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Platelet-rich plasma for tissue regeneration can be stored at room temperature for at least five days

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ABSTRACT

Background: Autologous platelet-rich plasma (PRP) is gaining increasing use as a wound healing promoter in a variety of clinical settings, including dentistry. Fresh PRP is often used, necessitating daily draws. The present study investigates the possibility of using stored PRP without having to freeze it by storing PRP under variable conditions and assessing growth factor release as a surrogate marker of continued viability.

Methods: Freshly drawn PRP was stored in oxygen permeable and non-oxygen permeable containers under conditions of constant agitation with or without added prostaglandin, intermittent agitation and no agitation, over an 8-day period. Serial platelet counts, mean platelet volume, platelet distribution width and platelet-large cell ratio, and collagen-induced aggregometry were undertaken. Once collagen-induced aggregation had gone to completion, the plasma was centrifuged to pellet platelet material and the supernatants separated and frozen for batched analysis of released platelet-derived growth factor-BB (PDGF-BB).

Results: As would be anticipated, platelet counts, percentage aggregation and PDGF-BB levels all reduced over time. Platelet parameters suggested that platelets were more stable in the non-oxygen permeable containers, possibly due to pH drift and a degree of microaggregate formation in the oxygen permeable containers.

Conclusion: Although platelet integrity and PDGF-BB fell over time, the intermittently agitated non-oxygen permeable container appeared to retain better platelet integrity and function, and PDGF-BB release, than other storage conditions, with potential for clinical use for 5–8 days.

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Introduction

Whilst platelets tend to be considered as having their primary role in haemostasis, they are intricately involved in the initiation of wound healing.[1] Indeed, haemostasis can be considered as the first stage of healing. Despite being 'mere' cell fragments, platelets have complex, specialised structure and physiology in order to fulfil their role duality.

An organelle crucial to effective platelet function is the α-granule, the most abundant platelet organelle. The α-granules contain an array of molecules essential to both haemostasis and wound healing. Once a platelet is activated, α-granules fuse with cytoplasmic canals to link them to the platelet surface and the exterior, and then release their contents into the surrounding medium or onto the platelet surface. This serves to both localise and concentrate vital molecules for haemostasis and wound healing.

Proteins secreted by activated platelets influence various aspects of wound healing. For instance, platelet-derived growth factor (PDGF) is chemotactic for macrophages, and together with transforming growth factor-β and insulin-like growth factor, it assists in chemotaxis and mitogenesis of stem cells and osteoblasts, angiogenesis for capillary in-growth, bone matrix formation and collagen synthesis.[1,2]

Platelet-rich plasma (PRP) is defined as a portion of the plasma fraction of autologous blood having a platelet concentration/platelet count above baseline.[3] The prominent role played by platelets in wound healing and the relatively easy generation and application of PRP in the clinic or surgical setting has led to an increase in the use of autologous PRP to promote soft and hard tissue regeneration.[2,4] Autologous PRP has been used in the treatment of chronic skin and soft tissue ulcerations, maxillofacial surgery, orthopaedic and trauma surgery, plastic surgery, spinal surgery, heart bypass surgery and burns.[1,2] It has also been used in a variety of settings in dentistry,[4] such as oral mucosa healing,[5] root canal revascularistaion [6] and scaffolding for regenerative endodontics.[7]

Local delivery of a single growth factor (GF), commonly recombinant PDGF-BB, has been employed.[8] However,

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use of PRP represents greater similarity to the natural healing process and permits application of multiple wound healing promoters in their biologically determined ratios.[1] Additionally, the short shelf life of recombinant PDGF-BB, cost and concerns about side effects [1] make PRP a more attractive and cost-effective proposition.

Whilst PRP is generally preferred to recombinant GFs in promotion of wound healing, there is a paucity of data on stability of PRP preparations in this clinical setting. Being anucleate, platelets have a short *in vivo* lifespan of 8–10 days, and platelet donations taken into citrate-based anticoagulants stored at 22 °C ± 2 °C with continuous, gentle agitation are generally assigned a five-day expiry.[9] Since platelet concentrates stored under these conditions are considered clinically viable for transfusion, the present study was instigated to investigate serial collagen-induced PDGF-BB release from normal donor platelets stored under variable conditions as a marker of viability for promoting tissue regeneration.

Materials & methods

Collection of donor platelets

A total of 125.0 mL of blood was taken from a consented, haemostatically asymptomatic, clinically well adult male donor into 25 BD Vacutainer® tubes (Bunzl Healthcare, Enfield, UK), each containing 1.0 mL of Acid-Citrate-Dextrose solution B (ACD-B) as the anticoagulant. The non-traumatic venepuncture was performed with minimal stasis using a 21-g butterfly needle. The donor had not received any drugs known to affect platelet function in the preceding two weeks, or any foodstuffs known to affect platelet function in the preceding five days.[10]

Blood for platelet diagnostics is normally taken into 0.105 M tri-sodium citrate in a ratio of nine parts blood to one part anticoagulant. However, this is based on diagnostic assays being performed within four hours of venepuncture to assess function on freshly drawn platelets before natural deterioration adversely affects diagnostic accuracy. The choice of ACD-B as anticoagulant for this study was based on the work of Lei *et al.* [11] who demonstrated that ACD-B was superior to tri-sodium citrate in maintaining platelet structure integrity and preventing spontaneous aggregation.

Preparation of platelet-rich plasma

The blood was allowed to cool for 15 min after the venepuncture to prevent formation of plasma clots. The tubes were then centrifuged at 22 °C in an IEC Centra CL3 centrifuge (Thermo Scientific, Basingstoke, UK) at 130 RCF (800 RPM) for 20 min to generate PRP. After centrifugation, the PRP was pooled by transferring into three 30 mL Sterilin™ polypropylene universal containers (Thermo Scientific) using plastic transfer pipettes and avoiding the white blood cell-rich buffy coat layer. The contents of the three containers were multiply intermixed to achieve homogeneity. The remaining whole blood was centrifuged at 2450 RCF for 10 min to obtain platelet poor plasma (PPP) for use as a blank in the collagen-induced platelet activation. The PPP was transferred into a separate universal container. A blood count was performed on the PRP using a Sysmex PocH 100i (Sysmex UK, Milton Keynes, UK) to check that the platelet count was between 150–600 $\times 10^9$/L and contamination with white blood cells (WBC) and red blood cells (RBC) was minimal.[12] Locally employed cut-offs are WBC $< 0.5 \times 10^9$/L and RBC $< 0.5 \times 10^{12}$/L.

Collagen-activated platelet aggregometry

Immediately after preparation, aliquots of the PRP were subjected to activation by Collagen Reagent HORM® (Takeda Austria, Linz, Austria), an equine tendon collagen suspension, in a PAP8 platelet aggregometer (Alpha Laboratories, Eastleigh, UK).[10] The platelets were activated with collagen at a final concentration in the PRP of 10.0 µg/mL. Once aggregation was complete, the aliquots were centrifuged to pellet the platelet aggregates and the supernatant removed and frozen at –80 °C.

Storage of platelet-rich plasma

The remaining PRP was stored under different conditions in two types of container. One was a 70 mL Nunc™ non-treated flask (NF) (Thermo Fisher Scientific, Langenselbold, Germany), a sterile, non-activating polystyrene tissue culture flask. Although the polystyrene body of the flask is not oxygen permeable, the filter cap permits a constant air flow. The other was a 30 mL Sterilin™ polypropylene universal container (Thermo Scientific), which is sterile but not oxygen permeable.

Gently agitating platelets during storage reduces activation and debris formation.[13] It has been shown that an interruption of one day has negligible, measurable effect, but longer periods can result in significant, deleterious changes.[14] For this reason, separate aliquots of PRP were stored constantly agitated (CA), intermittently agitated/mixed (IA) or kept stationary to assess the impact of variable agitation. An additional CA storage condition was introduced to include addition of prostaglandin E1 (PGE_1) (Sigma-Aldrich UK, Gillingham, UK), a natural platelet inhibitor that triggers an increase in cyclic adenosine monophosphate levels which counteracts platelet activation by reducing calcium flux. Addition of PGE_1 to PRP reduces platelet activation during storage whilst permitting a response to most agonists during *in vitro* analysis.[15] PGE_1 was added to the PRP at a final concentration of 10.0 µg/mL, the standard concentration for diagnostic use. All PRP was stored at room temperature since cold storage activates platelets.

A Luckham R100 Rotatest Shaker (Luckham Ltd, Cambridge, UK) was used to agitate the containers requiring CA. The platform was rotated on setting 5, a mid-point setting of approximately 90 rpm to approximately mimic that employed for platelets being stored for transfusion.[16] The IA aliquots were multiply inverted hourly during the laboratory core hours of 09.00–17.00 h for the first five days and left undisturbed for days six and seven as they were over a weekend. The 150 mL volume of donated blood plus anticoagulant yielded approximately 65 mL of PRP, which permitted storage of approximately 8.0 mL of PRP in each container.

Serial collagen-activated platelet aggregometry and platelet counting

At approximately the same time, each day as when the freshly prepared PRP was analysed, aliquots of mixed PRP from each of the eight containers were separately activated in the PAP8 aggregometer with collagen at a final concentration in PRP of 10.0 µg/mL and the aggregation patterns recorded.[10] Once aggregation was complete, the aliquots were centrifuged to pellet the platelet aggregates and the supernatants removed and frozen at –80 °C. This was undertaken on days 2–5 and day 8. Platelet counts were also performed each day as an ongoing assessment of PRP quality and platelet activation and spontaneous aggregation. Platelet counts were performed immediately prior to aggregometry. Additional platelet parameters of mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR) were recorded from each count.

Analysis of supernatants for PDGF-BB

To assess storage and release of GFs from stored platelets, measurement of PDGF-BB was chosen as a representative marker since it has been employed in similar studies for the same purpose,[17–20] Becaplermin is a recombinant version of PDGF-BB,[21] and reagents for analysing PDGF-BB levels are commercially available. PDGF-BB levels in the supernatants were quantified with Human PDGF-BB Platinum ELISA reagent kit (Affymetrix eBioscience, Hatfield, UK). Each reagent-employing step of the assay was performed manually whilst end-point detection, standard curve generation and calculation of final results were all performed on a Dynex DS2™ ELISA analyser (Werfen UK, Warrington, UK).

Results

Cell counting

The platelet count on the PRP immediately after harvesting and prior to collagen-induced platelet aggregation was 236 x 10^9/L, and no WBC or RBC were detected. The platelet count was within limits for analysis by aggregometry and no further manipulation was required.[11] Figures 1 and 2 plot the changes in platelet count over time in the variably stored PRP from both types of container. The serially estimated MPV, PDW and P-LCR parameters are shown in Table 1.

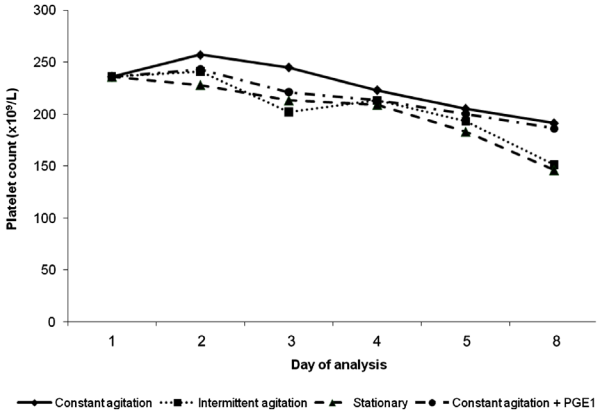

Figure 1. Serial platelet counts for Nunc™ flask-stored PRP.

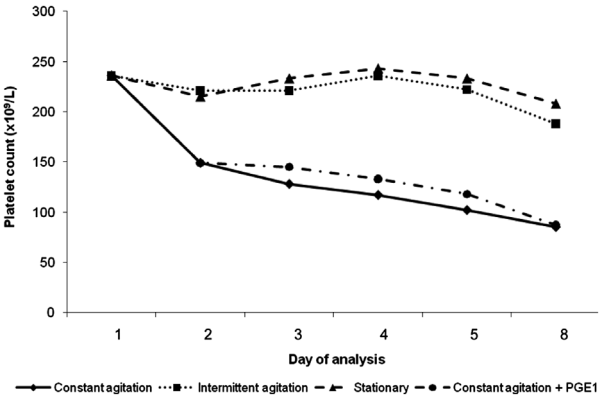

Figure 2. Serial platelet counts for universal-stored PRP.

Platelet aggregation and PDGF-BB release

Figures 3 and 4 plot the changes in final percentage collagen-induced aggregation (FPA) over time in the variably stored PRP from both types of container, and Figures 5 and 6 show the PDGF-BB levels in supernatants of collagen-activated PRP.

Discussion

The concentrated levels of GFs in platelet *α*-granules play a critical role in cell proliferation, chemotaxis, cell differentiation and angiogenesis.[18,22] PRP for promoting tissue repair can be directly applied to the wound site [23,24] or activated with thrombin to generate a gel for topical application or onto vehicles such as medical gauze.[1,2,25–27] Activated platelets are trapped in the fibrin network of the gel yet remain able to release their contents.[26] Collagen activation has the advantage of generating a more sustained GF release than the

Table 1. Serial platelet parameters in variably stored platelet-rich plasma.

Storage container	Storage conditions	Platelet parameters	Day of analysis 1	2	3	4	5	8
No storage	Fresh PRP	PDW (fL)	11.6					
		MPV (fL)	9.7	–	–	–	–	–
		P-LCR (%)	22.3					
Nunc™ flask	Constant agitation	PDW (fL)		16.2	15.2	14.0	12.2	8.8
		MPV (fL)	–	11.8	11.1	10.7	10.1	8.6
		P-LCR (%)		41.2	36.0	33.0	28.5	18.4
	Intermittent agitation	PDW (fL)		13.4	17.5	14.4	14.2	12.1
		MPV (fL)	–	10.7	11.8	11.3	11.0	10.4
		P-LCR (%)		29.7	41.8	36.4	34.7	30.5
	Stationary	PDW (fL)		12.5	16.4	16.1	15.2	13.9
		MPV (fL)	–	10.2	11.7	11.7	11.4	11.0
		P-LCR (%)		26.4	41.5	41.2	38.0	35.6
	Constant agitation + PGE_1	PDW (fL)		16.5	15.2	14.1	12.2	9.7
		MPV (fL)	–	11.7	11.1	10.9	10.2	8.9
		P-LCR (%)		41.3	36.4	34.4	29.1	20.2
Universal	Constant agitation	PDW (fL)		9.9	9.4	No analyser output	8.6	8.3
		MPV (fL)	–	8.8	8.6		8.6	8.4
		P-LCR (%)		16.9	17.9		19.2	18.3
	Intermittent agitation	PDW (fL)		11.3	11.6	12.6	12.0	13.8
		MPV (fL)	–	9.6	9.7	10.2	10.1	10.7
		P-LCR (%)		21.0	21.9	25.3	24.9	31.1
	Stationary	PDW (fL)		11.0	11.8	11.5	12.3	14.4
		MPV (fL)	–	9.5	10.1	10.1	10.1	11.4
		P-LCR (%)		19.6	23.8	23.8	23.5	36.6
	Constant agitation + PGE_1	PDW (fL)		9.8	8.7	8.5	7.8	8.9
		MPV (fL)	–	8.8	8.3	8.1	8.1	8.6
		P-LCR (%)		16.4	17.1	13.8	15.4	20.2

Notes: MPV, mean platelet volume; PDW, platelet distribution width, PGE_1, prostaglandin E1; P-LCR, platelet-large cell ratio (P-LCR) – the ratio of platelets smaller than 12 fL to those with a volume between 12–30 fL; PRP, platelet-rich plasma. Reference ranges for whole blood; MPV, 7.5 – 11.5 fL; PDW, 9.3–16.0 fL; P-LCR, 24.8–41.2%.

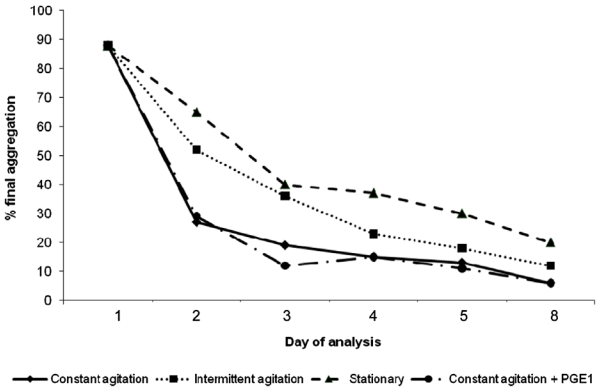

Figure 3. Final percentage aggregation plot for Nunc™ flask-stored PRP.

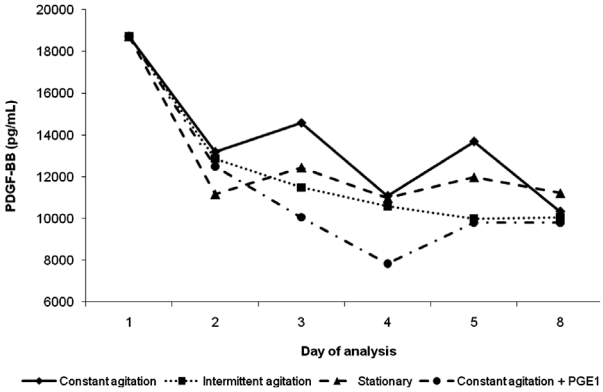

Figure 5. Serial PDGF-BB levels for supernatants of Nunc™ flask-stored PRP after collagen activation.

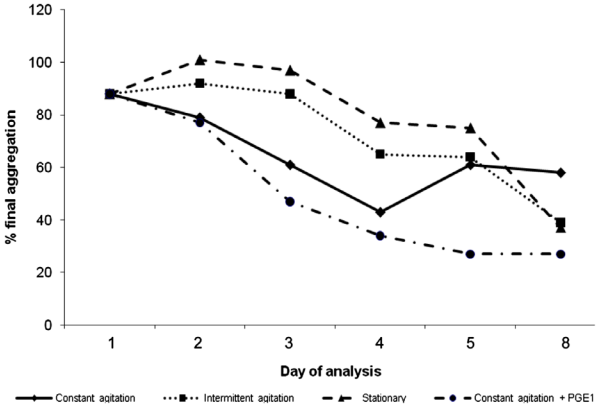

Figure 4. Final percentage aggregation plot for universal-stored PRP.

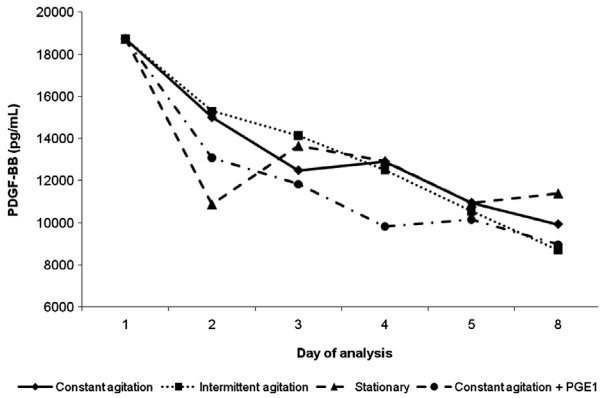

Figure 6. Serial PDGF-BB levels for supernatants of universal -stored PRP after collagen activation.

immediate release induced by thrombin [25] and was chosen as the activator for this study to parallel clinical practice at Stardent Dental and Facial Clinic. Freshly drawn PRP is commonly used for this purpose, or in some cases, PRP that has been frozen and thawed.[24] The aim of the present study was to evaluate GF release from PRP stored at room temperature over a number of days, using PDGF-BB as the marker.

One limitation of the study is that only one donor was employed. However, the aim was to empirically assess platelets from a haemostatically uncompromised donor as an 'ideal' example and achieve a guide to the potentiality of using stored platelets in place of daily draws. Donor PRP platelet count was within limits for performing aggregometry, although PRP for tissue regeneration should have a count of at least 1000×10^9/L to be clinically effective.[1,2] This level would have compromised aggregometry analysis and generated PDGF-BB levels beyond the measurable limits of the assay, and the aim was to assess relative loss of GF release over time.

All containers/conditions showed a steady platelet count fall over time except the two CA universals with a marked fall on Day 2, and then a steady decline. Both had lower PDW, MPV and P-LCR than stationary and IA universals on all days of analysis, suggesting larger platelets were missing. Possibly the agitation intensity was too high and promoted some activation, with the more reactive larger platelets being more susceptible,[28] yet this was not mirrored in the two CA NFs with apparently greater oxygen access. The otherwise steady platelet count decline in all containers was consistent with the platelet storage lesion.[29–31]

Remarkably, all PDW, MPV and P-LCR results from universal-stored PRP were lower than NF-stored counterparts, indicating container-induced effects. Furthermore, platelet parameters on Day 2 were closer to fresh PRP in universal-stored than NF-stored PRP. Minor MPV and PDW increases during storage have been reported [32,33] but not to the extent of the PDW results in the NF-stored PRP. If MPV does not normally increase significantly over time in stored PRP, the moderate increase on Day 2 in these flasks and marked PDW increase, particularly in CA flasks, suggests a degree of microaggregate formation. Thus, the P-LCR increase may have been due to microaggregates not larger cells. The moderately increased MPVs indicate they remained small enough for most to be counted as platelets. Interestingly, PDW, MPV and P-LCR reduced over time in CA NFs, probably due to increasing spontaneous aggregation over time such that larger aggregates were not aspirated into the blood count analyser. Further evidence of microaggregate formation was found from apparent low RBC counts in many NF-stored PRP results (data not shown). There were no RBC in PRP on Day 1 and these counts were likely due to small populations of aggregates too large to be counted as platelets. The only universal-stored PRP to register a low RBC count was the stationary universal on Day 8, which had the highest PDW, MPV and P-LCR values of any universal-stored PRP. MPV was more stable over time in IA and stationary NFs, suggesting CA exacerbated spontaneous aggregate formation in a storage situation where low-level activation was already more likely. There was little difference between the platelet parameters of CA NFs with and without PGE_1, or the universals, so it appears that PGE_1 did not suppress spontaneous aggregation. It may be that higher concentration was required, or PGE_1 is insufficiently stable for stored PRP.

Converse results were seen in universal-stored PRP in that PDW, MPV and P-LCR were lower in CA universals than other universals. The curious fall in platelet count, PDW, MPV and P-LCR parameters on Day 2 in CA universals may have been due to an immediate, more intense formation of aggregates that were not counted in the analyser, followed by relatively small PDW and MPV reductions over time. In contrast to the steep P-LCR falls in CA NFs, the counterpart universals exhibited mild increase over time, suggesting low-level aggregate formation.[29,34] Only IA and stationary universals mirrored baseline PDW, MPV and P-LCR values on Day 2, with the anticipated gradual increases of PDW and MPV. The P-LCR increases over time were more marked, suggesting a degree of aggregate formation here too. These observations indicate the IA universal maintained greater platelet integrity over time. Interruption of agitation for one day produces no measurable platelet damage,[14] so IA was sufficient to maintain comparable integrity.

There was an overall continuous fall in FPA over time [32,34–36] under all storage conditions. An unanticipated finding was the remarkable fall in FPA of NF-stored PRP on Day 2 and thereafter, in stark contrast to more gradual declines in universal-stored PRP. The minimal platelet count reductions, other than from CA universals on Day 2, indicate that relatively few platelets had formed aggregates and that aggregation responses were a result of activation of the stored platelets. In which case, storage conditions in NFs were detrimental to platelet function. The minimal platelet count reductions suggest that platelet binding to container walls via fibrinogen and von Willebrand factor [37,38] was minimal or absent. Thus, the container itself would not be expected to inhibit platelet function and conditions in the PRP itself the more likely culprit. The most probable explanation for such marked storage-induced differences in initially identical samples of PRP is plasma pH drift.[39,40] Storing PRP in a closed system to maintain pH better preserves platelet function than if stored in a controlled CO_2/air environment, likely related to the air/liquid interface in the latter.[39] The universal's lids were only removed once a day whilst the NFs permitted air flow. The 30 mL universals stored approximately 8.0 mL of PRP and likely retained sufficient oxygen in the 'dead space' until the next opening to not significantly impair platelet integrity. The anticoagulant

used for sample collection can also affect pH of PRP. All CA PRP showed reduced FPA compared to those IA or kept stationary. This is counter-intuitive as accepted dogma is that stored platelets should be constantly, gently agitated.[13] The agitation may have been too harsh or gentle for the PRP volumes and containers employed, or pH changes were possibly exaggerated by constant movement, even in universals since they retained an appreciable amount of air.

The amount of released PDGF-BB in fresh PRP is affected by numerous variables [17–20] and the PDGF-BB level in the fresh PRP in this study broadly mapped to levels reported elsewhere.[17,18] A marked fall in PDGF-BB levels was observed on Day 2 in all storage conditions. Most were followed by a steady, continuous decrease, some ostensibly reaching a plateau by Days 5 and 8. This is unsurprising as platelet secretory capacity falls during storage,[35] yet by Day 8, appreciable levels of PDGF-BB remained despite the expectation that most platelets would be dead or poorly functional. The PDGF-BB in the supernatant may have merely leaked from platelets as they aged and lost membrane integrity.[30] In view of minimal platelet count reductions under most storage conditions, and even the later steady fall in CA universals, this would seem unlikely to be the only causative factor. If the platelets were fragmenting over time there would likely have been gradual reductions in MPV, which was only observed in CA NFs, whilst others remained stable or increased over time. However, microaggregate formation could mask more subtle volume loss from membrane fragmentation. If the MPV reductions in the CA NFs were due to fragmentation, a concomitant increase in PDW over time would be expected, but the converse was the case. Interestingly, the relatively poor aggregation responses from Day 2 onwards in CA NF-stored PRP were not accompanied by similarly marked PDGF-BB reductions. This was true, albeit to a lesser extent, with the other paired storage conditions. It appears that some PDGF-BB in the supernatants was not derived from platelet release, which has been previously described unless platelets are washed prior to analysis.[17,41] From a clinical perspective, the PDGF-BB values in this study relate to a lower platelet count than would be used in clinical practice,[1,2] so differentiation between release from fresh and stored PRP would be greater.

Higher FPA over time was achieved by the PRP in IA and stationary universals compared to their CA partners, other than an apparent surge on Days 5 and 8 in the universal without PGE_1. However, the falls in aggregation (representing overall platelet reactivity), particularly in NF-stored PRP, were not accompanied by similarly dramatic reductions in supernatant PDGF-BB levels. It seems likely that although secretory capacity reduced over time,[35] PDGF-BB levels did not fall to near zero due to a mixture of leakage from senescing platelets, mechanical trauma and innate plasma levels. Although PRP stored in the IA universal maintained better overall stability, it did in fact generate the lowest PDGF-BB value on Day 8 despite achieving 39% FPA. An important consideration is that the study assessed PDGF-BB concentration not function, so the data do not reveal what levels of PDGF-BB are therapeutically efficacious, and therefore, at what point during storage the PRP should be discarded. Furthermore, the PDGF-BB itself may lose functionality over time and be therapeutically ineffective despite an apparently high level of availability.

Consideration of function aside, PDGF-BB levels from activation of PRP under all storage conditions were within previously reported ranges for fresh PRP with similar platelet count [17] up to Day 5, and up to Day 8 for all except the CA universal without PGE_1 and IA universal. Without knowledge of functionality, it would seem that freezing fresh PRP or post-activation supernatant might generate a more efficacious product. Frozen-thawed PRP without activation has shown comparable *in vitro* effects on cultured cells to those of fresh PRP despite reductions in GF levels.[24] The freeze/thaw cycle acts as a surrogate 'activator' by disrupting platelet membranes and inducing content release. Since GF levels are lower in freeze/thawed PRP than fresh PRP,[42] it may be a less efficient approach to increasing GF availability than direct activation since they are 'packaged' within α-granules rather than free in cytoplasm. This suggests that freezing the post-collagen activation supernatant is more likely to generate and preserve higher GF levels, with the possible additional value of excluding platelet debris from the final product.

Continuous reduction in PDGF-BB, platelet integrity and FPA were demonstrated in PRP stored under variable conditions. These observations were unremarkable, although the extent of some differences between storage conditions was unanticipated. Despite a mean percentage reduction of 32% in PDGF-BB levels after 24 h, appreciably higher levels than apparent Day 8 baselines were maintained for up to three days storage in some conditions, yet even the baseline levels matched previously reported levels for fresh PRP. The IA universal appeared to retain better platelet integrity and function, and PDGF-BB release, with potential for clinical use for 5–8 days. This work represents an advance in biomedical science because maintenance of PDGF-BB levels in non-frozen PRP suggests it can be stored at room temperature for use in tissue regeneration.

Disclosure statement

No potential conflict of interest was reported by the authors.

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