Normal platelet function in platelet concentrates requires non-platelet cells: a comparative in vitro evaluation of leucocyte-rich (type 1a) and leucocyte-poor (type 3b) platelet concentrates

William R Parrish,1 Breana Roides,1 Julia Hwang,1 Michael Mafilios,2 Brooks Story,1 Samir Bhattacharyya1

ABSTRACT

Background: Therapeutic success of platelet-rich plasma (PRP) may vary based on the composition and preparation method. The objective of this study was to evaluate the cellular components of platelet concentrates produced by a leucocyte-rich (LR-PRP) and a leucocyte-poor PRP systems (LP-PRP).

Methods: Parameters evaluated included platelet recovery, platelet concentration, red blood cell (RBC) and white blood cell (WBC) composition, platelet recovery, platelet concentration, red blood cell (RBC) and white blood cell (WBC) composition, platelet growth factor release and stimulation of human tendon cell proliferation in vitro.

Results: Platelet recoveries were 52% for LP-PRP and 89% for LR-PRP. LR-PRP demonstrated greater reproducibility with a 4.2% coefficient of variation (CV) compared with 19.4% for LP-PRP (p<0.001). LR-PRP demonstrated a greater increase in platelet concentration (7.9-fold) than LP-PRP (2.2-fold; p<0.001). LP-PRP showed 5.0-fold reductions in WBCs, while LR-PRP showed a 4.0-fold increase (p<0.001). LP-PRP reduced RBCs to a haematocrit of 0.25, while LR-PRP reduced haematocrit to 11.8. LP-PRP did not coagulate robustly on reactivation with CaCl2, and released significantly lower levels of epidermal growth factor (EGF) and transforming growth factor β1 (TGF-β1) than whole blood (p<0.03). LP-PRP also did not stimulate tendon cell proliferation greater than whole blood. In contrast, LR-PRP showed increases in each growth factor on activation with CaCl2 (p<0.01) and stimulated greater proliferation (p<0.05) compared with whole blood. Forced activation of LP-PRP with exogenous thrombin rescued the coagulation deficiency and induced greater growth factor release than comparable whole blood (p<0.03).

Conclusions: These data suggest that non-platelet cellular components in platelet concentrates are important for proper platelet function, including thrombin generation, growth factor release and clot retraction.

INTRODUCTION

Platelet-rich plasma (PRP) is an autologous, biologically active derivative of whole blood that contains therapeutic platelet concentrations elevated at least fivefold above baseline.1 By concentrating platelets into PRP, a large reservoir of autologous growth factors is generated, which if administered appropriately can establish an optimal environment for accelerated healing, including the reduction of pain and resolution of inflammation.2–7 There are currently more than 2 dozen commercially available systems in the worldwide market that concentrate whole blood into a platelet-rich substance. These various devices and techniques have resulted in a wide range of PRP final products that can vary greatly, especially in the concentration of platelets and the inclusion or exclusion of leucocytes and red blood cells.
Platelet-rich plasma classifications system

<table>
<thead>
<tr>
<th>Type</th>
<th>White blood cells</th>
<th>Activated?</th>
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<tbody>
<tr>
<td>1</td>
<td>Increased over baseline</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>Increased over baseline</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Minimal or no white blood cells</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Minimal or no white blood cells</td>
<td>Yes</td>
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</table>

A: Platelets >5x baseline.  
B: Platelets <5x baseline.
until assayed for growth factor content and the capacity to stimulate tendon cell proliferation in vitro. For forced PRP activation, 100 units of bovine thrombin were added in addition with 25 mM CaCl₂. Samples were immediately inverted several times to ensure mixing, and were incubated at room temperature for 60 min to allow complete coagulation. Serum samples were prepared as described above. No serum aliquots were freeze-thawed more than once prior to use in any assay.

**Tendon cell proliferation studies**

Normal healthy primary human patellar tendon fibroblasts (tenocytes) were purchased from Articular Engineering, LLC (Northbrook, Illinois) at passage #1. Cultures were expanded to passage #6 to ensure homogenous cell populations and appropriate acclimation to in vitro culture conditions. Cells were maintained at 37°C under 5% CO₂ in Dulbecco’s-Modified Eagle Medium-F12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS), 1×antibiotic/antimycotic and 2 mM L-glutamine. For studies, cells were seeded into tissue culture-treated black-walled clear bottom 96-well plates at 1×10³ cells/well in 100 µL volume. Cultures were washed once with 100 µL of DMEM-F12 24 h after seeding and synchronised for 24 h by serum restriction to 1% FBS. Eight wells per condition were treated in parallel (quadruplicate wells on duplicate plates) with serum derived from whole blood (positive control), PPP (negative control) or platelet concentrates from LP-PRP or LR-PRP. All serum dilutions were prepared in DMEM-F12 supplemented with 1×antibiotic/antimycotic, 2 mM L-glutamine and 0.25% FBS to maintain viability of the 0% serum-treated cultures. Cultures were incubated at 37°C under 5% CO₂ for 4 days. Wells were washed 1×with PBS to remove residual media, and relative proliferation was determined by CellTiter-Glo assay using a Molecular Diagnostics Spectramax M5 plate reader to measure luminescence.

**Growth factor measurements**

ELISA assay kits (Quantikine, R&D systems) were used to analyse the serum levels of five representative platelet growth factors (epidermal growth factor (EGF), transforming growth factor β1 (TGF-β1), platelet-derived growth factor AB (PDGF-AB), vascular endothelial growth factor (VEGF) and stromal-derived factor 1α (SDF-1α)). PPP that was prepared in parallel for each donor served as a negative control. Samples were assayed in duplicate and assay results were considered valid when r² values for the standard curves were greater than 0.99 and sample detection was within the linear range of the standard curve. Samples not within the linear detection range were diluted accordingly and reassayed.

**Statistical analyses**

Comparisons between groups were conducted using paired one-tailed Student t test, or by one-way analysis of variance (ANOVA) where indicated. Differences were considered significant when p<0.05. The coefficient of variation, a ratio of the SD to the mean expressed as a per cent (% CV=(SD/mean)×100), was also assessed for platelet recovery where a lower coefficient of variation (CV) indicates less variability.

**RESULTS**

**Platelet composition**

The mean whole blood platelet count was 224×10³/µL (table 2). Each system significantly increased the platelet count compared with baseline levels (p<0.001). LR-PRP demonstrated significantly greater increases in platelet concentration over baseline (7.9-fold) than LP-PRP (2.4-fold increase; p<0.001) (figure 1 and table 2). In addition, LP-PRP contained just over 50% of the total

<table>
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<th>Table 2 Platelet parameters</th>
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<tr>
<td><strong>Mean platelet count (x 10⁹/µL)</strong></td>
</tr>
<tr>
<td>Whole blood</td>
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<tr>
<td>LP-PRP</td>
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<tr>
<td>LR-PRP</td>
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<tr>
<td>PPP</td>
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LP-PRP, leucocyte-poor platelet-rich plasma system; LR-PRP, leucocyte-rich platelet-rich plasma system; NA, not applicable; PPP, platelet-poor plasma.
available platelets, while LR-PRP contained almost 90% (figure 2 and table 2). LR-PRP demonstrated a greater reproducibility in platelet recovery with a 4.2% CV compared with a 19.4% CV for LP-PRP (p<0.001) (table 2).

RBC composition
The mean whole blood baseline RBC count was 4.04×10^6/µL, resulting in a mean haematocrit of 32.9 (table 3). Each of the systems reduced RBCs compared with whole blood. Haematocrit levels for LP-PRP and LR-PRP were 0.25 and 11.8, respectively (p<0.01) (table 3).

WBC composition
The mean whole blood WBC count was 4.9×10^3/µL (table 4). WBCs were reduced by fivefold in LP-PRP (p<0.001), whereas LR-PRP showed a 4.0-fold increase in WBCs (p<0.001) (table 4). LP-PRP contained just below 5% of the total available WBCs, while LR-PRP contained approximately 45% (table 4).

Tendon cell proliferation
Human tendon fibroblast cells were grown in vitro and exposed to varying concentrations of serum derived from either whole blood, PPP or platelet concentrates generated by LP-PRP or LR-PRP. Untreated cultures gave a very consistent baseline measurement with an across group mean of 209±5 luminescence units, demonstrating excellent interassay and intra-assay repeatability and a common assay start point with respect to cell number per well (p>0.98 by one-way ANOVA) (table 5). PPP stimulated a linear concentration-dependent proliferative response in human tenocytes (r²=1) (table 5). Thus, to specifically compare the effects of platelet-derived growth factors on tendon cell proliferation, cell responses to serum were normalised to the PPP response and expressed as per cent (%) stimulation over PPP (figure 3).

Serum from whole blood, LP-PRP and LR-PRP each stimulated a concentration-dependent proliferative response from tendon cells (figure 3). In fact, although the LP-PRP system preparations contained a 2.2-fold increase in platelets over whole blood (figure 1), the serum derived from those platelets did not evoke an enhanced proliferation response compared with whole blood (p>0.7 by one-way ANOVA; figure 3). These results strongly suggest that the LP-PRP system preparations are deficient in platelet activation and growth factor release. In contrast, LR-PRP stimulated a proliferation dose–response that was significantly greater than that of either LP-PRP or whole blood (p<0.05 for each by one-way ANOVA; figure 3 and table 5). More specifically, the serum from LR-PRP at a concentration of 10% (volume:volume) in the culture medium stimulated robust proliferation that was sufficient to cause the cultures to grow to confluence, saturating the assay system. This finding is important because normal somatic cells such as the tendon fibroblasts used in these studies exhibit contact-dependent inhibition of proliferation when grown in culture, whereby cells that are surrounded by other cells can no longer respond to serum growth signals. This effect is evidenced by the apparent flattening of the slope of the serum response curve between 5% and 10% serum for LR-PRP (figure 3).

Growth factor analysis
Whole blood and LR-PRP achieved a robust activation of the coagulation cascade following reactivation with CaCl₂, including strong clot retraction. In contrast, LP-PRP and PPP displayed donor-dependent irregularity in the robustness and degree of clotting, and none of the LP-PRP or PPP clots demonstrated retraction to the

<table>
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<th>Table 3 Red blood cell parameters</th>
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<tbody>
<tr>
<td><strong>Mean RBC count (x10^6/µL)</strong></td>
</tr>
<tr>
<td>Whole blood</td>
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<tr>
<td>LP-PRP</td>
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<tr>
<td>LR-PRP</td>
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<tr>
<td>PPP</td>
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</table>

LP-PRP, leucocyte-poor platelet-rich plasma system; LR-PRP, leucocyte-rich platelet-rich plasma system; NA, not applicable; PPP, platelet-poor plasma; RBC, red blood cell.

Figure 2  Platelet recovery. Bars represent the mean ± SD of the percentage of total platelets recovered in platelet concentrates (n=12 donors). Per cent platelet recovery=(total platelet count of platelet concentrate (concentration x total volume)/total platelet count of whole blood (concentration x total volume))×100.

extent of whole blood (data not shown). This indicates a deficiency in activation of the coagulation cascade and also in platelet function, since clot retraction is a platelet-driven process.31 Further consistent with a platelet functional deficiency in LP-PRP, a lower level of growth factors compared with whole blood serum was found despite that LP-PRP contained 2.2-fold more platelets (figures 1 and 4 and table 6). In fact, the levels of EGF and TGF-β1 were significantly lower in the LP-PRP serum compared with whole blood (p<0.03 for both; figure 4 and table 6). In contrast, LR-PRP demonstrated significantly higher growth factor levels compared with serum from whole blood (p<0.01 for each; figure 4 and table 6).

The failure of LP-PRP to demonstrate robust coagulation and platelet growth factor release in response to reactivation with CaCl2 suggested that LP-PRP may be deficient in thrombin generation under physiological conditions. To test this, LP-PRP was directly activated with 100 units of bovine thrombin in addition to 25 mM CaCl2, and growth factor measurements were conducted on the serum samples. Robust coagulation was induced in each sample within seconds of the addition of thrombin, indicating that exogenous thrombin can indeed rescue the clotting deficiency observed for LP-PRP. Forced activation with exogenous thrombin also improved platelet growth factor release in LP-PRP compared with activation with CaCl2 alone, respective to whole blood controls (figure 5). Taken together, these data indicate that LP-PRP preparations are deficient in thrombin generation on reactivation with CaCl2.

**DISCUSSION**

Evidence in the literature suggests that clinical success of PRPs may vary based on the preparation method and PRP composition.9 13 32 Thus, there is a need to evaluate PRP formulations to discern the role of non-platelet cellular components in determining the potential efficacy of PRP generated by different systems. The primary objectives of the current study were to characterise the cellular components of LR-PRP (a type 1a PRP containing RBCs) and LP-PRP (a type 3b PRP, ie, RBC depleted) and to examine the contributions that RBCs and WBCs collectively have on platelet growth factor release and the efficacy of the releasate to stimulate tendon cell proliferation in vitro. LR-PRP contained almost 90% of the available platelets, resulting in approximately 7.9-fold increase over whole blood with a 4.2% coefficient of variation, which indicates a high degree of reproducibility in platelet concentration. WBCs were concentrated approximately four-fold over whole blood in LR-PRP, which was sufficient to maintain platelet to WBC ratios within a physiological range for each donor tested. The platelet to WBC ratio is a metric that has recently gained attention as a parameter that may be important for the anabolic activity of PRP,14 33 although the influence of this ratio on PRP bioactivity remains controversial. LR-PRP RBC levels were reduced to a haematocrit of 11.8, almost threefold lower than whole blood. The LR-PRP was type 1A, and demonstrated robust physiological responsiveness to reactivation with CaCl2. Complete coagulation with robust clot retraction was seen with LR-PRP, and this was evidenced by high serum growth factor content (table 6 and figure 4) and strong stimulation of tendon cell proliferation in vitro (table 5 and figure 3). Indeed, tendon cells stimulated with 10% serum from LR-PRP saturated the growth surface of the cell culture dishes, thus reaching the plateau for proliferation in vitro. Overall, these results indicate excellent platelet viability in the LR-PRP and suggest that robust platelet activity should be expected in a physiological setting.

**Table 4** White blood cell parameters

<table>
<thead>
<tr>
<th></th>
<th>Mean WBC count (x 10⁶/µL)</th>
<th>Per cent recovery</th>
<th>Mean fold increase</th>
<th>Coefficient of variation (%)</th>
<th>Mean platelet: WBC ratio</th>
<th>p Value (2-tailed paired t test) vs LR-PRP system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>4.9</td>
<td>100.0</td>
<td>1.0</td>
<td>NA</td>
<td>46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LP-PRP</td>
<td>1.0</td>
<td>4.1</td>
<td>0.2</td>
<td>100</td>
<td>486</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LR-PRP</td>
<td>19.3</td>
<td>44.9</td>
<td>4.0</td>
<td>26</td>
<td>92</td>
<td>NA</td>
</tr>
<tr>
<td>PPP</td>
<td>0.0</td>
<td>0.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
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<td></td>
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</table>
| LP-PRP, leucocyte-poor platelet-rich plasma system; LR-PRP, leucocyte-rich platelet-rich plasma system; PPP, platelet-poor plasma; WBC, white blood cells.

**Table 5** Tenocyte proliferation measurement (light units ± SEM)

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>0%</th>
<th>1%</th>
<th>2.5%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>208 (29)</td>
<td>271 (19)</td>
<td>515 (46)</td>
<td>1087 (139)</td>
<td>2622 (95)</td>
</tr>
<tr>
<td>LP-PRP</td>
<td>214 (19)</td>
<td>281 (19)</td>
<td>554 (62)</td>
<td>1240 (134)</td>
<td>2656 (92)</td>
</tr>
<tr>
<td>LR-PRP</td>
<td>203 (17)</td>
<td>384 (46)</td>
<td>1001 (91)</td>
<td>2517 (180)</td>
<td>4372 (481)</td>
</tr>
<tr>
<td>PPP</td>
<td>210 (16)</td>
<td>269 (10)</td>
<td>370 (11)</td>
<td>530 (23)</td>
<td>795 (58)</td>
</tr>
</tbody>
</table>

LP-PRP, leucocyte-poor platelet-rich plasma system; LR-PRP, leucocyte-rich platelet-rich plasma system; PPP, platelet-poor plasma.
The LP-PRP had a mean platelet increase of 2.2-fold over whole blood and mean platelet recoveries of 52% with a coefficient of variation of 19.4%, indicating a high degree of variability. LP-PRP had WBCs reduced fivefold compared with whole blood. RBCs were reduced 100-fold, to a haematocrit of 0.25. Platelet function in the LP-PRP was deficient, as evidenced by a failure to consistently coagulate and undergo clot retraction when reactivated with CaCl2 alone. Consistent with this, serum from LP-PRP did not demonstrate elevated growth factor levels (figure 4), and the stimulation of tendon cell proliferation was not significantly different than serum from whole blood (figure 3). This deficiency in platelet activation was at least in part attributable to a failure of the LP-PRP to generate sufficient thrombin because addition of exogenous thrombin rescued the coagulation deficit and improved platelet growth factor release (figure 5).

Recently published studies have demonstrated that the intimate interactions between platelets and other blood cells are crucial for the physiological mechanisms to prevent infection and promote healing in the event of an injury, and that the failure of these mechanisms can promote pathological tissue states that contribute to disease.8 11 17–25 34–37 The current study further contributes to this body of evidence, in that it uncovers a deficiency in platelet function with respect to growth factor release and the ability of the releasate to stimulate tendon cell proliferation when RBCs and leucocytes are lacking. A limitation of this study is that a detailed analysis of tendon cell viability was not conducted. Therefore, although the CellTiter-Glo assay that was used to determine cell proliferation measures the production of ATP specifically from viable cells, the relative contribution from prevention of apoptotic cell death and/or cellular senescence remains to be determined. In addition, it is well established that exposure of tendon fibroblasts to high levels of TGF-β1 in vitro can activate their trans-differentiation to myofibroblasts (review by Hinz38). Myofibroblasts play an integral role in wound healing reactions due to their induced expression of smooth muscle actin that results in an acquired ability to contract and to contribute to pathological tissue repair.38 Myofibroblast contraction also leads to the liberation of TGF-β1 from these cells,38 leading to the potential of a feed-forward mechanism to promote further fibroblast trans-differentiation. Further research will be required to determine the extent to which LR-PRP promotes fibroblast trans-differentiation, as this may represent an important mechanism through which PRP therapy may provide clinical benefit. Another important limitation of this study is that it is an in vitro study comparing two polar opposite platelet concentrate formulations (type 1a and type 3b).3 1 Therefore, the conclusions remain to be validated in animal models or the clinical setting. Further studies will be required to elucidate the precise individual contributions of platelets,
RBCs and WBCs to the beneficial effects of PRP that are emerging in the scientific literature for various clinical applications.

**CONCLUSION**

LP-PRP did not demonstrate robust coagulation and platelet growth factor release compared with whole blood and LR-PRP. In addition, serum derived from LP-PRP did not provide a significant advantage to tendon cell proliferation compared with whole blood serum, although the LP-PRP contained a 2.2-fold increase in platelet concentration. These findings suggest that the non-platelet cellular components that are excluded from the LP-PRP are important for optimal platelet function, including thrombin generation leading to robust coagulation, growth factor release and the resulting capacity of the serum to stimulate cell proliferation. Future studies should be conducted to validate these findings in vivo, as these findings may have profound implications for how platelet concentrates are prepared and utilised in clinical applications.

**Contributors** WRP, BR, JH and BS conceived and designed the experiments. WRP analysed the data. WRP and BR wrote the first draft of the manuscript. WRP, BR, JH and BS conceived and designed the experiments. BR, JH, BS, MM and SB made critical revisions and approved final version. WRP, BR, JH and BS jointly developed the structure and arguments for the paper. WRP, BR, JH, BS, MM and SB agree with manuscript results and conclusions. WRP, BR, SB, MM and BS jointly developed the structure and arguments for the paper. WRP, BR, JH, BS, MM and SB made critical revisions and approved final version.

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**Competing interests** WRP, BR, JH, BS and SB are employees of DePuy Synthes Mitek Sports Medicine, a Johnson & Johnson Company. MM is a paid consultant for DePuy Synthes Mitek Sports Medicine and an employee of Health Economics Associates, LLC.

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