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Xeno-Free Extraction, Culture, and Cryopreservation of Human Adipose-Derived Mesenchymal Stem Cells

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Key Words. Adipose • Adult stem cells • Xeno-free production • Human platelet lysate • Explant culture • Cryopreservation

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ABSTRACT

Molecules of animal or bacterial origin, which pose a risk for zoonoses or immune rejection, are commonly used for extraction, culture, and cryopreservation of mesenchymal stem cells. There is no sequential and orderly protocol for producing human adipose-derived stem cells (hASCs) under xeno-free conditions. After standardizing a human platelet lysate (hPL) production protocol, four human adipose tissue samples were processed through explants with fetal bovine serum (FBS)-supplemented or hPL-supplemented media for extracting the adipose-derived stem cells. The cells were cultivated in cell culture medium + hPL (5%) or FBS (10%). The cellular replication rate, immunophenotype, and differentiation potential were evaluated at fourth passage. Cellular viability was evaluated before and after cryopreservation of the cells, with an hPL-based solution compared with an FBS-based solution. The explants cultured in hPL-supplemented media showed earlier and faster hASC proliferation than did those supplemented with FBS. Likewise, cells grown in hPL-supplemented media showed a greater proliferation rate, without losing the immunophenotype. Osteogenic differentiation of xeno-free hASC was higher than the hASC produced in standard conditions. However, adipogenic differentiation was reduced in xeno-free hASC. Finally, the cells cryopreserved in an hPL-based solution showed a higher cellular viability than the cells cryopreserved in an FBS-based. In conclusion, we have developed a complete xeno-free protocol for extracting, culturing, and cryopreserving hASCs that can be safely implemented in clinical studies. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:358–365*

SIGNIFICANCE

This study was performed to standardize a complete ordered protocol to produce xeno-free human adipose-derived mesenchymal stem cells (hASCs) as a safe therapeutic alternative. Cells were extracted by adipose tissue explants and then cultured and cryopreserved using human platelet lysate (hPL). Different scientific journals have published data regarding the use of hPL as a safe fetal bovine serum substitute for hASC culture, using heparin to avoid clot formation. This article reports the use of hPL for extracting, culturing, and cryopreserving hASCs without anticoagulant.

INTRODUCTION

The proliferation and differentiation potential of mesanchymal stem cells (MSCs) has attracted worldwide attention in search of an alternative to replace lost cells in injured tissues [1]. MSCs can self-renew and differentiate into mesodermal and nonmesodermal cell lineages, including chondrogenic, osteogenic, adipogenic, myogenic, and neurogenic lineages [2]. They were initially isolated from bone marrow (bone marrow mesenchymal stem cells [BMSCs]) but can be isolated from various tissues [3], including adipose tissue (adipose-derived stem cells [ASCs]) [4].

BMSCs and ASCs have similar proliferation and differentiation potentials [5–7]. However, ASCs have certain characteristics that make them more attractive than BMSCs for therapeutic applications. They show greater morphological and genetic stability, retain their multipotentiality in culture and in vitro longer, have greater proliferation capability [8], are less prone to reach senescence [5], and support hematopoiesis more efficiently compared with BMSCs [9].

Moreover, more MSCs can be extracted from a primary sample of adipose tissue [10]. MSCs are rare in the bone marrow (approximately 1/100,000) [11], whereas in adipose tissue they are equivalent to nearly 2% of the nucleated cells [12]. In addition, the quantity of primary sample that can be obtained from bone marrow never exceeds a few milliliters, whereas it is very easy to obtain hundreds of milliliters of lipoaspirate. All these elements make it relatively easy to extract more than 5×10^5 ASCs per milliliter of adipose tissue sample [10].

Commonly used human ASC (hASC) extraction, culture, and cryopreservation protocols use

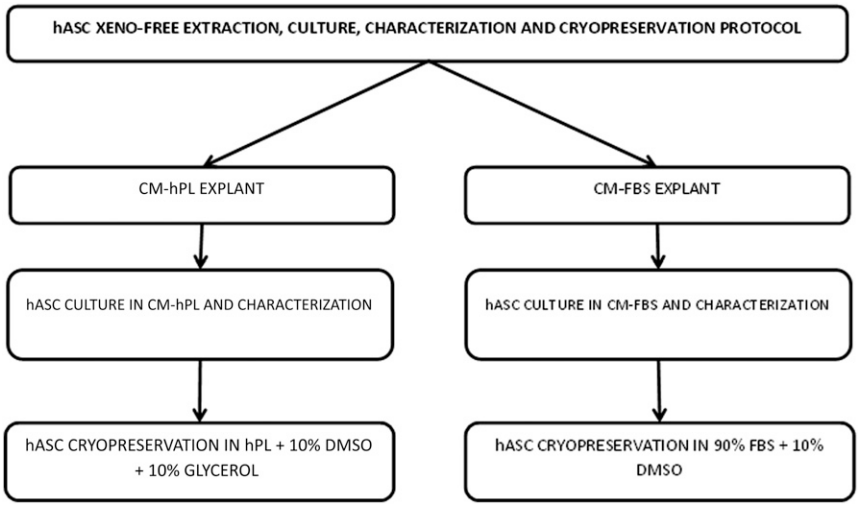

Figure 1. Flowchart of extraction, culture, characterization, and cryopreservation of adipose-derived stem cells with FBS and hPL. Abbreviations: CM-FBS, complete media fetal bovine serum; CM-hPL, complete media human platelet lysate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; hASC, human adipose-derived stem cell; hPL, human platelet lysate.

molecules of animal or bacterial origin, which pose different kinds of risks for humans [13, 14], thereby affecting the potential of these cells as a treatment option for regenerative medicine.

It has been suggested that ASCs are part of the nucleated cell population of the perivascular adipose tissue stroma [15–17]. Thus, enzymes such as collagenase (usually from animal or bacterial origin) are used to degrade the extracellular matrix and release the ASCs. A protocol for ASC extraction that does not use any enzyme but rather uses an adipose tissue explant technique has been reported [18–20]; this protocol can be used in a xeno-free protocol to produce hASCs.

MSC culture requires the use of a nutritional supplement, usually fetal bovine serum (FBS), in the culture medium. However, there are several concerns about FBS safety in the production of human therapeutic products. Immune rejection of FBS may be an important cause of treatment failures and adverse reactions reported in clinical trials [21]. In addition, it represents a risk for the transmission of infectious agents, such as the transmissible spongiform encephalopathies [22].

In recent years, human platelet lysate (hPL) has been proposed as a substitute for FBS in MSC production [23, 24]. In addition to lacking the risk of xenogeneic immune rejection or zoonosis transmission associated with FBS, hPL can be produced under good manufacturing practices at much lower costs than FBS [25, 26].

Previous studies have shown that hPL supports cellular viability, enhances proliferation [27, 28], delays senescence [29, 30], ensures cellular genomic stability [23], and preserves hASC phenotype. MSCs grown in media supplemented with hPL express surface markers commonly used to identify MSCs, even in advanced cell passages [31], in contrast to what happens with cells grown in media supplemented with FBS [32, 33]. It has also been shown that hASCs grown in media supplemented with hPL retain their adipogenic and osteogenic differentiation potential [24, 28, 31], although cells cultured in media supplemented with hPL may have less adipogenic differentiation potential [24] and greater capacity for osteogenic differentiation [34, 35].

Platelets are small enucleated structures of hematopoietic origin produced by the fragmentation of megakaryocytes in the bone marrow [36]. They have three different intracellular granules that store different soluble molecules, including proteins such as growth factors, cytokines, and chemokines [37]. Different protocols have been proposed for the production of hPL, but the complete set of platelet-released factors has not been described [38]. However, it is known that hPL contains substantial amounts of trophic factors such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), in addition to the three isoforms of platelet-derived growth factor (PDGF) (AA, AB, and BB) [31, 39]. It has been suggested that the effect of hPL on MSCs depends mainly on the activity of PDGF-BB, TGF-β, and bFGF [39]. The expression of TGF-β receptor in MSCs has been documented and is known to influence their proliferation, differentiation, and migration capacity [40, 41] and their angiogenic potential [42]. However, assays with mixtures of recombinant proteins have suggested that additional factors are responsible for the effects of hPL on MSCs [39, 43].

Finally, the implementation of MSC therapy for regenerative medicine requires the establishment of cryopreservation methods that can ensure cell viability and phenotypic stability for long periods. Cryopreservation protocols are based on cryoprotectants, such as dimethyl sulfoxide (DMSO) or glycerol and FBS. Although there are some commercial solutions for xeno-free cryopreservation, a cryopreservation protocol based on hPL has not been described [44]. Here we describe a protocol for xeno-free extraction, culture, and cryopreservation of hASCs, which could be implemented in clinical trials to evaluate the therapeutic potential of these cells (Fig. 1).

Materials and Methods

Preparation of hPL

In a modification of a previously reported protocol [27], 10 units of platelets from adult donors of both sexes and different blood

Table 1. Relative concentration of angiogenic factors, detected in the human platelet lysate with a human angiogenesis antibody array

Factor		Value (A.U./mg of total protein)
Enzymes	Angiogenin	0.37
	MMP-1	0.39
	MMP-9	0.27
Growth factors	EGF	0.42
	bFGF	0.30
	PDGF-BB	0.42
	VEGF	0.00
	VEGF-D	0.04
	Angiopoietin 1	0.40
	Angiopoietin 2	0.45
	PlGF	0.05
	IGF-1	0.00
Chemokines	CCL1 (I-309)	0.31
	CCL2 (MCP-1)	0.34
	CCL5 (RANTES)	0.45
	CCL7 (MCP-3)	0.37
	CCL13 (MCP-4)	0.40
	CXCL1-3 (GRO)	0.36
	CXCL5 (ENA-78)	0.37
	CXCL8 (IL-8)	0.28
	CXCL11 (I-TAC)	0.39
Hormones	Leptin	0.37
Cytokines	TGF-β1	0.15
	GM-CSF	0.33
	G-CSF	0.39
	TNF-α	0.39
	IFN-γ	0.08
	IL-1β	0.32
	IL-1α	0.28
	IL-2	0.16
	IL-4	0.07
	IL-6	0.13
	IL-10	0.44
	THPO	0.07
Adhesion molecules and receptors	PECAM-1	0.19
	uPAR	0.20
	Tie-2	0.03
	VEGFR-2	0.15
	VEGFR-3	0.09
Antioangiogenic factors	TIMP-1	0.29
	TIMP-2	0.32
	Angiostatin (PLG)	0.16
	Endostatin	0.00

Values indicate relative concentrations.
Abbreviations: A.U., arbitary units; bFGF, basic fibroblast growth factor; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; EGF, epidermal growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IGF-1, insulin-like growth factor-1; IL, interleukin; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; PDGF-BB, platelet-derived growth factor isoform BB; PECAM-1, platelet endothelial cell adhesion molecule-1; PlGF, placental growth factor; RANTES, regulated on activation, normal T-cell-expressed and secreted; TGF-β, transforming growth factor-β; THPO, thrombopoietin; Tie-2, soluble form of the angiopoietin receptor; TIMP, tissue inhibitor of metalloproteinases; TNF-α: tumor necrosis factor-α; uPAR, soluble form of the urokinase plasminogen receptor; VEGF, vascular endotelial growth factor; VEGF-D, vascular endotelial growth factor isoform D; VEGFR-2 and -3, soluble forms of the vascular endothelial growth factor receptor-2 and -3.

groups donated by the Hemocentro Distrital of the Secretaría Distrital de Salud de Bogotá (Blood, Tissue and Stem Cell Bank of the Secretariat of Health of Bogotá) were centrifuged at 1200 *g* for 15 minutes. The supernatant was discarded, and the pellets were frozen at −80°C after resuspension in the same volume of phosphate-buffered saline (PBS). This solution was then thawed at 4°C, homogenized, and centrifuged at 4,000*g* for 15 minutes; the supernatant was filtered through a 0.22-μm pore. The filtration product was separated into 10-ml aliquots and stored at −20°C. The hPL protein concentration was measured following the Micro BCA Protein Assay Kit standard protocol (Thermo Scientific Pierce, Rockford, IL, https://www.thermofisher.com) and the absorbance was measured at 660 nm on a plate reader Ultramark, Bio-Rad, Hercules, CA, http://www.bio-rad.com).

Protein Array

Two different hPL batches and one FBS batch were evaluated for the detection of 43 human proteins, including cytokines, growth factors, proteases, and soluble receptors, using the Human Angiogenesis Array C1000 (RayBiotech, Norcross, GA, http://www.raybiotech.com), according to the manufacturer's instructions. Briefly, 1 ml of solution was incubated with arrayed antibody membranes for 2 hours at room temperature; membranes were then washed and incubated with the mix of biotin-conjugated antibodies for another hour at room temperature. After washing, horseradish peroxidase-conjugated streptavidin was added to the membranes for 1 hour at room temperature. The signal was developed with detection buffer, and membranes were exposed to autoradiographic films. Signal optical densities were quantified using a program for digital image processing (ImageJ 1.410; National Institutes of Health, Bethesda, MD, https://www.nih.gov). Signal densities (pixels) were normalized against total protein concentration and then expressed as arbitrary units (A.U.) per milligram of protein.

Extraction of hASCs

In a modification of a previously described protocol [19], four biopsy specimens, approximately 5 g each, of abdominal adipose tissue from healthy donors (one man and three women) of different blood groups and age 10–50 years were washed twice with PBS supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), and neomycin (200 mg/ml) and cut into pieces of about 1 mm^3. The fragments were seeded into 40-mm flat-bottom wells and incubated in a humidified atmosphere at 37°C with 5% CO_2 for 1 hour. Then, 500 μl of Opti-MEM (Life Technologies, ThermoFisher Scientific, Grand Island, NY, https://www.thermofisher.com, Grand Island, NY) supplemented with penicillin (50 mg/ml), streptomycin (50 mg/ml), and neomycin (100 mg/ml) (Life Technologies, ThermoFisher Scientific), and 5% hPL (complete media hPL [CM-hPL]) or 10% FBS (complete media FBS [CM-FBS]) was added; the solution was incubated for 24 hours in a humidified atmosphere at 37°C with 5% CO_2. Finally, 500 μl of CM-hPL or CM-FBS was added to continue the incubation in a humidified atmosphere at 37°C with 5% CO_2; the culture media was changed twice a week with fresh medium.

Two weeks later, the explants were removed, and the cells were recovered with TrypLE Select (Life Technologies, ThermoFisher Scientific) after the cell monolayers were washed twice with PBS. The recovered cells were seeded in 750-mm^2 surface culture flasks and cultured in CM-hPL or CM-FBS.

Cell Proliferation Assay

The hASCs were seeded at 2,000 cells per well in triplicate by using 24-well flat-bottom plates and cultured in CM-hPL or CM-FBS for

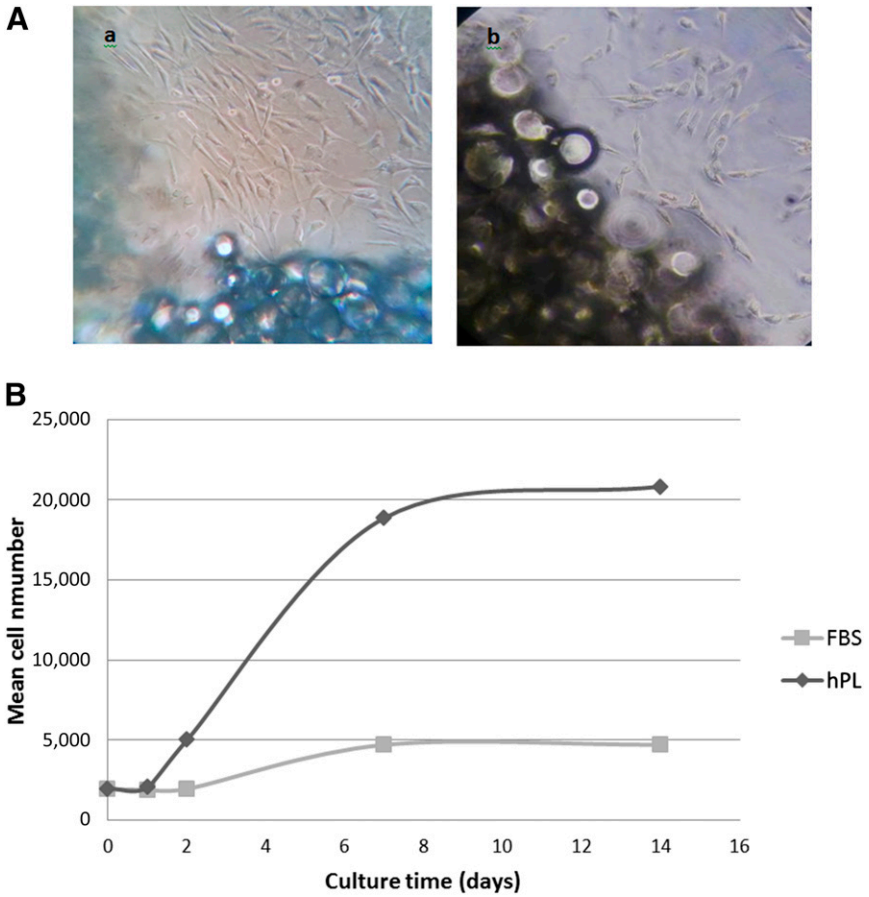

Figure 2. Proliferation of human adipose-derived stem cells (hASCs). **(A):** Proliferation of hASCs from adipose tissue explant. **(Aa):** Two weeks of incubation in complete media human platelet lysate (CM-hPL). **(Ab):** Two weeks of incubation in complete media fetal bovine serum (CM-FBS). Magnification, ×20. **(B):** Curves for hASC proliferation. The values correspond to the average of three replicates of hASC cultured in CM-hPL or CM-FBS. Abbreviations: FBS, fetal bovine serum; hPL, human platelet lysate.

12 hours, 1, 2, 7 and 14 days. After the culturing period, the hASCs per well were quantified with the CyQuant NF Cell Proliferation Assay Kit (Life Technologies, ThermoFisher Scientific), following the manufacturer's instructions. The absorbance was measured at 530 nm on a plate reader (Ultramark). The results were compared with a standard curve of known cellular concentrations in CM-hPL or CM-FBS.

The population doubling (PD) was calculated by using the following equation [45]:

$$X = \frac{[\log_{10}(N_H) - \log_{10}(N_1)]}{\log_{10}2}$$

where N_H corresponds to the final cell number in every time point and N_1 corresponds to the initial cell-plated number.

hASC In Vitro Differentiation Assay

To compare the xeno-free and standard-produced hASC differentiation potential (adipogenic and osteogenic), cells of the fourth pass were seeded at 70% of confluence, in triplicate, and cultured for 2 weeks (the culture media was changed three times per week) in StemPro Adipogenesis and Osteogenesis differentiation media (Gibco, Life Technologies, ThermoFisher Scientific), following the manufacturer's protocols. After the differentiation induction period, the cellular monolayers were stained with Oil Red O or alizarin red to identify the intracellular lipids deposits (adipogenic differentiation) or the interstitial calcium deposits (osteogenic differentiation). The percentage of the area covered by intracellular lipid deposits or extracellular calcium deposits was quantified by densitometric analysis using ImageJ 1.4 software as previously described [46].

hASC Immunophenotypification

Fourth-passage hASCs were recovered with TrypLE Select (Life Technologies, ThermoFisher Scientific) and 5×10^5 cells were incubated with monoclonal anti-human CD34-APC-conjugated (clone AC 136/ isotype IgG2A/Miltenyi Biotec, Bergisch Gladbach, Germany, http://*www.miltenyibiotec.com*), monoclonal anti-human CD45-RPE-Cy5-conjugated (clone T29/33/isotype IgG_1/Dako Cytomation, Glostrup, Denmark, http://www.dako.com), monoclonal anti-human CD73-PE-conjugated (clone D2/ isotype IgG_1/BD Pharmingen, San Jose, CA, http://www.bdbiosciences.com), monoclonal anti-human CD-90 APC-conjugated (clone F15-42-1/isotype IgG_1/AbD Serotec, Kidlington, U.K., *https://www.abdserotec.com)*, monoclonal anti-human CD105 PE-conjugated (clone SN6/isotype IgG_1, eBioscience, San Diego, CA, http://*www.ebioscience.com*), monoclonal anti-human HLA-ABC-FITC-conjugated (clone w6/32/isotype IgG2A/ AbD Serotec), monoclonal anti-human HLA-DR-RPE-conjugated (clone AB3/isotype IgG2A/Dako Cytomation), or specific isotype-negative controls (IgG2a/APC, IgG1/RPE-Cy5, IgG1/PE, IgG1/APC, IgG2A/FITC, IgG2A/RPE, Dako Cytomation). Readings and analysis

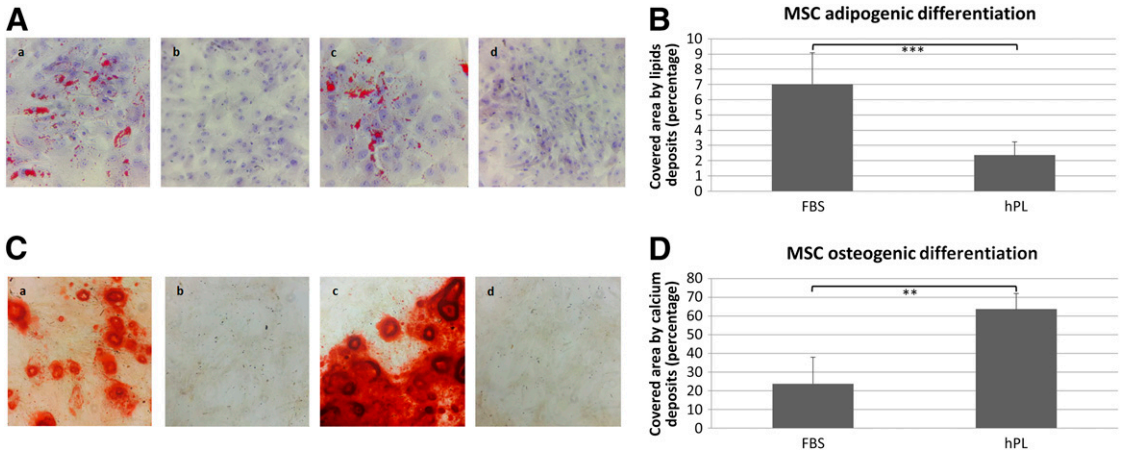

Figure 3. Differentiation potential of human adipose-derived stem cells (hASCs). **(A):** Adipogenic differentiation of fourth-passage hASCs was evaluated in cells cultured for 2 weeks in adipocyte differentiation medium stained with Oil Red O to detect intracellular fat droplets (red). All experiments ($n = 4$) were performed in triplicate. Shown are hASCs produced under standard conditions **(Aa)**, negative control **(Ab)**, xeno-free-produced hASCs **(Ac)**, and negative control **(Ad)**. Magnification, ×20. **(B):** Quantification of the adipogenic differentiation. Percentage of surface area covered by lipid intracellular deposits in hASCs, stained with O Red Oil. **, $p \leq 0.01$. **(C):** Osteogenic differentiation of fourth-passage hASCs was evaluated in cells cultured for 2 weeks in osteogenic differentiation medium stained with alizarin red to detect interstitial calcium deposits (red). All experiments ($n = 4$) were performed in triplicate. Shown are hASCs produced under standard conditions **(Ca)**, negative control **(Cb)**, xeno-free-produced hASCs **(Cc)**, and negative control **(Cd)**. Magnification, ×20. **(D):** Quantification of the osteogenic differentiation. Percentage of surface area covered by interstitial calcium deposits in hASC cultures, stained with alizarin red. ***, $p \leq 0.001$. Abbreviations: FBS, fetal bovine serum; hPL, human platelet lysate; MSC, mesenchymal stem cell.

were performed on an FACSCanto cytometer (BD Biosciences, Franklin Lakes, NJ, http://*www.bdbiosciences.com*) at the Banco de Sangre de Cordón Umbilical del Hemocentro Distrital de Bogotá.

hASC Cryopreservation in hPL

After recovery of the cells with TrypLE Select (Life Technologies, ThermoFisher Scientific), the hASCs were cryopreserved at −80°C at a concentration of 1×10^6 cells per milliliter. Different DMSO and glycerol (Sigma-Aldrich, St. Louis, MO, https*://www.sigmaaldrich.com*) concentrations (10%–30%) were assayed as hPL supplements for the cryopreservation solution. The solution of FBS (90%) and DMSO (10%) was used as a control.

Statistical Analysis

The protein quantification and cellular proliferation data are expressed as mean ± SEM of independent triplicates in every experiment. The statistical significance of the differences among groups was tested by using the Student's *t* test. The differences were considered to be significant at $p \leq .05$.

Results

hPL Characterization

The hPL produced with the described protocol is translucent and does not form a clot at 37°C. Thus, it was not necessary to add heparin or any other anticoagulant to the cell culture. The protein concentration was 2.53 ± 0.28 mg/ml.

The relative concentration of 43 different proteins, including EGF, bFGF, PDGF-BB, monocyte chemoattractant protein-1 (MCP-1), TGF-β1, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor (G-CSF), was quantified. The presence of IGF-1, VEGF, and endostatin was not detected. On the other hand, some other proteins, such as angiogenin, EGF, bFGF, MCP-1, angiopoietin, G-CSF, matrix metalloproteinase-9 (MMP-9), C-X-C motif chemokine ligand-5 (CXCL-5), C-C motif chemokine ligand-1, and platelet endothelial cell adhesion molecule-1 were found in high concentrations, with values between 0.12 and 0.45 A.U./mg of total protein (Table 1).

Isolation and Culture of hASC

Spindle-shaped cells began to proliferate from the explants after 2 days of culture and reached 80% confluence after 1 or 2 weeks of culture. These cells were uniform in shape and size and exhibited the characteristic morphology of hASC. However, migration and cell proliferation were greater in the explants cultured in CM-hPL (Fig. 2A).

Cell morphology was preserved as the cells were replicated throughout the study, and no differences were evident between the samples. Cells isolated from a sample, randomly selected, were used for the proliferation evaluation. The cellular differentiation potential was evaluated in cells from all samples, and the immunophenotype was evaluated in cells from two randomly selected samples.

hASC Characterization

After 7 days of culture, the cumulated number of population doublings had reached 3.39 for hASCs cultured in CM-hPL and 1.23 for hASCs cultured in CM-FBS. The population doubling time after 7 days of culture was 2.16 days for hASCs cultured in CM-hPL and 5.65 days for hASCs cultured in CM-FBS (Fig. 2B).

Both the xeno-free hASCs (CM-hPL) and the standard conditions (CM-FBS) produced hASC and showed osteogenic and adipogenic differentiation potential (Fig. 3A, 3C), but the xeno-free hASCs showed a significantly greater osteogenic differentiation (Fig. 3C, 3D; $p = .000001$) and less adipogenic differentiation than the hASCs produced under standard conditions (Fig. 3A, 3B; $p = .003$). Finally, a high percentage of the xeno-free hASCs

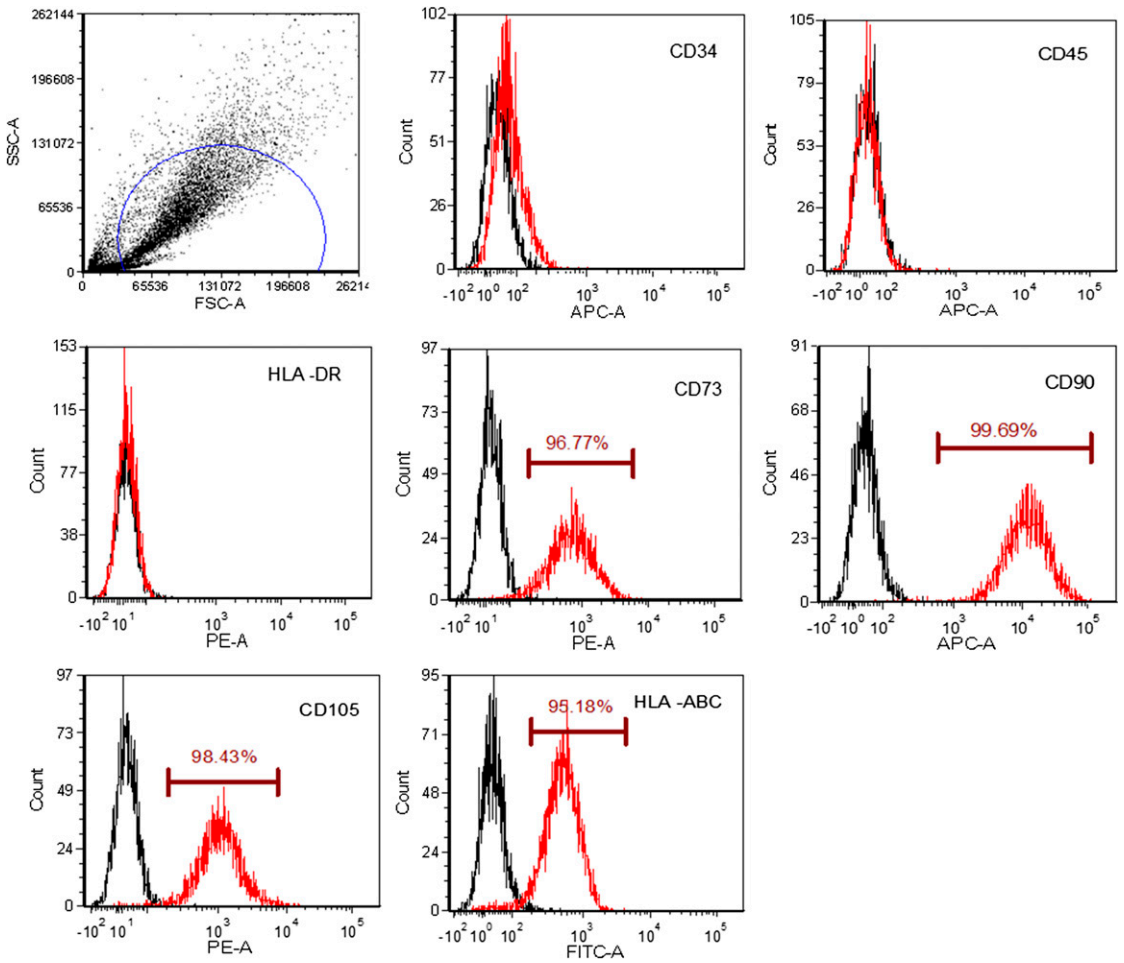

Figure 4. Immunophenotyping of human adipose-derived stem cells (hASCs). Representative flow cytometry analysis of fourth-passage hASCs. The cells were cultured in complete media human platelet lysate and incubated with monoclonal antibodies (CD34-APC, CD45-RPECy5, HLA II-RPE, CD73-PE, CD90-APC, CD105-PE, HLA I-FITC) or their respective isotype controls.

expressed CD73 (96.77%), CD90 (99.69%), CD105 (98.43%), and HLA-ABC (95.18%), without CD34, CD45, or HLA-DR expression, a characteristic hASC immunophenotype (Fig. 4).

hASCs Showed Higher Viability After Cryopreservation in hPL

The hPL produced with our protocol could also be used for hASC cryopreservation. The hASCs cryopreserved in the hPL 80% + DMSO 10% + glycerol 10% solution showed 82.5% ± 2.88% viability after cryopreservation compared with 72.5% ± 2.88% after cryopreservation in an FBS-based solution (FBS 90% + DMSO 10%) (Table 2).

Discussion

The production of xeno-free hASCs has been a factor hindering evaluation of its therapeutic efficacy. FBS has been used as supplement for the MSC production, including cells for clinical trials. There are several problems with FBS as a supplement for MSC production for human applications [21] besides the risk for zoonosis transmission [22, 47]. The MSCs can internalize components of the cell culture medium and those molecules could induce an immune rejection [14, 48, 49]. Stem cells cultured in FBS express a nonhuman immunogenic sialic acid form (Neu5Gc) [50], and the cell lysis is mediated by human antibodies against Neu5Gc [39]. Moreover, the immune reaction against FBS antigens in individuals who received several dose of MSC produced with FBS supplement [48] and the development of a diffuse urticariform reaction [13, 49] have been reported.

A xeno-free protocol to produce BMSCs using hPL [24] has already been reported, but there are no reports of alternatives to produce xeno-free ASCs. A previous study showed hPL is a feasible substitute for FBS [45]. hPL facilitates ASC proliferation more efficiently than does FBS [27, 28], favors genomic stability [23], and maintains the differentiation potential and expression of characteristic surface markers [29, 30].

Clot formation is the main problem seen with replacing FBS by hPL. There are different alternatives to avoid the fibrin clot in the culture medium. Perhaps the most used strategy is addition of heparin, but this anticoagulant has adverse effects on the cells, evidenced by a reduction of their proliferation [51]. In addition, seroconversion of the hPL has been proposed, inducing the coagulation and recovering the soluble proteins [52]. However, although the proliferation and phenotype conservation of the MSCs cultured in seroconverted hPL has been shown, a significant reduction in PDGF-BB concentration has been

Table 2. Cellular viability of human adipose-derived stem cells cryopreserved in human platelet lysate-based or fetal bovine serum-based solutions

	Viability (%)	
Sample	FBS 90% + DMSO 10%	hPL 80% + DMSO 10% + glycerol 10%
1	70 ± 2	85 ± 5
2	75 ± 5.5	81 ± 3.6
3	75 ± 8.1	80 ± 3
4	70 ± 5.2	85 ± 4.6

Values are expressed as mean ± SEM.
Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; hPL, human platelet lysate.

documented; this could have important effects on MSC phenotype in long-term cell cultures [40–42].

We have modified the hPL production protocol, replacing the plasma with PBS and avoiding the clot formation. Using this hPL, we have developed a xeno-free extraction, culture, and cryopreservation protocol for hASCs, which could be used for the production of BMSCs or umbilical cord blood-derived stem cells.

There is no standard protocol for hPL production, but it is known that growth factors such as PDGF-BB, EGF, TGF-β, bFGF, VEGF, and IGF-1 are among the most important components. However, assays with different combinations of the recombinant proteins have not reproduced hPL effects [39, 43].

Our results show some similarities and differences with previous reports. We have found high concentrations of proteins such as EGF, angiogenin, angiopoyetin-1, G-CSF, MMP-9, and CXCL-5 in the hPL; however, have not found IFG-1, VEGF, or endostatin [31, 39]. The absence of IGF-1 its not surprising, considering that it is mainly a plasma protein [53], and the platelet α granules have a very low concentration [54]. On the other hand, the absence of VEGF and endostatin could be a consequence of the level of sensitivity of the detection method or a different lysis efficiency for some subpopulations of α granules [55]

It has been reported that hASCs extracted from adipose tissue explants meet the identification criteria for MSCs, including their differentiation potential and the expression of commonly used markers for their identification [18, 19]. In addition, studies have documented that the hASCs cultured in hPL-supplemented medium retain their phenotype characteristics [32, 33]. Here we have shown that the hASCs extracted by explant and cultured in hPL-supplemented media retain all their phenotypic characteristics.

The xeno-free hASCs produced with our protocol have adipogenic and osteogenic differentiation potential. However, they vary in their differentiation potential in comparison with the hASCs produced under standard conditions. We documented a higher osteogenic differentiation of xeno-free hASCs, confirming previous reports [34, 35]. We also observed a lower adipogenic differentiation potential of xeno-free hASCs, which has also been suggested previously [24].

Conclusion

We have developed a sequential and ordered protocol for extraction, culture, and cryopreservation of xeno-free hASCs that can be safely applied as a therapeutic alternative for regenerative medicine.

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Author Contributions

C.H.E.: financial support, administrative support, collection and assembly of data, data analysis and interpretation, manuscript writing; O.C.: conception and design, financial support, administrative support, provision of study material, data analysis and interpretation, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

C.H.E. has compensated employment and research funding from Fundación Universitaria de Ciencias de la Salud. O.C. has compensated employment and has uncompensated intellectual property rights from Universidad Nacional de Colombia.

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