

Evaluation of platelet-rich plasma vs. platelet lysate: VEGF and PDGF concentration, stability, and shelf life

Shambhavi Prasad¹, Teena Mary Thomas², Jyothsna Rao², Gururaj Rao²

¹The International School Bangalore, NAFL Valley, Whitefield – Sarjapur Road, Bangalore, India

²CREST-International Stem Cell Services Limited, 9/1, Mission Road, Bangalore, India

SUMMARY

Cell-free biologicals are a novel method of treating clinical conditions which involve chronic inflammation such as tendonitis and osteoarthritis. Standard approaches for addressing such conditions involve steroids and powerful pain killers, which predispose patients to unfavorable clinical outcomes. Cell-free biologicals like platelet-rich plasma (PRP) and platelet lysate (PL) derived from blood provide a reliable approach to treat chronic inflammation. aPRP is the activated form of PRP and is assumed to contain higher concentration of cytokines. These blood derivatives are safe and can be administered with ease over long periods. Although this approach is widely popular among physicians, it requires scientific standardization of its growth factors and a complete analysis of its shelf life and stability. In this study, we compared platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) in PRP, activated PRP (aPRP), and PL. We analyzed the shelf life at different storage temperature over three weeks. We hypothesized that PL would contain higher concentrations of growth factors than PRP and that different storage temperatures for PL would diminish cytokine expression. Our results demonstrated PL had the highest concentrations of both cytokines, with concentrations slightly diminishing at -80C. aPRP and PRP demonstrated lower concentrations of PDGF and VEGF than PL.

INTRODUCTION

Inflammation is a common condition afflicting humankind in all age groups. The ailments accompanying it such as rheumatoid arthritis, psoriasis, and Crohn's disease can become chronic, as they lack sustained medical intervention barring steroids and pain killers (1). However, these interventions expose patients to severe, deleterious side effects due to prolonged usage. Exploring new biologic alternatives which could potentially overcome these shortcomings will facilitate better disease management with minimum side effects.

Platelet-rich plasma (PRP) is one such candidate. PRP has been explored extensively, as it is rich in growth factors such as platelet-derived growth factor (PDGF), vascular

endothelial growth factor (VEGF), transforming growth factor (TGF- β), fibroblast growth factor (FGF) and endothelial growth factor (EGF). By virtue of its chemical composition, PRP has proven to be effective in the wound healing applications: PRP limits inflammation, thereby promoting tissue regeneration (2).

PRP can either be lysed to form platelet lysate (PL) or can be activated with thrombin or CaCl₂ to form activated PRP (aPRP) (3). The use of CaCl₂ to lyse PRP in the formation of aPRP has proven to release PDGF and VEGF, important cytokines in re-vascularization of graft tissue, soft tissue healing, reduction of post-operative morbidity, and bone regeneration (4). These growth factors give aPRP key chemo-attractive, cell proliferative, matrix synthetic, and angiogenic properties, making it an attractive resource for future wound-healing investigations (5).

PL is a blood-derived supernatant. The freeze-thaw method used to derive PL results in the lysis of the platelets in the plasma that constitute PRP, which results in the release of the growth factors present in them. This freeze-thaw method employed to generate PL causes it to be rich in growth factors (6). Like PRP, PL has been proven to significantly enhance the proliferative properties of a range of *in vitro* cell cultures: adipocytes, mesenchymal stem cells (MSCs), human immortal keratinocyte cell line, and osteoblasts (7, 8). Studies have demonstrated the efficacy of PL in clinical medicine, including embedded scaffolds for skin regeneration due to the combination of the scaffold's tridimensional architecture and PL's enhanced wound healing properties (9). Furthermore, PL has also been studied in the treatment of oral mucositis in graft vs. host disease (GVHD) patients and in refractory ocular GVHD (10-12).

aPRP is defined as the PRP re-lysate, which involves a two-step mechanism: (i) degranulation of platelets to release growth factors and (ii) fibrinogen cleavage to initiate matrix formation, a clotting process which forms a platelet gel, confining the secretion of molecules to the chosen site (6, 7, 13).

Autologous PRP/PL is used most often in terms of its practical usage, although it poses certain logistic drawbacks. Obtaining PRP and PL involves blood draws and downstream processing, leading to waiting time for the patient. In addition, every enrichment will produce variable enrichment leading to significant variability in the resulting growth factor fraction. PL has consistently proven to be more stable than all forms of

PRP, making it a better candidate for clinical modalities (14).

Given the advantages PL has over PRP, we also investigated the impacts of temperature on their PDGF and VEGF concentrations in PL by storing it at different temperatures, including 4°C and -80°C. Standard operating procedure mandates the storage of proteins – cytokines included – at 4°C for optimal storage. However, storage in -80°C results in metabolic cessation, protecting the biomolecules in the supernatant, thus preventing the deterioration of the cytokines to a greater extent than storage at 4°C. It has previously been proven that the freeze-thaw procedure can be one of the most effective methods of releasing the growth factors in PRP (15). We employed this technique to facilitate growth factor (GF) release from all the blood supernatants used in this experiment.

The three-week time frame employed in this study was to reflect the optimum timeframe for potential clinical application. This rationale was justified by the extent to which growth factor contents, stability, and shelf-life variability between PRP and PL determine their clinical relevance. The cytokine concentrations were quantified using Enzyme-linked Immunosorbent Assay (ELISA).

PDGF and VEGF concentrations in PRP, aPRP, and PL from 15 human patients stored at different temperatures (4°C and -80°C) were measured during a three-week time interval.

RESULTS

We stored the isolated PRP and its derivatives 4°C to prevent degradation of the platelets present and to temporarily suspend metabolic activity within the extract, while the remaining was stored for later use. We determined the shelf life by calculating the rate of change of cytokine concentration over time – by measuring the differences between the initial concentration and the concentrations over three weeks via ELISA assay.

Shelf life with respect to PDGF concentration

The concentration of PDGF over time was maintained at a consistent high level over three weeks in the PL stored at -80°C and 4°C, reaching a maximum of 3370.8 ng/mL and 2892.7 ng/mL, respectively during week 2 (Figure 1). PRP and aPRP, with the expected decreasing trend for the growth factor concentration over time, reached highest concentrations of only 626.25 ng/mL and 1078.8 ng/mL, respectively (Figure 1). The aPRP exhibited a consistent higher concentration of GF than PRP, as was expected. The fastest rate of PDGF decomposition over the three weeks was observed in PL (4°C) which declined by more than 1000 ng/mL over the course of investigation. PL (-80°C), aPRP, and PRP had similar decomposition rate, despite low concentrations (Figure 1). Thus, we concluded the fractions with the highest PDGF concentrations were also the ones with the greatest rate of degradation as well.

Shelf life with respect to VEGF concentration

As with PDGF, the concentration of VEGF over time was maintained at a consistent maximum over the 3 weeks of the experiment in PL stored at -80°C and 4°C. The highest concentration of VEGF obtained in this series was 7801.8 ng/mL in the initial measurement of VEGF in PL (Figure 2). Following the trend established with PDGF, PRP and aPRP were consistently much lower in VEGF concentration than PL. The highest VEGF concentrations reached by these two solutions were 1924.2 ng/mL in aPRP and 1202.8 ng/mL in PRP, with the aPRP initial concentration being notably higher than that PRP.

The rate of VEGF degradation was the maximum in PL at -80°C for the duration of the experiment, falling at an average rate of 1261.5 ng/mL per week. The storage of PL at -80°C caused the development of significant, slightly differing trends in the VEGF measurement while the PL 4°C was relatively

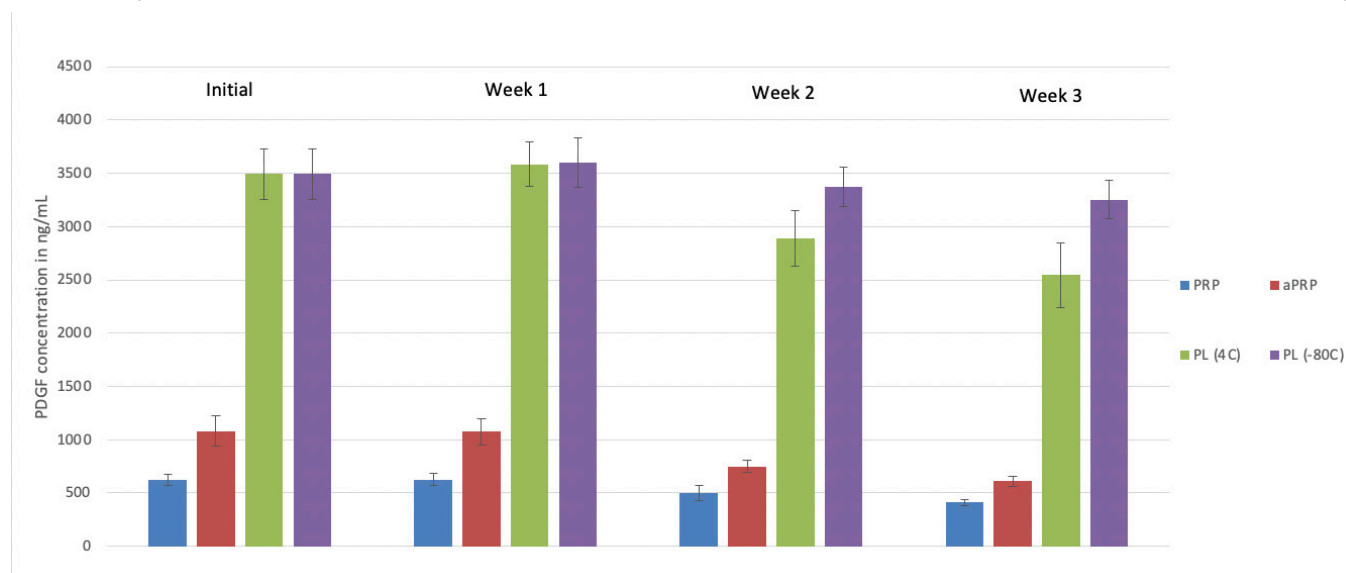


Figure 1. Bar graph depicting PDGF concentration (pg/mL) of PRP, aPRP, and PL determined using ELISA (n = 15, mean ± SD) across three weeks and (different) temperatures [PL only: -80°C, 4°C]. $p = 0.03$. As $p < 0.05$, results are statistically significant.

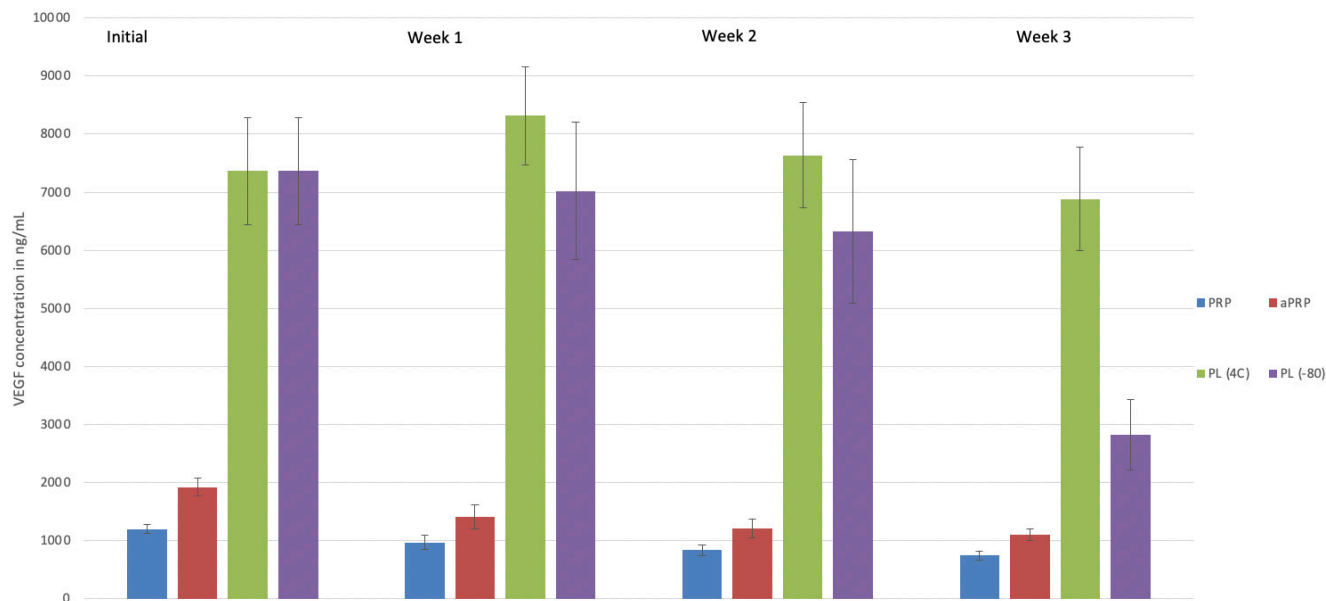


Figure 2. Bar graph depicting VEGF concentration (pg/mL) of PRP, aPRP, and PL determined using ELISA (n = 15, mean ± SD) across three weeks and (different) temperatures [PL only: -80°C, 4°C]. $p = 0.03$. As $p < 0.05$, results are statistically significant.

stable for both PDGF and VEGF. Unlike in PDGF, VEGF in PL at -80°C degraded faster than PL at 4°C. In both cytokines, however, it was observed that both aPRP and PRP expressed very low cytokine concentrations compared to PL ($p < 0.05$, t-test).

To underscore the differences between the initial and final concentrations, we have presented the normalized values for both PDGF and VEGF (Figures 3 and 4) as secondary substantiation to the obtained results.

DISCUSSION

We evaluated the growth factor content of PRP versus PL at different storage temperatures and time periods. We compared the relative stabilities of the blood-derived supernatants used by comparing the raw concentrations of growth factors (PDGF and VEGF) quantified using ELISA. We found that PL stored at both temperatures expressed the highest concentration of PDGF and VEGF, compared to PRP and aPRP. While the PL fractions had the highest concentrations of growth factors, they also underwent the most significant reduction over time.

Our results demonstrate PL to be more advantageous than PRP in terms of its stability and growth factor content to an extent. While the shelf life of PL (-80°C) regarding PDGF concentration was the highest among all blood fractions tested, PL (4°C) had the maximum shelf life regarding VEGF concentration. aPRP (activated with CaCl_2) demonstrated a similar trend in VEGF concentration across the three experimental points but with significantly lower concentrations than that of PL.

While PRP is very commonly used in clinical practices, this study demonstrates that PL may be a considerably better

potential option for regenerative therapies. However, this preliminary finding is in much need of further investigation by testing for consistent trends in other cytokines, such as IL-6, IL-8, and IFN- γ .

Given the employment of standard operating procedures for all the supernatants, the biochemical differences between PRP, PL and aPRP was the only possible explanation of the results obtained. The heterogeneity in the chemical composition was seen through the differences in the isolation methods, with PRP being derived from whole blood directly, PL derived from PRP, and aPRP activated by CaCl_2 .

A study comparing the release kinetics and concentration of growth factors between fresh PRP and frozen PRP claimed that freezing does not affect the release kinetics of growth factors by platelets (16). The repeated freeze-thawing procedure used to extract PL in this study could be an extension of the same logic. Given no significant change in the kinetics of the release mechanism, the resulting concentration of growth factors may be intrinsic to the supernatants themselves, substantiating our initial hypothesis (17).

Considering this, the findings in this study could further be explored by determining the concentration of other equally important growth factors, such as those in the TGF- β and FGF families. The reason behind the inconsistent decay of VEGF in PL at -80°C remains to be determined via further investigation. While this study demonstrated that PL has higher growth factor content than PRP or aPRP, the question of its optimal storage at 4°C or -80°C leaves considerable scope for further study.

Further investigation could also reveal different aspects of PRP, PL, and aPRP growth factor content, which would

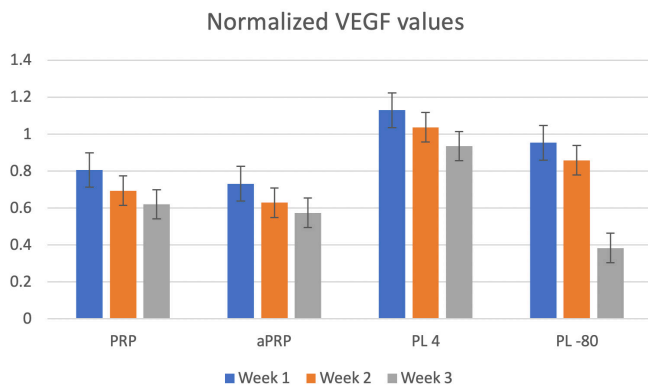


Figure 3. Bar graph depicting normalized VEGF values of PRP, aPRP, and PL (n = 15, mean ± SD) across three weeks and (different) temperatures [PL only: -80°C, 4°C]. $p = 0.03$. As $p < 0.05$, results are statistically significant.

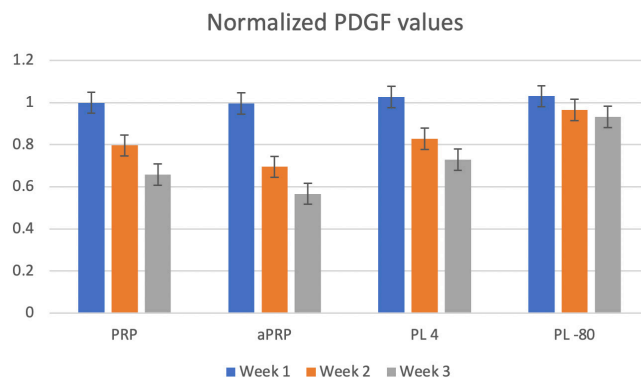


Figure 4. Bar graph depicting normalized PDGF values of PRP, aPRP, and PL (n = 15, mean ± SD) across three weeks and (different) temperatures [PL only: -80°C, 4°C]. $p = 0.03$. As $p < 0.05$, results are statistically significant.

be instrumental in determining the true significance of these results in clinical application – such as the potential, beneficial effects of storage of PRP and aPRP at -80°C (18, 19). Using larger sample sizes could be another source of further justification in subsequent investigations.

This preliminary study of standardization was performed to enable large-scale pooling of PRP/PL in terms of their growth factors. This technique could be then correlated to their clinical efficacy, especially in reducing inflammation and promoting healing during inflammation. From a wound-healing specific perspective, this investigation could be relevant in osteogenic applications and could be instrumental in facilitating the necessary biochemical cascade (20, 21). This approach will normalize variability in the samples enriched and will bring in the much-needed standardization required to scale up to a larger population.

MATERIALS AND METHODS

Blood samples were obtained from remains of blood bank collection at HCG (HealthCare Global Hospital). ELISA (Enzyme-linked immunosorbent assay) was performed to determine PDGF (Catalog#: ELH-PDGFAB) and VEGF (Catalog#: ELH-VEFG) concentrations according to the kit protocol (RayBio).

Isolation of PRP and PL

PRP was extracted by centrifuging whole blood from patients at 3000 rpm for 20 minutes. This resulted in the formation of PRP, a straw-colored supernatant in the separated layers post-centrifugation. The PRP supernatant was isolated using a long pipette, taking care to prevent mixing of the separate fractions, which could have resulted in contamination. PRP was treated with 0.1% calcium chloride (CaCl_2) (Fischer Scientific – CAS No: 10035- 04 -8) to form aPRP. aPRP was stored in a 1 mL Eppendorf tube at 4°C during the study.

PL was isolated by performing freeze-thaw disruption

of PRP three times. Freezing was conducted using liquid nitrogen obtained from a cryopreservation can while thawing was carried out by rapid swirling of PRP in a hot water bath at roughly 35°C-37°C.

Repeated freeze-thaw enabled the formation of PL (i.e., a solution containing the lysed platelet granules). As with PRP, the PL extract was stored. However, the PL extract was stored at two different temperatures at 4°C and at -80°C.

To test the shelf lives of PRP and PL, it was necessary to store them at their respective temperatures. For storage at 4°C, PRP, aPRP, and a part of PL were aliquoted and stored at 4°C during this study. This was accomplished by placing these solutions in a refrigerator pre-set to 4°C. For storage at -80°C, the PL aliquot that was not stored at 4°C was stored at -80°C using a deep freezer.

Analysis of concentrations

RayBio PDGF and VEGF ELISA kits were used over three weeks, including initial measurements pre-experiment of raw cytokine concentrations. Thereof, ELISAs were performed weekly. The technique used was sandwich ELISA. The trend formed between the initial and the subsequent concentrations were recorded and presented as results. The minimum concentration detectable for each of the assays was 10 ng/μl. The cytokine concentrations obtained in the experiment for PRP and aPRP far surpassed this basal level, deeming them detectable.

The normalized values were obtained from the formula (*average concentration [week-wise]*)/(*initial concentration*).

Statistical analysis

As there were 15 sets of individual PDGF and VEGF concentrations, the average concentrations were considered as the results, with data presented as mean ± standard deviation. Normalized values were achieved by further processing of the raw data obtained. Microsoft Excel software was used for data processing. Statistically significant

difference was determined by using one-way analysis of variance using the t-test function, where $p < 0.05$ was considered statistically significant.

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