Optimising Proliferation and Migration of Mesenchymal Stem Cells Using Platelet Products: A Rational Approach to Bone Regeneration

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ABSTRACT: This study investigates how mesenchymal stem cell's (MSCs) proliferation and migration abilities are influenced by various platelet products (PP). Donor-matched, clinical-, and control laboratory-standard PPs were generated and assessed based on their platelet and leukocyte concentrations. Bone marrow derived MSCs were exposed to these PP to quantify their effect on in vitro MSC proliferation and migration. An adapted colony forming unit fibroblast (CFU-F) assay was carried out on bone marrow aspirate using clinical-standard PP-loaded electrospun poly(ε-caprolactone) (PCL) membrane to mimic future clinical applications to contain bone defects. Clinical-standard PP had lower platelet (2.5 fold, $p < 0.0001$) and higher leukocyte (14.1 fold, $p < 0.0001$) concentrations compared to laboratory-standard PP. It induced suboptimal MSC proliferation compared to laboratory-standard PP and fetal calf serum (FCS). All PP induced significantly more MSC migration than FCS up to 24 h. The removal of leukocytes from PP had no effect on MSC proliferation or migration. The PP-loaded membranes successfully supported MSC colony formation. This study indicates that platelet concentrations in PP impact MSC proliferation more than the presence of leukocytes, whilst MSC migration in response to PP is not influenced by platelet or leukocyte numbers. Clinical-standard PP could be applied alongside manufactured membranes in the future treatment of bone reconstruction. © 2019 The Authors. *Journal of Orthopaedic Research*® Published by Wiley Periodicals, Inc. on behalf of Orthopaedic Research Society. J Orthop Res

Keywords: stem cells; platelet products; bone regeneration; fracture repair

Fracture nonunion persists as a prevalent complication, with the incidence in long bones reported to range between 5 and 10%.[1,2] Its etiopathogenesis remains multifactorial with a recent review identifying over 20 factors to be implicated in this process.[3] To address impaired fracture healing, Giannoudis et al. proposed the diamond conceptual framework for bone repair highlighting that, for a successful healing response both mechanical stability and biological factors must be present.[4] For biological stimulation, while autologous bone graft remains the gold standard, recently, other forms have gained popularity including bone marrow aspirates (BMA) and/or growth factors.[5,6] Since their introduction to the clinical setting, bone morphogenetic proteins (BMPs) have been extensively used for the treatment of nonunion fractures.[7] There are numerous publications reporting on the clinical results of BMP-2 and BMP-7.[8–10] However, since the withdrawal of BMP-7 from the market and the license limitation of BMP-2 to be used for the management of open tibial fractures, other inductive molecules gained popularity such as demineralized bone matrix (DBM),[11] teriparatide,[12] and platelet products (PP).[13,14]

PPs are highly enriched sources of autologous growth factors and cytokines that act as biological stimulants to accelerate osteogenesis and bone repair.[15,16] PPs are usually applied as platelet rich plasma (PRP), where the cells remain intact, or more recently, as platelet lysate (PL) where only the growth-factor containing plasma is used.[17] Both forms have been found to be highly effective in the treatment of orthopaedic trauma for decades,[18] and more recently for the treatment of fracture nonunion.[19] Several studies into PP loaded membranes are already proving to be successful at promoting regeneration via the delivery of growth factors in cartilage and bone repair.[20,21] When working with PP loaded membranes, maximizing bone marrow derived MSC (BM-MSC) proliferation, and migration is key. By ensuring these functions are enhanced, more viable cells are available to differentiate, as well as inducing more resident BM-MSC homing to the site of injury. While some studies found that the presence of leukocytes in PP are advantageous due to their antimicrobial properties[22] and high concentration of cytokines involved in bone repair such as VEGF,[23,24] there are also concerns related to leukocytes' effect of inducing excessive inflammatory and necrotic pathways from the surrounding tissue.[25,26] Collectively, this provides a strong case for their depletion.

This study investigates the in vitro effect of platelets and leukocytes on the proliferation and migration of MSCs from BMA by studying a range of PP compositions including a clinical-standard PRP

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(CPRP). A laboratory-standard PRP made using a two-spin centrifugation protocol to enrich platelets but deplete leukocytes was also used as control.[18,27]

To investigate the specific importance of leukocytes, PRP was processed further by syringe-filtration to remove leukocytes and produce a pure filtered platelet rich product (fPRP). These products were then lysed to ensure product consistency over the course of in vitro assays; thereafter referred to as clinical-standard platelet lysate (CPL), platelet lysate (PL), and filtered PL (fPL). These PLs were then evaluated in vitro utilizing primary BM-MSCs, including autologous BM-MSC-PP combinations, as well as loading onto a degradable electrospun membrane to achieve sustained localized delivery in a colony forming units-fibroblast (CFU-F) assay. The aim was to assess if high numbers of leukocytes would impede BM-MSC proliferation and migration and whether their depletion from clinical PPs would be desirable for bone regenerative approaches.

METHODS

This study was carried out in adherence with the Helsinki Declaration under ethics code 06/Q1206/127 following approval from the local National Health Service Research & Development Department, Leeds East Research Ethics Committee to harvest these samples. Written informed consent was obtained from each participant.

Sample Collection and Processing of CPRP, PRP, and fPRP

Whole blood was collected from 11 healthy volunteers (8 males and 3 females, 22–58 yrs) in acid-citrate dextrose solution A (ACD-A). Each sample was divided and used to prepare CPRP and PRP in a single-donor model. CPRP was made from whole blood using the BioCUE^{TM} device (Zimmer Biomet) which was centrifuged at 1100 g for 15 min. After centrifugation, the device was agitated and the CPRP fraction was extracted using a syringe. PRP was generated using a two-step centrifugation protocol[28] whereby the patients' whole blood was incubated at room temperature for 1 h before centrifugation at 400 g for 10 min. The platelet-containing supernatant was extracted and centrifuged at 2700*g* for 10 min before resuspending the pellet in 1/5th of the residual supernatant.

fPRP was generated from PRP that was filtered through a white blood cell (WBC) syringe filter (Acrodisc) which entraps leukocytes and allows platelets to pass through for collection. The division of PRP for the production of fPRP meant both their final volumes were much smaller than CPRP limiting the number of assays they could be used in. Platelet and leukocyte concentrations of CPRP, PRP, fPRP, and whole blood (1 ml) were determined using an automatic haematology analyser (Sysmex).

Generating Lysate Products (CPL, PL, and fPL)

To generate the lysed products for in vitro assays, CPRP, PRP, and fPRP were processed through three freeze-thaw cycles which involved freezing at −80 °C followed by thawing at 37 °C.[29] To remove contaminating cell debris, CPL, PL, and fPL were centrifuged at 2700*g* for 10 min and the supernatants were extracted for cell stimulation.

Sample Collection and Processing of BM-MSCs

Patients undergoing elective orthopaedic surgery with no underlying disease were recruited ($n = 11$, 6 males and 5 females, 17–69 yrs). 4 ml of BMA was collected from each donor's anterior iliac crest and was treated with ammonium chloride to lyse red blood cells. The remaining nucleated cells were plated into flasks at the cell seeding density of 5×10^5 cells/cm^2. BM-derived adherent cells were cultured in MSC expansion media (Miltenyi Biotec) for approximately 2 weeks until confluent.

Characterising BM-MSCs

Cultured BM derived cells (passage two) were tested for the surface expression levels of MSC markers as defined by the International Society for Cell Therapy (ISCT).[30] Following trypsinisation (Sigma Aldrich), cell cultures were stained with antibodies against positive markers of MSCs (CD90, CD73, and CD105) and negative hematopoietic-lineage markers (CD34, CD14, HLA-DR, CD19, CD45). All antibodies were used according to the manufacturer's recommendation (Miltenyi Biotec). Isotype controls (BD, Bio-Rad) were used to gate for positive expression. The data were acquired on an Attune flow cytometer (ThermoFisher Scientific).

Trilineage Differentiation Potential of BM-MSCs

BM derived cells were cultured up to passage four before being trypsinized and tested for multipotentiality as previously described.[31] Cells were seeded with AdipoDiff Media (Miltenyi Biotec) or OsteoDiff Media (Miltenyi Biotec) for adipogenesis or osteogenesis, respectively, with bi-weekly media changes. After 3 weeks, cell cultures were stained with oil red solution (adipogenesis)[32] or Alizarin Red (osteogenesis). For chondrogenesis, cells were added to Eppendorf tubes to create a pellet, resuspended in ChondroDIFF Media (Miltenyi Biotec) and cultured for 21 days after which the pellets were stained with toluidine blue (Sigma).

Proliferation Assay

The XTT assay was used to quantify BM-MSC proliferation in the presence of different PL products. Activity of mitochondrial dehydrogenase, and therefore cell number per well, is directly correlated to the amount of orange formazan formed, as monitored by the optical density (OD) at 450 nm. BM-MSCs were seeded in triplicate in a 96-well plate at a density of 500 cells/well with MSC expansion media and incubated for 24 h. The media was then replaced with basal Dulbecco's Modified Eagle's Media (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) of CPL, PL, fPL, or fetal calf serum (FCS) and incubated for 5 days with a half media change on day 3. Following exposure to the treatment media, cell proliferation was assessed on day 5 using an XTT cell proliferation kit (Roche) according to the manufacturer's instructions.

Migration Assay

The bottom wells contained basal DMEM (1% (v/v) Penicillin/Streptomycin and 2 I.U/ml sodium heparin solution) supplemented with 10% (v/v) of CPL, PL, fPL, or FCS. 10% was the selected concentration due to its frequent use in the literature.[33–35] Migration was analyzed over 24 h using an IncuCyte® (Essen) to collect phase-contrast images of the transwells as cells migrated along the chemotactic gradient. Images were captured every 30 min and a processing mask

was created for each BM-MSC culture to account for donor variation.

CFU-F Assay With CPL-Loaded Membrane

An adaptation of the CFU-F assay was used to quantify how a CPL-loaded membrane affected the proliferation and migration capacity of uncultured BM-MSCs and to further validate the results of the assays using cultured BM-MSCs. First, fresh BMA from three donors underwent red blood cell lysis after which a total of 5×10^5 cells were plated in 15 ml expansion media to adhere over 24 h. After initial attachment and a PBS wash the media was replaced with either 15 ml expansion media containing 2 I.U/ml sodium heparin solution (control) or 15 ml DMEM media (no serum) containing 2 I.U/ml sodium heparin solution (test). 1 cm^2 square sections, 400 μm thick, of UV-sterilized PCL that were made in-house[36] were soaked in CPL for 15 min until saturated before being placed in the centre of the test dishes. The dishes were incubated for a further 19 days with half-media changes once a week. After 21 days, the dishes were washed in PBS, fixed in 10% (v/v) formalin (Sigma) and stained in 1% (w/v) methylene blue (Sigma). All colonies were imaged using a plate scanner (Epson) at 1200 dpi. Colony area, integrated density and number per dish was quantified using ImageJ whereby scanned images were converted to 8-bit grayscale and a threshold mask was applied before particles were automatically analyzed and measured.

RESULTS

Characterising Cellular Content of PP

CPRP was generated using a BioCUE™ device whilst PRP was made following a previously optimised protocol[18] and their cell populations were quantified using a haematology analyzer. While both PP significantly enriched platelets compared to their whole blood counterparts, PRP also had

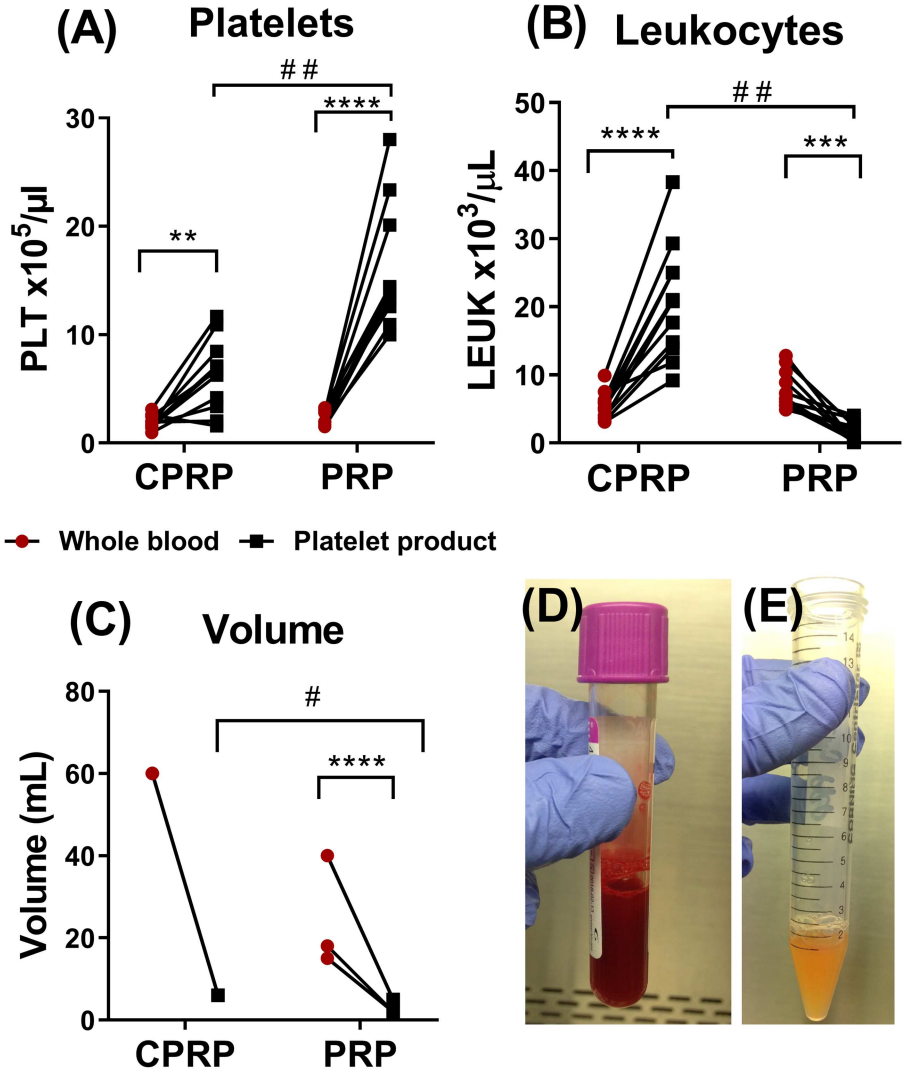

Figure 1. Characterising platelet rich plasma products. Using a haematology analyser, the change in platelets (A), leukocytes (B), and final volume (C) from whole blood to CPRP ($n = 11$) and PRP ($n = 11$) were quantified. The increase or decrease of each cell type indicates the change from whole blood (red) to the paired platelet product (black). A paired t-test was used to compare change in cell type before and after processing ($^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$) while a Mann-Whitney test was used to compare CPRP and PRP ($\# = p < 0.001$, $\# \# = p < 0.0001$). Statistics could not be conducted on CPRP's volume analysis (C) due to matching differences between each sample. The photographs show the CPRP fraction product (D) and the PRP product (E).

significantly higher numbers of platelets (15.9×10^5 PLT/μlL) (Fig. 1A) in comparison to CPRP (6.3×10^5 PLT/μl) as well as significantly lower leukocyte numbers (1.8×10^3 LEUK/μl) compared to CPRP (20.6×10^3 LEUK/μl) (Fig. 1B). Despite the significant difference in final volumes ($p = 0.0078$) (Fig. 1C), both products had comparable average fold decrease in volumes (10.0 ± 0.0 and 7.9 ± 0.5 CPRP and PRP, respectively). All CPRP replicates underwent identical reductions in volume causing their data points to overlap. The difference in the PP's cell populations is also visible in their appearance as CPRP is opaque with additional red blood cell contamination (Fig. 1D) while PRP had fewer contaminating red blood cells and was more translucent (Fig. 1E).

As previous studies have found that platelets and leukocytes are not stable at room temperature for the 5 days necessary for the proliferation and migration assays,[37] the PP were lysed after production to ensure consistency of samples. Lysis of the products changed their nomenclature from CPRP and PRP to clinical platelet lysate (CPL) and platelet lysate (PL).

Characterizing BM-MSCs

BMAs underwent red blood cell lysis, after which the remaining nucleated cells were plated into flasks, three randomly selected cultures were tested for MSC characterization according to the ISCT's definition.[38] Cell surface marker expression was analyzed using flow cytometry which found cells were negative for CD14, CD19, CD34, CD45, HLA-DR, and positive for CD73, CD90, and CD105 (Fig. 2A) consistent with the criteria for identifying MSCs. Their tri-lineage differentiation capacity was confirmed following 3-week culture in adipogenic, osteogenic, and chondrogenic stimulation medias. A representative culture is shown in Figure 2 which was stained with oil red o solution to visualize lipid droplets (Fig. 2B), alizarin red staining to visualize calcium depositions (Fig. 2C), and finally toluidine blue staining of the aggregated cell pellet indicative of glycosaminoglycan production. Nine of these cultures were used for subsequent PL proliferation and migration tests, each PL product was tested on at least three MSC cultures and the results were averaged.

Platelet Product Composition's Impact on BM-MSC Proliferation

Cultured cells were exposed to 10% PL, CPL, and control FCS containing media for 5 days, after which the cells were treated with XTT reagent causing a color change. BM-MSC proliferation was expressed as OD normalized to FCS. BM-MSCs treated with PL were found to proliferate significantly more than cells treated with CPL ($p = 0.0001$) and equal to cells treated with FCS (Fig. 3A). The same response was also seen from BM-MSCs tested with autologous PL and CPL (Fig. 3B). To investigate whether CPL's suboptimal support of BM-MSC proliferation was due to the high number of leukocytes, they were filtered out using a leukocyte syringe filter. The filtered PL's (fPL) platelet numbers were not significantly affected

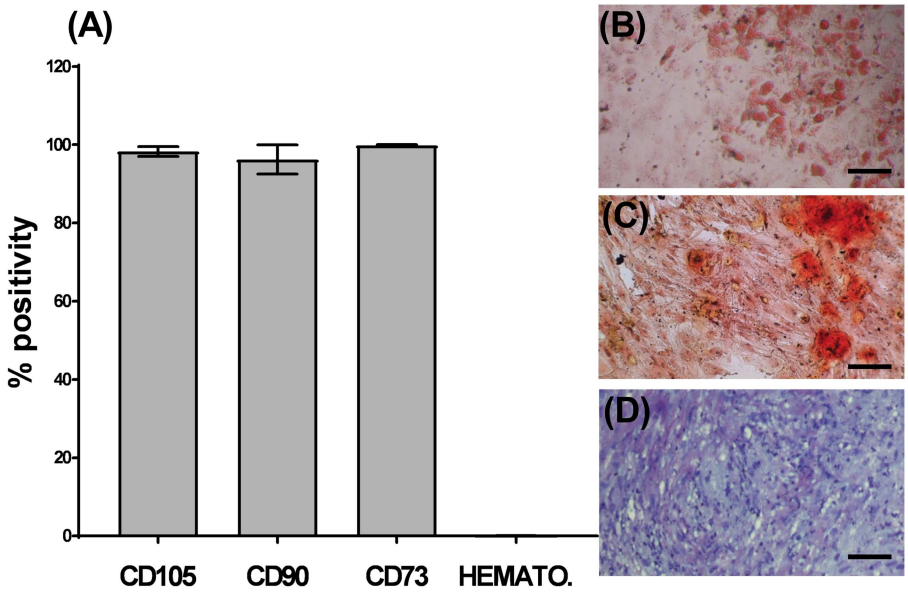

Figure 2. Platelet product composition's impact on BM-MSC proliferation. XTT assay quantifying BM-MSC proliferation following four day exposure to media containing 10% platelet products or FCS as control. Proliferation was represented as OD normalized to FCS. (A) Cultured BM-MSCs were exposed to 10% PL, CPL, and FCS. (B) Cultured BM-MSCs were exposed to autologous 10% PL and CPL as well as 10% FCS. (C) The change in platelets and leukocytes from whole blood to PRP and fPRP (compared using a paired *t*-test). (D) Cultured BM-MSCs were exposed to 10% PL, fPL and FCS from three PL donors. One-way ANOVA test was used to test significance between the platelet products effects on proliferation (3A, B, and D).($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$). Except for 3B, all experiments were performed on a minimum of three different PP donors and three BM-MSC cultures. Error bars indicate variation between PP except for 3B where error bars indicate technical variation between replicates.

by the filtration process while leukocytes were shown to be significantly depleted in fPL (Fig. 3C). Removal of the leukocytes was found to not significantly improve proliferation as it was shown that both PL and fPL induced the same BM-MSC proliferation as FCS (Fig. 3D).

Altogether, this data indicated that the leukocytes were not inhibitory for BM-MSC proliferation and suggested that differing performances of PL and CPL observed were likely due to the different numbers of platelets present.

Platelet Product Composition's Impact on BM-MSC Migration

To compare the chemotactic potential of the different PPs, the IncuCyte® transwell assay was used. Serum starved BM-MSCs were seeded on top of the transwells and exposed to different PPs below. Over the course of 24 h, the top and bottom of the transwells were imaged continuously to track their migration. Representative images of the top of the transwells are shown in Figure 4A where non-migrated cells are shown in focus (purple arrow), cells that have migrated through and identified by the software are highlighted in green for processing (blue arrow) and the pores are identified with black arrows. The migratory effects of PL and CPL were demonstrated as a time-course assay using one representative culture (Fig. 4B) which showed that, both PL and CPL appear to induce far more migration than 10% FCS. To enable statistical analysis, three independent PL preparations were each tested on three BM-MSC cultures and found that both PL and CPL induced significantly more migration than FCS at 12 h ($p = 0.0068$ and $p = 0.0434$, respectively) and at 24 h ($p = 0.0006$ and $p = 0.0008$) (Fig. 4C). Considering the higher levels of leukocytes in CPL, these findings suggest that the presence of leukocyte-derived proteins is not a detriment to BM-MSC migration and potentially contribute toward improved migration.

To further investigate the effect of leukocyte-derived proteins on BM-MSC migration, PL and fPL's chemotactic effects were investigated. A representative culture is shown in Figure 4D which showed that, while the complete removal of leukocytes did appear to reduce BM-MSC's migratory response this reduction was not statistically significant. In addition, PL and fPL both induced far more migration than 10% FCS. BM-MSCs were found to be significantly more migratory toward PL and fPL than 10% FCS at 12 h

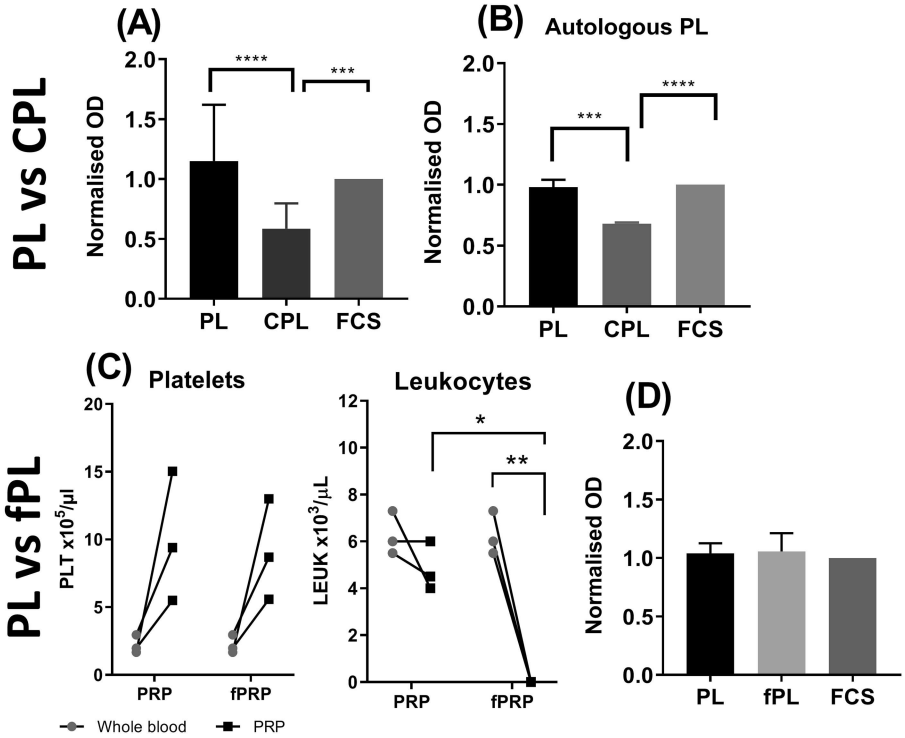

Figure 3. Platelet product composition's impact on BM-MSC migration. The IncuCyte® transwell assay quantifies BM-MSCs moving toward a chemotactic gradient. (A) Representative images of wells of 0.5% FCS (top), 10% FCS (middle), and 10% PL (bottom). Arrows indicate pores (black), static cells on the top of the transwell (purple) and cells that have migrated (blue). The processing mask that quantifies the migrated cells is shown in green. (B) Representative time-course response of the cells from one BM-MSC culture that have migrated through the transwell towards 10% PL, 10% CPL 10% FCS, and 0.5% FCS. Data are shown as the area of the bottom of the well occupied by cells. (C) Average object area of the underside of the transwell occupied by BM-MSCs and treated with 10% PL, 10% CPL, and 10% FCS was normalized to 10% FCS. (D) Representative time-course response of the cells from one culture that have migrated through the transwell towards 10% PL, 10% fPL, 10% FCS, and 0.5% FCS. (E) Average object area of the underside of the transwell occupied by BM-MSCs and treated with 10% PL, 10% fPL, and 10% FCS was normalized to 10% FCS. A one-way ANOVA was carried out using the Kruskal-Wallis test for normality ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). Error bars indicate variation between PL donors. Experiments in C and E were performed on a minimum of three different BM-MSC cultures.

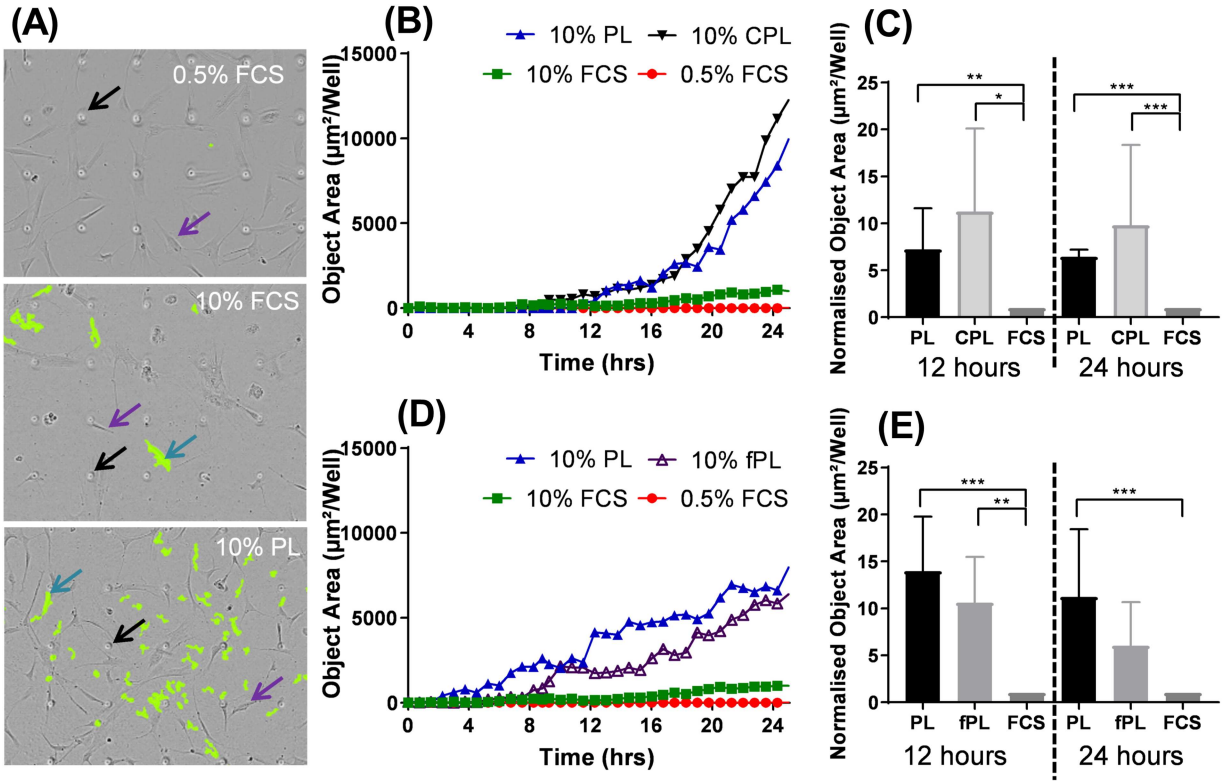

Figure 4. CPL-loaded membrane supports cell proliferation in CFU-F assay. (A) Representative CFU-F dishes of BM-MSCs grown in either standard expansion media (top dish) or serum-free DMEM with the additional CPL-loaded membrane as a source of released growth factors and cytokines (bottom dish). (B) Representative individual colonies of cells grown in expansion media (top) or CPL-loaded membrane and DMEM (bottom). Three BMA donors were tested on a single CPL product. Images were collected using a photo scanner at 1200 dpi. (C) Comparison of average colony area, density, and total number between cells treated with a CPL-loaded scaffold and expansion media (EM). An unpaired *t*-test found no significant difference between CPL of EM.

($p = 0.0015$ and 0.0198, respectively) and PL was also found to outperform 10% FCS at 24 h ($p = 0.0024$) (Fig. 4E). These findings suggest that while platelet and leukocyte numbers contribute toward BM-MSC migration, it is likely that most of the cytokines that induce migration are present in the plasma component of PP—and so any additional platelet or leukocytes make no significant difference.

Loaded Membrane Supports Colony Formation From Native MSCs Present in BMA

To more closely mimic clinical applications involving a PP-loaded biomaterial membrane as a MSC homing and containment device, experiments were performed using clinically approved PP. The aim of these experiments was to study if CPL released from a membrane could support colony formation from rare BM-MSCs without their culture-amplification. In standard CFU-F assays, single rare BM-MSCs give rise to individual colonies. The fresh BM-MSCs used for this assay were grown in either standard MSC expansion media or basal serum-free media with the addition of a CPL-loaded membrane (Fig. 5A). Colony formation was observed in all test dishes, with similar morphology to control dishes containing MSC expansion media (Fig. 5B). Furthermore, although there were trends for higher colony numbers in MSC expansion media, and higher colony areas and densities in CPL-loaded membrane dishes, the differences were not found to be statistically significant (Fig. 5C). This indicated that CPL-loaded membranes were able to release growth factors that induced colony formation and supported rare BM-MSC proliferation.

DISCUSSION

While the clinical effectiveness of PP is generally accepted, due to the lack of quality control, the variation in manufacturing, processing, delivery, and its different applications (e.g., rotator cuff repair and osteoarthritis[39,40]), the "optimal" composition is still hotly debated depending on its specific clinical application.

The main issue with lack of standardization is exemplified by one study which found that PRP had no significant effect on bone healing,[41] however, a closer look at the platelet numbers used in the study found that some of the PRP tested had over 16-fold more platelets than other PRP. Despite the vast differences in platelet numbers and omitted leukocyte numbers, these products were all classified under the same umbrella term "PRP." This is commonplace across the literature, whereby, the absence of standardized nomenclature, quality control, as well as thorough analysis of cellular contents, makes it difficult to draw clear

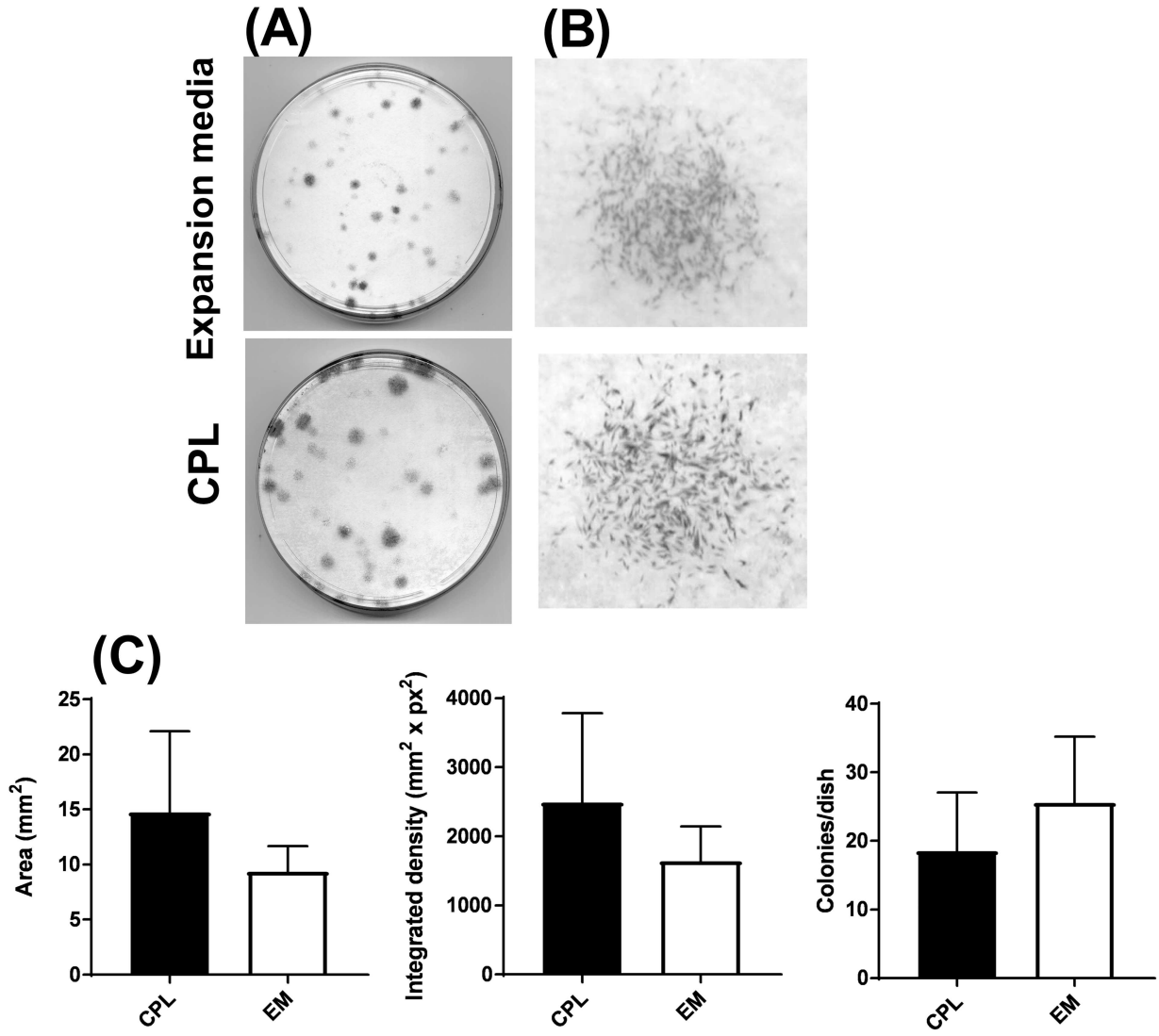

Figure 5. Characterizing platelet rich plasma products.

conclusions. According to Delong et al.'s classification system[42] platelet number, activation method, and white blood cell number (PAW), CPRP is classified as P2-A (which identifies the increase of platelets as "moderate" and the leukocytes as "enriched above baseline") while PRP is classified as P3-B (which identifies the platelet concentration as "highly enriched" above baseline and leukocytes depleted below baseline).

As well as variation in platelet concentrations, the lack of regulation also instils concern in the scientific community over the use of highly concentrated leukocytes, their associated pro-inflammatory cytokines (specifically TNF-α and IL-1β), and the risk that they could counteract the platelet's beneficial effects and impede bone regeneration.[26] TNF-α and IL-1β are known to induce inflammation causing a biphasic physiological response; whilst inflammation is necessary for healing,[43] in excess it is thought to activate the NFκB pathway[26] inhibiting osteogenesis and promoting osteoclastogenesis.[44] However, more recently, these proinflammatory cytokines have been linked to increased osteogenesis,[45] BM-MSC migration,[46] and proliferation[47] indicative of the lack of consensus in the field.

This study addressed these concerns by better defining platelets and leukocyte's effects in the two key physiological processes of bone regeneration: BM-MSC proliferation and BM-MSC migration. With regards to proliferation, when compared to PL and FCS, CPL induced significantly less BM-MSC proliferation than PL. This was also observed using donor-matched BM-MSCs and PP proving that the response from the cells was not due to the allogeneic nature of the PL. The cause of CPL's suboptimal ability to support BM-MSC proliferation compared to PL is either due to its lower number of platelets or higher number of leukocytes—both of which have been reported to reduce proliferation in the literature.[26,42,48,49] To identify which cell type is the key

player, leukocytes were filtered from PL while platelet numbers were kept the same to produce fPL. Direct comparison of PL and fPL showed that removing leukocytes did not improve proliferation. This suggests that in our experimental conditions, the cytokines released from leukocytes neither enhanced nor inhibited BM-MSC proliferation and that platelets are most likely responsible for releasing the predominant growth factors involved in supporting BM-MSC proliferation.

With regards to BM-MSC migration, all three platelet products (PL, CPL, and fPL) induced significantly more migration than FCS, likely due to the greater concentrations of cytokines such as VEGF and SDF-1.[50,51] Again, it was seen that removing leukocytes from PL did not further enhance BM-MSC migration, but even seemed to reduce it. While there is no precedent for the use of platelet products in BM-MSC migration studies, the current data supports previous literature of platelet products outperforming FCS.[26,34] The methods used are also unique in that, not only is the total migration shown, but also the increased rate of cell migration toward the platelet products.

Based on these findings it could be proposed that generating high quality platelet products should simply involve increasing platelet numbers as much as possible without regard for leukocytes—this could be easily achieved by decreasing the final volume to yield a more concentrated platelet product. However, the excessive enrichment of platelets faces the risk of paradoxically inhibiting cell proliferation, viability, and migration.[42] A therapeutically effective range of platelet concentration is likely to be the case rather than a specific pure concentration. Our results indicate that platelet concentrations in the range of $6.3–15.9 \times 10^5$ PLTs/μl (with and without leukocytes) were effective in supporting BM-MSC proliferation and migration without adverse effects on their attachment or morphology.

While several studies have already shown that PP increases the bone regeneration rate and prevention of non-union fractures in animal models[52,53] and human subjects,[14] as well as this work that aims to optimize PP's impact on BM-MSCs, it is also important to consider how PP should be delivered to the site of injury. To address this, a membrane was loaded with CPL and its discharge was found to support BM-MSC colony formation and BM-MSC proliferation. Due to the manufacturing process of PL and fPL (specifically the division of samples), low volumes prevented their loading onto a membrane for CFU-F analysis. In summary, this study supports the notion that the specific clinical application and desired outcome should be considered for defining best formulations of platelet products for bone regeneration. If cell proliferation is thought to be limiting regeneration, for example in elderly patients that have low numbers of autologous BM-MSCs,[54] then CPL will be sub-optimal and PP with higher concentrations of platelets should be used. If however, the surgeon's priority is to induce BM-MSC migration to the site of injury, for example to attract BM-MSCs toward an unpopulated bone scaffold, then the current clinical standard CPL may be sufficient. As well, CPL was found to support colony formation when delivered using a membrane, with a trend of increased colony size and density than MSC expansion media; providing encouraging insight toward future delivery alternatives and streamlined surgeries.

AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. PVG, EJ, GT, SR, and KM were responsible for the study conception and design. KM generated the platelet products and performed the platelet characterization, proliferation, migration, and CFU-F experimental work and analysis. JE conducted the flow cytometry experiments and analysis and HO conducted the tri-lineage characterization experiments and analysis. PVG was responsible for patient recruitment and sample collection. Data analysis and interpretation were performed by KM, EJ, JE, and HO. Manuscript preparation and writing was completed by KM and EJ with additional critical review by PVG, JE, HO, GT, and SR. All authors have read and approved the final submitted manuscript.

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