with Liquid -PRPs ($P$<0.05). B) Liquid-PRP showed higher *PDGFR* expression compared to other groups. Comparisons were restricted to treatments at the same concentrations (5% or 10%; *, significant difference compared with FBS; #, considerable difference between Liquid- and Lyophilized-PRP; $P$<0.05)
Angiopoietin-1 (Ang1), Platelet-Derived Growth Factor Receptor (PDGFR), Platelet-Rich Plasma (PRP)

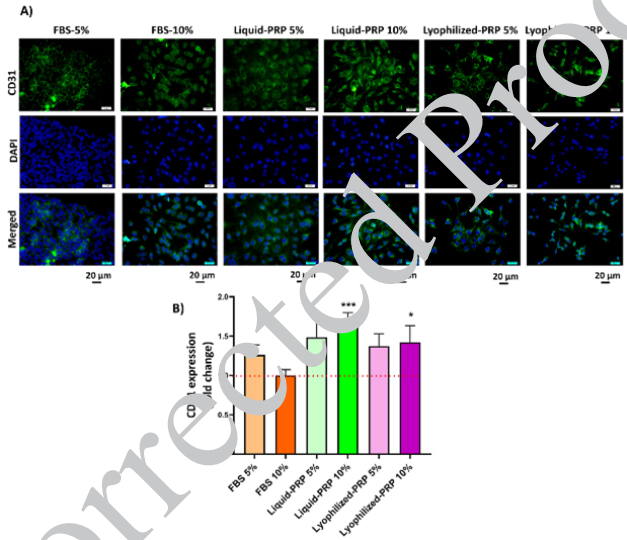


**Figure 5.** CD31 expression. A) ICC of experimental groups. B) Liquid-PRP showed higher CD31 expression compared to other groups, with no significant difference between Liquid- and Lyophilized-PRP. Comparisons were restricted to treatments at the same concentrations (5% or 10%; *, significant difference compared with FBS; #, significant difference between Liquid- and Lyophilized-PRP; $P$<0.05)
Cluster of Differentiation 31 (CD31), 4′,6-diamidino-2-phenylindole (DAPI), Platelet-Rich Plasma (PRP), Immunocytochemistry (ICC)

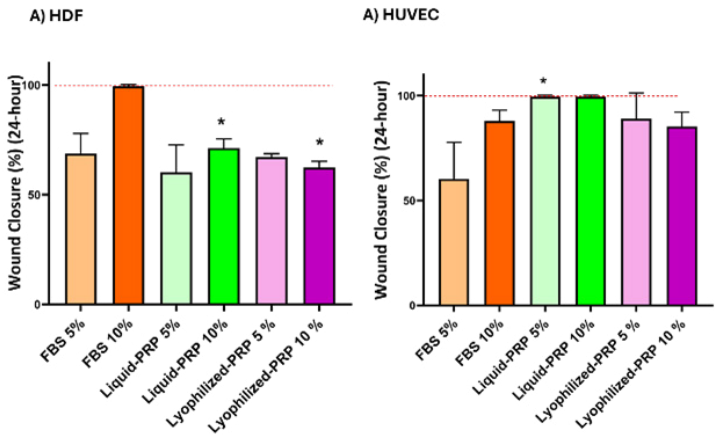


**Figure 6.** *In vitro* wound closure. A) HDF, in 24 hr, the FBS 10% reached complete wound closure. B) HUVECs, Liquid-PRP showed the fastest wound closure compared to other groups. Comparisons were restricted to treatments with the same concentrations (5% or 10%). No significant difference between Liquid- and Lyophilized-PRP (*, significant difference compared to FBS, $P$<0.05)
HDF: Human Dermal Fibroblast; HUVEC: Human Umbilical Vein Endothelial Cells; PRP: Platelet-Rich Plasma

## Discussion

Lyophilization offers complementary mechanistic and translational advantages, making the reuse of expired or non–transfusion-eligible platelet units particularly compelling for regenerative medicine (17). Controlled freeze-drying removes bulk water and stabilizes labile proteins, thereby limiting hydrolytic and oxidative degradation and enabling long-term stability during ambient or refrigerated storage. Notably, prior studies show that common platelet processing steps, such as centrifugation, freezing, and extended storage, often induce partial platelet activation, preserving the α-granule growth-factor payload and its biological activity. This observation helps explain why preserved growth-factor concentrations, rather than intact platelet aggregation, are the primary determinants of PRP's regenerative effects after reconstitution (17, 18).

Building on this mechanistic rationale, our findings extend prior evidence that freeze-drying stabilizes PRP's key trophic factors, including PDGF, VEGF, TGF-β, and EGF, while maintaining functional activity compared with fresh or frozen PRP. (11). Collectively, these insights support a practical and scalable workflow: platelet units otherwise destined for disposal can be pooled as isogroup PRP to reduce donor variability, lyophilized under optimized conditions, and released as standardized batches following sterility and growth-factor quality control. This strategy transforms low-value biomedical waste into a stable, accessible PRP-derived biologic suitable for topical or localized regenerative applications. Moreover, it highlights a significant and under-recognized opportunity within transfusion medicine, aligning with a growing body of literature demonstrating the therapeutic value of repurposing surplus blood components, such as platelet lysates, serum eye drops, and cryoprecipitate derivatives, into clinically relevant biologics (11, 18).

### *Growth factors*

PRP is widely recognized for its ability to enhance wound healing due to its concentrated reservoir of growth factors and cytokines released from platelet alpha granules upon activation. When platelets degranulate at a wound site, they release multiple bioactive molecules that act as signaling proteins, triggering and coordinating cellular responses essential for tissue repair. Among the most crucial are PDGF, VEGF, and FGF, each contributing to distinct yet interconnected aspects of the healing cascade. Through autocrine and paracrine signaling, these factors stimulate fibroblast proliferation, modulate ECM deposition, and promote angiogenesis, collectively establishing an environment conducive to tissue regeneration (19, 20).

PDGF is among the earliest growth factors released after injury and plays a central role in initiating the healing process. It acts as a chemotactic signal for neutrophils, macrophages, and fibroblasts, recruiting these cells to the wound site, where they proliferate and begin synthesizing ECM components such as collagen. PDGF also promotes endothelial cell proliferation and supports vessel formation, thereby contributing to both granulation tissue development and neovascularization (19, 21). VEGF is a potent pro-angiogenic factor that primarily acts on endothelial cells, inducing their proliferation, migration, and the formation of new capillary networks within developing granulation tissue. This angiogenic stimulation improves local blood flow, oxygen delivery, and nutrient supply to the regenerating tissue, processes indispensable for effective wound closure and the revitalization of damaged areas. FGF supports cell proliferation and re-epithelialization by enhancing fibroblast and keratinocyte activity. It also contributes to granulation tissue formation and works synergistically with VEGF to sustain neovascularization. FGF's role in regulating ECM remodeling and stimulating other growth factor pathways further underscores its importance in orchestrating the proliferative phase of wound healing.

Collectively, these growth factors promote fibroblast proliferation and ECM synthesis and influence angiogenesis, cell migration, and collagen deposition, which are key phases of the complex, multistep wound-healing process. Controlled delivery of platelet-derived growth factors has been shown to preserve regenerative efficacy while preventing excessive cellular stimulation (22). The ability of PRP to deliver this suite of bioactive factors at high local concentrations and in a coordinated, physiologically relevant manner provides a strong mechanistic basis for its regenerative effects observed in both experimental models and clinical applications of tissue repair (17, 23).

In our study, Liquid-PRP contained higher concentrations of FGF-1, PDGF, and VEGF than Lyophilized-PRP, and the lyophilized preparation showed statistically significant reductions in these growth factors. Although growth factor analysis revealed a reduction, lyophilization could preserve their activity as evidenced by functional assessments in cell-based studies.

One possible explanation for the observed decrease in PDGF and VEGF involves the physical and chemical stresses imposed during lyophilization. Lyophilization can subject proteins to physical and chemical stresses during freezing and dehydration, potentially destabilizing their native structure and leading to partial unfolding, aggregation, or loss of activity, especially for sensitive growth factors. These stresses include freeze-concentration effects, ice-water interfaces, and dehydration-induced conformational changes, which together can reduce measurable growth factor levels after reconstitution (24, 25).

Our growth factor analysis is broadly consistent with prior reports evaluating freeze-dried platelet derivatives. For example, Queiroz da Silva *et al.* observed that lyophilization significantly altered PDGF concentrations compared with fresh PRP ($p$=0.0464), despite reporting overall stability of VEGF, TGF-β, and EGF (18). Similarly, studies on freeze-dried PRP and cord-blood PRP report that trophic factors are mainly preserved; however, the amount of platelet-derived growth factor (PDGF) retained can vary depending on the preparation method and donor source (26). In contrast, long-term stability studies of lyophilized human platelet lysate report minimal loss of PDGF, VEGF, and TGF-β even after 9 months at 4 ˚C, indicating that factors such as stabilizing agents, lyophilization cycle, and protein-binding interactions can influence recovery efficiency (27).

Taken together, our results are consistent with the expected biological variability reported in the literature. Lyophilization reliably preserves the functional regenerative capacity of PRP, but moderate reductions in specific growth factors, particularly PDGF and VEGF, may occur under different processing conditions. Notably, despite these quantitative decreases, Lyophilized-PRP in our study remained biologically functional across all cellular

assays, suggesting that the retained growth-factor milieu is sufficient to support fibroblast angiogenic and regenerative responses.

The observed reduction in PDGF and VEGF levels following lyophilization in our study differs from several previous reports that describe near-complete preservation of growth factors in freeze-dried PRP. This discrepancy is likely attributable to significant methodological and biological differences between studies. Notably, many published investigations utilized freshly prepared PRP, platelet lysate, or cord-blood–derived products, often processed within 24-48 hr of collection and frequently supplemented with stabilizing agent before lyophilization (12, 27). In contrast, our work uniquely employed pooled-PRP units collected at the end of their storage period and deemed unsuitable for transfusion due to red blood cell contamination. Such units are known to exhibit partial platelet activation, oxidative stress, and baseline degradation of labile growth factors before freeze-drying, which may sensitize specific cytokines, particularly PDGF and VEGF, to additional losses during lyophilization (17, 18)

Furthermore, unlike several previous studies that used platelet activation or cryoprotective additives, our protocol relied solely on native plasma components. This approach more closely reflects real-world, resource-limited processing of discarded platelet concentrates. Together, these factors provide a possible explanation for the reduction in selected growth factors observed in our lyophilized preparations and highlight that growth-factor retention after lyophilization is strongly influenced by source material, platelet age, and processing conditions rather than by the freeze-drying process alone.

Despite these decreases, both PRP preparations retained biologically significant concentrations of these growth factors, sufficient to stimulate key biological processes, as evidenced by viability and functional wound-closure assays. These results align with other research showing that lyophilized PRP retains growth factor activity even after storage (12).

***Fibroblast responses***

In HDFs, both Liquid-PRP and Lyophilized-PRP preserved or enhanced cell viability compared with FBS. Notably, HDFs treated with Lyophilized-PRP (5%) exhibited nearly twofold higher cell viability than FBS-treated cells (FBS 5%), indicating that PRP, regardless of its form, supports fibroblast survival and proliferation. This is consistent with studies showing that PRP preparations, whether liquid or lyophilized, maintain cell activity and migration, which are critical for wound healing and tissue regeneration (28).

Wound closure assays in HDFs demonstrated comparable results between Liquid-PRP and Lyophilized-PRP, confirming that lyophilization did not negatively impact wound closure *in vitro*. These findings are supported by Valente *et al*, who reported that lyophilized PRP was similarly effective in promoting wound closure in fibroblast-based models (26). Consistent with our findings, a 2021 study (23) on allogeneic freeze-dried PRP demonstrated enhanced wound healing, characterized by increased fibroblast proliferation, collagen deposition, and neovascularization, indicating that regenerative function is preserved after lyophilization. Although that study assessed *in vivo* wound repair rather than *in vitro* scratch assays, both studies support the conclusion that lyophilization does not impair PRP's biological activity in fibroblast-mediated tissue repair. Although the FBS group showed slightly faster closure, no significant differences were observed between the two PRP preparations, further supporting the conclusion that lyophilization does not impair PRP's regenerative potential.

In HDFs, MMP-1 and MMP-3 expression was markedly higher in Liquid-PRP–treated cells than in FBS-treated cells and Lyophilized-PRP. Specifically, Liquid-PRP induced more than a 10-fold increase in MMP-1 and MMP-3, enzymes involved in ECM remodeling. This finding is consistent with previous studies showing that PRP-derived growth factors stimulate MMP expression, facilitating early ECM degradation and cell migration during wound repair (29).

MMP expression in fibroblasts is tightly regulated by platelet-derived growth factors, including PDGF, VEGF, and FGF-1, which activate downstream signaling pathways such as MAPK and NF-κB (30-32). In our study, lyophilization resulted in a moderate reduction in PDGF and VEGF concentrations, likely attenuating these signaling cascades and leading to decreased MMP-1 and MMP-3 transcription in Lyophilized-PRP-treated fibroblasts.

Importantly, the reduced MMP expression observed with Lyophilized-PRP does not appear to reflect impaired regenerative capacity, as supported by wound-closure and MTT assay results. Instead, it may indicate a more controlled remodeling response that limits excessive ECM degradation and promotes balanced matrix turnover during tissue repair. Excessive MMP activity is a hallmark of impaired wound healing, as elevated protease levels degrade ECM proteins, growth factors, and receptors, contributing to delayed closure and chronic wounds (33, 34). Therefore, the lower *MMP-1* and *MMP-3* expression induced by Lyophilized-PRP may help preserve matrix integrity while supporting effective tissue remodeling.

From a clinical perspective, fibroblast proliferation, controlled ECM remodeling, and balanced MMP expression are central to successful healing of chronic wounds, surgical incisions, and soft-tissue defects. The more moderate MMP response observed with lyophilized PRP may be advantageous in chronic or non-healing wounds, where excessive protease activity contributes to matrix degradation and impaired repair. Therefore, lyophilized PRP may provide a biologically tempered stimulus that supports regeneration while limiting the risk of protease-driven tissue breakdown (35-37).

***Endothelial responses***

In HUVECs, Lyophilized-PRP enhanced endothelial viability and metabolic activity relative to FBS, as demonstrated by the MTT assay, indicating that lyophilization did not compromise cytocompatibility or proliferative support. This finding aligns with in vitro studies showing that PRP and platelet-derived products stimulate endothelial proliferation via growth factors such as VEGF, PDGF, and EGF (38). Wound-closure assays further showed that both liquid and lyophilized PRP promoted HUVEC wound closure to a similar extent, supporting preserved function following lyophilization and aligning with reports of PRP-enhanced endothelial migration and angiogenic

behavior *in vitro* (39). Immunocytochemistry revealed increased CD31 expression in response to both Liquid- and Lyophilized-PRP, indicating maintenance of the endothelial phenotype and activation essential for neovascularization.

Cellular behavior is regulated by the mode of signal presentation and microenvironmental cues rather than solely by the absolute concentration of bioactive factors (13). At the transcriptional level, Liquid-PRP at 5% induced the highest expression of *Ang1* and *PDGFR*. In contrast, higher concentrations led to reduced transcription, consistent with a biphasic dose–response pattern characteristic of endothelial signaling. Lyophilized-PRP elicited lower *Ang1* and *PDGFR* expression compared with Liquid-PRP, likely reflecting reduced growth-factor abundance and altered release kinetics associated with lyophilization.

Despite these quantitative differences, key angiogenic functions, including endothelial viability, migration, and CD31 expression, were preserved. Although lyophilization modestly reduced PDGF and VEGF levels, these concentrations remained sufficient to support endothelial activation, indicating that PRP efficacy may depend on integrated growth factor signaling once an activation threshold is reached rather than on maximal cytokine abundance. This supports prior in vitro evidence that regulated, rather than excessive, PRP-mediated stimulation is sufficient to elicit endothelial angiogenic responses (40).

The observed reduction in MTT signal in HUVECs treated with Liquid-PRP likely reflects alterations in cellular metabolic activity rather than decreased viability. MTT assays measure mitochondrial dehydrogenase activity, which can be transiently suppressed by strong growth-factor signaling or cellular stress, even when cells remain viable and functionally active (41). High concentrations of platelet-derived cytokines such as PDGF and VEGF may induce negative feedback in endothelial signaling pathways, leading to reduced mitochondrial activity despite preserved angiogenic function (42, 43). Additionally, inflammatory mediators and oxidative products present in liquid PRP, particularly from storage-aged units, may further modulate metabolic readouts without indicating true cytotoxicity (44). In contrast, lyophilized PRP may attenuate excessive signaling, preserve baseline mitochondrial function, and support migration, CD31 expression, and angiogenic gene activation.

A limitation of the current study is that metabolic activity was assessed solely with MTT, which cannot distinguish between alterations in mitochondrial function and changes in proliferation or cell-cycle status. Future work should incorporate complementary viability and metabolic assays (e.g., ATP quantification, live/dead staining, real-time impedance measurements) to more precisely define endothelial cell health and bioenergetics in response to PRP formulations. This would further clarify how lyophilization influences endothelial metabolism under regenerative conditions.

Clinically, preservation of endothelial migration and CD31 expression indicates that lyophilized PRP maintains pro-angiogenic capacity, a key requirement for treating ischemic, diabetic, or radiation-impaired wounds where neovascularization is deficient. The observation that effective endothelial responses were achieved without maximal growth-factor concentrations supports the concept that therapeutic efficacy depends on coordinated signaling rather than supraphysiologic dosing, which may reduce the risk of aberrant angiogenesis or excessive inflammation (45, 46).

***Safety and endotoxin level***

Stable and standardized biological formulations are essential for integration into next-generation regenerative delivery platforms (47). In this regard, safety issues are considerable. The endotoxin analysis indicated that both Liquid-PRP and Lyophilized-PRP had negligible endotoxin contamination, confirming that the observed cellular responses were not influenced by inflammatory responses due to endotoxin contamination. This result is crucial, as endotoxin contamination in PRP preparations can trigger unwanted inflammatory pathways that would confound the therapeutic effects. The absence of endotoxins in both PRP types showed that high-quality PRP preparations can be produced with minimal endotoxin contamination, thereby enhancing their clinical safety (15, 16, 48).

Importantly, the ability to produce a stable, endotoxin-free, growth-factor–containing formulation from expired platelet units aligns with current trends in transfusion medicine and regenerative biologics manufacturing. Lyophilized PRP could be developed as an off-the-shelf biologic, subject to standardized release criteria such as sterility, endotoxin level, protein content, and growth-factor profiling. This shifts PRP from a procedure-based therapy to a quality-controlled biologic product, potentially facilitating regulatory pathways and broader clinical integration.

## Conclusion

In this study, we demonstrate that isogroup, pooled-PRP derived from platelet units initially deemed unsuitable for transfusion, can be lyophilized and reconstituted while retaining clinically relevant growth factor concentrations and biological activity. In conclusion, both Liquid-PRP and Lyophilized-PRP retain significant biological activity, enhancing fibroblast viability, wound closure, angiogenesis, and ECM remodeling *in vitro*, thereby supporting their potential for clinical wound-healing applications. Although lyophilization reduced growth factor concentrations (PDGF, VEGF, FGF), the functional regenerative capacity of Lyophilized-PRP was largely preserved, with no functionally relevant differences observed between Liquid- and Lyophilized-PRP in wound closure and angiogenesis-related outcomes. These findings confirm the suitability of lyophilized PRP as a stable alternative to liquid PRP, offering the advantages of an extended shelf life and easier storage without substantial loss of biological efficacy. The results are consistent with the growing body of literature supporting the clinical use of lyophilized PRP for tissue repair and regeneration, providing a practical and effective treatment option for wound healing in various clinical settings.

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## Ethical Approval

The study received ethical approval from the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1403.314).

## Data Availability

The datasets generated and analyzed in this study are available upon reasonable request.

## Authors' Contributions

A M handled investigation, data processing, collection, result analysis and interpretation, visualization, and original writing, along with review and editing. T M contributed to data collection, investigation, original draft writing, and visualization. MH M was responsible for draft manuscript preparation and supervision. HR R managed conceptualization, project administration, supervision, and validation. MH P was involved in study conception and design, supervision, funding acquisition, validation, and review & editing.

## Conflicts of Interest

The authors declare no conflict of interest.

## Declaration

The authors confirm that no AI-based tools were used in writing or preparing this manuscript.

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