

■ RESEARCH

Platelet lysates from aged donors promote human tenocyte proliferation and migration in a concentration-dependent manner



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Objectives

Platelet-rich plasma (PRP) is being used increasingly often in the clinical setting to treat tendon-related pathologies. Yet the optimal PRP preparations to promote tendon healing in different patient populations are poorly defined. Here, we sought to determine whether increasing the concentration of platelet-derived proteins within a derivative of PRP, platelet lysate (PL), enhances tenocyte proliferation and migration *in vitro*, and whether the mitogenic properties of PL change with donor age.

Methods

Concentrated PLs from both young (< 50 years) and aged (> 50 years) donors were prepared by exposing pooled PRP to a series of freeze-thaw cycles followed by dilution in plasma, and the levels of several platelet-derived proteins were measured using multiplex immunoassay technology. Human tenocytes were cultured with PLs to simulate a clinically relevant PRP treatment range, and cell growth and migration were assessed using DNA quantitation and gap closure assays, respectively.

Results

Platelet-derived protein levels increased alongside higher PL concentrations, and PLs from both age groups improved tenocyte proliferation relative to control conditions. However, PLs from aged donors yielded a dose-response relationship in tenocyte behaviour, with higher PL concentrations resulting in increased tenocyte proliferation and migration. Conversely, no significant differences in tenocyte behaviour were detected when increasing the concentration of PLs from younger donors.

Conclusion

Higher PL concentrations, when prepared from the PRP of aged but not young donors, were more effective than lower PL concentrations at promoting tenocyte proliferation and migration *in vitro*.

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Keywords: Ageing, Platelet lysate, Tenocyte, Proliferation, Migration

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.

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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°C .¹⁷ Upon thawing at 37°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°C and 37°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 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.

Table I. Platelet counts in concentrated platelet-rich plasma (PRP) from aged donors (> 50 years) prior to lysis

Age, yrs	Gender	Platelet count, plt/ μ l	Volume, %
51	Female	9.8×10^6	15.4
53	Male	8.1×10^6	5.0
54	Female	10.6×10^6	10.4
55	Male	8.0×10^6	23.2
64	Female	9.8×10^6	7.7
74	Female	7.7×10^6	6.6
74	Female	11.4×10^6	6.6
78	Female	6.8×10^6	25.1
Mean: 63 (SD 11)		Weighted mean: 8.6×10^6	100

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

Age, yrs	Gender	Platelet count, plt/ μ l	Volume, %
20	Male	11.8×10^6	5.8
22	Female	9.9×10^6	19.4
25	Male	7.2×10^6	17.5
30	Male	10.3×10^6	35
32	Male	13.9×10^6	9.7
33	Female	7.7×10^6	12.6
Mean: 27 (SD 5)		Weighted mean: 9.8×10^6	100

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

Experimental condition	Representative clinical PRP concentration
Platelet lysate (PL)	14 \times
1/2 PL	7 \times
1/4 PL	3.5 \times
1/8 PL	1.75 \times
1/16 PL	0.9 \times

A high-resolution image was acquired (Q-View Imager; Quansys Biosciences) and the protein concentration within each PL sample was determined by comparing chemiluminescent intensities with that of an eight-point standard curve (Q-View; Quansys Biosciences).

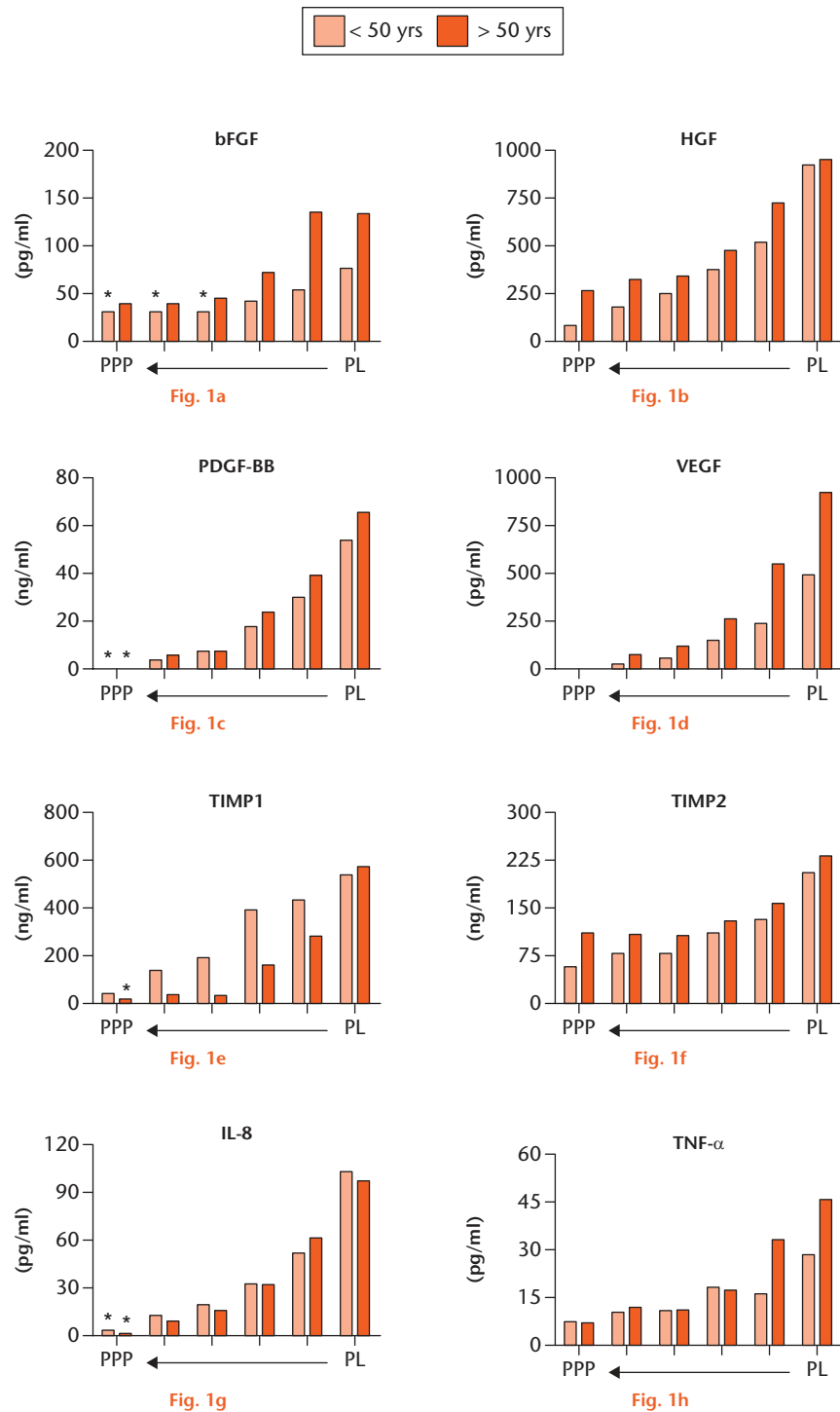
Culture of human tenocytes. Cryopreserved human tenocytes ($n = 3$) from the Achilles, patellar, and palmaris tendons of a 61-year-old man, a 65-year-old woman, and an 81-year-old man, respectively, were purchased from a commercial supplier (Zen-Bio Inc., Research Triangle Park, North Carolina). Upon thawing, tenocytes were plated in collagen-I coated tissue culture flasks (Corning Inc., Corning, New York) using expansion medium consisting of 88% minimal essential medium- α (α -MEM; Lonza, Basel, Switzerland), 10% foetal bovine serum (FBS; Corning Inc), 1% GlutaMAX (Thermo Fisher Scientific, Waltham, Massachusetts), and 1% penicillin-streptomycin (Thermo Fisher Scientific) by volume. Cultures were maintained in a humidified incubator at 37°C and 5% CO₂, with periodic media changes until ~80% confluent, at which point the tenocytes were trypsinized and replated for proliferation and migration assays. Tenocytes utilized for this study were not expanded beyond the fifth passage.

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 μ m 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 μ m 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 (ImageJ; NIH, Bethesda, Maryland) and the percentage of gap closure was calculated by the equation $((A_0 - A_T)/A_0) * 100$, where A_0 is the initial gap area and A_T 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.



Quantitative comparison of protein concentrations by multiplexed immunoassay technology. Concentrated platelet lysate (PL), serially diluted with platelet-poor plasma (PPP) to 1/2, 1/4, 1/8, and 1/16 of the initial concentration, and PPP from both age groups were evaluated for: anabolic growth factors, a) basic fibroblast growth factor (bFGF), b) hepatocyte growth factor (HGF), c) platelet-derived growth factor BB (PDGF-BB), and d) 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.

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

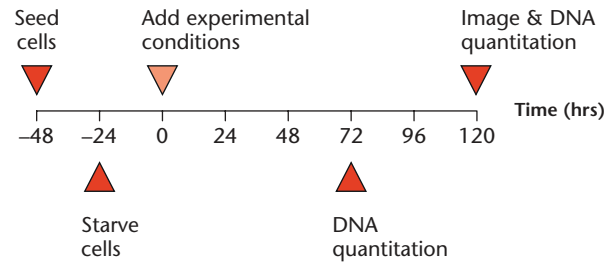


Fig. 2a

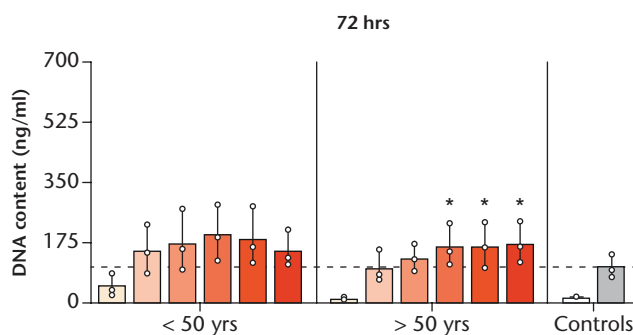


Fig. 2b

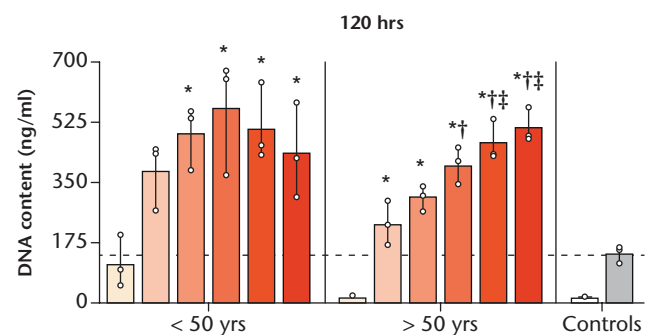


Fig. 2c

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.

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 maintained in cell culture medium containing PLs for 72 or 120 hours, at which point tenocyte proliferation was assessed by quantifying DNA (Fig. 2a). After 72 hours of culture, tenocyte growth was similar to, or better than, that of the positive control condition. Significant differences between higher PL concentrations and PPP were only observed in the aged donor group (Fig. 2b). After 120 hours of culture, PLs from the aged donors clearly

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

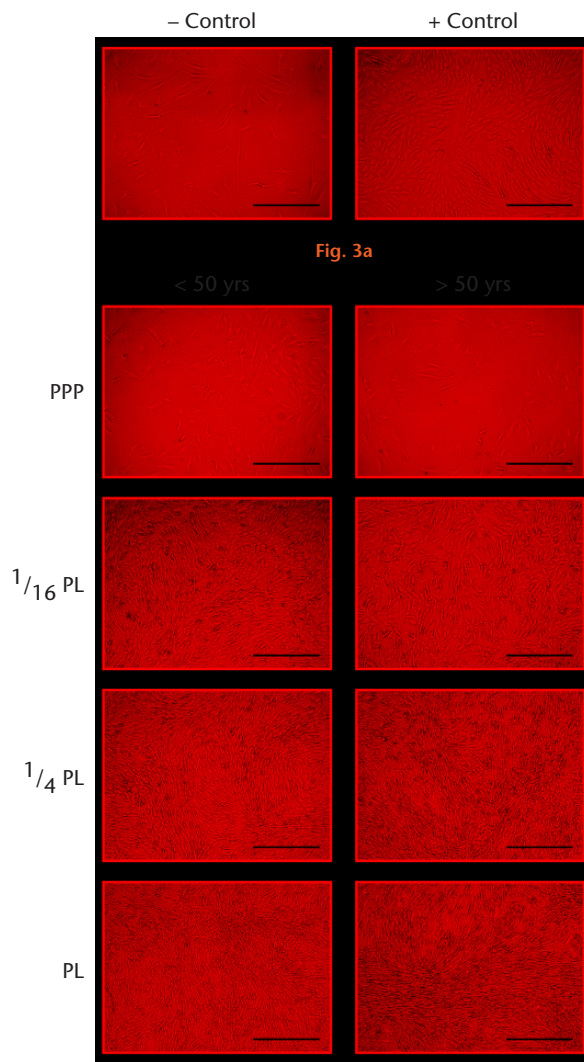


Fig. 3a

Characterization of tenocyte proliferation following culture with increasing concentrations of pooled platelet lysates (PLs) by phase contrast microscopy. a) Tenocytes cultured with negative (1% foetal bovine serum (FBS)) and positive (20% FBS + basic fibroblast growth factor (bFGF)) experimental control conditions after 120 hours. b) Tenocytes cultured with pooled PL (or platelet-poor plasma (PPP)) from different donor age groups after 120 hours. Representative images shown are from a single tenocyte donor (81-year-old male, palmaris tendon). Scale bar = 500 μ m.

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

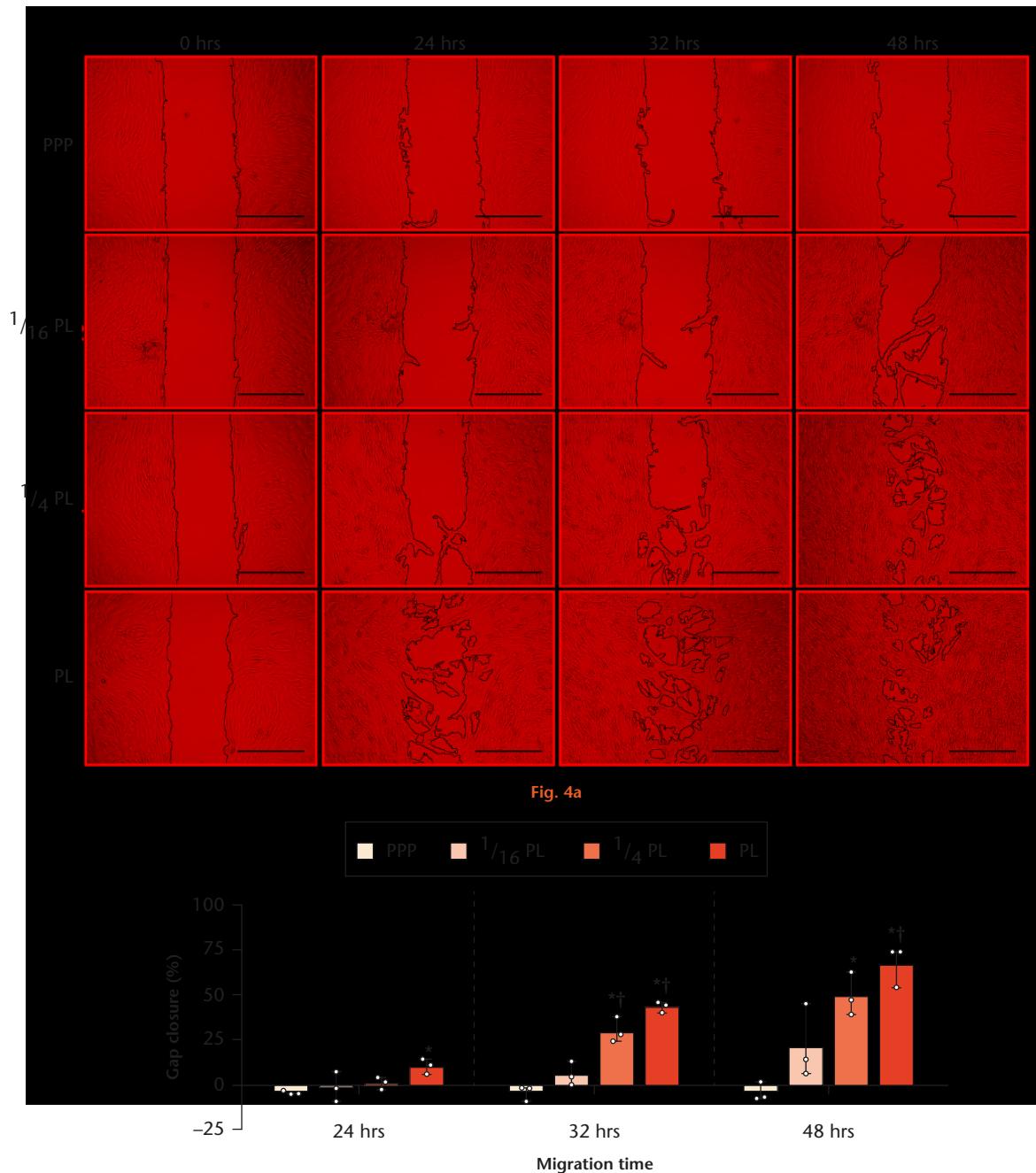
and 48 hours of culture (Fig. 4b). By comparison, Figure 5 demonstrates PL from young donors to promote tenocyte migration largely independent of concentration. Representative phase-contrast images reveal almost complete gap closure following 48 hours of culture (Fig. 5a). Significant differences in 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.⁵ Of the two anti-catabolic tissue inhibitors quantified, TIMP2 levels were less dependent on PL concentration, in accordance with previously published results.¹⁹ Low, but detectable, concentrations of the pro-inflammatory cytokines IL-8 and TNF- α were measured, and their presence could signify leucocyte contamination. However, other investigations have detected similar concentrations of these two cytokines in PRP with less than 0.3% of the initial white blood cells remaining.³

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,¹⁵ 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.²⁰ This patient population receives PRP therapy for musculoskeletal injuries with increased frequency²¹ and has an increased prevalence of tendon-related injuries.^{22,23}

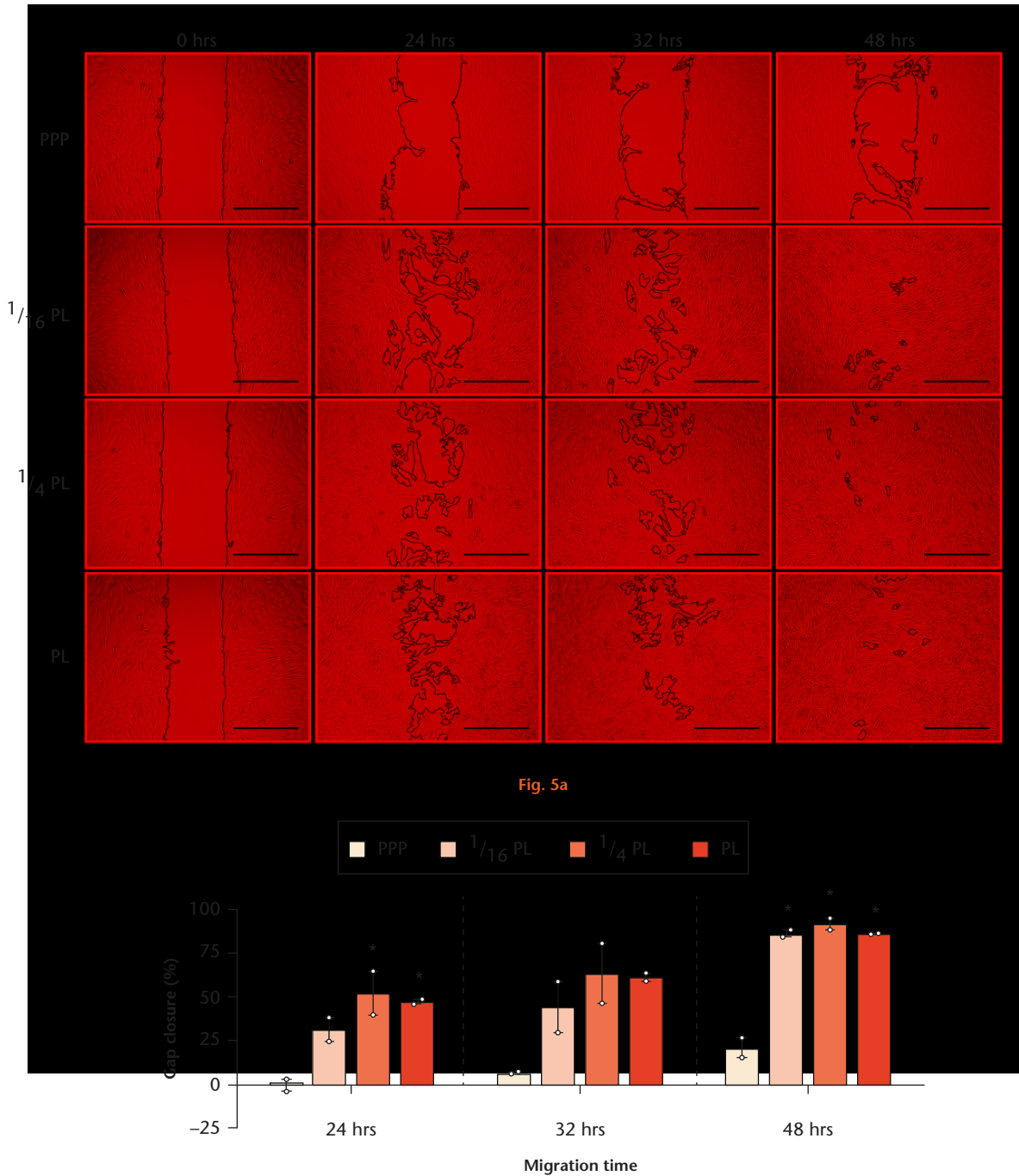
Interestingly, PPP from young donors was found to promote tenocyte proliferation and migration weakly, whereas PPP from their older counterparts did not.



Tenocyte migration is improved by increasing the platelet lysate (PL) concentration from aged donors. a) Representative images of tenocyte migration from a single tenocyte donor (81-year-old male, palmaris tendon). Cell-free regions are demarcated by a white line. Scale bar = 500 μ m. b) Tenocyte migration was quantified using imaging software following 24, 32, and 48 hours of culture. * $p < 0.05$ versus platelet-poor plasma (PPP); † $p < 0.05$ versus 1/16 PL.

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



Tenocyte migration is comparable across all platelet lysate (PL) concentrations from young donors. a) Representative images of tenocyte migration from a single tenocyte donor (81-year-old male, palmaris tendon). Cell-free regions are demarcated by a white line. Scale bar = 500 μ m. n = 2. b) Tenocyte migration was quantified using imaging software following 24, 32, and 48 hours of culture. *p < 0.05 versus platelet-poor plasma (PPP).

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.¹²⁻¹⁴ 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.²⁷ However, our study

included limitations one must consider when interpreting the results. Due to difficulties encountered when adding pure PRP to cell cultures, lysing via successive freeze and thaw steps was chosen as the optimal method to isolate the platelet-derived protein content, as recommended by others.¹⁸ Given that an *in vitro* culture model was used to measure the effects of concentrated PLs on tendon cell growth and migration from a small number of blood and tenocyte donors, we acknowledge that the cellular microenvironment lacked several physiologically relevant characteristics,²⁸ and that our study is limited in size.

In conclusion, this study reveals that higher PL concentrations promote increased tenocyte proliferation and migration when derived from ageing, but not young, donors. These results suggest that increasing the platelet concentration within PRP may be a useful biological strategy for helping tendon regeneration in an ageing patient population.

Supplementary material

 A table showing representative platelet, white blood cell (WBC), and red blood cell (RBC) counts in platelet-rich plasma (PRP); a table showing representative platelet count in PRP and concentrated PRP; and representative images of tenocytes from a single tenocyte donor

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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.

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