In vitro regenerative effects of a pooled pathogen-reduced lyophilized human cord blood platelet lysate for wound healing applications

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Background - Cord blood platelets, easily obtained from blood units not suitable for hematopoietic stem cell transplantation, represent an abundant source of growth factors for use in wound healing. Although several protocols have been described for platelet lysate production, no standard manufacturing protocol is available. The use of pooled cord blood platelets could thus facilitate standardization. In this study, the effect of varying concentrations (up to 20%) of a pooled pathogen-reduced lyophilized cord blood platelet lysate (PRL-CBPL) was investigated in different cell types involved in the wound healing process. The effect of heparin addition was also evaluated. In parallel, a comparison was performed with a single donor cord blood platelet lysate (SD-CBPL).

Materials and methods - The effect of PRL-CBPL on the viability and proliferation of different cell lines (L929 mouse fibroblasts and HaCaT keratinocytes) and human primary cells (fibroblasts-NHDF, coronary artery smooth muscle cells-HCASMC and coronary artery endothelial cells-HCAEC), on HaCaT migration and the chemotactic effect on human monocytes (THP-1) was evaluated.

Results - PRL-CBPL showed a lower PDGF-AB amount compared to SD-CBPL. Differing concentrations of both CBPL were necessary to influence cell viability and proliferation. 3% was the optimal concentration for L929 and HaCaT as well as for NHDF and HCASMC, while HCAEC required 10%. The effect of added heparin was more evident on SD-CBPL and in particular on NHDF and HCASMC proliferation. Keratinocyte scratch closure was obtained with 3 and 5% PRL-CBPL and SD-CBPL respectively. Both CBPLs caused an increase in the number of migrated THP-1 monocytes in a concentration-dependent manner up to 20% with a higher monocyte migration for SD-CBPL with respect to PRL-CBPL and in cells treated with heparin.

Discussion - The data obtained suggest that PRL-CBPL is an effective standardized alternative to SD-CBPL.

Keywords: *umbilical cord blood, platelet lysate, wound healing, pathogen reduction.*

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INTRODUCTION

Platelets are an abundant source of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF), which are involved in tissue healing and regeneration[1]. Platelet-rich plasma (PRP), which can be easily

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obtained from peripheral blood, has been investigated to promote healing in soft tissue injuries, bone fractures, orthopedic surgery, osteoarthritis, joint degeneration and wound care[2,3]. Kakudo *et al.*[4] cultivated human adipose-derived stem cells and dermal fibroblasts with different PRP concentrations, showing that 5% PRP added to the culture medium promoted maximum cell proliferation while 10 and 20% concentrations inhibited proliferation. Mishra *et al.*[5] and Wang *et al.*[6] found an increase in mesenchymal stem cell proliferation with up to 10% PRP, while 20% decreased proliferation.

Human platelet lysate (PL), an acellular product rich in growth factors and cytokines, obtained by freezing and thawing PRP, has been investigated as a source of growth factors alternative to fetal bovine serum (FBS), to avoid the drawbacks associated with the use of animal serum for *in vitro* cell proliferation[7]. Human PL was successfully used for human adipose tissue-derived stem cell culture[8,9]. Sovkova *et al.*[10] showed that 7% human PL added to serum-free culture media was sufficient to replace FBS for mouse fibroblasts and keratinocytes. Human PL was also used at 5% concentration for Vero and Hep-2 cell culture as an effective alternative to 10% FBS[11]. Recent reports have described the effectiveness of 10% PL from umbilical cord blood (CB) in promoting the growth and proliferation of dermal fibroblasts[12], human umbilical vein endothelial cells[13], and human mesenchymal stem cells[14].

As a growth factor source, CB can be extremely useful in cases of contraindications to or inconvenience for the production of autologous platelet derivatives[15]. Moreover, PRP and PL from CB contain higher levels of growth factors compared to platelets derived from peripheral blood[13,16,17].

PL manufacturing protocols have not been fully standardized[18], but allogeneic CBPL can facilitate standardization as it can be easily obtained from units of CB not suitable for hematopoietic stem cell transplantation due to an insufficient number of stem cells[19]. In addition, CBPL has been obtained from single-donor CB platelet units with a standard platelet count ranging from 800 to 1,200×10^9/L[3,20-22]. The biological variability observed in individual PL units may cause variability in composition and efficacy[23]. However, although using a pool of CB platelet units may reduce the natural variability and improve PL standardization[24-28], pooling increases viral risks. Therefore, the pathogen safety of pooled platelet-derived materials is closely correlated to the implementation of pathogen reduction treatment[25].

In this study, the wound healing effects of a pathogen-reduced, lyophilized PL manufactured from pooled CB platelet units (PRL-CBPL) were investigated in *in vitro* cultures of different cell lines and human primary cells involved in wound healing. Cell migration and the chemotactic effect were determined in accordance with the literature[29,30]. The efficacy of PRL-CBPL was compared with single donor CBPL (SD-CBPL), both with and without the addition of heparin due to the possibility of culture media clot formation.

MATERIALS AND METHODS

CBPL preparation

PRL-CBPL (Neofaster-Bio, Episkey S.r.l., Milan, Italy) was obtained from White Nest Pharma S.r.l. (Milan). Neofaster-Bio includes two glass vials containing the lyophilized powder obtained from 10 mL of pooled CBPL and from 10 mL of pooled CB platelet-poor plasma (CBPPP) respectively. Neofaster-Bio was manufactured in compliance with local regulations and good manufacturing procedures at the Banc de Sang i Teixits (Barcelona, Spain). The manufacturing comprised: 1) differential CB centrifugation to obtain CB platelet concentrates (CBPC) with a predefined platelet count of 800-1,200×10^9/L and CBPPP; 2) three freeze-thaw procedures of CBPC and CBPPP to promote platelet lysis and growth factor release from platelet alpha granules; 3) aseptic pooling of 35 CBPC (about 270-330 mL) and an equal volume of CBPPP into two 600 mL plastic bags; 4) hard spin centrifugation to sediment platelet stroma and collect CBPC and CBPPP supernatants; 5) pathogen reduction with the Mirasol® technology (Terumo BCT, Lakewood, CO, USA); 6) lyophilization of 10 mL CBPC and CBPPP supernatant aliquots in glass vials; 7) gamma sterilization at 25KGy. In accordance with the manufacturer's instructions, PRL-CBPPP was reconstituted with 10 mL sterile water, which was then transferred into the PRL-CBPL vial. Aliquots of 500 µL of the reconstituted PRL-CBPL were prepared and stored at –20°C until use.

SD-CBPL was prepared as previously described[13] at the Division of Transfusion Medicine and Transplant Biology, Pisa University Hospital (Italy), a structure accredited by the Regional Health Authority in The Italian

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Cord Blood Network, which has a programme to collect whole blood and prepare platelet concentrates. Written informed consent was obtained from parents, who were informed that the CB units were to be used for research. In brief, CB units were collected in plastic bags containing citrate-phosphate-dextrose-adenine-1 anticoagulant and processed within 48 hours of collection for CBPC preparation with a target platelet count of 800-1,200×10^9/L. Then the SD-CBPL units were cryopreserved without cryoprotectant in a –80°C mechanical freezer.

Figure 1 shows a schematic representation of the methods used for assessing CBPL efficacy.

PDGF-AB quantification

ELISA was performed on both PRL-CBPL and SD-CBPL to quantify the amount of PDGF-AB according to manufacturer's instructions (RAB0396, Merck KGaA, Darmstadt, Germany). The assay was performed on reconstituted, filtered, or centrifuged PRL-CBPL and on thawed, filtered, or centrifuged SD-CBPL. The PRL-CBPL and SD-CBPL were either sterile filtered using a 0.22 µm filter (ClearLine, Venice, Italy) or centrifuged at 2,500 RPM for 15 min (Eppendorf, Hamburg, Germany). The experiment was performed in triplicate using three different lots for both CBPLs.

Cell culture

L929 mouse fibroblast cell line (ICLCATL95001, Interlab Cell Line Collection, Genoa, Italy), THP-1 human acute monocytic leukaemia cell line (ICLC HTL97014, Interlab Cell Line Collection) and HaCaT keratinocytes (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini") were cultured at 37°C, 5% CO_2 in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% glutamine. Normal human dermal fibroblasts (NHDF, Promo Cell, Heidelberg, Germany) were cultured at 37°C, 5% CO_2 in high glucose Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 1% penicillin-streptomycin and 1% glutamine. The medium was changed every three days, and the L929, HaCaT, and NHDF cells were split at 70-80% confluence, while THP-1 was maintained at a cell density of 2 to 9×10^5 cells/mL. All the reagents for these cell cultures were purchased from Merck.

Human coronary artery smooth muscle cells (HCASMC, PromoCell) and human coronary artery endothelial cells (HCAEC, PromoCell) were cultured at 37°C, 5% CO_2 in smooth muscle cell growth medium added to growth medium supplement mix and 1% penicillin-streptomycin, and endothelial cell growth medium MV2, added to

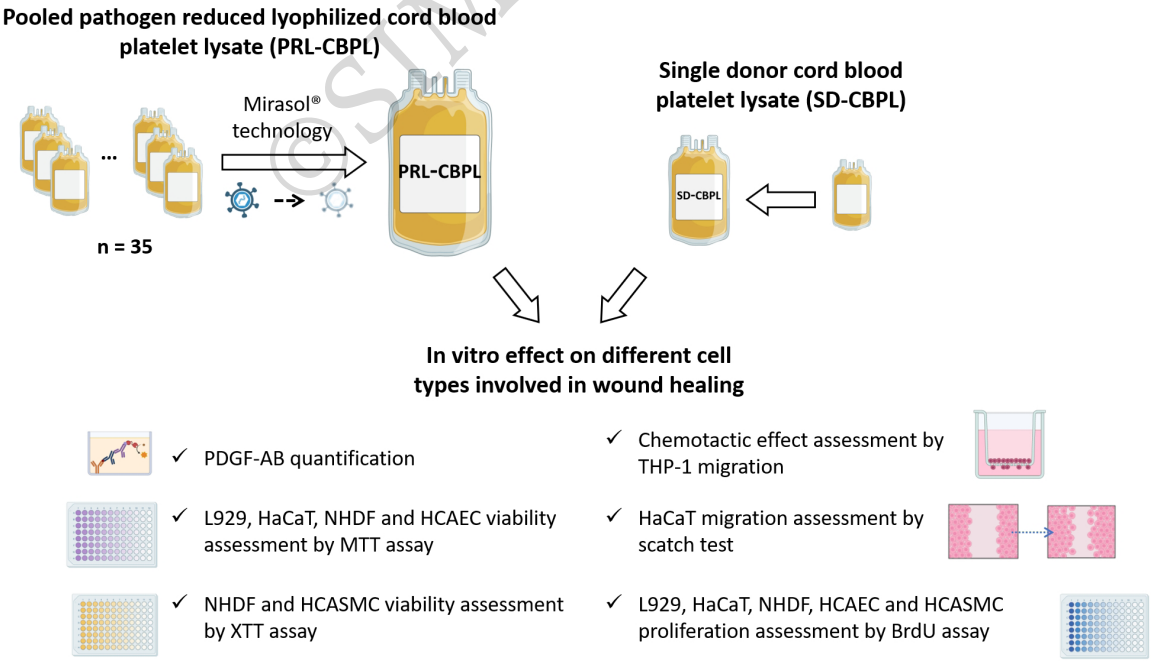


Figure 1 - Schematic representation of the study methods

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growth medium MV2 supplement pack and 1% penicillin-streptomycin, respectively. All the reagents for human coronary artery cells were purchased from PromoCell.

The medium was changed every three days, and the HCASMC and HCAEC cells were split at 70-80% confluence. Primary cells were used at passages 4-10.

Preliminary investigations were performed to identify the proper heparin concentration to avoid gel formation in *in vitro* cell cultures with CBPL (*Online Supplementary materials*).

Cell viability assay

The L929 and NHDF (4×10^3 cells/well), HaCaT (8×10^3 cells/well), HCAEC (8×10^3 cells/well) and HCASMC (3.5×10^3 cells/well) were seeded into 96-well plates. After 24 hours of incubation, the complete medium was replaced with 200 µL/well of serum-free medium containing 1, 3, 5, 10, or 20% (v/v) of PRL-CBPL or SD-CBPL with or without 4.88 U/mL of heparin (Epsoclar, Pfizer, New York, NY, USA). After 72 h of cell treatment at 37°C, an MTT assay was performed to assess the viability of L929, HaCaT, HCAEC, and HCASMC while an XTT assay was performed on NHDF cells.

For the MTT assay, 20 µL of MTT phosphate-buffered solution (0.5 mg/mL) was added to each well and incubated for 3 h at 37°C. The supernatant was removed and replaced with 100 µL/well of DMSO to solubilize the MTT tetrazolium dye. A microplate reader (Spectrafluor Plus; TECAN Austria GmbH, Grödig, Austria) was used to measure the optical density (OD) at 550 nm.

For the XTT assay, 100 µL of XTT solution was added to each well and, after 4 h of incubation at 37°C, and the absorbance of the supernatant was measured at 450 and 620 nm using the microplate reader.

For both of the assays performed, the percentage of cell viability was calculated vs. the complete medium (assumed as 100%) considered as positive control, while the serum-free medium was considered as a negative control.

Cell proliferation assay

Cell proliferation was evaluated by 5-bromo-2'-deoxyuridine (BrdU) assay (Roche Diagnostics, Mannheim, Germany). In short, L929 and NHDF (4×10^3 cells/well), HCASMC (3.5×10^3 cells/well), HaCaT and HCAEC (8×10^3 cells/well) were seeded into 96-well plates. After 24 h of incubation, the medium was replaced with 200 µL/well of serum-free medium containing 1, 3, 5, 10, or 20% (v/v) of PRL-CBPL or SD-CBPL with or without 4.88 U/mL of heparin. Cell proliferation was assessed in accordance with manufacturer's instructions after 48 hours of cell incubation with treatments. The OD was measured at 450 nm using the microplate reader. The percentage of cell proliferation was calculated vs. the complete medium (positive control) assumed as 100% of proliferating cells.

Cell migration assessment

Scratch closure assay was performed as previously reported[13] to evaluate the PL effect on cell migration ability. In short, HaCaT (2×10^5/well) were seeded into 24-well plates, cultured up to confluence, and scratched with a 10 µL pipette tip. Following PBS washes, cultures were treated with 500 µL/well of serum-free medium containing 1, 3, 5, 10, or 20% (v/v) PRL-CBPL or SD-CBPL with or without 4.88 U/mL heparin. Control wells received serum-free medium and complete medium (positive control) as references.

Digital cell images were captured 20 hours after scratching by a phase-contrast microscope (Axiovert 25, Zeiss, Milan, Italy; O.M. 50X) equipped with a digital camera (EOS 1000D, Canon, Milan) for a qualitative analysis of scratch closure with respect to time 0.

Chemotactic effect assessment

Polycarbonate membrane transwell inserts with 8.0 µm pore size (Corning Costar, Cambridge, MA, USA) were used to evaluate monocyte chemotaxis. The lower well chamber was filled with 400 µL of medium containing 1, 3, 5, 10, 20% of PRL-CBPL or SD-CBPL with or without 4.88 U/mL heparin while 1×10^5 THP-1 cells in 200 µL serum-free medium were transferred to the upper chamber of each transwell. THP-1 cells migrate through the polycarbonate filter pores in response to chemoattractant stimuli. After 4 hours of incubation at 37°C and 5% CO_2, the migrated cells were counted by a Bürker chamber under phase-contrast microscopy. Serum-free medium and medium added with 10% FBS were considered as negative and positive control, respectively.

Statistical analysis

Data are presented as the mean ± SD of at least three independent experiments. Statistical analysis was carried out using StatViewTM 5.0 (SAS Institute, Cary, NC, USA). Data were compared using the non-parametric Kruskal-Wallis test with Bonferroni correction as post

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hoc test. A non-parametric Mann-Whitney test was used for HCASMC due to the lack of SD-CBPL data. Values of p<0.05 were considered statistically significant.

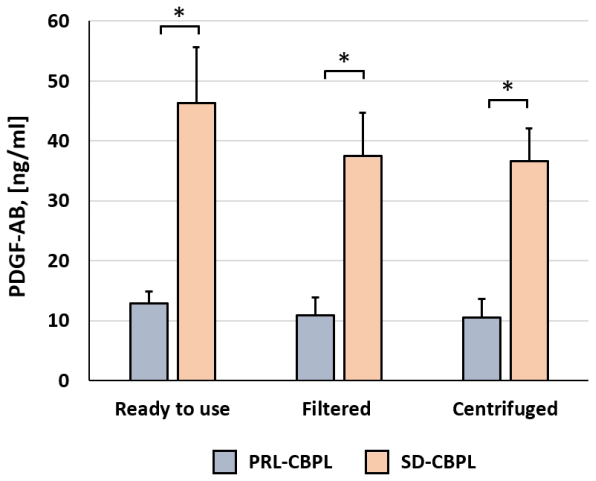


Figure 2 - Mean content of PDGF-AB in ready to use, filtered, or centrifuged PRL-CBPL or SD-CBPL
*p<0.05 PRL-CBPL *vs* SD-CBPL.

RESULTS

PDGF-AB quantification

Figure 2 shows the mean content of PDGF-AB in ready to use, filtered, or centrifuged PRL-CBPL and SD-CBPL.

The mean content of PDGF-AB in reconstituted, filtered and centrifuged PRL-CBPL was 13±2, 11±3 and 11±3 ng/mL respectively. For the thawed, filtered, and centrifuged SD-CBPL, the PDGF-AB mean content was 46±9, 38±7, and 37±5 ng/mL respectively. The mean content of PDGF-AB in SD-CBPL was significantly higher (p<0.05) than in PRL-CBPL.

Cell viability and proliferation assay

Figure 3 shows the effect of 1, 3, 5, and 10% PRL-CBPL or SD-CBPL in culture medium with and without heparin on viability and proliferation of L929 and HaCaT cell lines.

When added to the culture medium at low concentrations (1 and 3%) both PLs produced an increase in HaCaT keratinocyte viability and proliferation, as well as in L929 mouse fibroblast proliferation in comparison to the serum-free medium. However, when higher

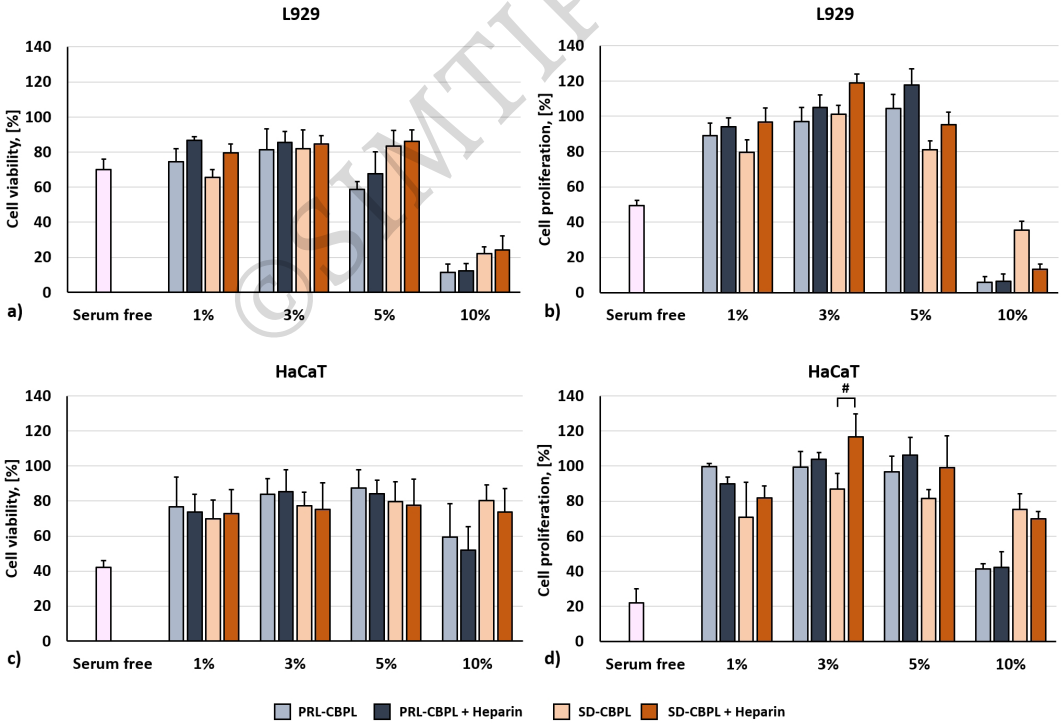


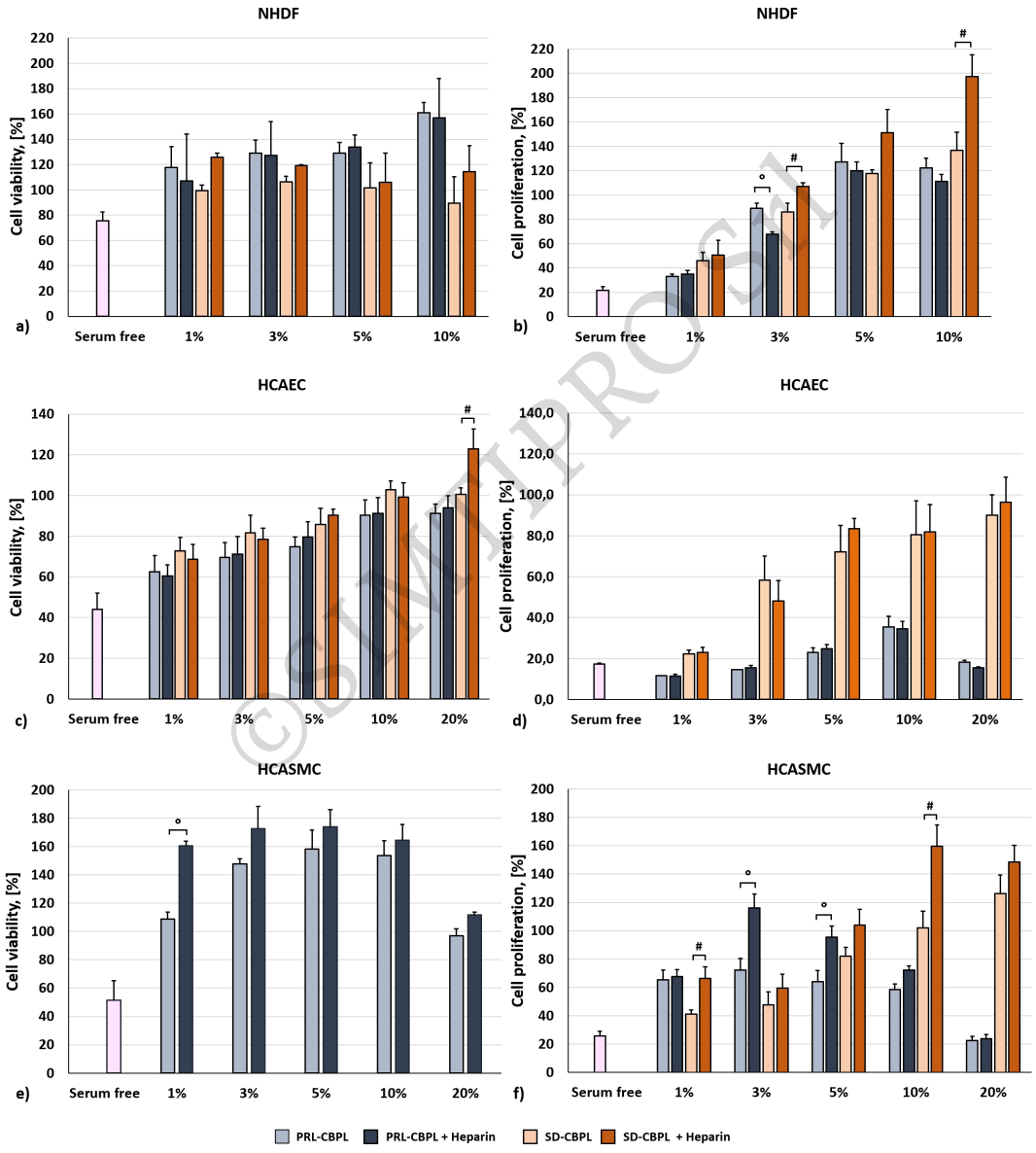
Figure 3 - Effect of 1, 3, 5, and 10% (v/v) PRL-CBPL or SD-CBPL, with and without 4.88 U/mL of heparin on a-b) L929 and c-d) HaCaT viability and proliferation
Cell cultured in medium with 10% FBS were assumed as positive control and 100% cell viability and proliferation. Data shown as mean + standard deviation of three independent experiments. °p<0.05 PRL-CBPL with *vs* without heparin; #p< 0.05 SD-CBPL with *vs* without heparin.

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concentrations of PRL-CBPL were added (5 and 10%), there was a reduction in L929 viability compared to the 3% and the serum-free medium while only the 10% concentration affected L929 cell proliferation. SD-CBPL added at 5% affected L929 proliferation while the 10% concentration resulted in a reduction in both viability and proliferation. The same trend was observed as regards HaCaT cell viability and proliferation for both CBPLs added at high concentrations.

The effect of heparin addition to CBPL was more evident with SD-CBPL. However, an increase in L929 and HaCaT cell viability and proliferation was also observed with PRL-CBPL. For both CBPLs, a 3% concentration could be used instead of 10% FBS for L929 and HaCaT cell culture.

Figure 4 shows the effect of CBPL on NHDF, HCAEC, and HCASMC cell viability and proliferation. These primary cells were responsive at 1% concentrations of both CBPLs,



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Figure 4 - Effect of 1, 3, 5, and 10% (v/v) PRL-CBPL or SD-CBPL, with and without 4.88 U/mL of heparin on viability and proliferation of a-b) NHDF, c-d) HCAEC and e-f) HCASMC

Data shown as mean + standard deviation of three independent experiments. Cells cultured in complete medium were assumed as positive control and 100% cell viability and proliferation; °p<0.05 PRL-CBPL with *vs* without heparin; #p<0.05 SD-CBPL with *vs* without heparin.

but for PRL-CBPL, the optimal concentration for NHDF and HCSMC was 3%, while 10% was required for HCAEC. Heparin addition to PRL-CBPL showed no significant increase in cell growth and proliferation except on HCASMC treated with 1 and 3% PRL-CBPL. Regarding SD-CBPL, the same percentage suggested for PRL-CBPL could be added in serum-free media for NHDF, HCAEC, and HCASMC culture. Unfortunately, due to clot formation, no data was available on SD-CBPL for HCASMC viability. However, NHDF and HCAEC showed increasing cell viability and proliferation up to 20% of CBPL added to serum-free culture media while HCASMC showed decreasing viability and proliferation for incremental concentrations higher than 3%.

Cell migration assessment

Figure 5 shows images captured after 20 h of cell exposure to the treatments. Both CBPLs promoted HaCaT cell migration in the scratch assay. Samples treated with 5% SD-CBPL without heparin were associated with scratch closure. A similar scratch closure rate was observed with 3% PRL-CBPL without heparin. Samples treated with 1% PRL-CBPL or SD-CBPL with and without heparin, with 3% PRL-CBPL or SD-CBPL with heparin, as well as the sample treated with 5 and 10% SD-CBPL with heparin, showed a scratch closure rate similar to 10% FBS. On the other hand, the samples treated with 5 and 10% PRL-CBPL with and without heparin showed a lower closure rate than the positive control. In particular, after 20 h of incubation, according to the MTT assay,

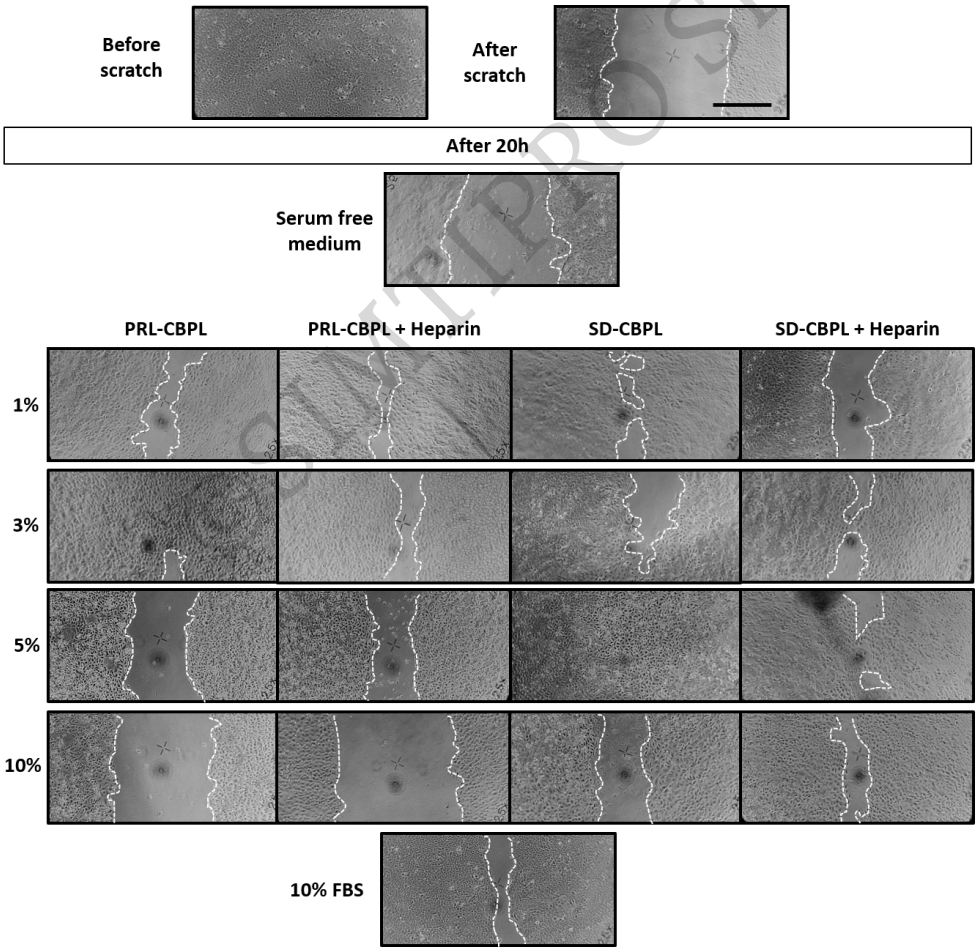


Figure 5 - Images were captured immediately before and after scratching, and after 20 h of HaCaT cell exposure to serum-free medium, culture medium with 1, 3, 5, and 10% of PRL-CBPL or SD-CBPL with and without heparin, and complete culture medium (10% FBS, assumed as positive control)
Images are representative of three independent experiments. Scale bar 500 µm.

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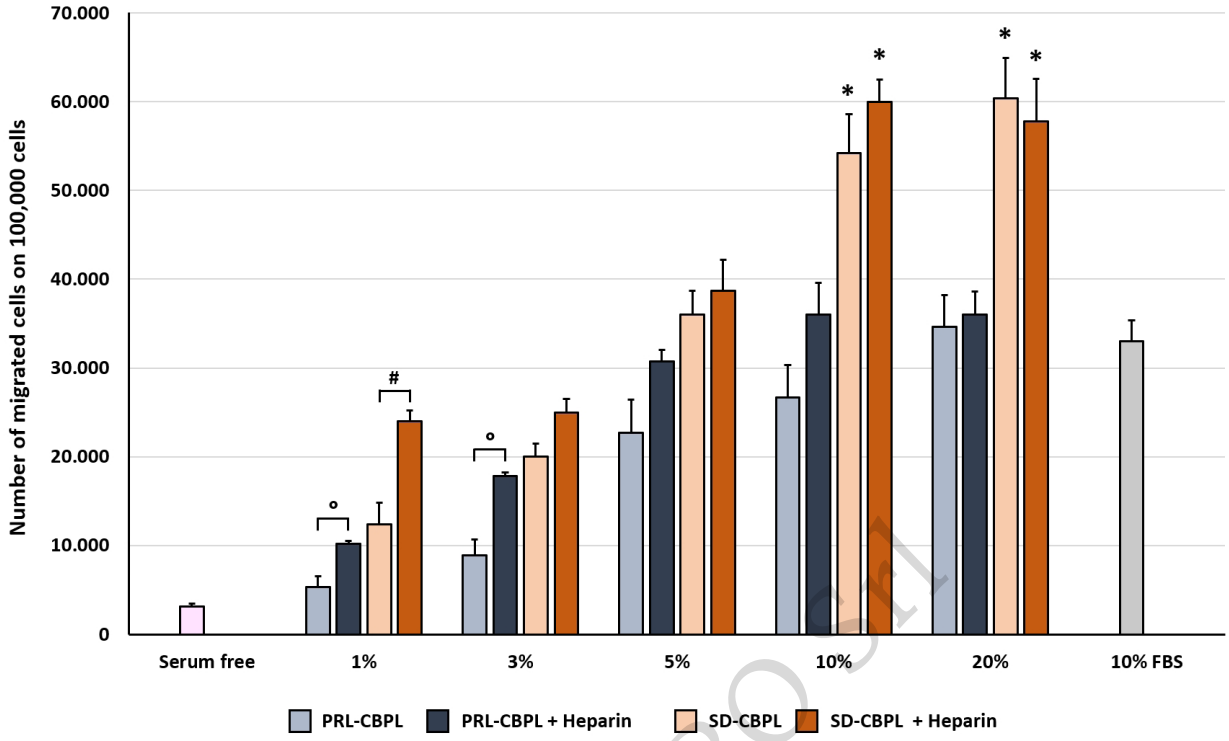


Figure 6 - Chemotactic effect of 1, 3, 5, 10, and 20% PRL-CBPL or SD-CBPL with and without heparin on THP-1 cells
Serum-free and complete medium (10% FBS) were considered as negative and positive controls, respectively. All samples showed statistically significant differences ($p<0.05$) *vs* serum-free medium; *$p<0.05$ *vs* complete medium; °$p<0.05$ PRL-CBPL with *vs* without heparin; #$p<0.05$ SD-CBPL with *vs* without heparin.

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the 10% concentration had a scratch closure rate similar to the negative control. Overall, the scratch closure rate was higher for the samples treated with the lower CBPL concentrations (1 and 3%) without heparin with respect to the samples with heparin added.

Chemotactic effect assessment

An increase in the number of THP-1 migrated cells was observed at up to 20% concentrations of both CBPLs. However, SD-CBPL showed a higher number of migrated cells with respect to PRL-CBPL. Moreover, SD-CBPL with and without heparin showed a significant increase ($p<0.05$) in migrated cells at all concentrations tested compared to serum-free samples, while 5, 10, and 20% concentrations also produced a significant increase ($p<0.05$) in comparison to 10% FBS. Heparin addition to both CBPLs caused an increase in migrated cells, which was significant ($p<0.05$) for 1, 3, 5 and 10% concentrations of PRL-CBPL and for 1 and 3% concentrations of SD-CBPL (**Figure 6**).

DISCUSSION

This study investigated the effect of concentrations of PRL-CBPL, a novel lyophilized PL obtained from pooled CB platelet units, on the viability and proliferation of different cell lines and primary cells, on keratinocytes migration, as well as its chemotactic effect on human monocytes. As a comparator, we tested a single donor PL from CB, whose efficacy in wound healing applications has already been reported in *in vitro* and clinical studies[12,13,31,32].

The amount of PDGF-AB was lower in PRL-CBPL with respect to SD-CBPL, whose PDGF-AB was in line with data reported in the literature[33,34]. This was probably an effect of the treatment performed for pathogen reduction[35]. However, in order to properly evaluate the effect of pathogen reduction on the amount of growth factor, more growth factors need to be considered, and quantification also needs to be carried out on untreated samples.

Both PRL-CBPL and SD-CBPL contain platelet fragments related to the freeze-thaw procedure used to promote

platelet activation and growth factor release. We investigated sterile filtration and centrifugation to remove platelet fragments and reduce the risk of clot formation[36]. The amount of PDGF-AB in PRL-CBPL was not affected by filtration and centrifugation while in SD-CBPL the additional physical process caused a significant reduction in the amount of PDGF-AB. Moreover, there was a great variation in the amount of PDGF-AB in the three production lots tested. These results point to an advantage in using PRL-CBPL with respect to SD-CBPL in terms of standardised product.

Since PL is also rich in fibrinogen, thrombocyte-derived factors, and clotting factors which can lead to fibrin clot formation[37], when it is used in culture medium instead of FBS, other substances like heparin must be added to prevent coagulation[38]. Moreover, heparin is a drug with a similar structure to heparan which binds and potentiates the signalling of angiogenic growth factors, including fibroblast growth factor (FGF) 2 and vascular endothelial growth factor (VEGF)-A[39].

Based on data reported in the literature[40], heparin was added at concentrations up to 4.88 U/mL to both CBPL. Gel formation was occasionally observed only for SD-CBPL even though 4.88 U/mL heparin was added to 1, 3, or 5% supplement concentrations in high glucose DMEM and smooth muscle cell growth medium leading to the choice of a different viability assay, the XTT, to quantify NHDF and HCASMC cell viability. Moreover, SD-CBPL showed clot formation in the same heparin and supplement concentration conditions observed among the three tested production lots. This difference in behaviour might be due to the concentration of calcium ions in different culture media. In particular, high glucose DMEM and smooth muscle cell growth medium are richer in calcium ions than other tested media, causing a reduced effect of citrate anticoagulant[26,11]. Therefore, PRL-CBPL has the additional advantage that it can be used without heparin.

The tested CBPL induced different biological effects in the cells involved in wound healing. In particular, the L929 mouse fibroblast cell line could be cultured using 3% PRL-CBPL or SD-CBPL instead of 10% FBS. Heparin had no significant effect on L929 viability and proliferation. The same effect was observed on HaCaT human keratinocytes. However, both cell lines were responsive in a positive way to higher CBPL concentrations up to 5%, while concentrations above that resulted in reduced cell viability and proliferation, probably due to an excess of nutrients. Our results are in line with previous data by Baik *et al.*[41], who used three pooled peripheral PL on HaCaT cells and found that the most effective supplement concentration was 5%, while 10% resulted in reduced cell proliferation.

NHDF cells were responsive to 1% concentrations of both CBPLs. However, the optimal concentration of both PRL-CBPL and SD-CBPL was 3%, showing cell viability and proliferation data comparable with 10% FBS. Hashemi *et al.*[12] reported 5 and 10% as being the most effective concentrations of CB PRP with regard to primary human skin fibroblast proliferation. These differences in optimal concentrations are probably due to the immediate availability of growth factor in CBPL with respect to PRP.

To the best of our knowledge, this is the first study to evaluate the effect of CBPL on HCAEC and HCASMC. The HCAEC and HCASMC primary cells were more susceptible to the CBPL concentrations added to the culture media than the previously described cell lines. Both primary cells were responsive to the lowest tested concentration (1%) of both CBPL, although 3% PRL-CBPL or SD-CBPL was the optimal concentration for HCASMC, while 10% was required for HCAEC. SD-CBPL required heparin addition to avoid clot formation in culture media for HCASMC. This effect was not observed for PRL-CBPL probably due to a smaller amount of fibrinogen or clotting factors. Moreover, HCASMC showed decreasing viability and proliferation for incremental CBPL concentrations higher than 3%. In L929, HaCaT, and HCASMC, reduced viability was observed at higher CBPL concentrations, as previously observed using SD-CBPL onto L929 and human umbilical vein endothelial cells[13]. However, more investigations need be performed taking into account more concentrations of both CBPLs, as well as other cell types to determine the optimal concentration for *in vitro* cell culture and regenerative applications.

With regard to the effect of CBPL on HaCaT cell migration, the optimum concentrations were 3 and 5% for PRL-CBPL and SD-CBPL respectively, confirming our previous results on SD-CBPL[13]. In this study, adding heparin to the culture medium caused a reduction in the scratch closure rate. PRL-CBPL proved to be more effective than

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SD-CBPL. The reduced HaCaT cell migration observed with 10% concentrations of both CBPLs is in accordance with data obtained on HaCaT cell viability and proliferation. Both CBPLs caused a significant increase in the number of migrated THP-1 cells in a concentration-dependent manner up to 20%. The chemotactic effect of SD-CBPL was more evident compared to PRL-CBPL, probably due to the higher amount of chemokines as demonstrated for PDGF content[42]. In particular, 5% PRL-CBPL added with 4.88 U/mL heparin achieved the same migration value as 10% FBS, while for SD-CBPL the optimum concentration was between 3 and 5% with added heparin.

CONCLUSIONS

This study points to the potential use of PRL-CBPL as a standardized alternative to SD-CBPL, which could reduce the biological variability between individual donors. In addition, CBPL would avoid the risk of animal protein carryover associated with the use of FBS.

FUNDING

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AUTHOR'S CONTRIBUTIONS

Conceptualization: MB, AC, PL; methodology: MB, AC, MF, SG, EC and AM; data curation: AC and PL. writing-original draft: AC and PL; manuscript review and editing: AC, PR and PL; funding acquisition: GS and PL.

CONFLICTS OF INTEREST

PR is scientific director of White Nest Pharma S.r.l. The other Authors declare no competing interests.

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REFERENCES

1. Guiotto M, Raffoul W, Hart AM, Riehle MO, di Summa PG. Human platelet lysate to substitute fetal bovine serum in hMSC expansion for translational applications: a systematic review. J Transl Med 2020; 18: 351. doi: 10.1186/s12967-020-02489-4.
2. Valentini CG, Nuzzolo ER, Bianchi M, Orlando N, Iachininoto MG, Pinci P, et al. Cord blood platelet lysate: in vitro evaluation to support the use in regenerative medicine. Mediterr J Hematol Infect Dis 2019; 11: e2019021. doi: 10.4084/MJHID.2019.021.
3. Orlando N, Pellegrino C, Valentini CG, Bianchi M, Barbagallo O, Sparnacci S, et al. Umbilical cord blood: Current uses for transfusion and regenerative medicine. Transfus Apher Sci 2020; 59: 102952. doi: 10.1016/j.transci.2020.102952.
4. Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, Kusumoto K. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. Plast Reconstr Surg 2008; 122: 1352-1360. doi: 10.1097/PRS.0b013e3181882046.
5. Mishra A, Tummala P, King A, Lee B, Kraus M, Tse V, et al. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. Tissue Eng Part C Methods 2009, 15: 431-435. doi: 10.1089/ten.tec.2008.0534.
6. Wang K; Li Z, Li J, Liao W, Qin Y, Zhang N, et al. Optimization of the platelet-rich plasma concentration for mesenchymal stem cell applications. Tissue Eng Part A 2019; 25: 333-351. doi: 10.1089/ten.TEA.2018.0091.
7. Burnouf T, Strunk D, Koh MB, Schallmoser K. Human platelet lysate: replacing fetal bovine serum as a gold standard for human cell propagation? Biomaterials 2016; 76: 371-387. doi: 10.1016/j.biomaterials.2015.10.065.
8. Kirsch M, Rach J, Handke W, Seltsam A, Pepelanova I, Strauß S, et al. Comparative analysis of mesenchymal stem cell cultivation in fetal calf serum, human serum, and platelet lysate in 2D and 3D systems. Front Bioeng Biotechnol 2021; 8: 598389. doi: 10.3389/fbioe.2020.598389.
9. Czapla J, Matuszczak S, Kulik K, Wiśniewska E, Pilny E, Jarosz-Biej M, et al. The effect of culture media on large-scale expansion and characteristic of adipose tissue-derived mesenchymal stromal cells. Stem Cell Res Ther 2019; 10: 235. doi: 10.1186/s13287-019-1331-9.
10. Sovkova V, Vocetkova K, Rampichova M, Mickova A, Buzgo M, Lukasova V, et al. Platelet lysate as a serum replacement for skin cell culture on biomimetic PCL nanofibers. Platelets 2018; 29: 395-405. doi: 10.1080/09537104.2017.1316838.
11. Mohamed HE, Asker ME, Kotb NS, El Habab AM. Human platelet lysate efficiency, stability, and optimal heparin concentration required in culture of mammalian cells. Blood Res 2020; 55: 35-43. doi: 10.5045/br.2020.55.1.35.
12. Hashemi SS, Mahmoodi M, Rafati AR, Manafi F, Mehrabani D. The role of human adult peripheral and umbilical cord blood platelet-rich plasma on proliferation and migration of human skin fibroblasts. World J Plast Surg 2017; 6: 198-205. PMID: 28713711.
13. Losi P, Barsotti MC, Foffa I, Buscemi M, De Almeida CV, Fabbri M, et al. In vitro human cord blood platelet lysate characterisation with potential application in wound healing. Int Wound J 2020; 17: 65-72. doi: 10.1111/iwj.13233.
14. Shirzad N, Bordbar S, Goodarzi A, Mohammad M, Khosravani P, Sayahpour F, et al. Umbilical cord blood platelet lysate as serum substitute in expansion of human mesenchymal stem cells. Cell J 2017; 19: 403-414. doi: 10.22074/cellj.2017.4886.
15. Ng SL, Azhar NA, Budin SB, Ibrahim N, Abdul Ghani NA, Abd Ghafar N, et al. Effects of platelet lysate gels derived from different blood sources on oral mucosal wound healing: an in vitro study. Gels 2023; 9: 343. doi: 10.3390/gels9040343.
16. Parazzi V, Lazzari L, Rebulla P. Platelet gel from cord blood: a novel tool for tissue engineering. Platelets 2010; 21: 549-554. doi: 10.3109/09537104.2010.514626.
17. Parazzi V, Lavazza C, Boldrin V, Montelatici E, Pallotti F, Marconi M, et al. Extensive characterization of platelet gel releasate from cord blood in regenerative medicine. Cell Transplant 2015; 24: 2573-2584. doi: 10.3727/096368915X687471.
18. González MB, Cuerva RC, Muñoz BF, Rosell-Valle C, López MM, Arribas BA, et al. Optimization of human platelet lysate production and pathogen reduction in a public blood transfusion center. Transfusion 2022; 62: 1839-1849. doi: 10.1111/trf.17045.
19. Azmi AF, Yahya MAAM, Azhar NA, Ibrahim N, Ghafar NA, Ghani NAA, et al. In vitro cell proliferation and migration properties of oral mucosal fibroblasts: a comparative study on the effects of cord blood- and peripheral blood-platelet lysate. Int J Mol Sci 2023; 24: 5775. doi: 10.3390/ijms24065775.
20. Rebulla P, Pupella S, Santodirocco M, Greppi N, Villanova I, Buzzi M, et al. Italian Cord Blood Platelet Gel Study Group (see Appendix 1). Multicentre standardisation of a clinical grade procedure for the preparation of allogeneic platelet concentrates from umbilical cord blood. Blood Transfus 2016; 14: 73-79. doi: 10.2450/2015.0122-15.
21. Cui X, Ma Y, Wang H, Huang J, Li L, Tang J, et al. The anti-photoaging effects of pre- and post-treatment of platelet-rich plasma on UVB-damaged HaCaT keratinocytes. Photochem Photobiol 2021; 97: 589-599. doi: 10.1111/php.13354.

22. Murphy MB, Blashki D, Buchanan RM, Yazdi IK, Ferrari M, Simmons PJ, et al. Adult and umbilical cord blood-derived platelet-rich plasma for mesenchymal stem cell proliferation, chemotaxis, and cryo-preservation. Biomaterials 2012; 33: 5308-5316. doi: 10.1016/j.biomaterials.2012.04.007.
23. Horn P, Bokermann G, Cholewa D, Bork S, Walenda T, Koch C, et al. Impact of individual platelet lysates on isolation and growth of human mesenchymal stromal cells. Cytotherapy 2010; 12: 888-98. doi: 10.3109/14653249.2010.501788.
24. Niemann M, Ort M, Lauterbach L, Streitz M, Wilhelm A, Grütz G, et al. Individual immune cell and cytokine profiles determine platelet-rich plasma composition. Arthritis Res Ther 2023; 25: 6. doi: 10.1186/s13075-022-02969-6.
25. Burnouf T, Chou ML, Lundy DJ, Chuang EY, Tseng CL, Goubran H. Expanding applications of allogeneic platelets, platelet lysates, and platelet extracellular vesicles in cell therapy, regenerative medicine, and targeted drug delivery. J Biomed Sci 2023; 30: 79. doi: 10.1186/s12929-023-00972-w.
26. Barro L, Burnouf PA, Chou ML, Nebie O, Wu YW, Chen MS, et al. Human platelet lysates for human cell propagation. Platelets 2021; 32: 152-162. doi: 10.1080/09537104.2020.1849602.
27. Viau S, Lagrange A, Chabrand L, Lorant J, Charrier M, Rouger K,et al. A highly standardized and characterized human platelet lysate for efficient and reproducible expansion of human bone marrow mesenchymal stromal cells. Cytotherapy 2019; 21: 738-754. doi: 10.1016/j.jcyt.2019.04.053.
28. Pierce J, Benedetti E, Preslar A, Jacobson P, Jin P, Stroncek DF, et al. Comparative analyses of industrial-scale human platelet lysate preparations. Transfusion 2017; 57: 2858-2869. doi: 10.1111/trf.14324.
29. Martinotti S, Ranzato E. Scratch wound healing assay. Methods Mol Biol 2020; 2109: 225-229. doi: 10.1007/7651_2019_259.
30. Teplicki E, Ma Q, Castillo DE, Zarei M, Hustad AP, Chen J, Li J. The Effects of aloe vera on wound healing in cell proliferation, migration, and viability. Wounds 2018 ; 30: 263-268.
31. Volpe P, Marcuccio D, Stilo G, Alberti A, Foti G, Volpe A, et al. Efficacy of cord blood platelet gel application for enhancing diabetic foot ulcer healing after lower limb revascularization. Semin Vasc Surg 2017; 30: 106-112. doi: 10.1053/j.semvascsurg.2017.12.001.
32. Lambadiari V, Kountouri A, Psahoulia F, Koliou GA, Lazaris A, Michalopoulos E, et al. Treatment with umbilical cord blood platelet lysate gel improves healing of diabetic foot ulcer. J Clin Med 2024; 13: 1310. doi: 10.3390/jcm13051310.
33. Barsotti MC, Losi P, Briganti E, Sanguinetti E, Magera A, Al Kayal T, et al. Effect of platelet lysate on human cells involved in different phases of wound healing. PLoS One 2013; 8: e84753. doi: 10.1371/journal.pone.0084753.
34. Pulcini S, Merolle L, Marraccini C, Quartieri E, Mori D, Schiroli D, et al. apheresis platelet rich-plasma for regenerative medicine: an in vitro study on osteogenic potential. Int J Mol Sci 2021; 22: 8764. doi: 10.3390/ijms22168764.
35. Valsami S, Grouzi E, Mochandreou D, Pouliakis A, Piroula-Godoy M, Kokori S, et al. Effect of mirasol pathogen reduction technology system on immunomodulatory molecules of apheresis platelets. Transfus Apher Sci 2023; 62: 103523. doi: 10.1016/j.transci.2022.103523.
36. Oota-Ishigaki A, Maruyama O, Sakota D, Kosaka R, Hijikata W, Nishida M. Quantitative investigation of platelet aggregation under high shear force for anti-platelet aggregation in vitro tests. Int J Artif Organs 2021; 44: 687-693. doi: 10.1177/03913988211020765.
37. Hemeda H, Giebel B, Wagner W. Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. Cytotherapy 2014; 16: 170-180. doi: 10.1016/j.jcyt.2013.11.004.
38. Kee LT, Lee YT, Ng CY, Hassan MNF, Ng MH, Mahmood Z, et al. Preparation of fibrinogen-depleted human platelet lysate to support heparin-free expansion of umbilical cord-derived mesenchymal stem cells. Biology (Basel) 2023; 12: 1085. doi: 10.3390/biology12081085.
39. Kim HN, Elgundi Z, Lin X, Fu L, Tang F, Moh ESX, et al. Engineered short forms of perlecan enhance angiogenesis by potentiating growth factor signalling. J Control Release 2023; 362: 184-196. doi: 10.1016/j.jconrel.2023.08.052.
40. Hemeda H, Kalz J, Walenda G, Lohmann M, Wagner W. Heparin concentration is critical for cell culture with human platelet lysate. Cytotherapy 2013; 15: 1174-1181. doi: 10.1016/j.jcyt.2013.05.006.
41. Baik SY, Lim YA, Kang SJ, Ahn SH, Lee WG, Kim CH. Effects of platelet lysate preparations on the proliferation of HaCaT cells. Ann Lab Med 2014; 34: 43-50. doi: 10.3343/alm.2014.34.1.43.
42. Fekete N, Gadelorge M, Fürst D, Maurer C, Dausend J, Fleury-Cappellesso S, et al. Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components. Cytotherapy 2012; 14: 540-554. doi: 10.3109/14653249.2012.655420.

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