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RESEARCH

Re-using blood products as an alternative supplement in the optimisation of clinical-grade adipose-derived mesenchymal stem cell culture

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Objectives

Adipose-derived mesenchymal stem cells (ADMSCs) are a promising strategy for orthopaedic applications, particularly in bone repair. *Ex vivo* expansion of ADMSCs is required to obtain sufficient cell numbers. Xenogenic supplements should be avoided in order to minimise the risk of infections and immunological reactions. Human platelet lysate and human plasma may be an excellent material source for ADMSC expansion. In the present study, use of blood products after their recommended transfusion date to prepare human platelet lysate (HPL) and human plasma (Hplasma) was evaluated for *in vitro* culture expansion and osteogenesis of ADMSCs.

Methods

Human ADMSCs were cultured in medium supplemented with HPL, Hplasma and a combination of HPL and Hplasma (HPL+Hplasma). Characteristics of these ADMSCs, including osteogenesis, were evaluated in comparison with those cultured in fetal bovine serum (FBS).

Results

HPL and HPL+Hplasma had a significantly greater growth-promoting effect than FBS, while Hplasma exhibited a similar growth-promoting effect to that of FBS. ADMSCs cultured in HPL and/or Hplasma generated more colony-forming unit fibroblasts (CFU-F) than those cultured in FBS. After long-term culture, ADMSCs cultured in HPL and/or Hplasma showed reduced cellular senescence, retained typical cell phenotypes, and retained differentiation capacities into osteogenic and adipogenic lineages.

Conclusion

HPL and Hplasma prepared from blood products after their recommended transfusion date can be used as an alternative and effective source for large-scale *ex vivo* expansion of ADMSCs.

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Keywords: Adipose-derived mesenchymal stem cells, Human plasma, Mesenchymal stem cells, Platelet lysate, Stem cell culture

Article focus

- This study examined the use of blood products beyond their recommended transfusion date taken from a blood bank for *in vitro* culture of adipose-derived mesenchymal stem cells (ADMSCs).

Key messages

- Compared with fetal bovine serum (FBS), The human platelet lysate (HPL) and human plasma (Hplasma) prepared from leukocyte-poor platelet concentrate (LPPC) after their recommended transfusion date, and fresh frozen plasma (FFP) could promote proliferation and reduce cellular senescence of ADMSCs, respectively.
- ADMSCs cultured in HPL and/or Hplasma retained typical cell phenotypes and differentiation capacity to osteogenic and adipogenic lineages.

Strengths and limitations

- Use of LPPC and FFP for *in vitro* culture of ADMSCs can promote added value of blood products after their recommended transfusion date in a blood bank.

- This study examines only mesenchymal stem cells (MSCs) from adipose tissue, therefore it cannot indicate that the outdated blood products can be used for culture of MSCs from other tissue types.

Introduction

Mesenchymal stem cells (MSCs) are currently of interest in orthopaedic tissue engineering and regenerative medicine. MSCs can be obtained from several types of tissue.[1-5] Adipose tissue is an attractive source of MSCs that has become increasingly popular in therapeutic applications. A high yield of MSCs can be isolated from adipose tissue via a minimally invasive procedure. Adipose-derived MSCs (ADMSCs) provide many advantages, not only for stem cell research, but also for clinical practice. Importantly, ADMSCs are considered to be a suitable autologous cell source for clinical application. Clinical trials have demonstrated the effectiveness of using ADMSCs for treating patients with a variety of orthopaedic complaints such as osteoarthritis[6] and bone defects.[7] However, the use of ADMSCs in therapeutic strategies requires a large number of cells, therefore it is extremely important that the process of *ex vivo* expansion of ADMSCs complies with the Good Manufacturing Practice (GMP) requirements. To achieve the GMP requirements, several procedures for stem cell preparation must be reviewed carefully, especially stem cell collection and expansion in order to obtain good quality stem cells in sufficient numbers.

Stem cell expansion is based on culture in a growth medium supplemented with xeno-derived serum, which may pose undesirable risks such as immunological reactions and cross-species pathogen infections transmittable to patients in clinical use.[8-10] Recently, human serum and human platelet-derived products, including platelet lysate and platelet release factors, have been suggested as a promising substitute for animal serum for clinical-scale MSC culture.[11-13] Platelets contain numerous bioactive molecules stored in distinct platelet granules such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β), and fibroblast growth factor-2 (FGF-2), which act by promoting MSC proliferation.[14,15] It is preferable to use human blood products prepared by a blood bank since donated blood must be examined for several infectious diseases in order to ensure that every single unit is free from blood-transmitted pathogens such as syphilis, human immunodeficiency virus, Hepatitis B, and Hepatitis C. Even though several studies have shown that it was possible to use human-derived products for stem cell culture, for sustainable application the sufficient supply of these products must be ensured. As a result of the short shelf life of platelet concentrate and the excess storage supply of fresh frozen plasma (FFP), the discarding of units of platelet concentrate and FFP after their recommended transfusion date has been reported, although a minimising strategy has been extensively studied.[16-19] However, the use of human blood products for stem cell techniques is still controversial and heterogeneous.[11-13] Expanding cultural acceptance of the use of human blood products in stem cell research is required prior to clinical translation.

In this study, we aimed to examine whether the units of platelet concentrate and FFP, which were obtained from the blood products after their recommended transfusion date, can be used for *ex vivo* expansion of ADMSCs. Several characteristics of ADMSCs were examined after being cultured in platelet lysate prepared from platelet concentrate and human plasma prepared from FFP, and compared with those cultured in fetal bovine serum (FBS).

Materials and Methods

Preparation of human platelet lysate (HPL). HPL in this study was prepared from leukocyte-poor platelet concentrate (LPPC) collected from the hospital blood bank. Only the group "O" pooled LPPCs with "out of date" labelling (five-day shelf life) were employed in our experiment. Platelet counts were performed by manual cell counting and the range of platelet number per LPPC unit was 7×10^8 cells/ml to 8×10^8 cells/ml before storage in a freezer at -80°C until use. HPL was prepared by freezing and thawing. The outdated LPPC that was stored in a freezer at -80°C was immersed in a water bath at 37°C for one hour to thaw. After thawing, the lysed platelet was centrifuged at 4000 g, 4°C for 20 minutes to remove cell debris. The supernatant was collected and filtered with a 40 μm cell strainer. The HPL was stored at 2°C to 8°C for up to four weeks. For medium preparation, 2 ml heparin was added to the medium to prevent clot formation. The complete medium was filtered with a 0.2 μm filter before use.

Preparation of human plasma (Hplasma). Hplasma in this study was prepared from FFP collected from the hospital blood bank. Only the group "AB" FFP with "out of date" labelling (one-year shelf life) was employed in our experiment. Five units of the outdated FFP were pooled, transferred to centrifuge tubes and stored in a freezer at -20°C until use. To prepare Hplasma, the FFP was thawed at 37°C in a water bath until completely dissolved. To prevent clot formation during culture, 20% (W/V) calcium chloride was added to the FFP at a ratio of 1:100, and FFP was then incubated at 37°C in a water bath for two hours to allow clot formation. After incubation, the fibrin clot was removed by centrifugation at 4000 g, 4°C for 20 minutes. The supernatant was collected, filtered with a 40 μm cell strainer, and stored at 2°C to 4°C for up to four weeks.

Isolation and culture of ADMSCs. ADMSCs were obtained from lipoaspiration of healthy donors. Ethical approval for the collection of tissue and subsequent research was granted by the Committee on Human Rights

Related to Research Involving Human Subjects (number MURA2015/149). All subjects were informed and signed a consent form. The lipoaspirate fraction was washed several times with phosphate buffer saline (PBS) and digested with 1 mg/ml collagenase type II (Sigma-Aldrich, St Louis, Missouri) for one hour at 37°C in a shaking water bath. After digestion, the stromal vascular fraction (SVF) was obtained by centrifugation at 2500 g for ten minutes. The SVF portion was collected and washed twice with basal medium. After centrifugation, the pellet was resuspended in complete medium, which was composed of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS (Merck Millipore, Temecula, California), 1X GlutaMAX (Gibco; Thermo Fisher Scientific, Waltham, Massachusetts), 100 U/ml penicillin and 100 µg/ml streptomycin, followed by plating in a T75 cell culture flask. All flasks were cultivated in a humidified incubator at 37°C with 5% CO_2. Non-adherent cells were removed after cultivation for 24 hours. The resulting plastic-adherent cell population was termed ADMSCs.

All of the experiments were performed using ADMSCs prepared according to the procedure that used FBS for the initial ADMSC isolation and expansion. Nevertheless, the collection and expansion of ADMSCs using HPL and Hplasma supplement can be done from an early stage of ADMSC production (supplementary material).

Cell growth. To determine the cell growth potential of MSCs, the periodic monitoring of cell numbers using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and population doubling time (PDT) was employed in this study. For the MTT assay, an indirect measurement of cell number is made by assessing mitochondrial activity of metabolically intact cells. Cells were seeded in 96-well plates at a density of 1×10^3 cells per well in 150 µl complete medium containing different supplements. On each day (day 0 to 8) of the MTT assay, 50 µl of 1 mg/ml MTT reagent (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts) was added to each well and the plate was incubated for four hours at 37°C with light protection. The supernatant was then carefully discarded and 100 µl dimethyl sulfoxide (DMSO) was added to each well to solubilise the formazan crystals. The plates were agitated for 15 minutes to dissolve the crystals completely, and the absorbance was measured at 570 nm using a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, Vermont).

PDT of the ADMSCs was assessed at passages P4-6. The cells were seeded at 1×10^5 cells per T25 culture flask with different supplements. After reaching 80% to 90% confluence, cells were trypsinised and counted. The PDT of ADMSCs cultured with different supplements was calculated according to the following formula: $PDT = (t \times \log 2)/(\log Nh - \log Ni)$, where t = culture time (h), Nh = number of harvesting cells, and Ni = number of initiating cells.[20]

Colony-forming unit – fibroblast (CFU-F) assay. The colony-forming capacity of ADMSCs cultured in FBS, HPL, and Hplasma was compared. Cells at passages four to seven were seeded at 100 cells per well in six-well plates in a medium containing different supplements, and cultured for 11 days. Colonies were fixed with methanol for five minutes, stained with 0.5% crystal violet solution for 30 minutes at room temperature, and washed with distilled water to remove excess dye. The colonies, which were defined as a group of more than 50 cells, were counted manually under an inverted microscope.

Immunophenotyping. The expression of ADMSC surface markers was assessed by flow cytometric analysis. Cells were trypsinised into a single cell suspension and incubated with the fluorochrome-conjugated antibodies against human antigens, including anti-CD34-PE, anti-CD45-PerCP, anti-CD73-PE/Cy7, anti-CD90-APC, and CD105-PE (BD Biosciences, San Jose, California), at 4°C for 30 minutes. The cells were then washed twice with cold PBS and fixed with 1% paraformaldehyde before analysis by FACSCanto II Flow Cytometer (BD Biosciences) using FACSDiva software.

Osteogenic and adipogenic differentiation. For osteogenic differentiation, 1×10^5 ADMSCs were seeded in a 35 mm dish and cultured in an osteogenic differentiation medium composed of DMEM, 10% FBS, 100 nM dexamethasone (Sigma-Aldrich), and 50 µg/ml ascorbic acid (Sigma-Aldrich). After the first week of culture, 10 mM β-glycerophosphate (Merck Millipore) was added to the medium. Cells were cultured for three weeks. To determine bone matrix mineralisation, cells were fixed with 10% formaldehyde for ten minutes and stained with 40 mM Alizarin Red S (Merck Millipore) for 20 minutes at room temperature. For adipogenic differentiation, 1×10^5 ADMSCs were seeded in a 35 mm dish and cultured in an adipogenic differentiation medium composed of DMEM, 10% FBS, 500 µM isobutyl methylxanthine, 1 µM dexamethasone, 10 µM insulin and 200 µM indomethacin (Sigma-Aldrich). Cells were cultured for two weeks. Adipogenic differentiation was detected by staining of lipid droplets with Oil Red O (Sigma-Aldrich). Cells were washed with PBS, fixed with formalin vapour for ten minutes, and stained with 0.5% Oil Red O in isopropanol for 20 minutes.

Senescence assay. Cellular senescence of ADMSCs was determined at the seventh passage of ADMSCs which had been cultured in medium containing different supplements including FBS, HPL, Hplasma, and HPL+Hplasma. Cells were seeded in a 35 mm dish at a density of 2.5×10^4 cells per dish in 2 ml of each medium and allowed to grow for three days. Cellular senescence was detected using a senescence-associated β-galactosidase (SA-β-gal) staining kit (Cell Signaling Technology, Inc., Danvers, Massachusetts) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS, fixed with

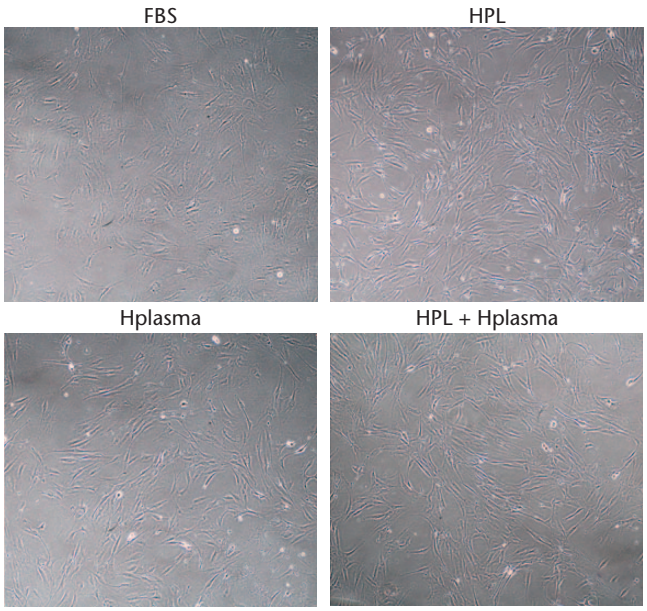

Fig. 1a

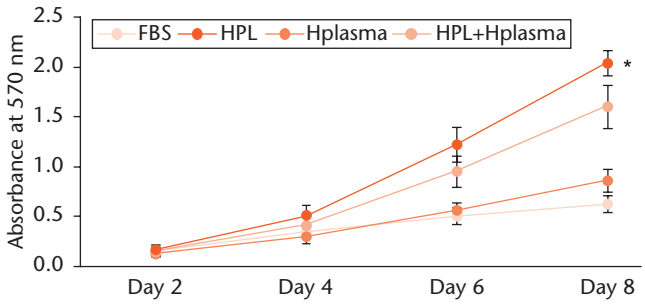

Fig. 1b

Morphology and growth of adipose-derived mesenchymal stem cells (ADMSCs) after culture in different supplements: a) ADMSCs showed fibroblast-like morphology after culture in each condition; b) the growth rates of ADMSCs after culture in different supplements for two, four, six, and eight days were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The growth of ADMSCs cultured in human platelet lysate (HPL) and HPL+human plasma (Hplasma) was significantly greater than that of those in fetal bovine serum (FBS). Data were presented as mean and standard error of the mean. * $p \leqslant 0.05$ compared with FBS. Statistical analysis was tested by Kruskal-Wallis test followed by Dunn's multiple comparison test.

1x fixative solution for 15 minutes, and stained with the freshly prepared β-gal staining solution at a pH of 5.9 to 6.1. Dishes were sealed with parafilm to prevent evaporation and incubated at 37°C overnight in the absence of carbon dioxide. The presence of a blue colour, i.e. senescent cells, was observed under an inverted microscope. Ten fields of 10× magnification per dish were taken, and senescent cells were manually counted in each field. Data were presented as a percentage of senescent cells over the total number of cells.

Statistical analysis. Data were presented as mean and standard error of the mean (SEM). Comparisons were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison test, and $p < 0.05$ was considered to be statistically significant. Statistical tests were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, California).

Results

Effect of human supplements on morphology and growth of ADMSCs. ADMSCs were cultured in medium containing different supplements: 10% FBS; 10% HPL; 10% Hplasma; and 5% HPL + 5% Hplasma. The morphology of ADMSCs from all conditions were spindle-shaped, fibroblast-like cells. However, ADMSCs cultured in HPL and/or Hplasma were more spindle-shaped and slightly smaller than those cultured in FBS (Fig. 1a). These characteristics could be observed throughout the long-term culture.

The growth of ADMSCs from these conditions was compared using MTT assay and PDT. As the MTT results show, HPL and HPL+Hplasma exhibited more stimulation of cell growth compared with FBS and Hplasma alone. Cell growth was significantly higher in HPL compared with FBS on day eight of the MTT assay. The growth of ADMSCs in Hplasma was comparable with that in FBS (Fig. 1b). Population doubling time was calculated from three passages of ADMSCs cultured in each group. The mean PDTs of ADMSCs in FBS, HPL, Hplasma, and HPL+Hplasma were 132 (SEM 8), 50 (SEM 1), 65 (SEM 8) and 65 hours (SEM 4), respectively ($n = 3$). The ADMSCs cultured in HPL, Hplasma, and HPL+Hplasma had faster growth kinetics than those cultured in FBS. The PDT was significantly lower in HPL conditions compared with FBS conditions ($p < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparison test).

Human supplements maintain typical characteristics of ADMSCs. The influence of different supplements in the maintenance of MSC characteristics was examined. ADMSCs were cultured with different supplements for several passages until reaching the tenth passage, when they were harvested for investigation of several characteristics of MSCs. ADMSCs cultured in HPL and/or Hplasma expressed typical MSC surface markers (similar to FBS conditions): CD73; CD90; and CD105, but not haematopoietic markers; CD34 and CD45.(Fig. 2a, Table I). The differentiation properties of ADMSCs cultured in each supplement were determined by inducing differentiation towards osteogenic and adipogenic lineages. Osteogenic differentiation was indicated by formation of bone matrix mineralisation which was demonstrated by Alizarin Red S staining (Fig. 2b). Adipogenic differentiation was indicated by detection of lipid droplets in the cells with Oil Red O staining (Fig. 2c). Results showed that ADMSCs cultured in human supplements were able to differentiate into both osteogenic and adipogenic lineages similar to those cultured in FBS.

To compare the osteogenic differentiation potential of ADMSCs in each supplement, ADMSCs during osteogenic differentiation at days 18, 24 and 30 were stained

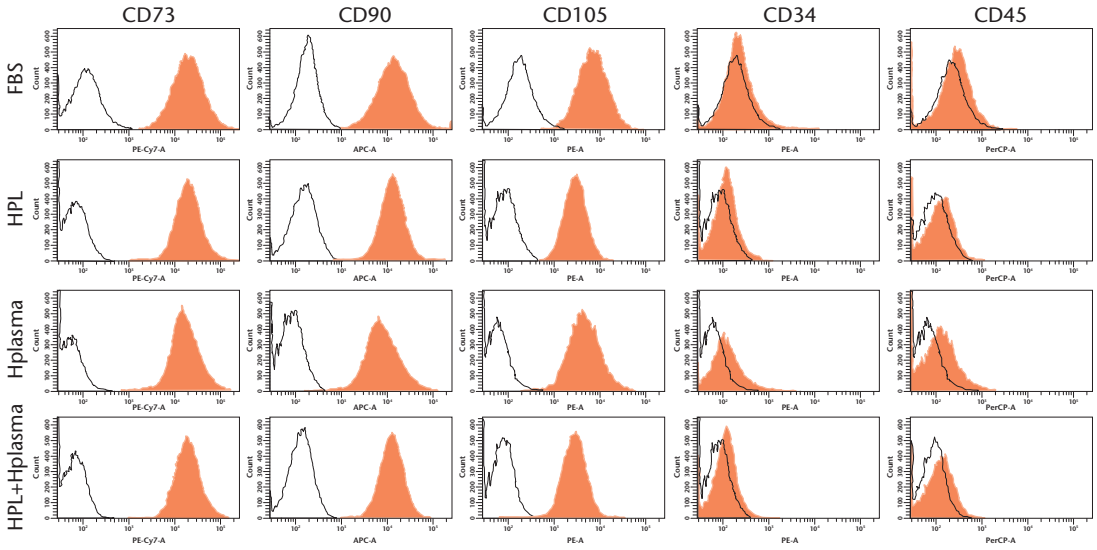

Fig. 2a

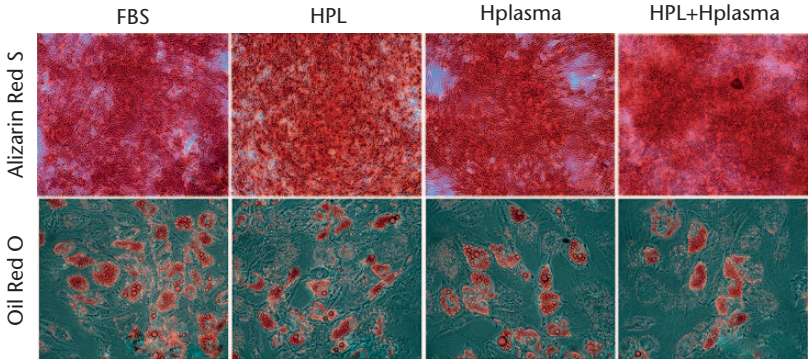

Fig. 2b

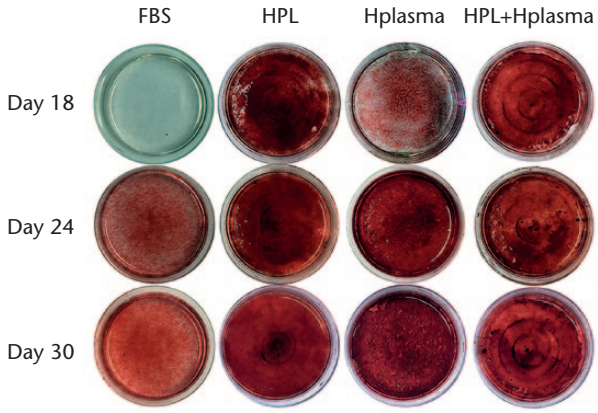

Fig. 2c

Characterisation of Adipose-derived mesenchymal stem cells (ADMSCs) after long-term culture in different supplements: a) immunophenotype of ADMSCs was determined by flow cytometry; b) osteogenic and adipogenic differentiation of ADMSCs was assessed by Alizarin Red S and Oil Red O staining, respectively; c) macroscopic examination of osteogenic differentiation which was assessed by Alizarin Red S staining at days 18, 24, and 30 (FBS, fetal bovine serum; HPL, human platelet lysate; Hplasma, human plasma).

by Alizarin Red S. ADMSCs cultured in HPL and/or Hplasma exhibited more robust osteogenic differentiation compared with those cultured in FBS (Fig. 2c).

Effect of human supplements on self-renewal property of ADMSCs. The self-renewal property of ADMSCs was determined by CFU-F assays that were performed in four

Table I. Surface marker expression of adipose-derived mesenchymal stem cells after long-term culture in different supplements assessed by flow cytometric analysis.

Surface marker (%)	CD73	CD90	CD105	CD34	CD45
FBS	99.3 (*0.2*)	97.7 (*0.4*)	95.8 (*1.0*)	1.1 (*0.1*)	0.2 (*0.1*)
HPL	99.8 (*0.1*)	99.2 (*0.4*)	97.3 (*1.2*)	0.7 (*0.3*)	0.1 (*0.0*)
Hplasma	99.4 (*0.3*)	98.7 (*0.2*)	96.2 (*1.0*)	1.5 (*0.3*)	0.3 (*0.1*)
HPL+Hplasma	99.8 (*0.1*)	99.2 (*0.3*)	96.5 (*1.5*)	0.6 (*0.1*)	0.1 (*0.1*)

Data presented as mean and standard error of the mean
FBS, fetal bovine serum; HPL, human platelet lysate; Hplasma, human plasma

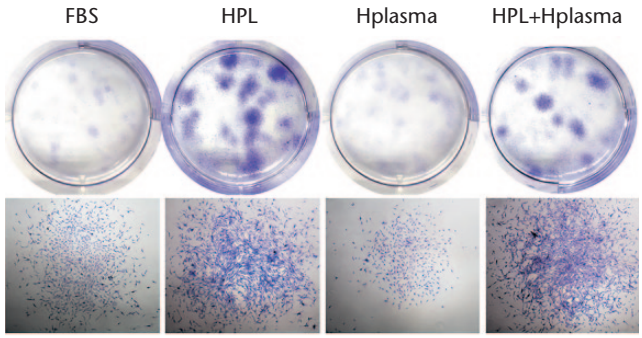

Fig. 3a

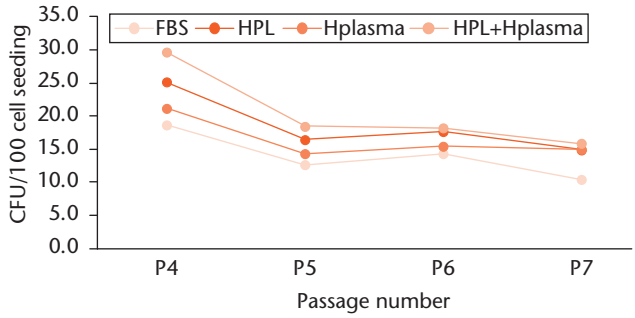

Fig. 3b

Colony-forming unit – fibroblast (CFU-F) assay of adipose-derived mesenchymal stem cells (ADMSCs) after culture in different supplements: a) macroscopic (upper panel) and microscopic (lower panel) examination of CFU-F revealed the variation in size and cell density of colonies generated from ADMSCs of different culture conditions. The large and dense colonies were frequently observed in ADMSCs of human platelet lysate (HPL) and HPL+human plasma (Hplasma) condition; b) number of CFU-Fs generated from ADMSCs at passages four to seven was counted and presented as CFU per 100 cell seeding. Data were presented as a mean generated from three independent experiments (FBS, fetal bovine serum).

consecutive passages after culture in each condition. Variation in size and cell density of CFU-F generated from ADMSCs of different conditions was observed. ADMSCs in HPL and HPL+Hplasma condition generated similar characteristics of CFU-F which were larger and denser than those of Hplasma or FBS (Fig. 3a). Although the number of CFU-Fs declined with further cell passages, ADMSCs in HPL, Hplasma, and HPL+Hplasma conditions generated more CFU-Fs than FBS, indicating that human supplements could efficiently maintain the self-renewal property of ADMSCs after long-term culture (Fig. 3b).

Human supplements prevent cellular senescence of ADMSCs. The effect of different supplements on cellular senescence of ADMSCs was evaluated by the detection of senescence-associated β-galactosidase (SA-β-gal). ADMSCs were cultured with different supplements for several passages until reaching the seventh passage. These cells were stained with SA-β-gal and the senescent cells appeared as a blue colour that could be observed under an inverted microscope (Fig. 4a). As revealed by the quantitative analysis, the number of senescent cells in ADMSCs cultured in HPL, Hplasma, and HPL+Hplasma was lower than that cultured in FBS (Fig. 4b). Thus, human supplements could prevent cellular senescence of ADMSCs after long-term culture.

Discussion

MSCs are as a promising source of cells for cell-based therapy in orthopaedic applications. In recent years, numerous clinical trials using MSCs have been carried out to treat a variety of conditions such as fracture non-unions and osteoarthritis. The number of cells has been shown to be associated with treatment outcomes.[21] In order to generate a sufficient quantity of cells for clinical application, *ex vivo* expansion of MSCs is needed. Generally, a culture of MSCs relies on the use of FBS, however, to prepare cells for clinical application, xenogenic products should be avoided. The risk of viral transmission from animal serum and the potential of immunological induction against bovine antigen in recipients have been reported and these raise concerns about using FBS for the preparation of MSCs in a clinical setting.[8-10] Even though serum-free medium is an optional culture condition for the preparation of clinical-grade MSCs and a commercially available resource, the effect of serum-free medium on MSC characteristics appeared to be variable among the available brands.[22,23]

The culture supplements derived from humans have been investigated with a view to replacing the use of FBS in MSC culture. It has been demonstrated that human serum collected from peripheral blood and umbilical cord blood could promote MSC proliferation and maintain MSC trilineage differentiation comparable with that of FBS.[12,24,25] Human platelet-derived products have been found to be powerful in promoting MSC proliferation.[11,26] Those effects were likely the result of several

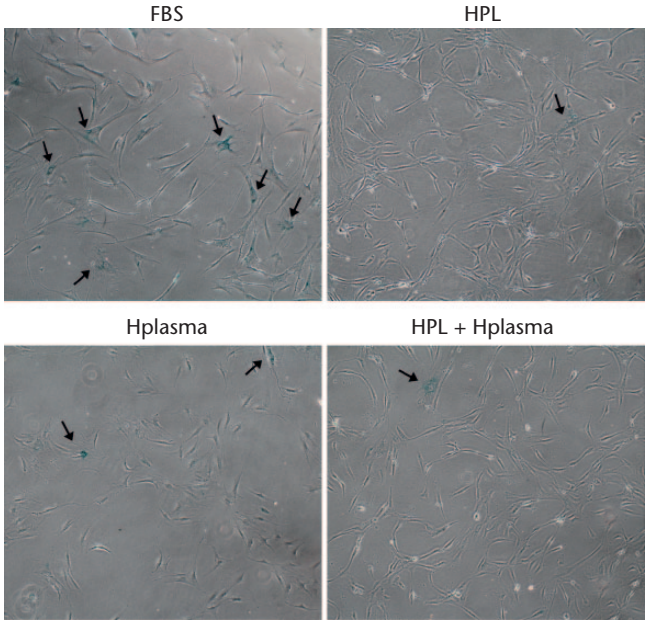

Fig. 4a

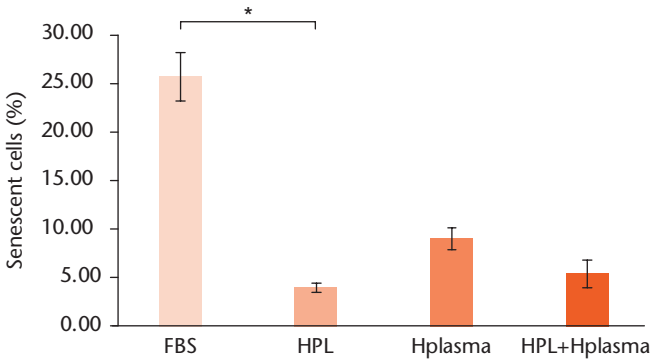

Fig. 4b

Cellular senescence assay of adipose-derived mesenchymal stem cells (ADMSCs) after long-term culture in different supplements: a) cellular senescence of ADMSCs was determined by SA-β-gal staining. The senescent cells appeared as a blue colour (arrow); b) senescent cells were counted and presented as a percentage of total cell number. The graph represents mean and standard error of the mean. * $p \leqslant 0.05$; statistical analysis was tested by Kruskal-Wallis test followed by Dunn's multiple comparison test. (FBS, fetal bovine serum; HPL, human platelet lysate; Hplasma, human plasma).

growth factors that were released from the granules residing in the platelets.[14,15] In particular, PDGF, TGF-β, and FGF-2 have been found to be the essential stimuli for MSC proliferation[27] and these factors are predominant in platelet lysate.[28] This evidence reveals that human alternatives can be used as a substitute for FBS in MSC culture and may contribute to the preparation of MSCs for clinical application. However, the preparation procedures of human serum/plasma and platelet-derived products are highly variable and may be a risk for disease transmission. FFP and LPPC are blood components collected by blood banks that provide a safe source of human supplements for MSC culture. The reason for this is that these products have been prepared in accordance with the strict regulations of the blood bank, and all units of the blood products have been screened for several infectious diseases. Currently, some human blood units are discarded by the blood bank due to expiration of the usability period, which is defined by the blood bank for transfusion purposes.[29] According to general standard guidelines for blood banks, platelet concentrate and FFP have shelf lives of five days and one year, respectively, when stored in the appropriate conditions. It has been reported that the risk of bacterial contamination in platelet concentrate after storage for more than five days was increased,[30] thus platelet concentrate units must be used for transfusion within five days of collection. Considering the short shelf life of platelet concentrate, a number of platelet units expire before they can be used for transfusion and are discarded. There is an excess storage of FFP units in the blood bank, and thus, a number of FFP units are unused and reach the expiration time. Use of these blood products after their recommended transfusion date for other applications such as plasma fractionation can add value to otherwise wasted blood products in the blood bank.

In this study, the use of HPL prepared from LPPC and Hplasma prepared from FFP, as a supplement to ADMSC culture, was compared with FBS. Comparing the growth-promoting effect of HPL and Hplasma on ADMSC, Hplasma exhibited similar a proliferative effect to that of FBS, while HPL had a significantly greater proliferative effect than FBS. A combination of HPL and Hplasma showed a smaller proliferative effect than that of HPL alone, suggesting that the proliferative effect of HPL was dose-dependent. Moreover, after long-term culture in HPL, Hplasma, and a combination of HPL and Hplasma, ADMSCs retained the typical characteristics of MSCs, including cell phenotypes and the capacity to differentiate into osteogenic and adipogenic lineages comparable with those cultured in FBS. Clonogenic assays revealed that CFU-F generated by ADMSCs of different conditions showed that variation in size and cell density could represent the differences in cellular function.[31,32] The large and dense colonies which were observed in ADMSC cultured in HPL and HPL+Hplasma may be related to the high proliferation rate and high mobility of the cells.[31] We also demonstrated that HPL and Hplasma could sustain the self-renewal properties of ADMSCs after long-term culture *in vitro*. The maintenance of self-renewal in MSCs is regulated by many mechanisms involving both genetics and epigenetics and have not been clearly elucidated.

Cellular senescence is one of the major hurdles that limits the *ex vivo* expansion of MSCs. Long-term culture of MSCs has been shown to induce cellular senescence and loss of differentiation capacity, as well as the propensity to develop into a fibroblast phenotype.[33] The time taken to reach the senescence phase of MSCs appears to vary depending on tissue types and culture conditions.[20,34] Here, we demonstrated that long-term culture in HPL and/or Hplasma was able to slow down the cellular

senescence of ADMSCs compared with those cultured in FBS. The mechanisms underpinning the effects of HPL and Hplasma have not been clarified. Modulation of cell cycle regulation may be the possible mechanism. The replicative senescence of MSCs has previously been associated with increased cell cycle inhibitory proteins such as p53 and p16INK4a, which were found to be increased in late passage MSCs and senescent MSCs.[34,35]

In conclusion, the use of HPL and Hplasma prepared from LPPC and FFP, respectively, could promote cell growth and reduce cell senescence, while retaining the cell phenotype and differentiation capacity of ADMSCs. Considering the dire clinical need for human blood products in blood banks, it is not necessary to use fresh human plasma and platelet concentrate which are prepared for blood transfusions in the production of MSCs. The use of blood products after their recommended transfusion date as growth supplement for MSC culture may be advantageous. A well-organised system between the laboratory and blood bank could lead to an efficient long-term application of potentially otherwise wasted blood products. Moreover, the use of HPL alone and HPL combined with Hplasma yielded the same results in this study. Therefore the selection of culture conditions for MSC expansion could be further optimised in future studies.

Supplementary material

Figure showing adiposed-derived mesenchymal stem cells is available alongside this article online at www.bjr.boneandjoint.org.uk

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Conflicts of Interest Statement
- The authors declare that they have no conflict of interests.

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