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Human AB Serum and Thrombin-Activated Platelet-Rich Plasma Are Suitable Alternatives to Fetal Calf Serum for the Expansion of Mesenchymal Stem Cells from Adipose Tissue

ASLI KOCAOEMER, SUSANNE KERN, HARALD KLÜTER, KAREN BIEBACK

Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg – Hessen, Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany

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ABSTRACT

MSCs are currently in focus regarding their clinical potential in cell therapy and tissue engineering. However, most isolation and expansion protocols for clinical-scale production of MSCs use fetal calf serum (FCS) as a supplement, which poses a potential risk for infections as well as immunological reactions. To find a suitable FCS substitute, we investigated the effects of pooled human AB serum (AB-HS) and thrombin-activated platelet-rich plasma (tPRP) on adipose tissue MSCs (AT-MSCs) with FCS as the standard control medium. AT-MSCs of 10 donors were cultured under three different conditions: (a) 10% FCS, (b) 10% AB-HS, and (c) 10% tPRP. Colony-forming units, cumulative population doubling rates, and differentiation capacity toward the adipogenic and osteogenic lineages were assessed, along with immunophenotype. We demonstrated that AB-HS and tPRP provide a significantly higher proliferative effect on AT-MSCs than does FCS. In the first six passages, AB-HS and tPRP MSCs exhibited a fold expansion of 66.6 ± 15.7 and 68.1 ± 6.7, respectively, compared with 24.4 ± 0.7 for FCS. Differentiation capacity was preserved throughout long-term culture. Immunophenotype was characteristic for MSCs and comparable for all culture conditions with the exception of a distinct CD45-/CD14-positive side population for AB-HS and tPRP that tended to diminish with prolonged culture. We showed that pooled human AB serum and thrombin-activated platelet-rich plasma are alternatives to FCS for AT-MSCs. These human sources are better characterized regarding potential infectious threats, while providing a higher proliferation rate and retaining differentiation capacity and mesenchymal stem cell marker expression throughout long-term culture. STEM CELLS *2007;25: 1270–1278*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

MSCs are multipotent, nonhematopoietic stem cells that are typically obtained from bone marrow [1] but can also be isolated from several other tissues such as umbilical cord blood [2] and adipose tissue [3]. Human MSCs are currently in focus regarding their clinical potential in cell therapy and tissue engineering. They are attractive candidates for clinical use because of their ease of isolation, their extensive proliferation and differentiation capacity, and their hypoimmunogenic nature [4]. MSCs display the ability to differentiate into multiple mesoderm-derived cells, such as adipocytes, osteocytes, and chondrocytes [5], but they may also give rise to cells of nonmesodermal origin, such as hepatocyte-like [6] and neuronal-like cells [7]. A variety of ongoing clinical trials use human MSCs in search of a treatment for diseases such as myocardial infarction [8] and graft-versus-host disease [9], and to promote engraftment of bone marrow transplants [10]. At the moment, however, most isolation and expansion protocols for clinical-scale production of MSCs use culture media supplemented with fetal calf serum (FCS). FCS contains xenogeneic proteins that are internalized by MSCs [11]. Consequently, a host of potential problems such as viral and prion transmission [12] or immunological reactions [13–15] can arise. Our goal was to develop a manufacturing process for MSCs that does no depend upon FCS and complies with current regulations of good manufacturing practice (GMP). Recently, human serum and platelet lysate have been identified as promising substitutes [16, 17]. Platelet factors such as platelet-derived growth factor, transforming growth factor, and epidermal growth factor that promote increased migration and proliferation of MSCs have been studied extensively [18–20]. The effectiveness of human serum is based mainly on studies using autologous serum [16, 21], whereas the reports on allogeneic serum have been contradictory [22, 23]. Our aim was to compare the effectiveness of human serum and platelet factors in promoting MSC growth with FCS. We decided to use pooled human AB serum (AB-HS) instead of autologous serum because, for clinical use, an "off-the-shelf" product that is available in large quantities is more desirable. After testing several protocols for the derivation of a platelet-factor rich supernatant, we chose thrombin-activated platelet-rich plasma (tPRP) pooled from AB donors as the most suitable supplement. In the present study, we investigated the effects of pooled AB-HS and tPRP on the isolation, expansion, and differentiation capacities, as well as the immuno-

Correspondence: Karen Bieback, Ph.D., Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg – Hessen, Medical Faculty Mannheim, University of Heidelberg, Friedrich-Ebert-Str. 107, 68167 Mannheim, Germany. Telephone: +49-621-3706-8216; Fax: +49-621-3706-851; e-mail: k.bieback@iti-ma.blutspende.de Received October 4, 2006; accepted for publication January 12, 2007; first published online in STEM CELLS *EXPRESS* January 25, 2007. 1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2006-0627

phenotype, of adipose tissue-derived MSCs (AT-MSCs) using FCS as the standard control medium.

Materials and Methods

Preparation of Supplements and Media

AT-MSCs were cultured under three different culture conditions. Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Cambrex, Walkersville, MD, http://www.lonzabioscience.com) with 4 mM L-glutamine (PAA, Pasching, Austria, http://www.paa.com) and 0.05 units of penicillin per 0.05 μg of streptomycin (PAA) served as the basal medium, which was supplemented with (a) 10% fetal calf serum (preselected lot; mesenchymal stem cell growth supplement; Cambrex), (b) 10% AB-HS, or (c) 10% tPRP.

AB-HS

Human serum was derived from whole-blood donations of 10 AB-blood-group-typed donors. Blood donations were drained into blood bags without anticoagulants and allowed to clot overnight at 4°C. Subsequently, the serum was separated by centrifugation at 2,000*g* for 15 minutes. The serum was aliquoted into 15-ml sterile tubes (Greiner Bio-One, Frickenhausen, Germany, http://www.gbo.com/en) and frozen at −30°C. After thawing, AB-HS of at least five donors was pooled and sterile filtered through 0.2-μm pores (Nalgene filtration device; Nalge Nunc International, Rochester, NY, http://www.nuncbrand.com).

Preparation of Platelet-Rich Plasma and Platelet Activation

Whole-blood donations of four AB-blood-group-typed donors were used to prepare one pooled platelet concentrate out of buffy coats as described in Janetzko et al. [24]. The protocol was slightly modified by suspending the pooled platelet concentrate in AB plasma of one donor instead of using a platelet stabilizer such as T-sol. Platelet count was between 2×10^6 and 3×10^6 platelets per microliter for all platelet concentrates as determined by CellDyn (3200; Abbott, Wiesbaden, Germany, http://www.abbott.de). The resulting pool of platelets in plasma was termed platelet-rich plasma (PRP). Two different protocols to derive platelet factor rich supernatants were tested. First, a platelet lysate protocol derived from Doucet et al. [17] in which PRP was shock frozen twice in liquid nitrogen was developed. After 30 minutes centrifugation at 3,000*g*, the platelet-factor rich supernatant was sterile filtered through 0.2-μm pores and stored in aliquots of 5 ml at −80°C. Alternatively, platelets were activated by (a) 1 unit of human thrombin [19] (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) per milliliter of cell suspension, (b) 5 μM adenosinediphosphate (ADP) (Sigma-Aldrich)/5 μM epinephrine [25] (Sigma-Aldrich), or (c) 1 mM thrombin receptor activated peptide (TRAP)-6 [26] (Sigma-Aldrich) for 45 minutes at room temperature with gentle agitation. Calcium was not used in the activation protocols to prevent increased osteogenic differentiation [18]. This was followed by centrifugation and sterile filtration as described earlier herein. After thawing, the aliquot was centrifuged again for 5 minutes at 1,500*g* to remove any developing clots. To prevent in vitro gel formation, 2 units of heparin (Liquemin 5000; Roche, Basel, Switzerland, http://www.roche.com) per milliliter of medium were added to the basal medium before adding the platelet factors. We selected tPRP as the best option of all platelet-activation protocols (see Results), and therefore used this for MSC isolation and expansion in repetitive experiments.

Isolation and Culture of Adipose Tissue MSCs

AT-MSCs were obtained from lipoaspirates of 10 donors (two males and eight females) ranging in age from 25 to 64 years, with a median age of 31.5 years. Lipoaspirates were obtained in accordance with the ethical standards of the local ethical committee. Two hundred milliliters of each raw lipoaspirate were processed as described previously [3]. Briefly, lipoaspirates were washed with phosphate buffered saline (PBS; Biochrom AG, Berlin, Germany, http://www.biochrom.de) and digested with 0.075% collagenase type I (Sigma-Aldrich) for 30–45 minutes at 37°C. After neutralization of collagenase activity with basal medium and supplements, the stromal vascular fraction (SVF) was derived by centrifugation at 1,200*g* for 10 minutes. The pellet was resuspended and filtered through a 100-μm nylon cell strainer (Falcon; Becton Dickinson, Heidelberg, Germany, http://www.bd.com). After one more centrifugation at 1,200*g* for 10 minutes, the resuspended SVF cells were plated into T175 cell culture flasks (Greiner Bio-One) using basal medium with (a) 10% FCS, (b) 10% AB-HS, or (c) 10% tPRP. All flasks were cultivated at 37°C in a humidified atmosphere containing 5% CO_2. Nonadherent cells were removed 12–18 hours after plating by extensive washing. The resulting plastic-adherent cell population was termed AT-MSCs.

AT-MSCs were maintained under the three culture conditions described in the preceding paragraph with twice-weekly media changes until a confluence of 70%–80% was reached. At this time, cells were passaged using Trypsin/EDTA (Promocell, Heidelberg, Germany, http://www.promocell.com) and replated at a density of 200 cells per square centimeter for all passages. Viability was determined by Trypan blue exclusion (Sigma-Aldrich), and cells were counted using a Neubauer chamber (Marienfeld GmbH, Lauda-Königshofen, Germany, http://www.superior.de).

Proliferation Kinetics

AT-MSCs of all 10 donors were expanded using three different supplements. Cells were counted and passaged at a confluence of 70%–80%. If cells did not reach a minimum confluence of 50% after 21 days, the culture was discontinued. At each passage, the population doubling rate was determined by using the formula $x = [\log10(NH) - \log10(N1)]/\log10(2)$ [3], where N1 is the plated cell number and NH is the cell number at harvest. To calculate the cumulative population doubling rate, the population doublings for each passage were determined and then added to the previous passages. Because the cell number of plastic-adherent cells could first be counted at passage 1, the cumulative doubling rates were calculated starting at passage 2. Fold expansion rates were also determined for passages 2–6 by dividing the cell number at harvest by the plated cell number (NH/N1).

At the same time, a growth curve of AT-MSCs from three different lipoaspirates was started at passage 3. Two hundred cells per square centimeter were plated in five T25 flasks (Greiner). At days 3, 5, 7, 9, and 11, cells from one T25 flask were harvested and counted. The fold expansion from seeding at day 1 was calculated and compared for all supplements.

The use of heparin and thrombin in tPRP might have an additional proliferative effect; therefore, separate heparin and thrombin controls were started at passage 2. AT-MSCs from four lipoaspirates were cultured in basal medium with 10% FCS until passage 2, and then plated at a density of 200 cells per square centimeter in three different T75 flasks (Nunc) using basal medium with either 10% FCS, 10% FCS + 2 units of heparin per milliliter, or 10% FCS + 1 unit of thrombin per milliliter. Cells were counted and passaged at days 8, 16, 26, and 36.

Fibroblastoid Colony-Forming Unit Assays

AT-MSC frequency in primary culture at P0 was determined for all 10 donors by counting fibroblastoid colony-forming units (CFU-Fs). After isolation from raw lipoaspirates, SVF cells were plated in six-well plates (Falcon) at low densities of 1×10^3, 5×10^3, and 1×10^4 per well using the three different culture conditions. The medium was replaced twice weekly, and cultures were stopped on day 10. Then, the cell layer was fixed with methanol and stained with a Giemsa solution (Merck, Darmstadt, Germany, http://www.merck.de). AT-MSC frequency was calculated as the mean number of colonies per 1,000 cells seeded.

In Vitro Differentiation Assays

Differentiation assays into the adipogenic and osteogenic lineages were performed for AT-MSCs of all 10 donors and of all culture conditions at passage 1 and then again at passage 7 to assess whether differentiation capacities were retained throughout long-

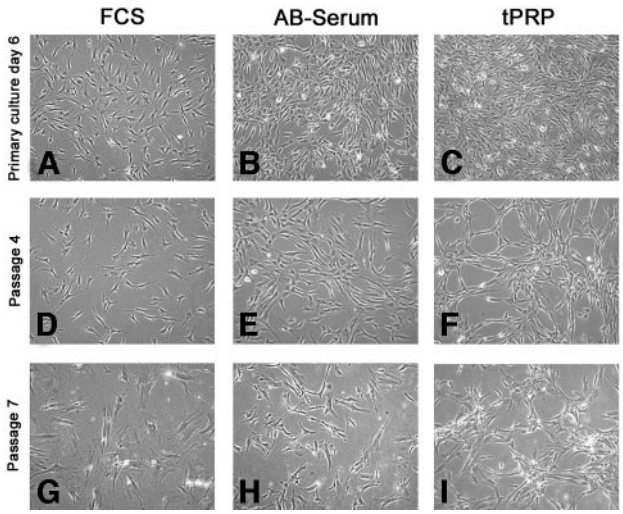

Figure 1. Morphology of adipose-tissue mesenchymal stem cells of one representative donor at primary culture and passages 4 and 7 for FCS **(A, D, G)**, AB serum **(B, E, H)**, and tPRP **(C, F, I)**. Examples are representative of all 10 donors. Magnification, ×100. Abbreviations: FCS, fetal calf serum; tPRP, thrombin-activated platelet-rich plasma.

term culture. Osteogenic differentiation was confirmed by the increase of alkaline phosphatase (AP) expression by histochemical staining following manufacturer's instructions (leukocyte alkaline phosphatase kit 85L-3R; Sigma-Aldrich). The formation of a hydroxyapatite matrix was confirmed by the van Kossa stain as described in Kern et al. [3].

Adipogenic differentiation was induced in 100% confluent AT-MSC cultures with three cycles of alternating induction and maintenance medium. The developing lipid vacuoles were stained with Oil Red. All osteogenic and adipogenic media consisted of bullet kits by Cambrex, which included osteogenic and adipogenic basal media in addition to the supplements.

Flow Cytometry Analysis

Immunophenotypic analyses with flow cytometry were performed for AT-MSCs at passage 1 for four donors and again at passage 7 for three of the same donors. The cells used for analyses at passage 1 were cryopreserved in FCS with 10% dimethyl sulfoxide (DMSO) for cells cultured in FCS, or in 4% Albumin-isotonic sodium-chloride solution and 10% DMSO for all cells cultured in either AB-HS or tPRP. Before flow cytometry analysis, cells were thawed and cultured for at least 1 week.

At passage 1, the following mouse anti-human antibodies were used: CD31-phycoerythrin (PE), CD34-PE, CD29-fluorescein isothiocyanate (FITC; Becton Dickinson), CD45-PE (Diatec, Oslo, Norway, http://www.diatec.com), CD144-PE (Beckman Coulter, Krefeld, Germany, http://www.beckmancoulter.com), CD14-PE, CD73-PE, CD90-Cy5, CD44-FITC, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-FITC (Immunotech-Coulter, Marseille, France, http://www.beckmancoulter.com/products/pr_immunology.asp), CD133/1-PE (Miltenyi Biotech, Bergisch Gladbach, Germany,http://www.miltenyibiotec.com), and vascular endothelial growth factor receptor (VEGFR)-2-PE (R&D Systems, Minneapolis, http://rndsystems.com). CD14-PE, CD31-PE, CD44-FITC, CD45-PE, CD73-PE, CD90-Cy5, HLA-ABC-FITC, and HLA-DR-FITC were again reassessed at passage 7. Mouse isotype antibodies served as control (Becton Dickinson; Beckman Coulter). Ten thousand labeled cells were acquired and analyzed using a FACScan flow cytometer running CellQuest software (Becton Dickinson).

Human Cytokine Antibody Array

Human AB-HS of all 10 donors and tPRP of all preparations were pooled, respectively, and their cytokine profiles were analyzed with a semiquantitative human cytokine antibody array that detects 174 cytokines in one experiment (RayBio Human Cytokine Antibody Array G series 2000; Tebu-bio GmbH, Berlin, http://www.tebu-bio.com). Total protein content of all supplements was determined with the Bradford reaction. The array consisted of three glass slides (array 6, 7, and 8) that were pretreated according to the manufacturer's instructions and incubated with 10-fold diluted AB-HS or tPRP for 2 hours. All sample measurements were performed in duplicate. Because of the human specificity of the array, FCS was not tested. The array glass slides were washed, incubated with a biotin-conjugated anti-cytokine mix for 2 hours, washed again, and developed for 2 hours with Cy3-conjugated streptavidin. The signals were detected using a laser scanner (GMS 418 array scanner; Affymetrix, Santa Clara, CA, http://www.affymetrix.com) and analyzed with ArrayVision version 7 (Imaging Research Inc., St. Catharines, ON, Canada, http://www.imagingresearch.com). Signals were normalized using positive, negative, and internal controls included on the array.

Statistical Analysis

Data are presented as arithmetic mean ± SD. A two-sided paired Student's *t* test was used to compare the mean values of cumulative population doublings and cell numbers, as well as to analyze flow cytometry data. Differences were considered to be significant at $p < .05$. All statistical tests were performed using the SAS software package (version 9; SAS Institute, Cary, NC, http://www.sas.com).

RESULTS

Choice of Alternative Supplement

Human AB-HS and tPRP were identified as alternatives to FCS, and their effect on AT-MSCs was studied in long-term culture. To determine the optimal way to prepare a platelet-factor-rich

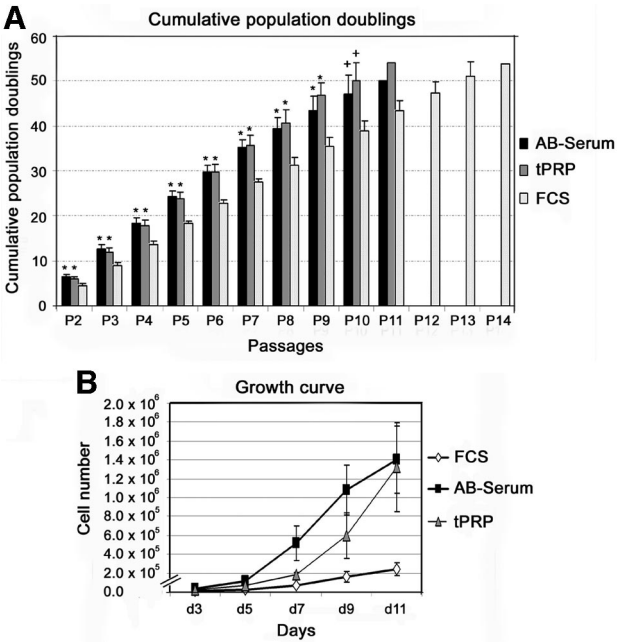

Figure 2. Expansion capacity of adipose tissue MSCs cultured in FCS or human alternatives. **(A):** Mean cumulative population doublings for FCS (white), AB serum (black), and tPRP (gray). For AB-HS, $n = 10$ until P8, $n = 8$ for P9, $n = 5$ for P10, and $n = 1$ for P11; for tPRP, $n = 10$ until P8, $n = 5$ for P9, $n = 3$ for P10, and $n = 1$ for P11; and for FCS, $n = 10$ until P10, $n = 6$ for P11, $n = 5$ until P13, and $n = 1$ for P14. **(B):** Growth curve assessing the proliferation kinetics of adipose-tissue mesenchymal stem cells in FCS, AB serum, and tPRP at days 3, 5, 9, and 11. ∗, in comparison to FCS ($p < .0001$); +, in comparison to FCS ($p < .05$). Abbreviations: FCS, fetal calf serum; P, passage; tPRP, thrombin-activated platelet-rich plasma.

Table 1. Comparison of fold expansion per passage for passages 2 through 6

Passage	FCS (n = 10)	AB-HS (n = 10)	tPRP (n = 10)
P2	24 ± 6	78 ± 30[a]	62 ± 24[b]
P3	24 ± 8	85 ± 38[b]	68 ± 24[b]
P4	24 ± 5	65 ± 23[a]	74 ± 43[c]
P5	26 ± 5	57 ± 13[a]	73 ± 38[b]
P6	24 ± 4	47 ± 13[a]	59 ± 14[a]
Average	24.4 ± 0.7	66.6 ± 15.7	68.1 ± 6.7

This table shows the mean fold expansion of cells ± SD for passages 2–6 for the culture conditions FCS, AB-HS, and tPRP. Cells cultured with AB-HS or tPRP have a significantly higher expansion rate than cells cultured with FCS, whereas there is no significant difference observed between AB-HS and tPRP.
[a] $p < .001$.
[b] $p < .01$.
[c] $p < .05$.
Abbreviations: AB-HS, human AB serum; FCS, fetal calf serum; tPRP, thrombin-activated platelet-rich plasma.

supernatant, several platelet-activation protocols were compared to a platelet-lysate protocol published by Doucet et al. [17]. Platelet lysate was not considered an alternative to FCS because, although it provides a comparable proliferative effect, the handling was decidedly more difficult. It was not possible to purify the platelet-factor rich supernatant of platelet membranes that formed huge aggregates and adhered to AT-MSC surfaces (not shown). At the same time, centrifugation at a high g rate to clear the membranes resulted in a decreased proliferative effect (not shown). Compared with thrombin-activated PRP, ADP/epinephrine or TRAP-6-activated PRP were inferior in numbers of AT-MSCs isolated and their expansion during passages 1 and 2. For this reason, thrombin was chosen as the optimal agent for releasing platelet factors into plasma and was used for all subsequent experiments. All preparations of AB-HS and tPRP were used as a pool of at least five donors to minimize donor-related variations and to mimic an off-the shelf product for clinical-grade MSC manufacturing.

Morphology and Growth Pattern

AT-MSCs of all culture conditions displayed a characteristic fibroblast-like morphology (Fig. 1A–1C). However, cells cultured with AB-HS and tPRP appeared smaller and more spindle-shaped with fewer cytoplasmic processes than did cells cultured with FCS, and had a pronounced clustering of vesicles around the nucleus. In primary culture, AB-HS (Fig. 1B) and tPRP (Fig. 1C) cells had a much denser growth on day 6 compared with FCS cells (Fig. 1A). This resulted in higher cell numbers harvested from the same culture area compared with FCS. Starting at passage 3–4, AT-MSCs cultured in AB-HS and tPRP showed a distinct mesh-like growth pattern (Fig. 1E, 1F), which was even more pronounced in later passages, as seen in Figure 1H and 1I. This mesh-like growth pattern was most evident for tPRP cells and prevented the formation of a confluent monolayer.

Starting at passage 4, FCS cells lost their spindle-like shape and assumed a more flattened, spherical morphology (Fig. 1G), whereas AB-HS and tPRP cells retained their spindle-shaped morphology throughout long-term culture (Fig. 1H–1I), assuming a spherical morphology only at the end of their proliferative phase after 40–50 population doublings.

Furthermore, AT-MSCs cultured with AB-HS and tPRP had decreased adhesion potential compared with AT-MSCs cultured with FCS. In the FCS condition, cells detached from plastic after

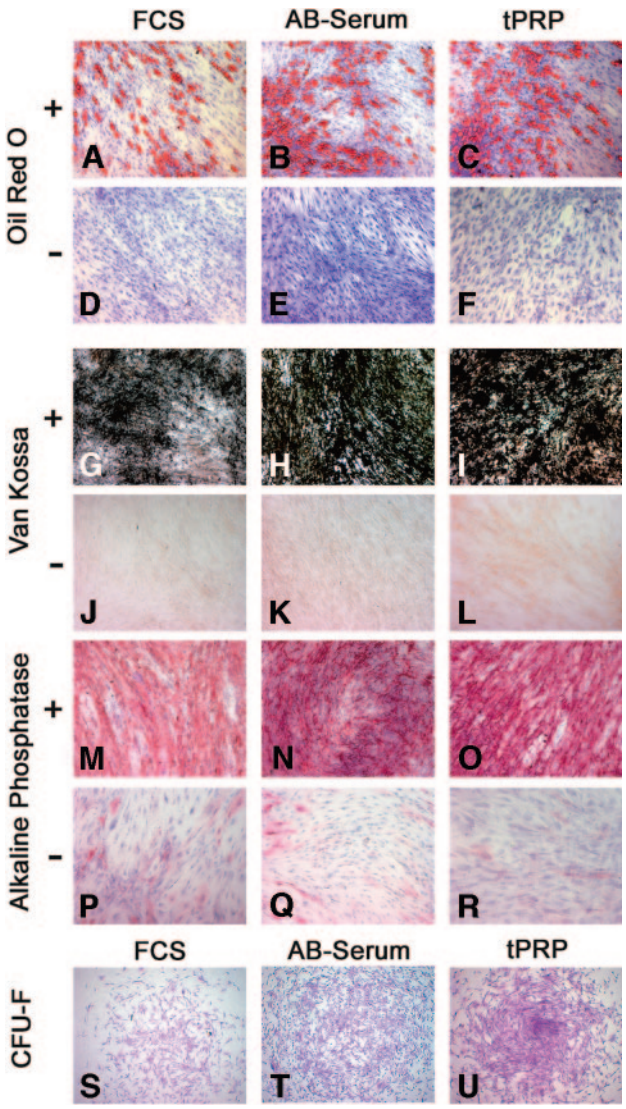

Figure 3. Cells of all culture conditions differentiate consistently into the adipogenic lineage (**[A–C]**, induced; **[D–F]**, noninduced) and osteogenic lineage (**[G–I]**, **[M–O]**, induced; **[J–L]**, **[P–R]**, noninduced). Stains shown are representative for passages 1 and 7. **(S–U):** CFU-F assay shows larger and denser colonies for AB serum **(T)** and tPRP **(U)**. Magnification, ×100. Abbreviations: CFU-F, fibroblast colony forming unit; FCS, fetal calf serum; tPRP, thrombin-activated platelet-rich plasma.

5–6 minutes of trypsination, whereas AB-HS and tPRP cells had already detached after 3–4 minutes. Even without trypsination, tPRP cells tended to detach from the middle of the cell clusters on reaching higher cell densities (Fig. 1I).

Isolation and Expansion Potential

The isolation of AT-MSCs from lipoaspirates was successful for all donors (n = 10) irrespective of culture condition. Cumulative population doublings were calculated for all passages to compare the effect of different supplements on the proliferative capacity of AT-MSCs. We showed that cumulative expansion rates were significantly higher when MSCs were cultured in AB-HS or tPRP rather than in FCS (Fig. 2A; $p < .001$). However, we did not observe a significant difference between AB-HS and tPRP ($p = .37$). AB-HS and tPRP exerted an early and strong proliferative effect on AT-MSC growth that leveled

Table 2. Comparison of the surface marker expression of mesenchymal stem cells cultured with either FCS, AB-HS, or tPRP as analyzed by flow cytometry at passages 3 and 7 (mean percentage ± SD)

	FCS		AB-HS		tPRP	
Antibody	P3 (n = 4)	P7 (n = 3)	P3 (n = 4)	P7 (n = 3)	P3 (n = 4)	P7 (n = 3)
CD29	99.5 ± 0.6		100.0 ± 0.1		99.8 ± 0.3	
CD44	99.9 ± 0.1	99.9 ± 0.1	100.0 ± 0.1	99.9 ± 0.1	99.9 ± 0.2	99.9 ± 0.1
HLA-I	93.5 ± 7.0	94.3 ± 9.13	94.4 ± 9.1	73.5 ± 28.7	89.2 ± 16.4	90.1 ± 13.5
HLA-II	1.2 ± 0.6	0.9 ± 0.7	0.7 ± 0.4	0.1 ± 0.0	4.9 ± 8.1	0.2 ± 0.0
CD34	2.3 ± 1.6		8.7 ± 7.9		5.3 ± 4.5	
CD45[a–d]	11.6 ± 11.9	3.0 ± 1.3	61.0 ± 2.1	5.0 ± 4.1	30.5 ± 8.3	10.5 ± 10.0
CD73	95.6 ± 3.6	89.7 ± 10.2	98.2 ± 3.2	91.1 ± 14.8	99.8 ± 0.2	99.8 ± 0.1
CD133/1	0.4 ± 0.3		0.6 ± 0.6		0.4 ± 0.3	
CD144	0.8 ± 0.5		0.2 ± 0.1		0.5 ± 0.3	
CD31	0.8 ± 0.3	1.0 ± 0.9	0.3 ± 0.3	0.4 ± 0.2	0.3 ± 0.3	0.3 ± 0.1
VEGFR-2	1.2 ± 0.2		0.3 ± 0.2		0.7 ± 0.5	
CD14[a,b]	9.5 ± 12.1	2.8 ± 3.6	56.2 ± 9.9	12.4 ± 15.1	30.6 ± 13.0	14.1 ± 14.4
CD90	99.8 ± 0.1	99.9 ± 0.1	99.9 ± 0.0	100.0 ± 0.0	99.9 ± 0.0	100.0 ± 0.0

[a] Significant differences observed in P3 between AB-HS compared with FCS and tPRP ($p < .01$).
[b] Significant differences observed in P3 between tPRP and FCS ($p < .05$).
[c] Significant differences observed between P3 and P7 of AB-HS ($p < .001$).
[d] Significant differences observed between P3 and P7 of tPRP ($p < .05$).
Abbreviations: AB-HS, human AB serum; FCS, fetal calf serum; P, passage; tPRP, thrombin-activated platelet-rich plasma.

off after 40 ± 2.5 population doublings at passage 8 and proceeded at a slower rate until a maximum population doubling of 54 for tPRP and 50 for AB-HS, both at passage 11. On the contrary, AT-MSCs cultured in FCS exhibited a slow but continuous proliferation, reaching 31 ± 1.3 population doublings only at passage 8 and proceeding to the same maximal population doubling of 54 at passage 14.

Looking at the span of high proliferation between passages 2 and 6, we demonstrated that AB-HS and tPRP have a significantly higher fold expansion rate compared with FCS. In these five passages, AB-HS and tPRP had an average fold expansion rate of 66.6 ± 15.7 and 68.1 ± 6.7, respectively, both inducing an expansion of AT-MSCs 2.8 times higher than that of FCS, with a mean fold expansion rate of 24.4 ± 0.7 (Table 1).

Besides the addition of thrombin, tPRP required heparin to prevent gel formation. To assess whether thrombin and heparin provide an additional proliferative effect, we performed heparin and thrombin controls for AT-MSCs of four lipoaspirates starting at passage 2 and performed cell counts on days 8, 16, 26, and 36. Looking at cumulative population doublings, there was no significant difference between FCS compared with FCS/thrombin. However, heparin slightly slowed MSCs growth on days 26 and 36 ($p < .05$ compared with FCS and FCS/thrombin). Consequently, the proliferative stimulus provided by tPRP is due neither to the addition of thrombin nor to that of heparin.

Colony Frequency

The CFU-F frequency of AB-HS and tPRP determined in primary culture did not differ significantly from that of FCS. We calculated the CFU-F frequency to be 1 in 1,000 seeded cells, independent of the supplement used (FCS, 0.7 ± 0.5; AB-HS, 0.7 ± 0.6; tPRP, 0.6 ± 0.5). However, the colonies differed in size and cell densities instead of number, indicating an increased proliferation of cells within the colonies (Fig. 3S–3U).

Growth Curve

Because of the high proliferative effect and mesh-like growth pattern of AB-HS and tPRP cells, it was more difficult to judge the confluency of each passage. To look more closely at the period of highest proliferation, we started a growth curve of AT-MSCs (n = 3) for all culture conditions at passage 3 (Fig. 2B). Cells were seeded at the same density of 200 cells per square centimeter, with an initial cell number of 1,000 cells per 25-square-centimeter flask. Starting on day 3, cell numbers were counted and compared on every other day until day 11. Proliferation kinetics of the growth curve show a much more explosive exponential growth of AB-HS and tPRP cultures starting on day 5 compared with FCS. AB-HS and tPRP reached a final cell count of $1.4 \times 10^6 \pm 3.5 \times 10^5$ and $1.32 \times 10^6 \pm 4.7 \times 10^5$, respectively, on day 11. In comparison, FCS exhibited a cell count of only $2.41 \times 10^5 \pm 6.5 \times 10^4$ on day 11. Thus, AB-HS and tPRP have a high stimulatory effect on AT-MSC causing them to multiply 1,320- to 1,400-fold within 11 days compared with a 254-fold expansion for FCS in the same time span. Considering that AB-HS and tPRP flasks already reached postconfluency on day 9, the proliferation of these cells might be even higher given a lower seeding density and a larger growth area.

Differentiation Capacity

The differentiation potential of AT-MSCs from all donors and culture conditions was tested for the adipogenic and osteogenic lineages at passages 1 and 7. Most importantly, we demonstrated that AB-HS and tPRP did not adversely affect the differentiation capacity of AT-MSCs.

Adipogenic differentiation was confirmed by the formation of lipid vacuoles that were stained by the oil red O (Fig. 3A–3C). Osteogenic differentiation was indicated by deposition of a mineralized matrix stained with van Kossa (Fig. 3G–3I) and the increased expression of alkaline phosphatase (Fig. 3M–3O).

We showed that all samples within the adipogenic and osteogenic differentiation assays were consistently positive defined by the appropriate stain. The differentiation capacities for FCS, AB-HS, and tPRP were comparable and was maintained throughout long-term culture.

Characterization of Surface Marker Expression

The expression of cell surface markers was analyzed by flow cytometry at passages 3 (n = 4) and 7 (n = 3) for all culture conditions (Table 2). All AT-MSCs were highly positive for CD29, CD44, CD73, and CD90, although FCS cells had a significantly higher CD44 expression intensity in passage 3 compared with AB-HS and tPRP ($p < .05$). The mean fluorescence intensity (MFI) for FCS was 843.4 ± 245.7, whereas

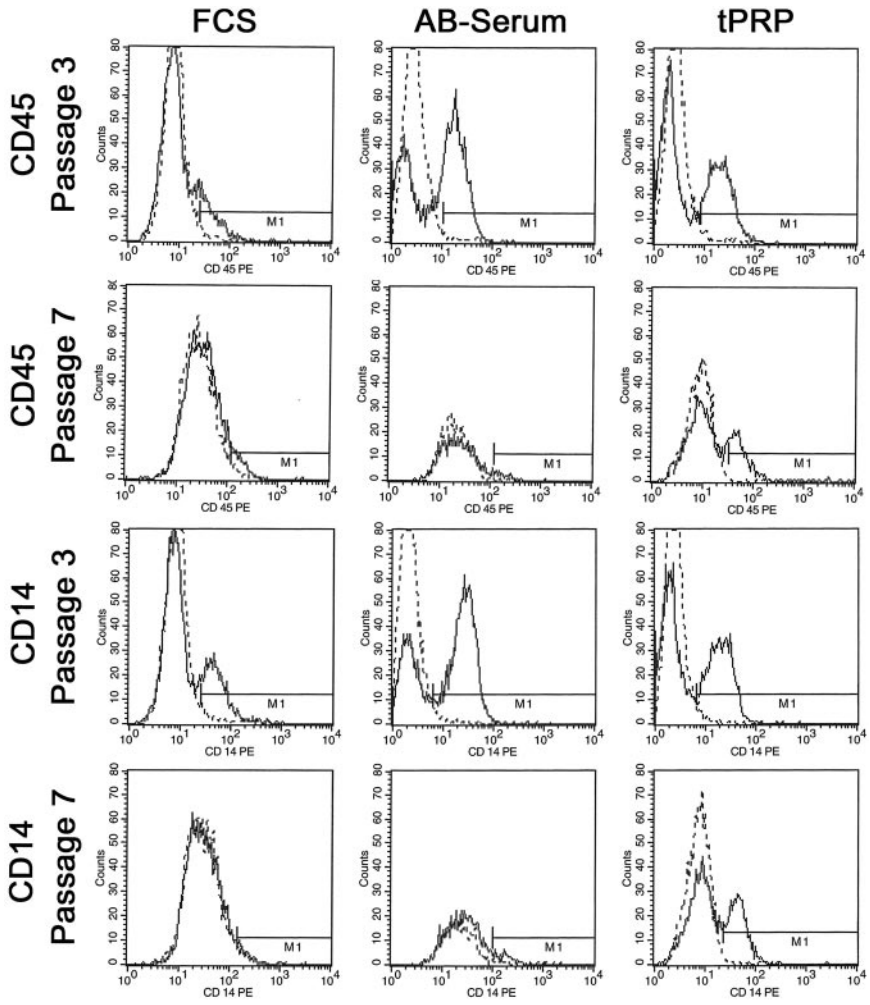

Figure 4. Comparison of CD45 and CD14 expression for all culture conditions at passages 3 and 7 as analyzed by flow cytometry. Exemplary histograms are all derived from the same donor with isotype controls indicated by the stippled line. Bars represent percentage of cells expressing the surface marker. Abbreviations: FCS, fetal calf serum; PE, phycoerythrin; tPRP, thrombin-activated platelet-rich plasma.

AB-HS had an MFI of 317.7 ± 61.2 and tPRP an MFI of 416 ± 98.4. In addition, AT-MSCs of all culture conditions stained positive for HLA-class I and negative for HLA-class II, CD133, and endothelial markers CD31, CD144, and VEGFR-2. All AT-MSCs were negative for the hematopoietic marker CD34; however, there was a marked side population of CD45- and CD14-positive cells in passage 3 (Fig. 4) of AB-HS (CD45, 61% ± 2.1%; CD14, 56.2% ± 9.9%) and tPRP cells (CD45, 30.5% ± 8.3%; CD14, 30.6% ± 13%), compared with FCS (CD45, 11.6% ± 11.9%; CD14, 9.5% ± 12.1; $p < .05$). This CD45-/CD14-positive population was extremely heterogeneous, varying widely between AT-MSCs of different donors (also for FCS cultures). Subsequent passaging tended to diminish this population ($p < .05$). At passage 7, we found 3.0% ± 1.3% CD45- and 2.8% ± 3.6% CD14-positive cells for FCS, but still 5.0% ± 4.1% CD45- and 12.4% ± 15.1% CD14-positive cells for AB-HS and 10.5% ± 10.0% CD45- and 14.1% ± 14.4% CD14-positive cells for tPRP.

There was no significant difference in either percentage expression and expression intensity between passages 3 and 7 for all culture conditions with the exception of CD90 and the afore-mentioned hematopoietic markers. All culture supplements displayed a much higher mean fluorescence intensity of CD90 at passage 7 compared with passage 3 (FCS, P3: 2,090.2 ± 544.25; P7: 5,956.8 ± 2,294.5; AB-HS, P3: 2,006.1 ± 727.7; P7: 8,023.5 ± 130.3; tPRP, P3: 2,471.3 ± 942.2; P7: 8,237.6 ± 835.4; $p < .05$).

Cytokine Profile

To characterize the cytokine and growth factor profile of AB-HS and tPRP, we used a semiquantitative human cytokine antibody array that detects 174 cytokines in one experiment. First, we determined total protein content of all culture supplements with the Bradford reaction. AB-HS contained 90 mg/ml total protein, tPRP 97.2 mg/ml, and FCS 87 mg/ml. Only AB-HS and tPRP were used in the cytokine antibody array because of the anti-human antibody specificities. For analysis, the internal negative controls were used to determine the cut-off rate for a positive signal. Intensities up to 20,000 were regarded as a weak signal, 20,000–40,000 as a moderate signal, and > 40,000 as a strong signal. If the difference of signal intensity between a factor in AB-HS and tPRP differed by more than twofold, it was considered to be a significant difference. In total, 43 of 174 chemokines were not detectable in AB-HS or tPRP (for a complete list, see supplemental online data). Another 83 factors had a weak signal intensity for both AB-HS and tPRP. Thirty-two factors were either negative or weakly positive in one or the other. There were 16 factors that had a moderate or strong signaling intensity. Of all 174 factors tested, only four chemokines had a significantly higher signal in AB-HS than in tPRP: angiogenin, PARC (pulmonary and activation-regulated chemokine), HCC-4 (hemofiltrate CC chemokine 4), and IL (interleukin)-13 receptor $\alpha 2$. Conversely, another five factors had a higher signaling intensity in tPRP compared with AB-HS: MCP-2 (monocyte chemoattractant protein 2), GRO (growth

Table 3. Comparison of the processing steps in the production of AB-HS and tPRP

AB-HS	tPRP
1. Alloquation of each 10 ml and freezing at −30°C	1. Aliquot for platelet count
2. Pooling and sterile filtration	2. Addition of 1 U/ml thrombin
3. Once medium is prepared, it can be kept at 4°C–8°C for up to 4 weeks	3. 30-minute incubation
	4. Centrifugation of resulting clot at 3,000*g* for 30 minutes
	5. Pooling and sterile filtration of supernatant
	6. Alloquation of each 5-ml aliquot and freezing at −80°
	7. After thawing, centrifugation at 1,500*g* for 5 minutes to discard clots
	8. Addition of 2 U/ml heparin to the medium before adding tPRP
	9. Medium supplemented with tPRP has to be freshly prepared before usage

Compared with preparation of AB-HS, it is apparent that the preparation of tPRP involves more processing steps as well as addition of further reagents such as thrombin and heparin, possibly hampering a good manufacturing practice-compliant manufacturing.
Abbreviations: AB-HS, human AB serum; tPRP, thrombin-activated platelet-rich plasma.

related oncogene), uPAR (urokinase plasminogen activator receptor), ICAM-2 (intercellular adhesion molecule 2), and LAP (liver activator protein).

DISCUSSION

There are a number of clinical trials exploring the use of MSCs for the treatment of various diseases, most of which use FCS as a culture supplement [9, 10, 27–29]. However, it is paramount to substitute FCS with a supplement free of xenogeneic proteins to ensure adherence to GMP and the delivery of MSCs as a clinically safe cell product [30].

This is the first study that compared the in vitro effects of pooled human AB serum and thrombin-activated PRP on AT-MSCs with FCS. Isolation and expansion of AT-MSCs were successful for all donors independent of medium supplement, and morphology was comparable, although AB-HS and tPRP exhibited a more spindle-shaped morphology. Kobayashi et al. [21] have shown that this could be an indication of increased motility.

We show that human AB serum as well as tPRP have a significantly higher proliferative effect on AT-MSC than FCS providing a 1,320–1,400-fold expansion in 11 days compared with only 254-fold expansion with FCS. The fact that both alternative supplements trigger such an early explosive growth rate makes them attractive candidates for MSC expansion in clinical settings, since the large number of MSC required for transplantation [31] can be achieved in a shorter period of time.

We also demonstrated that AT-MSC cultured with AB-HS or tPRP consistently differentiated into the adipogenic and osteogenic lineages and retained their differentiation capacity throughout long-term culture. Gruber et al. observed that thrombin-activated platelets decreased osteogenic differentiation of bone marrow derived MSC [19], whereas Doucet et al. postulated that platelet lysate stimulated osteogenic differentiation [17]. We did not find a decrease in the number of samples displaying osteogenic differentiation. Van Kossa and AP staining seemed to be even more intense for AB-HS and tPRP, however, this could be due to the increased proliferation of AT-MSC rather than a true increase in differentiation. Future studies will have to quantify whether human substitutes affect the number and intensity of differentiation or bias differentiation into one direction or the other.

Surface marker expression did not differ markedly between the different cell culture conditions and remained stable throughout long-term culture with the exception of a distinct CD45-/CD14-positive side population for AB-HS and tPRP cells in comparison to FCS which contained a much smaller number of CD45-/CD14-positive cells. The increased CD45-/CD14-positive population in AB-HS and tPRP could be due to cytokines such as HCC-4 and Flt-3-ligand which are present in serum/plasma and are chemoattractants for monocytes. The presence of this population was extremely donor dependent for all culture conditions and could relate to the AT-MSC isolation technique. Yoshimura et al. have shown that the fluid portion of the lipoaspirate contains a higher proportion of CD45+ cells than the fatty portion [32]. Although this CD45-/CD14-positive population diminishes with increased passaging time, we consider it essential to eliminate these contaminating cells before clinical application.

We also observed that the mean fluorescence activity of CD44 was decreased for AB-HS and tPRP in the early passages, possibly due to the presence of bFGF in serum/plasma which has been shown to downregulate CD44 [33]. CD44 is involved in cell-cell and cell-matrix interactions, therefore being involved in extravasation and homing [34]. CD44 expression may relate to the proliferative strength as when expansion of AT-MSC in HS-AB and tPRP ceases after long-term culture, levels of CD44 expression are similar across culture conditions.

There has been much controversy about what constitutes a suitable FCS supplement. Most studies have concluded that serum-free media cannot promote MSC growth without the addition of cytokines [35, 36]. This is possibly due to the fact that serum induces intracellular Ca^{2+} oscillations which are vital to MSC proliferation and differentiation [37]. Recently, several studies have concentrated on the use of autologous serum [11, 16, 23, 36]. Yet, this approach is limited by the amount of autologous serum necessary to expand MSC for clinical use [38] and the variability of serum, especially for patients receiving prior chemotherapy. Some studies have been successful in isolating and expanding MSC using AB serum [23, 39]; however, others have reported growth arrest of MSCs after the first passage [11, 22]. We demonstrate that AT-MSC can be efficiently kept throughout long-term culture, with AB serum reaching the same final cumulative population doublings as FCS. In our study, we used serum donors of the German Red Cross Blood Service who were all carefully screened for the absence of HLA allo-antibodies. Possibly, this quality control contributed to the effectiveness of AB-HS in our study. Alternatively, there is evidence that tissue source (i.e., adipose tissue vs. bone marrow) may play a role as well (A. Hecker, H. Lannert, unpublished data).

Besides human serum, platelet-factor rich supernatant has been described as a potential substitute for FCS [17–19]. On the basis of our study results, we conclude that thrombin-activated

PRP is the optimal way to achieve a platelet-factor-rich supernatant. Platelet activation with thrombin closely imitates the physiologic activation of platelets, ensuring the bioactivity of secreted growth factors [40]. Also the release of small-membrane vesicles, termed microparticles [41], has an additional mitogenic effect [42]. Furthermore, the presence of thrombin-cleaved osteopontin could have an effect as well, because it promotes cell attachment and spreading [43]. In comparison, platelet lysate does not provide physiologic activation and contains aggregates of platelet membranes that are difficult to remove without compromising the proliferative effect. Furthermore, membrane-associated platelet antigens pose the threat of immunological responses [44]. However, clearing AT-MSC of platelet membranes before clinical administration seems to be technically difficult. Therefore, we favor the use of tPRP even though there is evidence that platelet lysate promotes MSC growth as well [17].

In conclusion, we show that pooled human AB-HS as well as tPRP enhance MSC proliferation without compromising differentiation capacity or immunophenotype. Our results provide a further step in the direction of producing an off-the-shelf animal-free medium supplement for that is widely available and prepared in as few processing steps as possible. We promote pooled AB serum as the optimal choice on the basis of the ease of preparation and the fact that further additives such as heparin and thrombin in tPRP are not included (Table 3). In addition, AB-HS in and of itself provides a good enough proliferative stimulus such that further addition of growth factors such as fibroblast growth factor-2 does not seem necessary. Furthermore, the decreased adhesion potential of tPRP cells may have an impact on the clinical application of platelet-derived alternatives. The premature culture surface detachment of tPRP cells could possibly be a sign of a change in the in vivo homing and docking ability of these cells. Therefore, we are currently addressing this issue in molecular and functional experiments. Although we consider it necessary to conduct further studies, our study is a first step toward a GMP-compliant manufacturing protocol of AT-MSCs.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9:641–650.
2 Bieback K, Kern S, Kluter H et al. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. STEM CELLS 2004;22:625–634.
3 Kern S, Eichler H, Stoeve J et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. STEM CELLS 2006;24:1294–1301.
4 Kassem M, Kristiansen M, Abdallah BM. Mesenchymal stem cells: Cell biology and potential use in therapy. Basic Clin Pharmacol Toxicol 2004;95:209–214.
5 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
6 Lee KD, Kuo TK, Whang-Peng J et al. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40:1275–1284.
7 Munoz-Elias G, Woodbury D, Black IB. Marrow stromal cells, mitosis, and neuronal differentiation: Stem cell and precursor functions. STEM CELLS 2003;21:437–448.
8 Chen SL, Fang WW, Ye F et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92–95.
9 Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439–1441.
10 Koç ON, Gerson SL, Cooper BW et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 2000;18:307–316.
11 Spees JL, Gregory CA, Singh H et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9:747–756.
12 Medicinal and other products and human and animal transmissible spongiform encephalopathies: Memorandum from a WHO meeting. Bull World Health Organ 1997;75:505–513.
13 Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood 1997;89:776–779.
14 Tuschong L, Soenen SL, Blaese RM et al. Immune response to fetal calf serum by two adenosine deaminase-deficient patients after T cell gene therapy. Hum Gene Ther 2002;13:1605–1610.
15 Drach G, Maret A, Richard MF et al. Transfer and induction of delayed hypersensitivity to methylated bovine serum albumin in the absence of adjuvant. C R Acad Sci Hebd Seances Acad Sci D 1977;284:2435–2437.
16 Stute N, Holtz K, Bubenheim M et al. Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 2004;32:1212–1225.
17 Doucet C, Ernou I, Zhang Y et al. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 2005;205:228–236.
18 Kilian O, Flesch I, Wenisch S et al. Effects of platelet growth factors on human mesenchymal stem cells and human endothelial cells in vitro. Eur J Med Res 2004;9:337–344.
19 Gruber R, Karreth F, Kandler B et al. Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in vitro conditions. Platelets 2004;15:29–35.
20 Tamama K, Fan VH, Griffith LG et al. Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells. STEM CELLS 2006;24:686–695.
21 Kobayashi T, Watanabe H, Yanagawa T et al. Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. J Bone Joint Surg Br 2005;87:1426–1433.
22 Shahdadfar A, Fronsdal K, Haug T et al. In vitro expansion of human mesenchymal stem cells: Choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. STEM CELLS 2005;23:1357–1366.
23 Anselme K, Broux O, Noel B et al. In vitro control of human bone marrow stromal cells for bone tissue engineering. Tissue Eng 2002;8:941–953.
24 Janetzko K, Klüter H, van Waeg G et al. Fully automated processing of buffy-coat-derived pooled platelet concentrates. Transfusion 2004;44:1052–1058.
25 Gaarder A, Jonsen J, Laland S et al. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961;192:531–532.
26 Rand ML, Perry DW, Packham MA et al. Conditions influencing release of granule contents from human platelets in citrated plasma induced by ADP or the thrombin receptor activating peptide SFLLRN: Direct measurement of percent release of beta-thromboglobulin and assessment by flow cytometry of P-selectin expression. Am J Hematol 1996;52:288–294.
27 Bang OY, Lee JS, Lee PH et al. Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol 2005;57:874–882.
28 Mazzini L, Fagioli F, Boccaletti R et al. Stem cell therapy in amyotrophic

lateral sclerosis: A methodological approach in humans. Amyotroph Lateral Scler Other Motor Neuron Disord 2003;4:158–161.
29 Chen SL, Fang WW, Qian J et al. Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. Chin Med J 2004;117:1443–1448.
30 Dimarakis I, Levicar N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. STEM CELLS 2006;24:1407–1408.
31 Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: Characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 2004;8:301–316.
32 Yoshimura K, Shigeura T, Matsumoto D et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol 2006;208:64–76.
33 Sotiropoulou PA, Perez SA, Salagianni M et al. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. STEM CELLS 2006;24:462–471.
34 Ponta H, Sherman L, Herrlich PA. CD44: From adhesion to signaling regulators. Nat Rev Mol Cell Biol 2003;4:33–45.
35 Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 1995;85:929–940.
36 Kuznetsov SA, Friedenstein AJ, Robey PG. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 1997;97:561–570.
37 Foreman MA, Smith J, Publicover SJ. Characterisation of serum-induced intracellular Ca2+ oscillations in primary bone marrow stromal cells. J Cell Physiol 2006;206:664–671.
38 Sotiropoulou PA, Perez SA, Salagianni M et al. Cell culture medium composition and translational adult bone marrow-derived stem cell research. STEM CELLS 2006;24:1409–1410.
39 Yamaguchi M, Hirayama F, Wakamoto S et al. Bone marrow stromal cells prepared using AB serum and bFGF for hematopoietic stem cells expansion. Transfusion 2002;42:921–927.
40 Marx RE. Platelet-rich plasma: Evidence to support its use. J Oral Maxillofac Surg 2004;62:489–496.
41 Barry OP, FitzGerald GA. Mechanisms of cellular activation by platelet microparticles. Thromb Haemost 1999;82:794–800.
42 Gruber R, Varga F, Fischer MB et al. Platelets stimulate proliferation of bone cells: Involvement of platelet-derived growth factor, microparticles and membranes. Clin Oral Implants Res 2002;13:529–535.
43 Senger DR, Perruzzi CA, Papadopoulos-Sergiou A et al. Adhesive properties of osteopontin: Regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. Mol Biol Cell 1994;5:565–574.
44 Santoso S. Human platelet alloantigens. Transfus Apher Sci 2003;28:227–236.


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