Article focus

Motivated by the emerging use of platelet-rich plasma (PRP) for the treatment of tendon-related pathologies, the primary focus of this article is to determine whether human tenocyte behaviour is enhanced when cultured with increasing concentrations of platelet-derived proteins, and whether the cell behaviour changes with blood donor age.

The present hypothesis is that increasing the platelet-derived protein levels within platelet lysate (PL) promotes tenocyte proliferation and migration in vitro, and that differences may exist between the donor age groups investigated.

Key messages

- There is a clear dose-response relationship between PL concentration and cell behaviour in PL obtained from aged donors.
- In contrast, tenocytes displayed similar behaviour irrespective of PL concentration in PL obtained from young donors.
**Strengths and limitations**

- This study shows that increasing the platelet-derived protein content from aged donors can have a positive effect on tenocyte behaviour.
- However, it is limited in size and scope, necessitating additional in vivo investigation.

**Introduction**

Platelet-rich plasma (PRP) is gaining popularity in a variety of clinical settings as an autologous source of bioactive molecules for promoting tissue repair. The supraphysiological concentration of platelet-derived growth factors and cytokines within PRP has been thought to support the regeneration of tendons, the connective tissue structures responsible for anchoring muscle to bone and facilitating mechanical motion.\(^1\)\(^-\)\(^3\) Ageing tendons have limited healing potential and undergo both cellular and extracellular changes, including reductions in tenocyte proliferation, volume, and metabolic activity, as well as a loss of vascularization, decreased levels of non-collagenous matrix components, and low collagen turnover.\(^1\)\(^,\)\(^4\) Together, these degenerative tissue changes culminate in diminished strength, increasing the prospect of tendinous injury.\(^4\) Pathological tendons may well benefit from the growth factors found in PRP preparations, which have been shown to promote cellular proliferation and support angiogenesis.\(^5\) However, the clinical efficacy of PRP for the treatment of tendinopathies has been questioned, as several systematic reviews of the current literature have drawn opposing conclusions.\(^6\)\(^-\)\(^10\)

Reported discrepancies among clinical trials investigating the use of PRP for treating tendinopathies may be attributed in part to inconsistencies in PRP preparation and treatment protocols,\(^8\)\(^,\)\(^10\) as different methodologies for creating PRP have been reported to affect the kinetics of growth factor release.\(^11\) Furthermore, consensus as to how the most elementary of PRP components, platelet concentration, affects tendon healing is lacking. For instance, multiple human studies have reported higher platelet concentrations to have inhibitory effects on cell proliferation in vitro,\(^12\)\(^-\)\(^14\) whereas others have demonstrated PRP to stimulate tenocyte proliferation in a dose-dependent manner, with platelet concentrations of more than 50 times greater than physiological levels having no inhibitory effects on cell growth.\(^15\) Consequently, it is currently unclear if increasing the platelet concentration in PRP is beneficial for treating tendinopathies. Moreover, whether the optimal platelet concentration to promote tendon healing changes with patient age remains to be seen.

In the current study, we cultured human tenocytes within increasing concentrations of platelet lysate (PL), a derivative of PRP, to determine whether higher platelet-derived protein levels enhance cell proliferation and migration in vitro, and whether the mitogenic properties of PL change with donor age. Concentrated PLs, generated from the pooled PRP of both young and aged donors, were combined with cell culture medium using a fixed volumetric ratio to model a range of PRP preparations used clinically. We hypothesized that increasing the platelet-derived protein levels within PL would enhance tenocyte behaviour, and that differences would exist between donor age groups.

**Materials and Methods**

**Preparation of concentrated platelet lysates (PLs).** After informed consent was obtained, whole blood from healthy adult donors (\(n = 14\)) was collected into a standard blood bag (Teruflex; Terumo BCT, Lakewood, Colorado) or blood tubes (BD Vacutainer; Becton Dickinson, Franklin Lakes, New Jersey) containing anticoagulant citrate dextrose solution A (Baxter Healthcare Corporation, Deerfield, Illinois). Platelet-rich plasma (PRP) was manually separated from other blood components following centrifugation at \(200 \times g\) for ten minutes. Care was taken during PRP isolation to avoid disturbing the leucocyte-rich buffy coat, thereby minimizing nucleated cell contamination. The leucocyte-poor nature of the PRP isolated when using this methodology was confirmed using a haematological analyzer (Micros 60; Horiba, Irvine, California), with all leucocyte counts falling below the recommended reportable limit (Supplementary Table i). Isolated PRP was subjected to a second round of centrifugation at \(2300 \times g\) for ten minutes to form a platelet pellet, and the platelet-poor plasma (PPP) supernatant was completely aspirated. The remaining platelet pellet was resuspended in \(1/35\) of the initial plasma volume (\(~70\) times over the baseline), resulting in a concentrated PRP solution having about \(20\) times more platelets than the original PRP (Supplementary Table ii).\(^16\) To account for the potential of platelet clumping, platelet counts were obtained manually from the concentrated PRP using a haemocytometer prior to storage at \(-80^\circ C\).\(^17\) Upon thawing at \(37^\circ C\), the PRP preparations were combined into two different pooled platelet products based on an age cut-off of 50 years (Tables i and ii), and were subjected to two additional freeze/thaw cycles at \(-80^\circ C\) and \(37^\circ C\) to generate concentrated PLs.\(^18\) Fragmented platelet bodies were pelleted and removed by centrifugation at \(4500 \times g\) for ten minutes, and sodium heparin (Sagent Pharmaceuticals, Inc., Schaumburg, Illinois) was added to the recovered supernatant at \(100 \text{ IU/ml}\) to prevent hydrogel formation. Finally, the concentrated PLs were serially diluted two-fold in pooled PPP in order to prepare less concentrated forms with \(1/2, 1/4, 1/8,\) and \(1/16\) of the starting PL volume.

**Protein analysis.** Quantitative protein analysis was performed using a multiplex, antibody-based assay (Q-Plex Human Angiogenesis; Quansys Biosciences, Logan, Utah) in accordance with the manufacturer’s recommendations.
Platelet counts in concentrated platelet-rich plasma (PRP) from aged donors (>50 years) prior to lysis

<table>
<thead>
<tr>
<th>Age, yrs</th>
<th>Gender</th>
<th>Platelet count, plt/µl</th>
<th>Volume, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>Female</td>
<td>9.8 x 10^6</td>
<td>15.4</td>
</tr>
<tr>
<td>53</td>
<td>Male</td>
<td>8.1 x 10^6</td>
<td>5.0</td>
</tr>
<tr>
<td>54</td>
<td>Female</td>
<td>10.6 x 10^6</td>
<td>10.4</td>
</tr>
<tr>
<td>55</td>
<td>Male</td>
<td>8.0 x 10^6</td>
<td>23.2</td>
</tr>
<tr>
<td>64</td>
<td>Female</td>
<td>9.8 x 10^6</td>
<td>7.7</td>
</tr>
<tr>
<td>74</td>
<td>Female</td>
<td>7.7 x 10^6</td>
<td>6.6</td>
</tr>
<tr>
<td>74</td>
<td>Male</td>
<td>11.4 x 10^6</td>
<td>6.6</td>
</tr>
<tr>
<td>78</td>
<td>Female</td>
<td>6.8 x 10^6</td>
<td>25.1</td>
</tr>
<tr>
<td>Mean: 63 (sd 11)</td>
<td>Weighted mean: 8.6 x 10^6</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Platelet counts in concentrated platelet-rich plasma (PRP) from young donors (<50 years) prior to lysis

<table>
<thead>
<tr>
<th>Age, yrs</th>
<th>Gender</th>
<th>Platelet count, plt/µl</th>
<th>Volume, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Male</td>
<td>11.8 x 10^6</td>
<td>5.8</td>
</tr>
<tr>
<td>22</td>
<td>Female</td>
<td>9.9 x 10^6</td>
<td>19.4</td>
</tr>
<tr>
<td>25</td>
<td>Male</td>
<td>7.2 x 10^6</td>
<td>17.5</td>
</tr>
<tr>
<td>30</td>
<td>Male</td>
<td>10.3 x 10^6</td>
<td>35</td>
</tr>
<tr>
<td>32</td>
<td>Male</td>
<td>13.9 x 10^6</td>
<td>9.7</td>
</tr>
<tr>
<td>33</td>
<td>Female</td>
<td>7.7 x 10^6</td>
<td>12.6</td>
</tr>
<tr>
<td>Mean: 27 (sd 5)</td>
<td>Weighted mean: 9.8 x 10^6</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table III. Simulated clinical platelet-rich plasma (PRP) concentrations

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Representative clinical PRP concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet lysate (PL)</td>
<td>14×</td>
</tr>
<tr>
<td>1/2 PL</td>
<td>7×</td>
</tr>
<tr>
<td>1/4 PL</td>
<td>3.5×</td>
</tr>
<tr>
<td>1/8 PL</td>
<td>1.75×</td>
</tr>
<tr>
<td>1/16 PL</td>
<td>0.9×</td>
</tr>
</tbody>
</table>

Preparation of experimental conditions. Concentrated PLs (PL, 1/2 PL, 1/4 PL, 1/8 PL, and 1/16 PL) were added to culture medium using a fixed volumetric ratio to simulate typical PRP preparations used in the clinical setting. For example, the PL condition in this study is representative of a clinical PRP that is approximately 14 times more concentrated than baseline (Table III). All experimental media conditions contained 20% PL, 78% α-MEM, 1% GlutaMAX, and 1% penicillin-streptomycin by volume. Culture medium containing 1% FBS, 20% PPP, and 20% FBS supplemented with 1 ng/ml recombinant human basic fibroblast growth factor (hbFGF; Gold Biotechnology, Inc., St. Louis, Missouri) were used as negative, PPP, and positive controls, respectively. All culture media preparations were filter sterilized using 0.22 µm syringe filters (EMD Millipore, Billerica, Massachusetts).

Proliferation assay. Tenocytes were plated at a density of 1000 cells/cm² within collagen-coated, multi-well plates (Corning), using expansion medium. Following an initial 24-hour attachment period, the cultures were starved with serum-free medium for an additional 24 hours, after which the experimental and control media conditions were added. Tenocyte cultures were maintained in a humidified incubator at 37°C and 5% CO₂ for 72 or 120 hours. After each timepoint, cell proliferation was qualitatively assessed by phase-contrast microscopy (EVOS FL; Thermo Fisher Scientific), and the genomic DNA was quantified using a fluorometric-based DNA assay in accordance with the manufacturer’s recommendations (Quant-iT PicoGreen; Thermo Fisher Scientific).

Migration assay. Tenocytes were plated at a density of 30 000 cells/cm² within specialized inserts (Ibidi GmbH, Martinsried, Germany) that were previously adhered to the culture surfaces of multi-well plates (Corning). Cells were grown in expansion medium until confluence, and reproducible cell-free gaps, approximately 500 µm in width, were created upon careful removal of the inserts. The tenocyte cultures were washed once with phosphate-buffered saline (PBS) prior to addition of experimental media conditions. Phase-contrast images of the gap region were acquired (EVOS FL; Thermo Fisher Scientific) immediately following insert removal, and after 24, 32, and 48 hours of culture in experimental media. The gap area was measured using imaging software (Image; NIH, Bethesda, Maryland) and the percentage of gap closure was calculated by the equation \( \frac{(A_o - A_f)}{A_o} * 100 \), where \( A_o \) is the initial gap area and \( A_f \) is the area remaining at the timepoint of interest.

Statistical analysis. Unless stated otherwise, experiments were performed in triplicate for each of the three tenocyte donors. Statistical differences between means within each age group were determined by one-way analysis of variance (ANOVA) followed by Tukey correction for multiple comparisons using GraphPad Prism software (GraphPad Software Inc., La Jolla, California), and \( p < 0.05 \) was considered statistically significant.
**Results**

Protein levels increase with PL concentration. Quantification of multiple anabolic growth factors, anti-catabolic tissue inhibitors, and pro-inflammatory cytokines are shown in Figure 1. Protein levels were confirmed to increase concomitantly with PL concentration, with higher levels typically presenting within the PLs from aged donors. Anabolic growth factors measured included bFGF, platelet-derived growth factor BB (PDGF-BB), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF); anti-catabolic tissue inhibitors, e) tissue inhibitor of metalloproteinase 1 (TIMP1) and f) tissue inhibitor of metalloproteinase 2 (TIMP2); and pro-inflammatory cytokines, g) interleukin (IL)-8 and h) tumour necrosis factor alpha (TNF-α). *Samples with protein levels below the lower limit of detection.
growth factor (VEGF). With a more than ten-fold increase between the lowest and highest PL concentrations in both donor groups, protein levels of PDGF-BB and VEGF changed the most (Figs 1c and 1d). Of the two anti-catabolic tissue inhibitors quantified, tissue inhibitor of metalloproteinase 1 (TIMP1) was more affected than tissue inhibitor of metalloproteinase 2 (TIMP2) with respect to changes in PL concentration (Figs 1e and 1f). While detected at lower overall levels, the pro-inflammatory cytokines interleukin 8 (IL-8) and tumour necrosis factor alpha (TNF-α) also went up with PL concentration (Figs 1g and 1h).

Aged PLs promote tenocyte proliferation in a concentration-dependent manner. Increasingly concentrated PLs stimulated tenocyte proliferation differently depending on donor age, as shown in Figure 2. Tenocytes were plated in expansion medium for 24 hours, starved in serum-free medium for an additional 24 hours, and then maintained in experimental conditions for either b) 72 or c) 120 hours prior to DNA quantitation. *p < 0.05 versus platelet-poor plasma (PPP); †p < 0.05 versus 1/16 PL; ‡p < 0.05 versus 1/8 PL.

Tenocyte proliferation is significantly enhanced by increased pooled platelet lysate (PL) concentrations when derived from aged donors. a) Tenocytes were plated in expansion medium for 24 hours, starved in serum-free medium for an additional 24 hours, and then maintained in experimental conditions for either b) 72 or c) 120 hours prior to DNA quantitation. *p < 0.05 versus platelet-poor plasma (PPP); †p < 0.05 versus 1/16 PL; ‡p < 0.05 versus 1/8 PL.

Promoted cell growth in a dose-dependent manner, with significant increases observed between the highest and lowest PL concentrations. In contrast, fewer significant differences were observed between tenocytes cultured in PLs from the young donor group, with only the midrange PLs resulting in significantly greater proliferation than PPP (Fig. 2c). Although the highest PL concentration from the young donors resulted in lower tenocyte proliferation relative to the midrange PLs, the culture medium within this condition tended to undergo sporadic hydrogel formation, which likely affected the results (Supplementary Figure a).

Tenocyte proliferation was assessed qualitatively using phase-contrast microscopy. Representative images from a single tenocyte donor after 120 hours of culture are shown in Figure 3. Cellular proliferation was restricted within the negative control medium, as tenocytes tended to increase in length and display limited spreading, whereas tenocytes within the positive control medium proliferated to cover the available surface area (Fig. 3a). When cultured in PPP, tenocytes exhibited limited proliferation, with cell growth only observed in PPP from the young donors. Increasing the PL concentration caused tenocytes to adopt more of a linear morphology and...
PLATELET LYSATES FROM AGED DONORS PROMOTE HUMAN TENOCYTE PROLIFERATION AND MIGRATION IN A CONCENTRATION-DEPENDENT MANNER

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Tenocytes pack tightly together in dense bundles, with differences in cell densities being most apparent between PL concentrations from the aged donors (Fig. 3b).

**Aged PLs promote tenocyte migration in a concentration-dependent manner.** Tenocyte migration in PLs from aged donors was markedly different depending on the PL concentration, as shown in Figure 4. Representative phase-contrast images demonstrate an absence of cellular migration when tenocytes are cultured in PPP. However, as the PL concentration is increased, the extent of tenocyte migration is noticeably improved (Fig. 4a).

Quantification of the cell-free area revealed significant differences between different PL concentrations after 36 and 48 hours of culture (Fig. 4b). By comparison, Figure 5 demonstrates PL from young donors to promote tenocyte migration largely independent of concentration. Representative phase-contrast images reveal almost complete gap closure following 48 hours of culture (Fig. 5a). Significant differences in tenocyte migration were measured between the PLs and PPP, but not between any of the PL concentrations investigated (Fig. 5b). Similar to the proliferation results, tenocyte migration was weakly promoted in PPP from young, but not aged, donors.

**Discussion**

Determining the optimal platelet concentration to facilitate PRP-induced tendon repair in an ageing patient population remains a clinical challenge. In the present study, PLs, prepared from concentrated PRP and pooled based on donor age, were added to tenocyte cultures to determine the effects on cell behaviour. Quantification of the PL preparations revealed greater platelet-derived protein levels with increasing PL concentration and, generally, greater protein levels were found in PLs from the aged donor group. Platelet-derived growth factor BB and VEGF, two growth factors involved in the early inflammatory and reparative stages of tendon healing, were most impacted by changes in PL concentration. Of the two anti-catabolic tissue inhibitors quantified, TIMP2 levels were less dependent on PL concentration, in accordance with previously published results.

Tenocyte proliferation and migration were found to increase significantly with platelet-derived protein levels when cultured in pooled PLs derived from aged, but not young, donors. The results seen with the aged PL largely support the findings of Jo et al, in which tenocyte proliferation is stimulated by PRP in a dose-dependent manner without detrimental effects at higher platelet concentrations. The general trend of improved tenocyte proliferation and migration with increased PL concentrations suggests that higher PRP concentrations (up to $14 \times$) may be more beneficial than lower PRP concentrations for promoting tendon repair in an ageing patient population. Indeed, a recent clinical study has proposed increasing the platelet concentration of PRP for treating tendinopathies among the elderly patient population.

This patient population receives PRP therapy for musculoskeletal injuries with increased frequency and has an increased prevalence of tendon-related injuries.

Interestingly, PPP from young donors was found to promote tenocyte proliferation and migration weakly, whereas PPP from their older counterparts did not.
Emerging data demonstrating that increased volumes of PPP inhibit angiogenic responses in vitro, including cell proliferation and migration, suggests that optimal activity is obtained by reducing the presence of inhibitory plasma proteins.24 Cell culture studies investigating thrombospondin-1 (TSP1) and TIMP2 have shown these plasma proteins to reduce cell growth.25,26 In the present study, quantified TIMP2 levels were found to be almost two times greater than those in aged PPP, consistent with the observed lack of tenocyte growth and migration. Moreover, the dose-response in tenocyte behaviour with increasing PL concentration may result from shifting the balance between inhibitory factors in the plasma in favour of mitogenic factors within platelets. Although several studies have concluded that high platelet concentrations are inhibitory to cell growth, they often rely upon
dosing protocols that increase platelet concentration by adjusting the volumetric ratio of PRP to cell culture medium, thereby increasing the presence of inhibitory plasma proteins and potentially confounding the results. Additional investigations are necessary to better understand how the interplay between different platelet concentrations, the presence of plasma proteins, and patient age impacts tendon healing.

A renewed approach towards improving our understanding of PRP through basic scientific research can help to safeguard patient safety and further optimize PRP formulations for specific indications. However, our study
D. R. Berger: Collected, analyzed, and interpreted the data, Wrote and edited the manuscript. N. J. Steinmetz: Designed and supervised the study, Wrote and edited the manuscript. C. J. Centeno: Designed the study, edited the manuscript.

The author or one or more of the authors have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article.

Author contribution

D. R. Berger: Collected, analyzed, and interpreted the data, Wrote and edited the manuscript.

C. J. Centeno: Designed the study, edited the manuscript.

N. J. Steinmetz: Designed and supervised the study, Wrote and edited the manuscript.

Funding statement

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References


20. Salini V, Vanni D, Pantalone A, Abate M. Platelet-rich plasma therapy in non-insertional achilles tendinopathy: the efficacy is reduced in 60-years old people compared to young and middle-age individuals. Front Aging Neurosci 2016;7:228.


