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Comparison of human platelet lysate alternatives using expired and freshly isolated platelet concentrates for adipose-derived stromal cell expansion

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Abstract

Pooled human platelet lysate (pHPL) has been used to expand adipose-derived stromal cells (ASCs) and can be formulated using fresh or expired buffy coats (BCs) which are then resuspended in either plasma or an additive solution. Not much is known about the effects that expired products and additive solutions have on ASC expansion, and the need for quality control and release criteria has been expressed. This pilot study compared proliferation, cell size, morphology and immunophenotype of ASCs expanded in the different pHPL alternatives versus foetal bovine serum (FBS). Quality control criteria were assessed prior to and during the manufacture of the pHPL alternatives. ASCs were then expanded in 1%, 2.5%, 5% or 10% of the different pHPL alternatives or in 10% FBS. Cell size, morphology, cell number and immunophenotype were measured using microscopy and flow cytometry. The majority of the pHPL alternatives were within the recommended ranges for the quality control criteria. ASCs expanded in the pHPL alternatives were smaller in size, displayed a tighter spindle-shaped morphology, increased cell growth and had a similar immunophenotype (with the exception of CD34 and CD36) when compared to ASCs expanded in FBS. Here we report on the effects that expired BC products and additive solutions have on ASC expansion. When taken together, our findings indicate that all of the pHPL alternatives can be considered to be suitable replacements for FBS for ASC expansion, and that expired BC products can be used as an alternative to fresh BC products.

Introduction

Adipose-derived stromal cells (ASCs) are multipotent cells with immunomodulatory properties that can be isolated from adipose tissue (1). These properties have resulted in the use of ASCs for cellular therapies, as evidenced by the fact that their therapeutic effect is being assessed in over 180 clinical trials (2,3). Of those clinical trials that have listed their expansion conditions and media preparation (4), the majority make use of foetal bovine serum (FBS), which has had adverse reactions on patients receiving the cells (4). While FBS is essential for ASC attachment, expansion and maintenance, it does harbour the potential for xenoimmunisation and the transmission of zoonotic contaminants (5). In an attempt to circumvent this, many research groups have moved away from FBS as the

traditional serum supplementation in ASC expansion. Furthermore, the preparation of ASCs for cellular therapies needs to be compliant with good manufacturing practices (GMP). This can be achieved, in part, by changing to chemically defined, xeno-free or human blood-derived alternatives (4,6). These human blood-derived alternatives include human serum, platelet-rich plasma, platelet-poor plasma and platelet lysate (7–10). Studies comparing these alternatives make use of guidelines set out by the International Society of Cellular Therapy (ISCT) and International Fat Applied Technology Society for the characterisation of ASCs (11,12). These guidelines propose a set of criteria that need to be met when expanding ASCs which include their ability to adhere to plastic, express a predefined surface marker profile (immunophenotype) and have the ability to differentiate into adipose tissue, bone and cartilage (11,12). *In vitro* studies that have used human alternatives for ASC expansion have described faster proliferation, better morphology and a comparable immunophenotype for certain surface markers (7–9,13,14). In contrast, these studies have also reported differing trilineage differentiation capacities. It has therefore been suggested that the choice of human alternative should be determined on the basis of the proposed downstream application of the ASCs (8,15–17). Human platelet lysate (HPL) has been used in the past few years as an alternative to FBS for GMP-compliant ASC expansion and is being used in several clinical trials (3,15,18,19). Both autologous (recipient-derived) and allogeneic (donor-derived) HPL have been investigated for ASC

expansion with favourable outcomes (20,21). While autologous platelet lysate negates the need for testing for disease-causing agents, it is not always possible to produce sufficient volumes for expansion of ASCs. As an alternative, allogeneic platelet lysate can be pooled (pooled human platelet lysate, pHPL) in order to reduce batch-to-batch variation and to create larger quantities for ASC expansion (9,22). HPL is produced from platelet concentrates (PCs) which have been subjected to a series of freeze–thaw cycles and centrifugation, which leads to the release of bioactive molecules. One PC unit is comprised of four buffy coat (BC) units (the white blood cell and platelet fraction obtained following centrifugation), and is resuspended in either one unit of platelet-rich plasma or another additive solution (22,23). Expired PCs, which are usually discarded by blood banks after 4 or 5 days, can also be used to manufacture HPL, although there are very few reports on the use of expired PC products for the expansion of ASCs (19,24). In this study, we have investigated the effects that different pHPL alternatives (fresh versus expired BCs resuspended in either platelet-rich plasma or an additive solution), produced using quality controlled manufacturing protocols, have on ASC proliferation, morphology and immunophenotype.

Materials and methods

pHPL manufacture

Four different PC formulations were used in this study and are summarised in Table I. Two PCs for each of the alternatives (Table I) were obtained from the South African National Blood Service (SANBS). The expired PCs were stored on a platelet shaker at 20–24°C for 5 days after collection, whereafter they were considered expired. The fresh PCs were manufactured directly after collection. The pHPL alternatives were manufactured (Figure 1) as previously described (22). The bags were frozen at −25°C for 24 hours after which the PCs were thawed in a protective cover (SteriZip™, OriGen Biomedical, Texas, USA) using a water bath (EcoBath, Labotec, South Africa) at 37°C. Once completely thawed, the two PC bags were pooled in a double pooling bag (Sterile 3 litre waste bag, Haemonetics Corporation, Massachusetts, USA) using a sterile connection device (TCD® Sterile Tube Welder with wafers, Genesis, New Jersey, USA) and sealed using a sterile tube sealer (Rapid Seal II™ SE540, Genesis, New Jersey, USA). The contents were mixed thoroughly and an aliquot was taken into a small Baxter bag (PEDI-PAK® Single 75 mL Transfer Bags, Genesis, New Jersey, USA) for sterility and pH testing. Roughly 250 mL of the pooled PCs was transferred from the double pooling bag into 600 mL Baxter bags (Platelet Transfer and Storage Bags, Macopharma, Mouvaux, France) and frozen at −25°C for 24 hours. The bags were thawed again and the contents of the bags were then transferred, under sterile conditions in a laminar flow cabinet, into 50 mL conical tubes (Corning, NY, USA). The 50 mL tubes were centrifuged at 4000g at 4°C for 15 min after which the remaining supernatant (pHPL) was transferred to new 50 mL tubes and stored at −25°C for future use.

Table I. Different types of PCs, their composition and their designated name.

pHPL alternative	BC unit	Resuspension unit
BCs + Plasma	4× freshly isolated BCs	Platelet-rich plasma
BCs + TSOL	4× freshly isolated BCs	Additive solution (T-Sol[a])
Exp BCs + Plasma	4× expired BCs	Platelet-rich plasma
Exp BCs + TSOL	4× expired BCs	Additive solution (T-Sol)

[a]Thrombocyte solution (Adcock Ingram, Johannesburg, South Africa).

Quality control criteria for the pHPL alternatives

Quality control criteria (Table II) were assessed on the PCs prior to and during the manufacture of the different pHPL alternatives (22). The criteria that were measured included platelet content, platelet volume, sterility, the presence of residual leukocytes and pH.

Platelet count

Before the first freeze step, an aliquot was taken from each PC bag and used to determine the initial platelet count using a Gallios flow cytometer (Beckman Coulter, Miami, USA). The absolute number of platelets was obtained by adding 20 μL of the pHPL alternatives, 20 μL Flow-Count™ fluorospheres (Beckman Coulter, Miami, USA) and either 5 μL mouse anti-human CD42a-FITC or 5 μL mouse anti-human CD61-PE monoclonal antibodies to separate flow cytometry tubes (Beckman Coulter, Miami, USA). The cells were incubated for 10 min in the dark at room temperature (RT), whereafter they were analysed on a Gallios flow cytometer (Beckman Coulter, Miami, USA). Flow cytometry data were analysed post-acquisition using the Kaluza Post-Acquisition Flow Cytometry Analysis software (Version 1.3; Beckman Coulter, Miami, USA). The flow cytometric protocols and gating strategies (Figure S1) are summarised in the supplementary data.

Volume

Prior to the manufacture of the pHPL alternatives, each PC bag was weighed and the volume was calculated using the formula $V = m/SG$, where V is the volume, m is the mass and SG is the specific gravity (1.0.32, as used by the SANBS).

Sterility test

Sterility tests were performed on the PCs by the SANBS by making use of the Bactec9120 and the BactAlert 3D blood culture systems using their in-house protocols.

Residual leukocytes

Residual leukocytes were measured using the DNA Prep kit (Beckman Coulter, Miami, USA). Briefly, 100 μL cell suspension from each pHPL alternative and 100 μL LPR lysing buffer were added to a flow tube, vortexed and incubated at RT for 5 min. A 500 μL DNA Prep stain containing 50 μL/mg propidium iodide was added and incubated in the dark at RT. After 10 min, 100 μL Flow-Count™ fluorospheres was added to the tube and analysed on a Gallios flow cytometer. The flow cytometry protocols and gating strategies (Figure S2) are summarised in the supplementary data section.

pH

pH was measured in the aliquots taken during the pooling step of the pHPL manufacturing process. Aliquots were stored at 4°C and after 5 days (the clinically accepted shelf-life of PCs) and pH was measured using a CRISON micropH 2001 pH metre at 22°C (Crison Instruments, Barcelona, Spain).

Preparation of media

Media containing four concentrations (1%, 2.5%, 5% and 10%) of each of the pHPL alternatives was prepared as previously described with minor modifications (22). Prior to the preparation of the media, the pHPL alternatives were heated to 37°C. The following reagents were added to alpha Modified Eagle's Medium (αMEM) (GIBCO, Life Technologies™, New York, USA): 2%

Figure 1. A schematic illustration of the pHPL manufacturing process. Adapted from Schallmoser and Strunk (2013) (22).
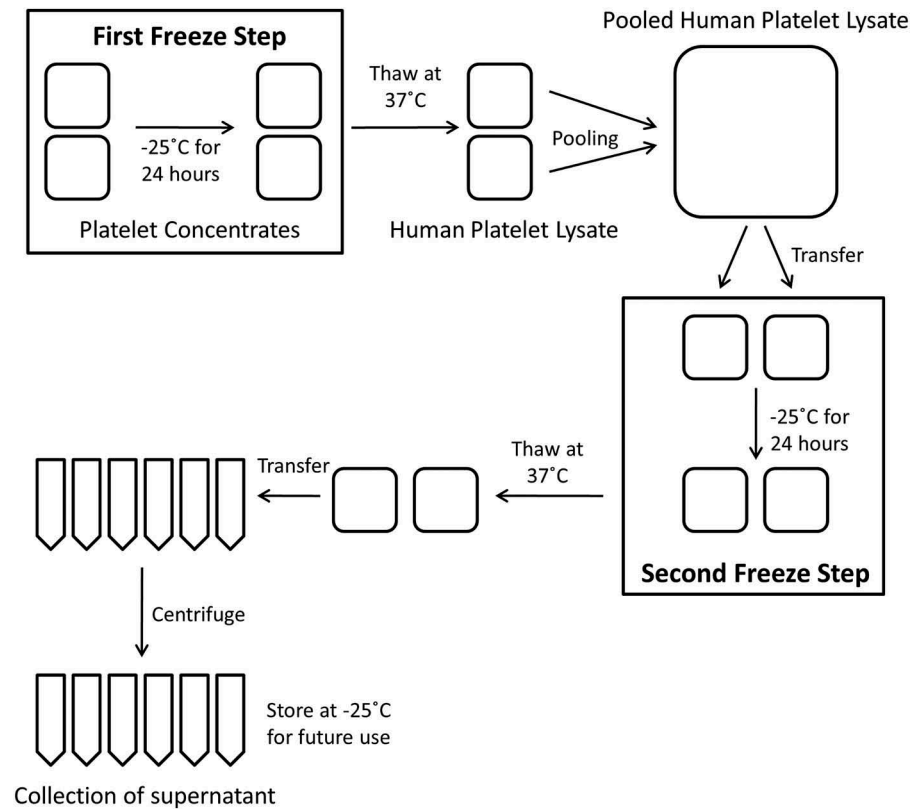

Table II. Quality control criteria, requirements and their outcomes in the pHPL alternatives.

Quality control criteria and requirements (the latter in parenthesis)	BCs + Plasma	BCs + TSOL	Exp BCs + Plasma	Exp BCs + TSOL
Platelet content ($>240 \times 10^9$ per four pooled units)	1179.64×10^9 $\pm 167.53 \times 10^9$	633.27×10^9 $\pm 36.88 \times 10^9$	269.40×10^9 $\pm 3.49 \times 10^9$	76.45×10^9 $\pm 7.47 \times 10^9$
Volume (>40 mL per 60×10^9 platelets)	290.12 ± 56.29	348.45 ± 10.17	328.98 ± 13.54	349.90 ± 3.59
Sterility testing (Negative)	Negative	Negative	Negative	Negative
Residual leukocytes ($<1.0 \times 10^6$ per PC unit)	130 ± 25.22	62.70 ± 0.18	182 ± 7.50	11.6 ± 0.12
pH measured after 5 days at 22°C (>6.4)	6.4	6.6	7.5	6.6

(v/v) penicillin [10 000 U/mL]-streptomycin [10 000 μg/mL] (p/s; GIBCO, Life Technologies™, New York, USA) and preservative-free Heparin ([2 U/mL]; Biochrom, Merck Millipore, Berlin, Germany). Thereafter, different volumes of pHPL and FBS were added to the media to produce the different concentrations of pHPL (1%, 2.5%, 5% or 10%), and FBS (10%) (Biochrom, Merck Millipore, Berlin, Germany). After the addition of pHPL or FBS, the medium was filtered through 0.22 μm sterile filtration units (Corning, New York, USA) and stored at 4°C until future use.

Cell culture and expansion

Three previously isolated (25) cryopreserved ASC samples were thawed by adding pre-warmed (37°C) αMEM containing 10% (v/v) FBS and 2% (v/v) p/s to a cryopreservation tube. The liquid portion containing the cellular fraction was then transferred to a 15 mL conical tube (Corning, New York, USA). The above-mentioned step was repeated until the ASC sample was completely thawed. Once thawed, the cell suspension was centrifuged at 184g for 5 min. The pellet was resuspended, seeded into an 80 cm^2 culture flask (NUNC™, Roskilde Site, Kamstrupvej, Denmark) and cultured in 10% FBS supplemented medium at 37°C in a 5% CO_2 incubator (Thermo Forma CO_2 water jacketed incubator, 3111TF). When the cells reached 70–80% confluence, they were dissociated using trypLE (Life Technologies™, New York, USA) and counted in a Gallios flow cytometer. The ASCs were seeded in duplicate at a density of 5000 cells/cm^2 into six well plates for cell counts and microscopy, and seeded into 80 cm^2 culture flasks for immunophenotyping, after which the respective media were added to the wells and flasks. The ASCs in the six well plates were incubated for 7 days, whereas the cells in the 80 cm^2 culture flasks were cultured to confluence at 37°C in a 5% CO_2 incubator. Fresh medium was added to the wells and flasks every second day.

ASC morphology and size

Fluorescence microscopy and adherent cell size

During the 7-day culture period, the size and morphology of the adherent ASCs was assessed using fluorescent staining

techniques. At roughly 40–50% confluence, the medium was removed and the ASCs were washed twice with PBS. Thereafter, the cytoplasm and nuclei were stained by adding 2 mL of PBS, 20 μL carboxyfluorescein succinimidyl ester (Cell Trace® CSFE, Thermo Fischer, Waltham, Massachusetts, USA) and 2 μL Vybrant® DyeCycle™ Violet (VDC violet; Invitrogen/Molecular Probes®, Life Technologies, Eugene, Oregon, USA) to each well followed by incubation for 15 minutes at 37°C in a 5% CO_2 incubator. After the incubation, the ASCs were washed twice with PBS and resuspended in 1 mL PBS. Immediately thereafter, micrographs (five per condition) were captured using a 10× magnification objective lens in an AxioVert A1 inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany) equipped with an AxioCam Cm1 camera (Carl Zeiss, Gottingen, Germany). Each image represents an overlay converted from two single channel images, where the first channel (yellow-green fluorescence; filter set 9; excitation: bandpass filter 450–490; emission: longpass filter 515) captured the CSFE staining (visualisation of cytoplasm) and the second channel (DAPI fluorescence; filter set 49; excitation: green 365; emission: bandpass filter 445/50) captured VDC Violet staining (visualisation of nuclei). Images were captured using AxioVision software (Version 4.8.2, Carl Zeiss, Gottingen, Germany) and analysed post-acquisition using Image J imaging software (Version 1.49; Schneider et al., 2012) (26). Images were enhanced for contrast and brightness. Thereafter, the images and the ASC sizes were analysed in CellProfiler (27) using the Human Cells pipeline. Pixels were converted to micrometre using a conversion factor of 1 pixel = 0.212 μm^2 as per the microscope specifications.

Light microscopy

After 7 days, the morphology of the adherent ASCs was assessed using a Zeiss AxioVertA1 microscope. Micrographs were taken at 5× magnification using a Zeiss Axiocam digital camera. Images were captured using AxioVision and analysed post-acquisition using Image J imaging software. Images were enhanced for contrast and brightness.

ASC absolute cell number

Absolute ASC number was assessed using flow cytometry after 7 days. Cells were dissociated using trypLE, and cells from each well were prepared for counting by preparing two duplicate flow cytometry tubes containing 50 μL of the cell suspension and 0.5 μL Vybrant® DyeCycle™ Ruby (VDC Ruby; Invitrogen/Molecular Probes®, Life Technologies, Eugene, Oregon, USA). After 5 min incubation in the dark at RT, 50 μL Flow-Count™ fluorospheres and 500 μL PBS were added to each tube. Each of the flow cytometry tubes was then analysed twice (technical replicates) on a Gallios flow cytometer. The flow cytometric protocols and gating strategies (Figure S3) are summarised in the supplementary data section.

Immunophenotype

ASC immunophenotype was measured using a panel of seven fluorochrome-conjugated monoclonal antibodies. The panel consisted of the following antibodies: CD73-FITC (eBiosciences, San Diego, USA); CD105-PE (BioLegend, San Diego, USA); CD34 PE-Cy7 (BioLegend, San Diego, USA); CD36 APC (BioLegend, San Diego, USA); CD44 APC/Cy7 (BioLegend, San Diego, USA); CD90 BV421 (BD Biosciences, San Jose, USA) and CD45 BV510 (BD Biosciences, San Jose, USA). At confluence, the ASCs were dissociated and 100 μL of cell suspension was simultaneously stained with all seven monoclonal antibodies after adding 5 μL of each antibody. The tubes were incubated for 15 minutes in the dark, whereafter 1 mL PBS was added to the tubes and centrifuged at 184g for 5 min. The PBS was aspirated and 700 μL PBS was added to the tube and analysed on a Gallios flow cytometer. Flow cytometry data were analysed post-acquisition using Kaluza Post-Acquisition Flow Cytometry Analysis software (Version 1.3). The intact ASC population was identified using forward scatter vs side scatter two parameter plots. All subsequent flow cytometric plots were gated on the intact ASC population. The positive expression of the different surface markers was identified using a one parameter log histogram plot, and the co-expression profiles were obtained using tree plots.

Statistical analysis

Data are expressed as mean ± SEM. To compare the means between FBS and pHPL expanded ASCs, a Kruskal–Wallis test, followed by a Dunn's post hoc multiple comparisons test with a Bonferroni correction was employed. The significance level for all statistical analyses was set at $\alpha = 0.05$ and a value of $p < 0.05$ was considered to be significant. Data and statistical analysis were performed in RStudio (Version 3.2.3) (28).

Results
Quality control criteria for the pHPL alternatives

Quality control criteria were assessed for all of the alternatives and are summarised in Table II. The platelet content for the Exp BCs + TSOL group (76.45 × 10^9 ± 7.47 × 10^9 platelets) was below the recommended range (>240 × 10^9 platelets). The remaining groups were above the recommended range, and the BCs + plasma group had the highest number of platelets (1179.64 × 10^9 ± 167.53 × 10^9) in the smallest volume (290.12 ± 56.29 mL). All the groups had a volume higher than the recommended volume (>40 mL per 60 × 10^9 platelets). The freshly isolated BC groups (BCs + Plasma and BCs + TSOL) had a greater platelet concentration (platelets/mL, 2.03 × 10^9 ± 0.29 × 10^9 and 0.91 × 10^9 ± 0.05 × 10^9, respectively) when compared to the Exp BC (Exp BCs + Plasma and Exp BCs + TSOL) groups (0.41 × 10^9 ± 0.005 × 10^9 and 0.19 × 10^9 ± 0.15 × 10^9, respectively). No bacterial contamination was found in any of the PCs which were confirmed to be negative by the sterility tests. A few residual leukocytes were detected in each of the pHPL alternatives; however, their number was below the recommended range. The pH for the BCs + Plasma, BCs + TSOL and Exp BCs + TSOL groups was 6.4–6.6, respectively and the Exp BCs + Plasma group was 7.5; however, they were all within the recommended range (>6.4).

ASC morphology and adherent cell size

ASCs expanded in the pHPL alternatives displayed a smaller, tighter elongated shape (Figure 2(B–Q)) when compared to ASCs expanded in FBS (Figure 2(A)). When the cell size was quantitatively assessed, all the pHPL alternatives, except for the 1% BCs + TSOL and 1% Exp BCs + TSOL, were significantly smaller in size when compared to ASCs expanded in FBS (Figure 3). There was no significant difference in cell size between 2.5%, 5% and 10% concentrations in the BCs + TSOL expanded ASCs, Exp BCs + Plasma expanded ASCs and the Exp BCs + TSOL expanded ASCs, where the opposite is true for BCs + Plasma. The 1% concentrations were significantly larger in size when compared to the 10% concentrations in all the pHPL alternatives (Table SI). The pHPL alternatives that were resuspended in plasma had the greatest effect on cell size; whereas the
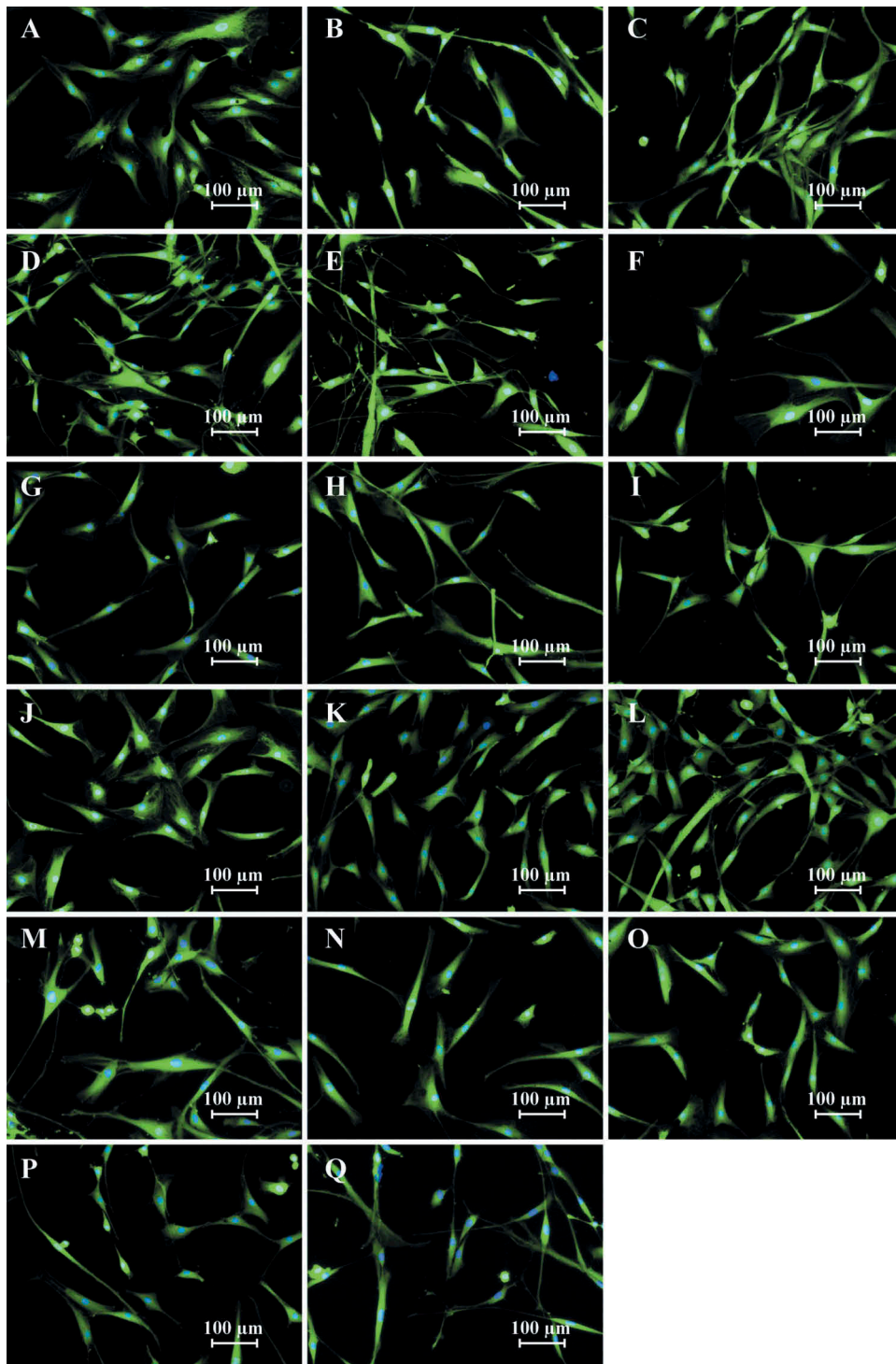
Figure 2. Fluorescence micrographs of ASCs expanded in FBS and the pHPL alternatives at different concentrations. ASCs expanded in (A) 10% FBS; (B) 1% BCs + Plasma; (C) 2.5% BCs + Plasma; (D) 5% BCs + Plasma; (E) 10% BCs + Plasma; (F) 1% BCs + TSOL; (G) 2.5% BCs + TSOL; (H) 5% BCs + TSOL; (I) 10% BCs + TSOL; (J) 1% Expired BCs + Plasma; (K) 2.5% Expired BCs + Plasma; (L) 5% Expired BCs + Plasma; (M) 10% Expired BCs + Plasma; (N) 1% Expired BCs + TSOL; (O) 2.5% Expired BCs + TSOL; (P) 5% Expired BCs + TSOL; (Q) 10% Expired BCs + TSOL; captured at 10× magnification. The cytoplasm was stained with CSFE and the nuclei were stained with VDC Violet. Scale bars represent 100 μm.

alternatives contacting TSOL had the least effect on cell size (Table SI).

ASC absolute cell number

Of the 4 pHPL alternatives, the BCs + Plasma and the Exp BCs + Plasma group had the greatest effect on cell number (Figure 4 (A) & (C)). Cell numbers in the 2.5%, 5% and 10% concentrations for both the BCs + Plasma and the Exp BCs + Plasma were significantly higher than in ASCs expanded in FBS. In contrast, only the 10% concentrations of the BCs + TSOL and Exp BCs + TSOL groups showed a significant increase in cell number when compared to ASC number in FBS (Figure 4 (B) & (D)).
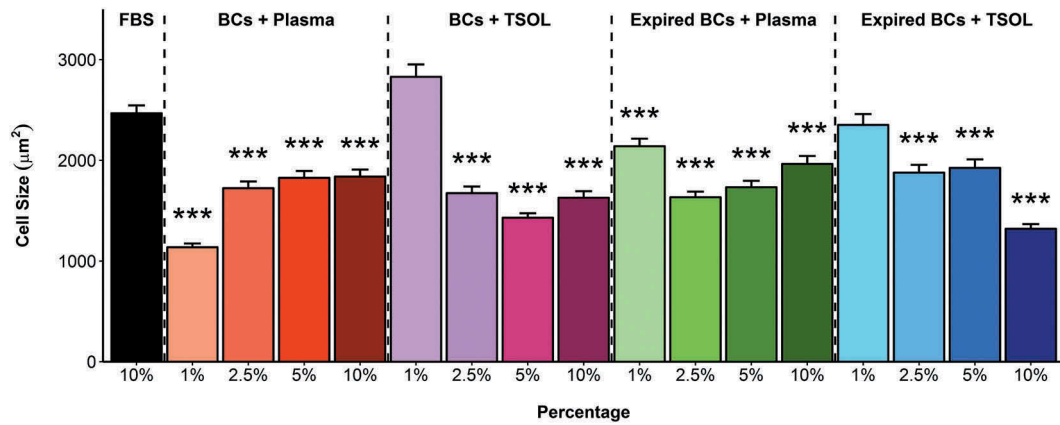
Figure 3. Size (μm^2) of adherent ASCs expanded in FBS and pHPL alternatives. Bars represent mean ± SEM. Data are derived from three ASC biological replicates ($n = 3$) and statistical significance is represented by $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Statistical significance is only indicated for groups that were significantly different to FBS.
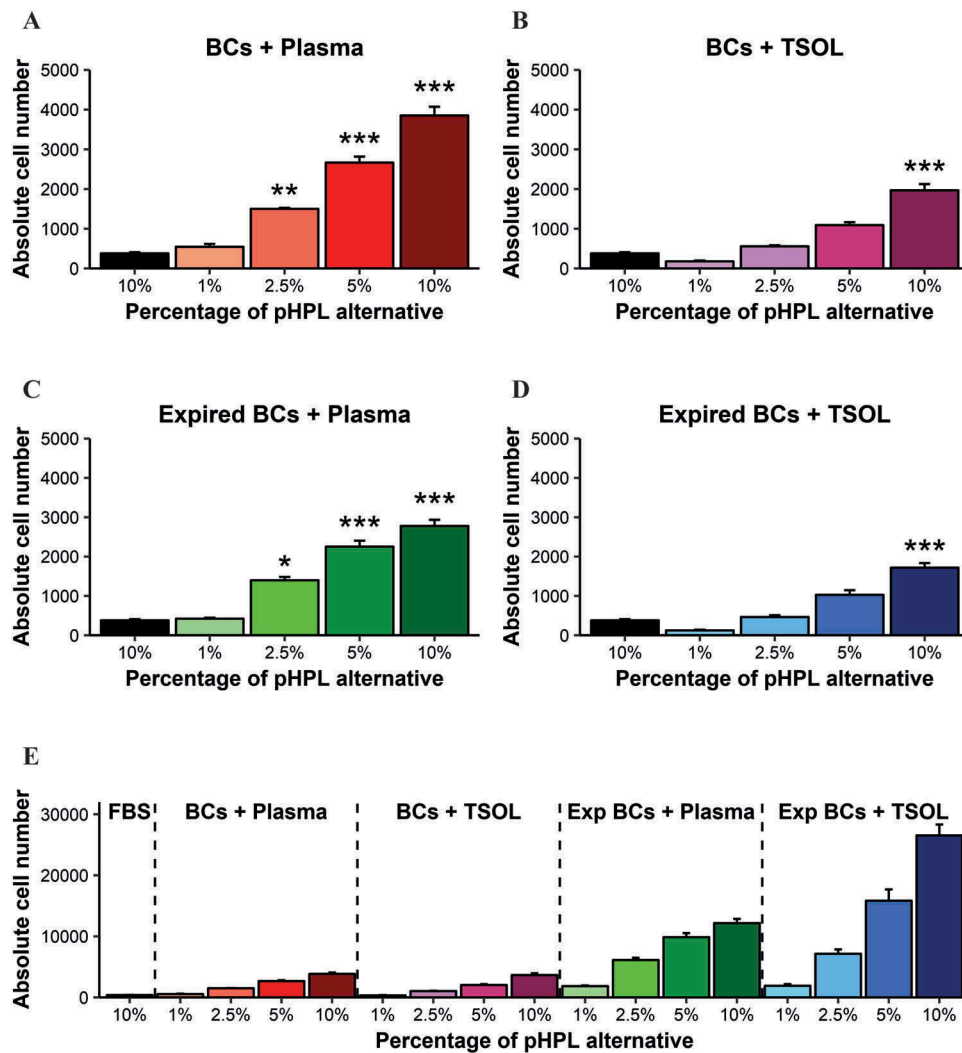

Figure 4. Absolute number of ASCs (cells/μL) expanded in FBS and the pHPL alternatives at different concentrations. ASCs expanded in (A) BCs + Plasma; (B) BCs + TSOL; (C) Expired BCs + Plasma and (D) Expired BCs + TSOL. Bars represent mean ± standard error. Data are derived from three ASC biological replicates ($n = 3$) and statistical significance is represented by $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Statistical significance is only indicated for the groups that were significantly different to FBS. (E) The relative effect on absolute cell count when normalised to the starting platelet material of BCs + Plasma (significance is not indicated on the graph).

When examined under a light microscope, ASCs expanded in 10% pHPL alternatives (Figure 5(E), (I), (M) & (Q)) as well as the 5% BCs + Plasma and Exp BCs + Plasma groups (Figure 5(D) & (L)) were almost 100% confluent after 7 days
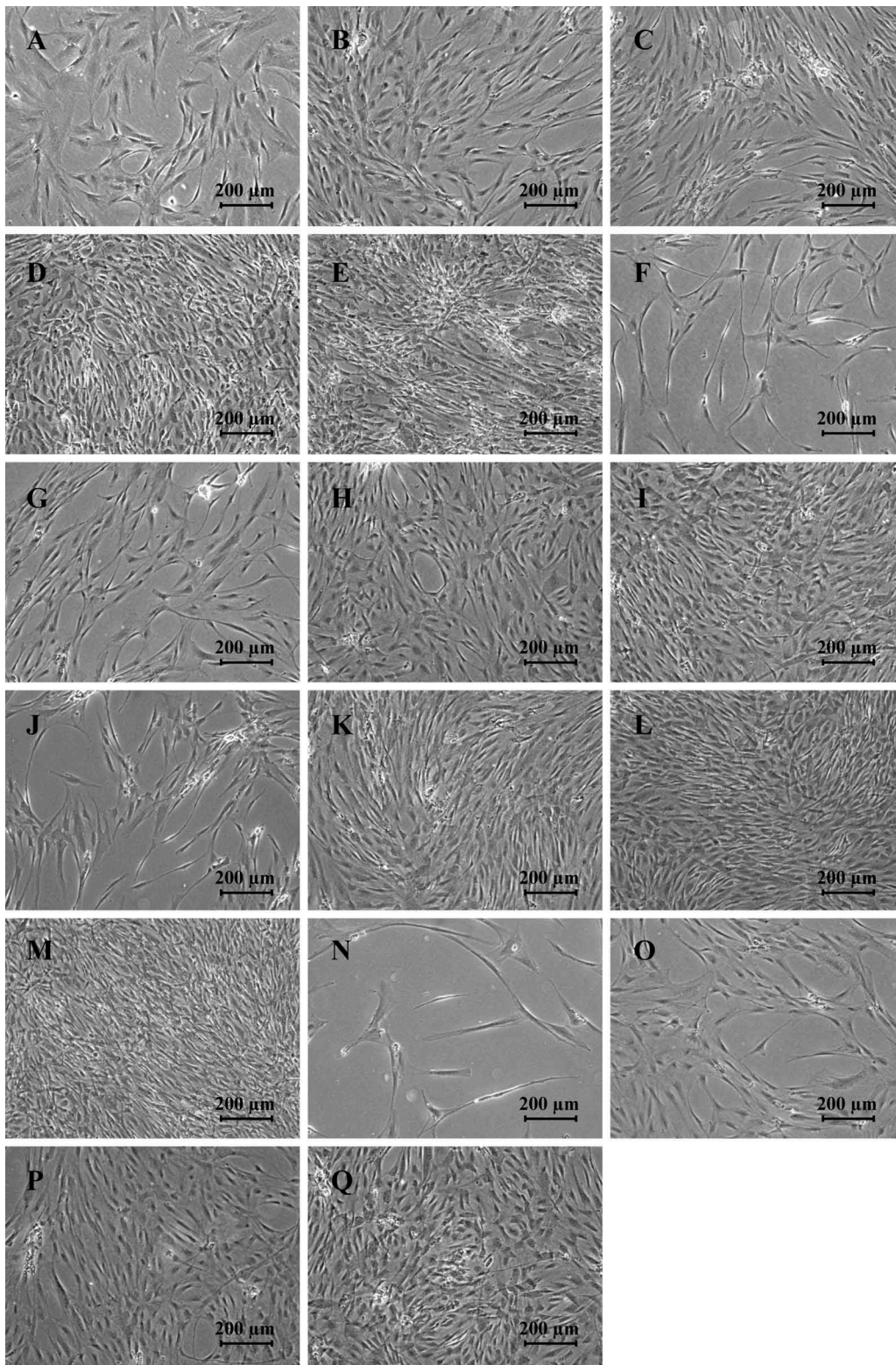
Figure 5. Light micrographs of ASCs expanded in FBS and the pHPL alternatives at different concentrations after 7 days in culture. ASCs expanded in (A) 10% FBS; (B) 1% BCs + Plasma; (C) 2.5% BCs + Plasma; (D) 5% BCs + Plasma; (E) 10% BCs + Plasma; (F) 1% BCs + TSOL; (G) 2.5% BCs + TSOL; (H) 5% BCs + TSOL; (I) 10% BCs + TSOL; (J) 1% Expired BCs + Plasma; (K) 2.5% Expired BCs + Plasma; (L) 5% Expired BCs + Plasma; (M) 10% Expired BCs + Plasma; (N) 1% Expired BCs + TSOL; (O) 2.5% Expired BCs + TSOL; (P) 5% Expired BCs + TSOL; (Q) 10% Expired BCs + TSOL; captured at 5× magnification. Scale bars represent 200 μm.

in culture, whereas the remaining ASC expanded in pHPL alternatives and the ASCs expanded in FBS (Figure 5(A)) had not reached confluency by day seven.

Immunophenotype

Changes in the co-expression profiles of extracellular cell surface markers (immunophenotype) were assessed using
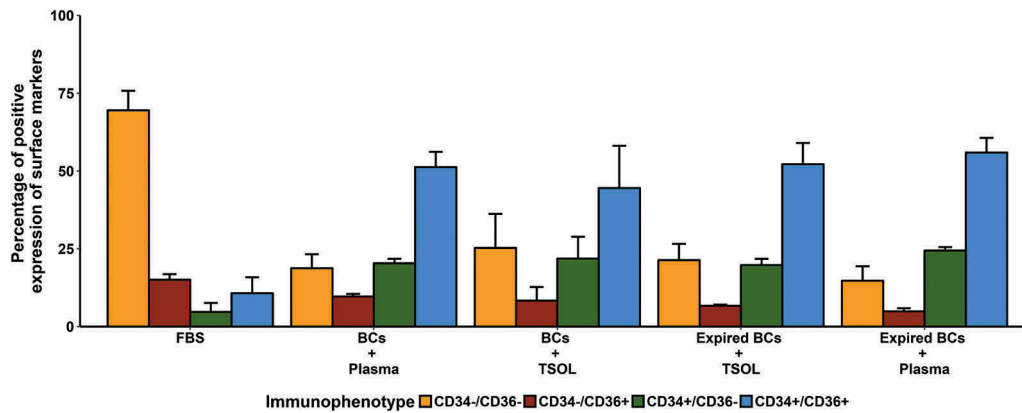
Figure 6. Changes in CD34 and CD36 immunophenotypic expression (percentage positive cell surface marker expression) in ASCs expanded in FBS and the pHPL alternatives. Data are displayed as the percentage of $CD44^+CD45^-CD73^+CD90^+CD105^+$ ASCs that are either negative for both CD34 and CD36 ($CD34^-CD36^-$; yellow bar); negative for CD34 and positive for CD36 ($CD34^-CD36^+$; red bar); positive for CD34 and negative for CD36 ($CD34^+CD36^-$; green bar); or positive for both CD34 and CD36 ($CD34^+CD36^+$; blue bar). No significant differences were found in the subpopulations between ASCs expanded in the pHPL alternatives and ASCs expanded in FBS. Bars represent mean ± SEM. Data are derived from three ASC biological replicates (n = 3).

flow cytometry. The immunophenotype was analysed at confluence for ASCs expanded in FBS and the pHPL alternatives. Only the 10% concentrations were used to monitor changes in the immunophenotypic profiles (Figure 6). ASCs expanded in FBS and the different pHPL conditions had similar immunophenotypic profiles ($CD44^+CD45^-CD73^+CD90^+CD105^+$). The only surface markers affected by the exposure to the different pHPL alternatives were CD34 and CD36. The ASCs expanded in FBS displayed the classic immunophenotypic profile where 69.5 ± 6.3% of the ASCs were $CD34^-CD36^-$ (Figure 6). In contrast, approximately 50% of the ASCs expanded in the different pHPL alternatives were $CD34^+CD36^+$. Although the different subpopulations varied slightly between the different pHPL alternatives, they all shared a similar immunophenotypic profile. No significant differences in the immunophenotype were found between the freshly isolated BCs + Plasma and BCs + TSOL expanded ASCs when compared to the Exp BCs + Plasma and Exp BCs + TSOL expanded ASCs.

Discussion

The use of GMPs is essential for providing safe and regulated cell therapy products to patients. Therefore, the process of obtaining ASCs and any downstream manipulation or modification of these cells (expansion and differentiation) needs to adhere to GMP guidelines (6). In the research setting, FBS is the standard serum supplement used for tissue culture and the *in vitro* expansion of cells. However, FBS has many disadvantages, such as batch-to-batch variation, xenoimmunisation and zoonotic transmission of infectious agents, and may therefore affect the outcome of experiments and ultimately render the cell product unacceptable for clinical use from a safety perspective (1,5,9,29–34). Before ASCs can be used in the clinical setting, GMP-compliant standardised operating procedures as well as isolation and expansion protocols need to be in place (9,24). One of the key adjustments that need to be made during the transition from the research to the clinical setting is the replacement of FBS with alternatives that adhere to GMP standards, such as human-derived alternatives.

pHPL is currently used for the expansion of ASCs in clinical trials (3,4,18). When compared to FBS, platelet-rich plasma and human serum, pHPL has been shown to produce ASCs with greater spindle-like morphology, proliferation and differentiation

capacity, while the cells remain untransformed and retain their immunophenotype (35,36).

PCs are manufactured by blood donation services worldwide and are routinely used to treat patients that suffer from conditions associated with defective and/or decreased platelet function or count. Currently, the recommended shelf-life of PCs is 5 days, whereafter the expired PCs are routinely discarded. Expired PCs are therefore readily available and represent an attractive alternative for ASC expansion when compared to fresh PCs, which may be more expensive and difficult to obtain (19,37,38).

In this study, the expansion of ASCs was compared in four different pHPL alternatives (BCs + plasma, BCs + TSOL, expired BCs + plasma and expired BCs + TSOL), at four different concentrations (10%, 5%, 2.5% and 1%) as well as to 10% FBS. Each of these alternatives was manufactured using the same protocol and subjected to the same quality control tests (22). Cryopreserved ASCs were used to investigate the effect of the different pHPL alternatives and FBS supplemented media on ASC cell number, morphology, cell size and immunophenotype. The expansion of the cells in FBS prior to cryopreservation is not expected to affect the results, as it has been shown that ASCs initially expanded in FBS experience no priming effect when subsequently expanded in pHPL (9). We acknowledge however that prior expansion of ASCs in FBS contradicts the need for homologous products, and freshly isolated ASCs expanded in the different alternatives from isolation would be ideal.

It has been suggested by Astori *et al.* that certain release criteria should be considered prior to using pHPL (19). These criteria include the quality, composition, endotoxin levels and viral and bacterial safety of the pHPL product. In this study, this was achieved in part by using the quality control criteria (Table II) as set out by the by Schallmoser & Strunk in their pHPL protocol (22). All of the pHPL alternatives used adhered to the above-mentioned quality control criteria. Although the total platelet content of the pHPL alternatives fell within the recommended ranges, we observed that the platelet concentrations (platelets/mL) were lower in the expired BC groups (Exp BCs + Plasma and Exp BCs + TSOL) compared to the fresh BC groups (BCs + Plasma and BCs + TSOL). This might be due to degradation of the platelets during storage and may explain why the proliferation of ASCs was slightly less in the expired BC groups when compared to their fresh BC group counterparts (Figure 4). Furthermore, a platelet concentration below 1.5×10^9 platelets/mL has been shown to negatively impact the proliferation of ASCs (16,39). In this study, the platelet concentrations were

approximately 2.03×10^9, 0.91×10^9, 0.41×10^9 and 0.19×10^9 for BCs + Plasma, BCs + TSOL, Exp BCs + Plasma and Exp BCs + TSOL. This major difference in platelet concentration would best explain the disparity in the expansion of the ASCs in the different alternatives. In addition, it has been suggested that platelet additive solutions may affect ASC expansion due to the presence or the lack of plasma components (38). In future, to compare the fresh and expired alternatives more accurately, and to truly distinguish the effect of the fresh vs expired PCs and TSOL vs Plasma resuspension, the concentration of platelets (platelets/mL) should be the same in the starting PC prior to pHPL manufacture. Nonetheless, robust expansion of ASCs was achieved when the Exp BC alternatives were used, suggesting that Exp BC products might be an attractive alternative to freshly prepared BC products.

ASCs have a classic spindle-like morphology similar to that of fibroblasts (40,41). Differences in morphology between FBS and pHPL have been noted: ASCs expanded in FBS lose their spindle-like shape and change into a flattened, spread out shape, whereas ASCs expanded in pHPL display a tighter spindle-like shape and are smaller in size (9,40). Various studies have measured the size of non-adherent ASCs using flow cytometric techniques (Supplementary data) such as mean fluorescent intensities (9,40). We measured cell size in both adherent (Figure 3) and non-adherent (Figures S4 and S5) ASCs, and found that the size of adherent ASCs did not correlate with the size of non-adherent ASCs. Adherent ASCs expanded in FBS were significantly larger in size when compared to all of the pHPL alternatives except for the 1% BCs + TSOL and 1% Exp BCs + TSOL groups. These two groups had a similar morphology to that of FBS (large and spread out) which could be due to the cells becoming senescent (42). It is well known that various components of serum and plasma promote spreading of adherent cells on tissue-culture plastic (43,44). TSOL is a chemical formulation (sodium citrate-acetate-chloride) and does not contain any of the biochemical compounds present in plasma (45); the absence of these compounds may contribute to the decreased spreading observed when the cells were treated with the plasma-free additive, TSOL.

It has been well established that ASCs expanded in pHPL proliferate faster than ASCs expanded in FBS (9,14,15). Although most studies have made use of pHPL that was manufactured from fresh BCs and resuspended in platelet-rich plasma, very little is known about the effects that expired BCs and TSOL have on ASC expansion (19). Here we have shown that ASCs expanded in either of the 10% pHPL alternatives proliferate significantly faster than ASCs expanded in FBS. BCs + Plasma and Exp BCs + Plasma had the greatest effect on cell number (Figure 4(A) and (C)). Although not investigated in this study, the difference in proliferation could be due to the presence of growth factors and other bioactive molecules present in plasma that are known to effect cell proliferation (19). Previous studies have shown that ASCs expanded in either 10% or 5% pHPL display a greater proliferation and growth capacity compared to 10% FBS; however, no difference was found between 10% and 5% pHPL (22,46,47). Contrary to the above-mentioned observations, we found significant differences between 10% and 5% within each of the pHPL alternatives (Supplementary data, Table S2). Because the platelet content of starting material of the different pHPL alternatives was different, comparison is limited. In an attempt to more accurately compare the alternatives, ASC counts were normalised to the platelet count of BCs + Plasma. Here we found that the weakest of the alternatives (Exp BCs + TSOL) would have had the greatest effect on ASC expansion in comparison to the rest (Figure 4(E)). However; in future studies the

starting material would need to be the same in all of the alternatives in order to be able to truly compare results between different alternatives.

We also recognise that ASC proliferation is not just a measure of cell number. Therefore in future studies, not only will absolute cell counting assays include cell count, but also apoptotic and cell cycle measurements undertaken at more regular intervals. In addition, in order to be able to truly detect changes in proliferation rates, the cells would need to be expanded and measured for a period longer than 7 days. As cells proliferate *in vitro* they undergo three prominent phases of growth, namely a lag/latent phase, an exponential phase and a plateau phase (7,48–51). Depending on how rapidly the cells grow, 7 days be enough to capture all the cells in the exponential and plateau phase.

ASC immunophenotype is a widely studied and controversial topic. Studies have found that the immunophenotypic profiles differ significantly between ASCs derived from different donors, between ASCs obtained from different tissue sources, between ASC populations at different passages and to a large extent between individual cells within a given ASC population (52–56). To date, no immunophenotypic marker has been identified that is specific to ASCs. Numerous immunophenotypic panels have been proposed; however, no consensus exists as to which of these is ideal for the characterisation of ASCs *in vitro* (12,53,55,56). According to the ISCT, the classic ASC immunophenotype includes the surface markers CD36 (weakly positive), CD44, CD73, CD90, CD105 while CD34 and 45 should be negative (11,12). Studies comparing the classic immunophenotype (CD34⁻CD44⁺CD45⁻CD73⁺CD90⁺CD105⁺) of ASCs expanded in pHPL and FBS have shown that there is no significant difference between the surface markers investigated (8,9,38,57) or between the percentages of pHPL that were used for tissue culture (15). In our study, we obtained similar findings, namely that ASCs expanded in FBS and the different pHPL alternatives had a CD44⁺CD45⁻CD73⁺CD90⁺CD105⁺ phenotype. CD36 is a multifunctional class B scavenger receptor present on various cell types including ASCs, adipocytes, phagocytes, platelets and others. It has been reported to act as a negative regulator of angiogenesis and is a known fatty acid translocase, due to its role in the binding and transportation of long chain fatty acids into cells (58). CD36 is one of the few markers suggested by the ISCT that allows investigators to distinguish between bone marrow derived mesenchymal stem/stromal cells (MSCs) and ASCs (12). Bone marrow-derived MSCs are reported to be negative for CD36, while ASCs express CD36 weakly (12). In a recent study, Durandt *et al.* showed that a subpopulation of ASCs expanded in FBS inherently expresses higher levels of CD36 *in vitro* (59). In the current study, we observed that the subpopulation of ASCs with higher levels of CD36 increases when ASCs are expanded in any of the pHPL alternatives (Figure 6). The physiological relevance of the observed increase in CD36 expression was not studied and requires further investigation. Interestingly, immunophenotyping of ASCs expanded in the different pHPL alternatives revealed two additional subpopulations that were strongly positive for CD34. It is well known that all freshly isolated ASCs have increased CD34 expression which is lost during the expansion and culture process. This has led to several reports indicating that CD34 expression is variable and unstable in ASCs (12,52,53,60). Camilleri *et al.* suggested in a recent study that ASCs express CD34 weakly and that its expression can be modulated by confluency, whereby increased confluency resulted in increased expression of CD34. In our study, cells grown in the 10% pHPL alternatives were almost 100% confluent (Figure 5(E), (I), (M) and (Q)) prior to being dissociated, which may explain the strongly positive CD34 expression. These findings are contradictory to a study comparing expired HPL resuspended in platelet additive solutions, in which the CD34 positivity was less than 5% (38). Although the function of CD34 in

ASCs is not clear, Suga *et al.* suggested in their study that it may play a role in the proliferative capacity, angiogenic potential, differentiation capabilities and stemness of these cells (61).

The Schallmoser and Strunk (2013) protocol indicates that either fresh or expired BCs may be used, and these may be resuspended in an additive solution such as TSOL instead of platelet-rich plasma (22). To the best of our knowledge, previous studies using pHPL were performed using fresh BCs + Plasma, or it was not stated which alternative was used. We believe our study is the first to perform a direct comparison between pHPL alternatives – BCs + TSOL, expired BCs + Plasma and expired BCs + TSOL – and FBS. Furthermore, it has been highlighted that there is a lack of data regarding expired pHPL alternatives (19); here we present data that has revealed large differences in ASC proliferation, morphology, cell size and immunophenotype. Our experiments formed part of a pilot study, and we recognise that more in depth studies on these alternatives are needed. This is due to differences in the trilineage differentiation capacity that have been reported between ASCs expanded in different blood products (8) as well as differences in growth factor concentrations between pHPL alternatives (38). This study would also have benefitted from growth factor analysis as platelet-derived growth factors are known to play pivotal roles in cellular proliferation, differentiation and migration (62–64). Furthermore, ASCs expanded in FBS have had adverse immunogenic effects (4,65) when transplanted back into patients. In order to assess the immunomodulatory effects of ASCs expanded in the different pHPL alternatives, functional experiments should be performed both *in vitro* and *in vivo*.

In summary, we provide in-house protocols that can aid in establishing release and quality control criteria for pHPL manufacture. We found that 10% pHPL alternatives had the greatest effect on ASC proliferation; however, rapid proliferation and spindle-like morphology is maintained at decreased concentrations. We also found that immunophenotype was comparable between the pHPL alternatives. However, in order to prove the safety and efficacy of these pHPL alternatives, future studies should assess their effect on differentiation capacity, immunomodulation, an investigation of the transcriptome, proteome and secretome, and finally these cells should be studied in appropriate animal models.

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Declaration of interest

The authors declare that they have no conflict of or competing interests.

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Supplementary material

Supplemental data for this article can be accessed here.

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