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ORIGINAL ARTICLE

Comparative release of growth factors from PRP, PRF, and advanced-PRF

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Abstract
Objectives The use of platelet concentrates has gained increasing awareness in recent years for regenerative procedures in modern dentistry. The aim of the present study was to compare growth factor release over time from platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and a modernized protocol for PRF, advanced-PRF (A-PRF).
Materials and methods Eighteen blood samples were collected from six donors (3 samples each for PRP, PRF, and A-PRF). Following preparation, samples were incubated in a plate shaker and assessed for growth factor release at 15 min, 60 min, 8 h, 1 day, 3 days, and 10 days. Thereafter, growth factor release of PDGF-AA, PDGF-AB, PDGF-BB, TGFB1, VEGF, EGF, and IGF was quantified using ELISA.
Results The highest reported growth factor released from platelet concentrates was PDGF-AA followed by PDGF-BB, TGFB1, VEGF, and PDGF-AB. In general, following 15–60 min incubation, PRP released significantly higher growth factors when compared to PRF and A-PRF. At later time points up to 10 days, it was routinely found that A-PRF released the highest total growth factors. Furthermore, A-PRF released significantly higher total protein accumulated over a 10-day period when compared to PRP or PRF.
Conclusion The results from the present study indicate that the various platelet concentrates have quite different release kinetics. The advantage of PRP is the release of significantly higher proteins at earlier time points whereas PRF displayed a continual and steady release of growth factors over a 10-day period. Furthermore, in general, it was observed that the new formulation of PRF (A-PRF) released significantly higher total quantities of growth factors when compared to traditional PRF.
Clinical relevance Based on these findings, PRP can be recommended for fast delivery of growth factors whereas A-PRF is better-suited for long-term release.

Keywords Platelet-rich plasma · Platelet-rich fibrin · Choukroun's PRF · Platelet concentrates · Growth factor release

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Introduction

A number of techniques have been utilized in modern dentistry to speed the regeneration of either hard or soft tissues [1–4]. While a great deal of effort in recent years has been focused on the use of biologics as key mediators of tissue regeneration, some of the disadvantages of utilizing recombinant growth factors include high supra-physiological doses as well as high costs associated with their use [5–7]. Nevertheless, the use of growth factors such as recombinant platelet-derived growth factor (PDGF) has been shown to positively increase tissue formation across a wide variety of clinical procedures in both dentistry and medicine [8].

While the use of recombinant growth factors have demonstrated significant advantages, the use of autologous platelet concentrates has also been shown to support tissue regeneration [9, 10]. Platelet-rich plasma (PRP) is an autologous concentration of growth factors derived from patient whole blood centrifuged to reach super-natural concentrations [9, 10]. In the 1970s, it was introduced as "fibrin glue" and has gained popularity in the medical and dental fields for the regeneration of both hard and soft tissues [11–16]. Early experiments revealed the ability for several key growth factors found in the blood (including PDGF) to significantly modulate tissue repair and wound healing events [11–16].

The use of PRP has since been utilized by both oral surgeons and periodontists alike demonstrating advantages associated with its use for a variety of extensive dental regenerative procedures [16–21]. Furthermore, reports have demonstrated that PRP may also successfully be combined with various biomaterials including collagen membranes and bone grafting materials [22–27]. One of the reported drawbacks of PRP is that it contains anticoagulants, an event that interferes with the natural healing process despite containing a number of growth factors implicated in tissue repair [9, 10].

Following numerous research utilizing PRP, further investigation found that a platelet concentrate made from whole blood without the use of coagulants could also be further utilized to improve wound healing [28–32]. This protocol has been termed platelet-rich fibrin (PRF, also more recently referred to as leukocyte-PRF or L-PRF) and involves the use of a fibrin clot which may be utilized as a membrane containing autologous growth factors hypothesized to slowly release growth factors to the surrounding environment during wound healing [21, 33–36].

Recently, these investigators have formulated a new protocol for PRF where centrifugation procedures have been altered to further improve tissue regeneration [37]. While standard PRF is centrifuged at 2700 rpm for 12 min, the advanced platelet-rich fibrin (A-PRF) is centrifuged at slower speeds (1500 rpm, 14 min). This modification to centrifugation protocol has previously been shown to increase platelet cell numbers and monocytes/macrophages behavior [37]. Despite these findings, little is known about the release of growth factors from the various platelet concentrates over time with no data available to date on A-PRF. Therefore, the aim of the present study was to compare growth factor release from PRP, PRF, and A-PRF over time and investigate the in vitro release of seven growth factors at 15 min, 60 min, 8 h, 1 day, 3 days, and 10 days post incubation. The growth factors investigated include PDGF-AA, PDGF-AB, PDGF-BB, transforming growth factor beta 1 (TGFB1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF).

Materials and methods

Platelet concentrations

Three samples of blood were collected with the informed consent of six volunteer donors (18 total samples) and the blood was then processed for PRP, PRF, or A-PRF centrifugation. All blood samples were obtained from members of our laboratory between the ages of 30 and 60. PRP platelet concentration was prepared via a protocol as previously described [38]. Briefly, 10 mL of whole blood was centrifuged for 7 min at 1000 rpm (45 g) at room temperature without brake (centrifuge 5702; Eppendorf, Darmstadt, Germany). The plasma rum was decanted up to the erythrocyte sediment and then centrifuged again for 10 min at 3,000 rpm (400*g*) at room temperature. Finally, the PRP was decanted and the final PRP sediment was suspended in 6-well in vitro plastic culture dishes with 5 mL of culture media and processed as further described.

The PRF and A-PRF were also isolated as previously described [37]. Ten milliliters of whole blood without anticoagulant was centrifuged at 2,700 rpm (325*g*) for 12 min whereas A-PRF was centrifuged at 1,500 (100*g*) for 14 min. The lack of anticoagulants in these samples allows for a fibrin clot formation to form which can then be collected in the middle of the tube between the red corpuscles at the bottom and acellular plasma at the top. Thereafter, PRP, PRF, and A-PRF clots were then transferred to 6-well in vitro plastic culture dishes with 5 mL and processed for further investigation.

Protein quantification with ELISA

In order to determine the amount of released growth factors at 15 min, 60 min, 8 h, 1 day, 3 days, and 10 days, samples were placed into a shaking incubator at 37 °C to allow for growth factor release into the culture media. At each time point, the 5 mL of culture media was collected, frozen, and replaced with 5 mL of additional culture media. Protein quantification was carried out using ELISA. At desired time points, PDGF-AA (DY221, range = 15.60–1000 pg/mL), PDGF-AB (DY222, range = 15.60–1,000 pg/mL), PDGF-BB (DY220, 31.20–2,000 pg/mL_, TGFβ1 (DY240, range = 31.20–2,000 pg/mL), VEGF (DY293B, range = 31.20–2,000 pg/mL), IGF (DY291, range = 31.20–2,000 pg/mL) and EGF (DY236), range = 3.91–250 pg/mL) were quantified using an ELISA assays (RND Systems, Minneapolis, MN, USA) according to manufacturer's protocol as previously described [39]. Briefly, 100 μL of assay diluents and 100 μL of sample were incubated for 2 h at room temperature in antibody-precoated 96-well plates. Wells were washed four times with washing buffer, incubated for 2 h with peroxidase-conjugated antibody solution, washed again, followed by addition of 100 μL of substrate solution for 20 min and 50 μl of stopping

solution for 20 min. Absorbance was measured at 450 and 570 nm on an Infinite 200 microplate reader (Tecan Group LTD, Männedorf, Switzerland) and subtracted at 570 nm from the readings at 450 nm. All samples were measured in triplicate and three independent experiments were performed for each platelet concentrate. Statistical analysis was performed by two-way ANOVA with Bonferroni test.

Results

Total protein released from PRP, PRF, and A-PRF after 10 days

All proteins were quantified for both growth factor release at desired time points as well as the total accumulated protein quantities (Figs. 1–3, Table 1). It was found that A-PRF released the highest total amount of growth factors when compared to either PRF or PRP (Table 1). Subsequently, release of PDGF-AA was found highest for all platelet concentrates followed by TGF-beta1, PDGF-BB, PDGF-AB, VEGF, EGF, and IGF with slight differences observed between groups (Table 1). It was found that A-PRF released 11048.19 ng/mL of total protein after 10 days, significantly more than PRF 9261.89 ng/mL and PRP 6176.15 ng/mL. Interestingly, PDGF-AA was found released from all platelet concentrations at 6–10-fold higher concentrations when compared to PDGF-AB and PDGF-BB. Furthermore, significantly lower levels of EGF and IGF were found when compared to PDGF, TGF-beta1, and VEGF concentrations (Table 1).

PDGF-AA, PDGF-AB, and PDGF-BB growth factor release from PRP, PRF, and A-PRF over time

Analysis of growth factor release of PDGF-AA, PDGF-AB, and PDGF-BB at each time point as well as accumulated over time is displayed in Fig. 1. It was found that after 15 min, significantly higher levels of PDGF-AA was released from PRP when compared to PRF or A-PRF. Interestingly, significantly lower levels were observed at 60 min demonstrating that PRP rapidly releases PDGF-AA between 0 and 15 min and thereafter significantly less release is observed comparatively up to 10 days (Fig. 1a). No significant differences between A-PRF and PRF at times points ranging from 15 min to 1 day; however, at 3 days, A-PRF showed significantly higher growth factor release of PDGF-AA when compared to either PRP or PRF (Fig. 1a). The total PDGF-AA accumulated proteins over time (Fig. 1b) demonstrated that while PRP showed significantly higher levels at an early time point of 15 min, thereafter significantly lower levels could be observed from 8 h till 10 days. In contrast, significantly higher levels were found for A-PRF from 1 to 10 days when compared to PRP or PRF (Fig. 1b). Similar trends were also observed for PDGF-AB and PDGF-BB (Fig. 1c–f). Interestingly, however, the total protein content for growth factor PDGF-BB was significantly higher in PRP samples when compared to PRF and A-PRF at all time points (Fig. 1f).

TGFB1 and VEGF growth factor release from PRP, PRF, and A-PRF over time

Thereafter, the release of TGFB1 and VEGF were quantified over time (Fig. 2). Once again, it was observed that PRP demonstrated significantly higher levels at early time points of 15 min and 8 h when compared to PRF and A-PRF (Fig. 2a, c). Thereafter, PRP levels dropped to significantly lower levels when compared to PRF or A-PRF with A-PRF demonstrating the significantly highest levels at 1, 3, and 5 days for both TGFB1 and VEGF concentrations (Fig. 2a, c). Parallel to the results obtained with PDGF, total protein accumulation was significantly highest at early time points for PRP and significantly dropped to lower levels by 10 days when compared to PRF and A-PRF (Fig. 2b, d). Total protein release was significantly highest for A-PRF at 3 and 10 days for TGFB1 and 1, 3, and 10 days for VEGF when compared to PRP and PRF (Fig. 2b, d).

EGF and IGF growth factor release from PRP, PRF, and A-PRF over time

Different trends were observed for the release of EGF and IGF (Fig. 3). Significantly highest levels for PRP were found only at 15 days for PRP (Fig. 3a). Thereafter, significantly highest protein release was found on A-PRF for EGF at 60 min, 8 h, 1 day, and 3 days (Fig. 3a). While no differences in PRF and A-PRF could be observed for protein release of IGF at almost all time points, significantly lower levels were observed for PRP at 15 min, 60 min, 8 h, and 1 day comparatively (Fig. 3c). Total protein accumulation demonstrated the highest total growth factor of EGF for A-PRF with the lowest being PRP whereas slightly higher PRF results were observed for IGF when compared to A-PRF (Fig. 3d).

Discussion

The aim of the present study was to compare growth factor release from three different platelet concentrates including PRP, PRF, and a new protocol established termed advanced-PRF. While the advancements made in terms of platelet concentrates have been hypothesized to improve tissue regeneration [37], no information is available to date regarding the growth factors released from these three platelet concentrates over time. Therefore, the aim of the present study was to investigate in detail five different growth factors including

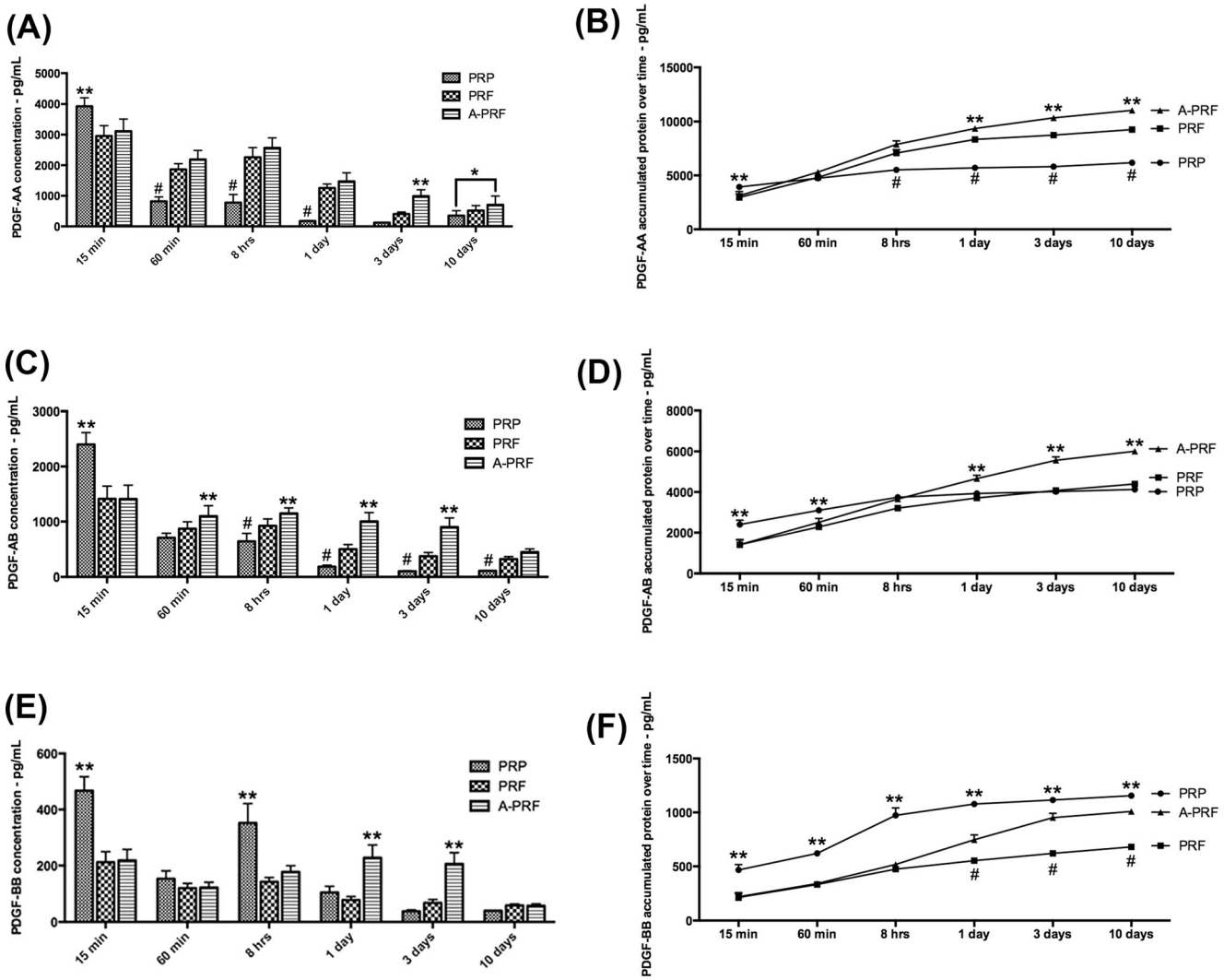

Fig. 1 ELISA protein quantification at each time point of **a** PDGF-AA, **c** PDGF-AB, and **e** PDGF-BB over a 10-day period. Total accumulated growth factor released over a 10-day period for **b** PDGF-AA, **d** PDGF-AB, and **f** PDGF-BB. (*$p < 0.05$ signifies significant difference between groups, **$p < 0.05$ signifies significantly higher than all other groups, #$p < 0.05$ signifies significantly lower than all groups.) Assay performed in triplicate from six different blood samples

three isomers of PDGF (AA, AB, and BB) on protein release over time from PRP, PRF, and A-PRF.

Three things stand out from the results in the current investigation. First, it was found that PRP released the highest amount of growth factors at early time points when compared to either PRF or A-PRF. The fast action of released proteins found in PRP concentrates may be hypothesized to speed the recruitment of incoming progenitor cells in defect locations

Table 1 Final growth factor released over a 10-day period from the various platelet concentrates including PRP, PRF, and A-PRF

	PRP	PRF	A-PRF
PDGF-AA	6176 (2812–9184)	9262 (2877–13839)	11048 (5036–18817)
PDGF-AB	4131 (1837–5492)	4396 (862–7563)	6007 (3455–10298)
PDGF-BB	1155 (531–1371)	680 (220–1147)	1010 (643–1803)
TGF-beta1	1105 (619–1453)	1110 (302–1714)	1589 (1052–2315)
VEGF	847 (693–1009)	732 (537–914)	847 (814–1063)
EGF	363 (210–497)	512 (146–715)	659 (447–795)
IGF	54 (44–67)	166 (55–252)	129 (81–179)

Data represents averages (pg/ml) with ranges (minimum to maximum values)

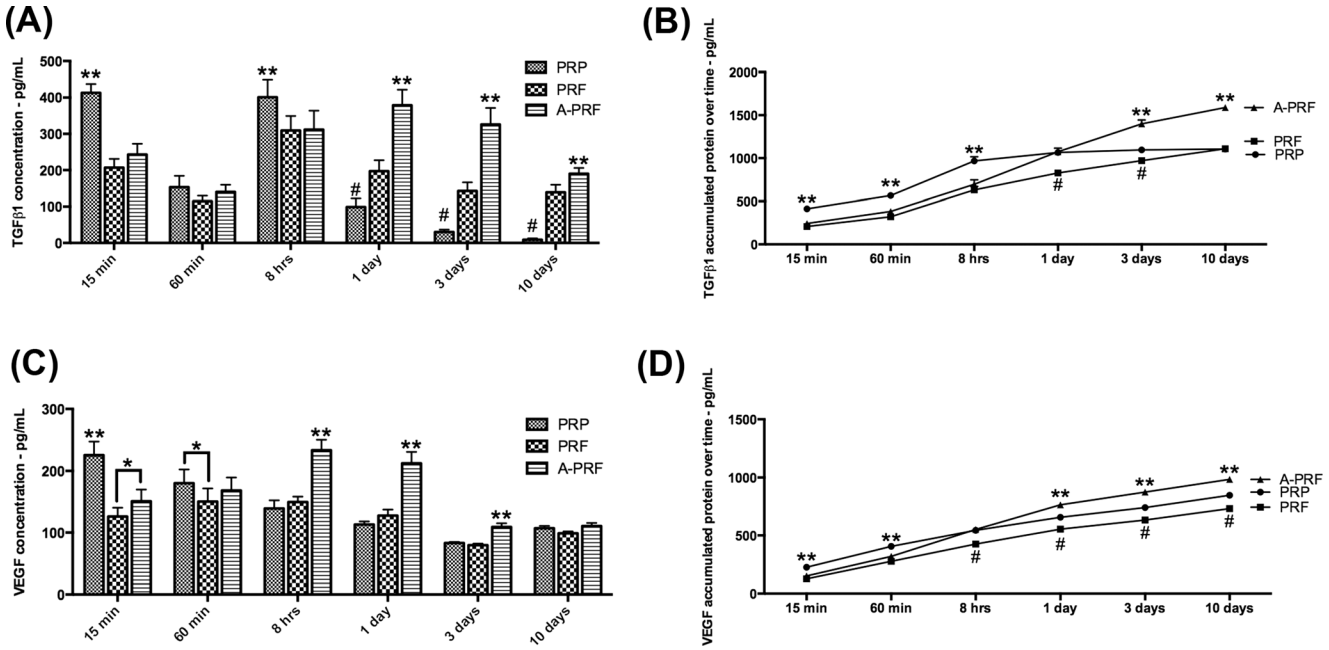

Fig. 2 ELISA protein quantification at each time point of **a** TGFB1 and **c** VEGF over a 10-day period. Total accumulated growth factor released over a 10-day period for **b** TGFB1 and **d** VEGF. (*$p < 0.05$ signifies significant difference between groups, **$p < 0.05$ signifies significantly higher than all other groups, $^{\#}p < 0.05$ signifies significantly lower than all groups.) Assay performed in triplicate from six different blood samples

and could prove a valuable means in medical and dental procedures requiring rapid incoming recruitment of regenerative cells. Secondly, while the release of PRP had rapid release of growth factors, it was interesting to note that over time, PRF not only had more growth factor released at later time points but also contained more growth factors as a whole from within their fibrin matrix. One of the hypothesized reasons for this is the fact that PRF and A-PRF have been shown to contain more living cells [37]. Therefore, these cells are likely the contributing difference between the results observed between PRF, A-PRF, and PRP. Lastly, one of the surprising findings from the present study was the significant increase in total

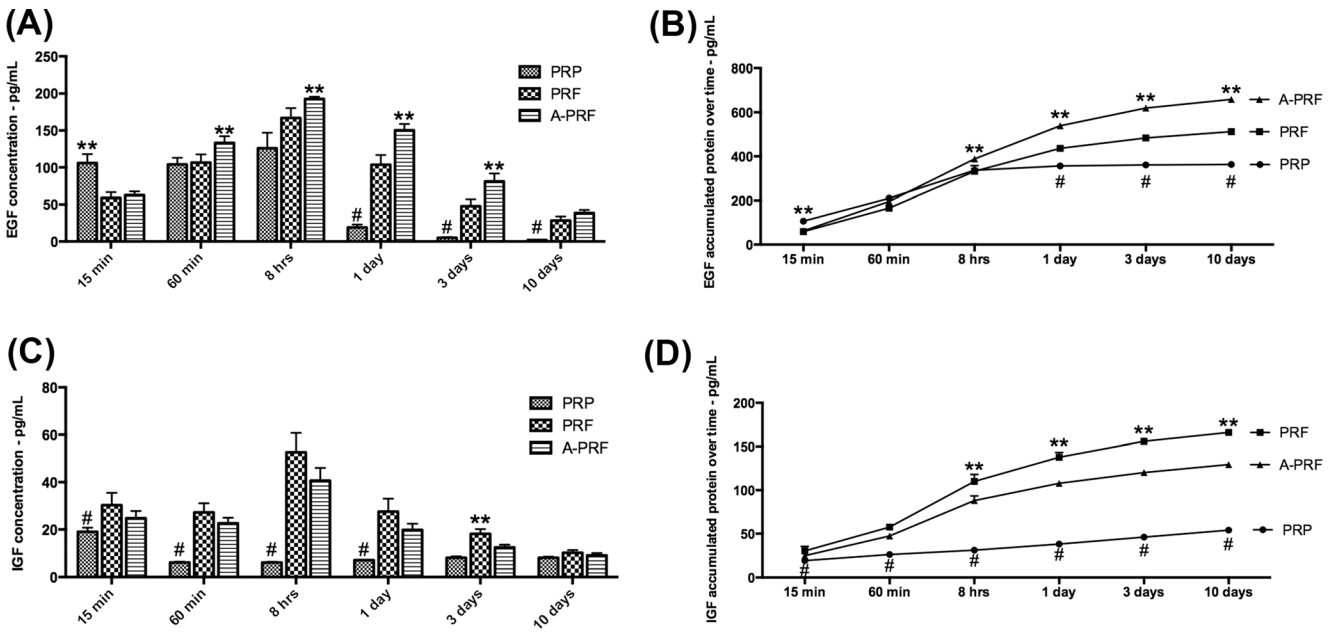

Fig. 3 ELISA protein quantification at each time point of **a** EGF and **c** IGF over a 10-day period. Total accumulated growth factor released over a 10-day period for **b** EGF and **d** IGF. (*$p < 0.05$ signifies significant difference between groups, **$p < 0.05$ signifies significantly higher than all other groups, $^{\#}p < 0.05$ signifies significantly lower than all groups.) Assay performed in triplicate from six different blood samples

protein released between PRF and A-PRF. While it has been shown that A-PRF contains more living progenitor cells and platelets when compared to PRF [37], the subsequent significant increase in total protein release may therefore present additional advantages for clinical use.

Although this is the first report to investigate release of growth factors from A-PRF, previous authors have investigated protein release from either PRP or PRF and further investigated its subsequent effect on cell activity. In a first study, El-Sharkawy et al. demonstrated that PRP was able to promote increases in PDGF-AB, PDGF-BB, TGF-B1, VEGF, and EGF when compared to whole blood [40]. Furthermore, in another previous study it has been shown that PRF could release various growth factors including PDGF-AB, TGF-B1, VEGF, EGF, and IGF-1 [41]. The results from that study demonstrate an increase in growth factor over time from 5 to 300 min but did not look at later time points. Furthermore, the release of growth factors was not compared to a second platelet concentrate making it difficult to investigate the potency of PRF in comparison to either PRP or A-PRF [41].

It has also previously been shown that PRP has high levels of secreted PDGF and TGFB [42] which subsequently stimulates collagen synthesis in PDL cells and gingival fibroblasts [43, 44], induces cell proliferation [45] and mineralization potential in osteoblasts [46], and increases endothelial cell activity [47] in vitro. Gassling et al. showed that osteoblasts and fibroblasts that were cultured with PRP or PRF demonstrated varying expression of various growth factors with those cultured with PRP favoring significantly higher levels of PDGF-AB and TGFB1 expression [38]. Furthermore, a second report by this group compared PRF (as a membrane) to bovine-derived collagen membranes (BioGide) and tested osteoblast response to the two biomaterials [48]. It was found that cell growth was significantly higher on PRF when compared to the bovine collagen membrane [48].

Another aspect that needs to be considered when comparing in vivo work with in vitro studies is the variability in growth factor concentrations between donors. In the present study, the patient age range was between 30 and 60. We found reported differences between minimum and maximum growth factor accumulation between donors as reported in Table 1. Furthermore, with an increasingly aging population continuously requiring regenerative procedures, one can only expect that with advanced age (and the likelihood of increased systemic diseases and medications), a much larger variability may also be expected. Therefore, ongoing research investigating the optimal concentrations may be required to further optimize this avenue of research. It was also found in the present study that the slower spinning protocols of A-PRF released more growth factor than the prototype PRF. As one previous report demonstrated that A-PRF contains more platelets and neutrophilic granulocytes [37], it may be hypothesized that these cells contributed to the slight increase in total growth factor accumulation after a 10-day period. This hypothesis however requires further investigation.

There remain several aspects of research necessary to further compare the various platelet formulations investigated in this study. First, it is unclear how the release of the various platelet concentrates including PRP, PRF, and A-PRF will affect cell behavior over time. Therefore, a comparative in vitro cell study further investigating the use of PRP, PRF, and A-PRF on cell behavior of various cell types including osteoblasts, gingival fibroblasts, and periodontal ligament cells could further provide rationale for which treatment modalities stimulates a higher cell response. Furthermore, it is known that platelet concentrates are often combined with various biomaterials such as collagen membranes and bone grafting materials. Therefore, it would also be worthwhile to compare growth factor release from a variety of biomaterials following coating with either PRP, PRF, or A-PRF. Future research comparing the various platelet formulations in a clinical setting would also be valuable to compare which indications may serve better for various clinical scenarios.

Conclusion

The results from the present study demonstrated that PRP, PRF, and A-PRF were able to release growth factors over time from their respective platelet formulations. Interestingly, PRP demonstrated the ability to release significantly higher levels of growth factors at very early time points whereas PRF and A-PRF had a more gradual release of growth factors up to a 10-day period. The new formulation of PRF (called advanced-PRF) stimulated significantly higher growth factor release over time when compared to standard PRF and may prove clinically beneficial for future regenerative procedures. Future investigations studying the effects of each platelet formulation on cell behavior as well as in vivo study would further enhance our understanding for how A-PRF compares to previously utilized PRP and A-PRF.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Ethical approval Blood was drawn from patients (lab members) with informed signed consent. An ethical approval was not required for this purpose. No animals were used in this study.

Informed consent All blood was drawn from patients (lab members) with informed signed consent.

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