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Isolation and expansion of adipose-derived stem cells for tissue engineering

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1. ABSTRACT

For treatment of cardiac failure with bone marrow-derived mesenchymal stem cells, several clinical trials are ongoing. However, more attention is gathering on the use of adipose tissue-derived stem cells (ASCs). This paper describes the optimization of isolation and propagation of ASCs for subsequent clinical use. In the isolation step, several enzymes were compared with respect to yield of nucleated cells and precursor cells. Our results showed, that the interdonor variablility was greater than differences between individual enzymes. For propagation of cells, different types of media, sera and serum replacers were evaluated regarding their ability to support cell growth and preserve differentiation potential. Most of serum replacers proved inferior to fetal calf serum. Among the media tested, modified Eagle's media alpha was superior in promoting cell growth while preserving the ability to differentiate. Also, the effect of cell seeding density and hypoxic culture was evaluated. In this study, we show that it is possible to maximize cell yield regardless of donor individual characteristics by simple manipulations of media composition, cell seeding density and gaseous environment.

2. INTRODUCTION

For the treatment of patients with heart failure, the use of autologous mesenchymal stem cells (MSCs) appears promising. Numerous clinical studies have been performed, where the safety and feasibility of stem cell therapy of cardiac disorders have been confirmed. While most studies have been performed on MSCs derived from the bone marrow (1), a few studies have recently been launched where the regenerative properties of adipose tissue-derived stem cells (ASCs) are being assessed (2). Similarly to bone marrow-derived MSCs, ASCs have a broad differentiation potential into as varied lineages as adipogenic, chondrogenic, osteogenic, myogenic, angiogenic, and cardiomyogenic cell types (3). Adipose tissue contains a higher number of stem cells / mL tissue than bone marrow does (4), and, importantly, the amount of stem cells do not appear to decrease with age (5), thus making this cell type appealing. While the isolation of stem cells from bone marrow is a relatively standardized procedure, the isolation and expansion of ASCs in the most efficient and reproducible manner has yet to be determined. To release the ASCs from the adipose aspirate, typically either crude collagenase or a more purified collagenase is

used. The different types of collagenases may be supplemented with various other proteases and often vary between batches (6). It is thus a challenge to identify the enzymatic conditions that will yield the maximum number of ASCs. Furthermore, although ASCs may be harvested in large quantities, an expansion *in vitro* may still be necessary prior to clinical use, as current protocols call for large numbers of cells to be injected. Currently a study is ongoing, where between 2 x 10^7 and 2 x 10^8 mesenchymal cells are to be used for each patient patient (Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS), registered at www.clinicaltrials.gov). It is thus necessary to develop methods to expand the cells while preserving the differentiation capability. Different combinations of sera and media and plating densities have been tested on bone marrow-derived stem cells (7). A final parameter to be considered for the optimal expansion of ASCs, is the growth under controlled oxygen tensions. It has been shown that for a number of cell types, including endothelial cells, the cells proliferate faster when grown under reduced oxygen conditions (8, 9). In this paper, we describe the selection of the optimal enzyme for ASC yield, followed by identification of the best culture conditions, including media, sera, plating densities, and gaseous mixture to give the highest number of stem cells while retaining their differentiation potential.

3. MATERIAL AND METHODS

3.1. Isolation of ASCs with different collagenases

All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. Subcutaneous fat was obtained from five female patients, undergoing liposuction at Grymer Private Hospital, Skejby, Denmark in accordance with protocols approved by the regional Committee on Biomedical Research Ethics in Northern Jutland. Within 12 hours of surgery, the adipose tissue was processed at the laboratory. Human ASCs were isolated essentially according to methods previously described by Zuk *et al.* (10). Briefly, adipose tissue was washed 3-4 times in equal volumes of prewarmed sterile PBS, after which the tissue was divided into 6 equal volumes that were enzymatically dissociated through incubation for 2 h at 37°C with either Blendzyme 1 to 4 (B1 to 4), Human Liberase 1 (L1) (Roche Applied Sciences, Hvidovre, Denmark), or crude collagenase mixture (CCM) (lot. no. LTQ5230; Wako, Neuss, Germany). The final collagenase activity of each enzyme was 0.28 Wünsch U/ml. The dissociated tissue was subjected to centrifugation at 400 x g for 10 min after which the pelleted cells were filtered through a 70 µm mesh cell strainer (BD Bioscience, Broendby, Denmark) to remove debris. Contaminating erythrocytes were lysed using sterile water and the remaining nucleated cells were further purified through a second round of centrifugation and filtration. The total yield of nucleated cells was determined in a hemocytometer after the cells had been stained with acetic methylene violet. Cells were either immediately used for analysis in the colony-forming assays or cultured overnight in (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12) DMEM/F12 medium supplemented with 10% fetal calf serum (FCS), after which non-adherent cells were removed.

3.2. Colony-forming unit assays

The number of colony forming units (CFUs) / mL adipose tissue was determined by a limiting dilution assay. The freshly isolated cells were suspended in DMEM/F12 medium supplemented with 10% FCS at a concentration of 50 000 cells/ml. The cells were subjected to a two-fold serial dilution across the 12 columns of a 96-well culture plate resulting in 8 replicate rows containing 10 000 to 4 cells / well. One plate was prepared for each enzyme and each donor. The cells were cultured for 11 days, after which the cells were fixed with 4% formaldehyde and stained with methylene blue. For each dilution series, the number of wells containing only colonies of less than 20 cells were counted. All plates were scored by two independent observers. The number of CFUs was calculated according to the equations $F_0 = e^{-u}$ and $u = -\ln F_0$, where F_0 is the fraction of empty wells within the dilution series and u is the average number of precursors or colony-forming units per well (11). Based on the yield of nucleated cells, the absolute yields of CFUs / mL adipose tissue were calculated.

3.3. Cell proliferation in different media, sera and serum replacers

To determine the effect of different growth media, sera and serum replacers on cell growth and differentiation potential of the stem cells, the ASCs in passage 2 were were plated in 24-well plates (Corning, Schiphol-Rijk, The Netherlands) at a concentration of 300 cells/cm^2 in DMEM/F12 with 10% FCS. When the cells had attached, the medium was replaced with DMEM/F12 supplemented with 5 or 10% FCS, 1x serum replacements SR1 or 3 (Sigma-Aldrich, Brøndby, Denmark), or 10% or 20% knockout serum replacement (KOSR) for the studies of the effect of different types of sera and serum replacers. To determine the effect of different media on the ASCs, the medium was replaced with either DMEM/F12, Dulbecco's Modified Eagle Medium with 4,500 mg/ml glucose (DMEM HG), Dulbecco's Modified Eagle Medium with 1,500 mg/ml glucose (DMEMLG), Nutrient Mixture F-12 (F12), or Alpha Modified Eagle Medium (alpha-MEM), all supplemented with 10% FCS, or with complete medium LP02 (MacoPharma, Mouvaux, France). All combinations of media and sera was supplemented with penicillin (10 IU/ml), streptomycin (10 micro g/ml), and gentamicin (5 micro g/ml). Media were changed on days 3 and 6. On day 8 the cells were counted using PicoGreen dsDNA Quantitation Kit (Invitrogen, Leiden, NL) according to manufacturer´s instructions. Briefly, cells were washed twice with PBS, lysed in 0.02% SDS in DNase-free water, and incubated at -18°C for 2 hrs. Aliquots of 100 micro l cell lysate were transferred to duplicate wells in a black microtiter plate and mixed with 100 micro l PicoGreen dye diluted 1:200 in 1x TE buffer. The mixture was incubated at room temperature for 15 min under continuous agitation, after which the fluorescence was measured using Wallac Victor^2 multilabel counter (PerkinElmer, Hvidovre, Denmark) with center excitation

and emission at 485nm and 535nm, respectively. The amount of DNA was calculated on the basis of a standard curve made from known concentrations of Lambda DNA and the number of cells was calculated based on 6 pg DNA/cell. Experiments were performed in duplicate with cells from 5 donors.

3.4. Induction of osteogenic differentiation

As a marker for preservartion of differentiation potential we determined the capacity of the cultures to undergo osteogenesis. The cultures were initiated at a density of 300 cells/cm^2 in 24-well plates, and maintained in the different growth media, and with the different serum replacements as described above. After 14 days of cell expansion to test the basal media or 21 days to test serum replacements, the media were changed to osteogenic induction medium consisting of DMEM/F12 supplemented with 10% FCS, 0.1 microM dexamethasone, 50 microM L-ascorbic acid 2-phosphate, 0.5 microM calcitriol, 10 mM glycerol 2-phosphate (all Sigma-Aldrich). After a two-week induction period, the degree of osteogenesis was evaluated by staining of calcium deposits with alizarin red. In brief, the cells were washed with PBS, fixed in ice-cold 70% ethanol for 15 min, and incubated with 2% alizarin red solution (Bie & Berntsen, Rødovre, Denmark) at room temperature for 15 min. The stained plates were photographed, and a histomorphometric analysis of the images was carried out with the aid of AxioVision software package (Zeiss, Gottingen, Germany). The images in RGB mode were inverted, and the average pixel intensity was determined for the whole image area. The degree of differentiation was normalized to the values obtained using DMEM/F12 supplemented with 10% FCS. The differentiation experiments were performed in duplicate with cells from 3 donors.

3.5. Cell proliferation as a function of initial seeding density

For seeding density experiments, cells were seeded in densities of 50, 100, 200, or 800 cells/cm^2 in 24-well plates (Corning) in alpha-MEM supplemented with 10% FCS, with medium changes at days 3 and 6. The cells were counted using pico green as described above at days 2 and 8. The doubling time (Td) was calculated according to the equation $Td = t(\log 2)/(\log N_t - \log N_0)$, where $t = 6$, and N_0 and N_t are the cell numbers at day 2 and 8, respectively. Experiments were performed in duplicate with cells from 5 donors.

3.6. Short term culture at 5% oxygen

For the study of effect of reduced oxygen tension on the cell growth, cells were seeded in densities of 300 cells/cm^2 in 24-well plates in alpha-MEM supplemented with 10% FCS. Half the cultures were transferred to a hypoxic workbench/incubation chamber (X-Vivo, Biospherix, Lacona, NY) and incubated in an atmosphere of 5% oxygen and 5% CO_2 balanced with N_2 the other half of the cultures were cultured at ambient oxygen tension. The culture media was changed at days 3 and 6. At days 4, 7, and 8, the cells were counted using picogreen as described above. The experiments were performed in duplicate with cells from 5 donors.

3.7. Long term culture at 5% oxygen

To study the effect of long term culture in hypoxia, the cells were seeded in T25 culture flasks in alpha-MEM supplemented with 10% FCS and cultured either at 5% oxygen or at ambient oxygen tensions for 35 days. The cells were monitored daily, and when cultures were approximately 90% confluent, cells were trypsinized, counted using a hemocytometer, and 1/3 of the cells reseeded for further culturing. The X-Vivo incubator system allowed for both monitoring of the cultures with an inverted microscope and for media changes in a constant low atmosphere, thus avoiding reoxygenation of the cultures. The long-term experiments were performed on cells from three donors.

3.8. Statistics

To assess the statistically significant differences, one-way multiparameter analysis of variance (ANOVA) or paired t-testing (SPSS software version 15.0; SPSS, Chicago, IL) was performed. The level of significance was set to $p = 0.05$. The data are presented as a mean + standard error of mean (SEM).

4. RESULTS

4.1. Effect of collagenase on the yield of ASCs from adipose tissue

The total yield of nucleated cells obtained after digestion of the adipose tissue with various enzymes was determined (Figure 1A). It is evident, that large interdonor variability exists. However, enzyme Liberase 1 gives the highest number of cells for three patients and Blendzyme 3 yields the lowest number from three patients. As for the suitability of the enzymes for the isolation of cells supporting colony growth (Figure 1B*)*, again there is clear interdonor variability. However, Blendzymes 1 and 2 and Liberase 1 yield the highest number of CFUs per mL adipose tissue and again Blendzyme 3 yields the lowest numbers.

4.2. Selection of serum or serum replacements for expansion of human ASCs

To find possible alternatives to FCS for the expansion of human ASCs, cells were cultured for 8 days in different concentrations of FCS and serum replacements, after which the cell numbers were determined (Figure 2A, black bars). The results show that 5% FCS supported cell growth to the same extent as 10% FCS and both 10% and 20% KOSR. Notably, none of the serum replacements, SR1 and SR3, supported the expansion of ASCs to the same degree as the FCS did. Incubation in the presence of either of the SRs led to a significantly lower ($p < 0.05$) cell proliferation than in media containing the other types serum supplements. Also the differentiation potential towards the osteogenic lineage was determined (Figure2A, open bars*)*. The histochemical analysis showed that after three weeks of culture with the different sera, only media with 10%, 5% FCS and 20% KOSR preserved the osteogenic capacity of the cells. Based on the capacity to support expansion and differentiation potential of ASCs, 10% FCS was selected for subsequent experiments.

Table 1. Cell doubling time (Td) as a function of initial cell plating density

Plating density (cells/cm^2)	Doubling time (Td)
50	3.21
100	1.45
200	1.51
800	3.18

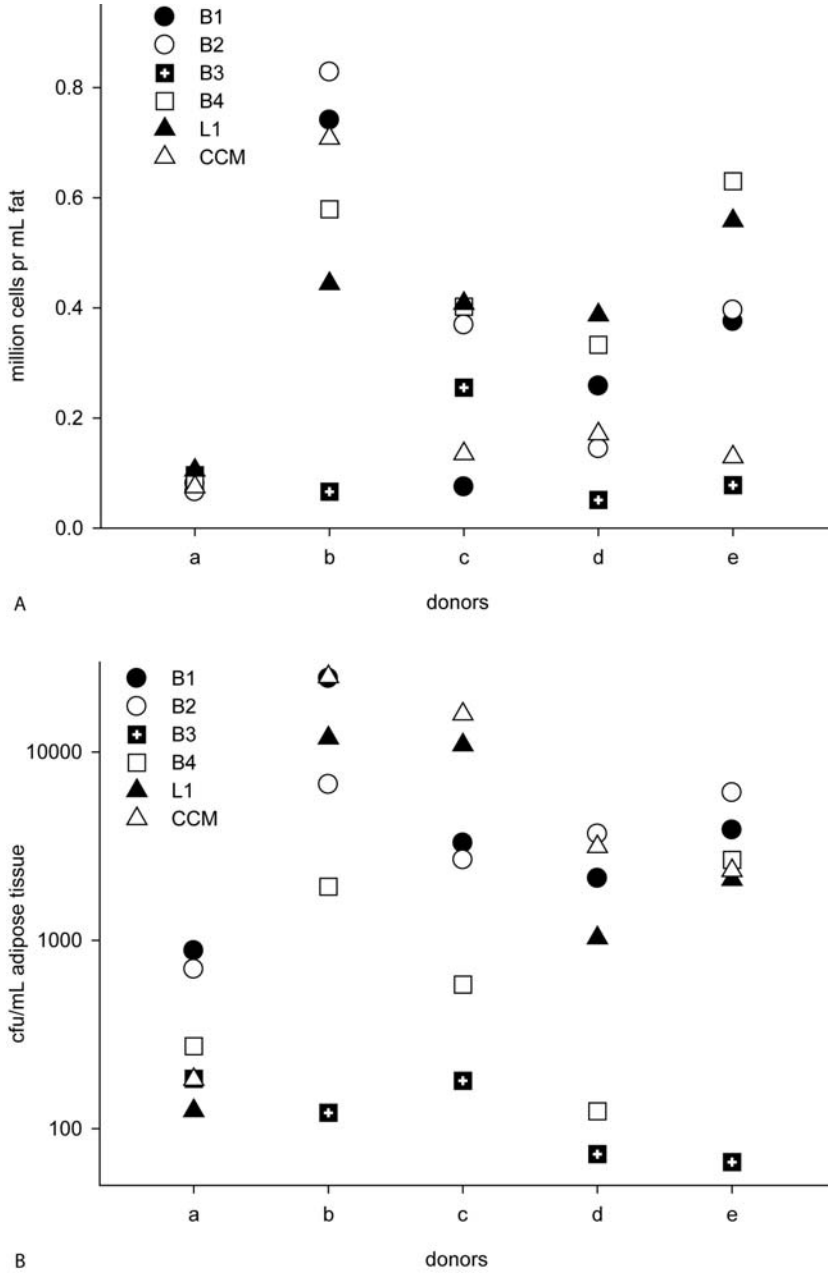

Figure 1. Absolute yield of nucleated cells and colony forming units from 5 different donors. (A) The yield of nucleated cells pr mL of adipose tissue from the use of different enzyme preparations. (B) The yield of colony-forming units pr mL of adipose tissue using different enzymes. B: Blendzyme, L1: Liberase H1, CCM: crude collagenase mix, cfu: colony-forming units.

4.3. Selection of optimal basal medium for expansion of human ASCs

To find optimal basal medium for expansion of ASCs, cells were cultured for 8 days in 5 different basal media supplemented with 10% FCS or the complete medium LP02, after which the cell number was determined. The results demonstrated that all media but LP02 were capable of supporting cell growth, and that alpha-MEM performed significantly better (2.5 fold than DMEM/F12) (Figure 2B, black bars). When the osteogenic potential of

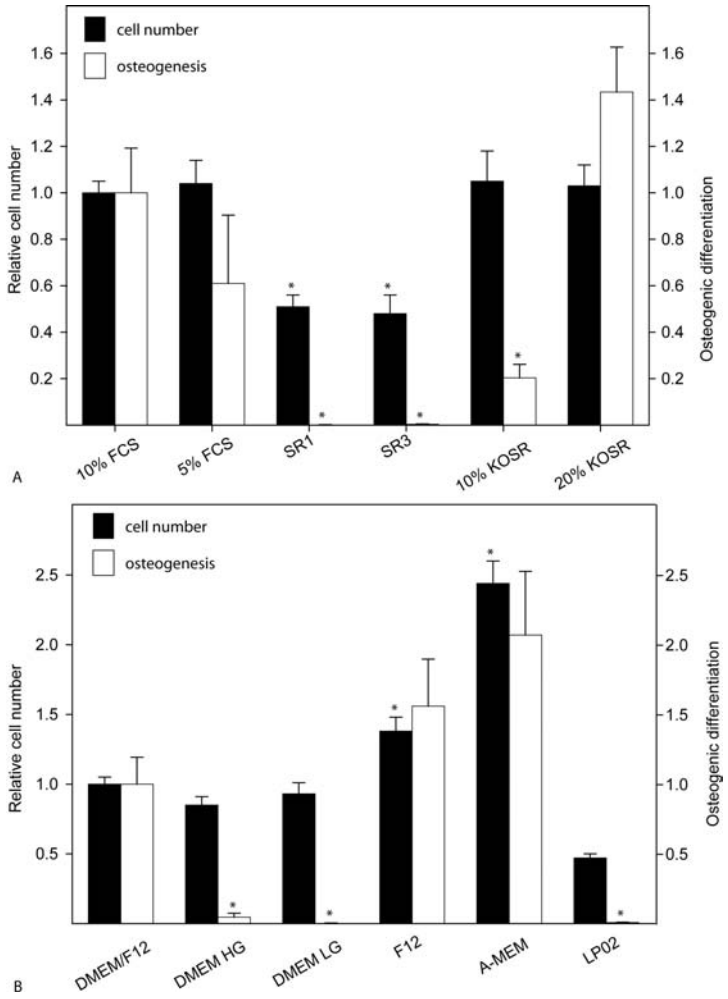

Figure 2. Effect of serum, serum replacements and media on growth and osteogenic differentiation of human ASCs. The cells were cultured in DMEM/F12 supplemented with different amounts of serum or serum replacements (Panel A) or in in different basal media supplemented with 10% FCS (Panel B). The numbers of cells on day 8 were normalized to those cultured in DMEM/F12 10% FCS (Black bars). After culture in the respective media/sera the cells were induced to undergo osteogenesis. Deposition of calcium deposits were quantified and normalized to values from cells cultured in DMEM/F12 10% FCS (open bars). The data are presented as a mean + standard error of mean from five different donors. Asterisks denote statistically significant difference ($p < 0.05$) when compared to DMEM/F12 10% FCS. FCS: Fetal calf serum; SR1: Serum replacement 1; SR3: Serum replacement 3; KOSR: Knockout serum replacement; DMEM/F12: Dulbecco's Modified Eagle Medium Nutrient Mixture F-12; DMEM HG: Dulbecco's Modified Eagle Medium with 4,500 mg/ml glucose; DMEM LG: Dulbecco's Modified Eagle Medium with 1,500 mg/ml glucose; F12: Nutrient Mixture F-12; A-MEM: Alpha Modified Eagle Medium; LP02: LP02 complete medium.

he cells was determined after 14 days of culture in the selected media, only cells cultured in DMEM/F12, F12, and alpha-MEM were capable of differentiation (Figure 2B, open bars). Based on the capacity to support proliferation of the ASCs and preservation of their differentiation potential, alpha-MEM was selected for the subsequent experiments.

4.4. Effect of plating density on expansion of human ASCs

ASCs were plated at 4 different densities, including 50, 100, 200, and 800 cells/cm^2 in alpha-MEM containing 10% FCS and cultured for 8 days, after which the Td was determined (Table 1). It is clear that optimal seeding density for rapid cell proliferation is roughly 100 to 200 cells/cm^{2}with a doubling time of approximately 1.5 days. Lowering or increasing the concentration of seeded cells, as exemplified by 50 and 800 cells/cm^2, resulted in a doubling of the Td.

4.5. Effect of lowered oxygen tension on cell growth

The short term hypoxic treatment demonstrated a quite dramatic difference between the proliferation rate of cells grown at either 5% oxygen or 21% oxygen (Figure 3A), with an approximately 7-fold higher cell density in the hypoxic culture after only seven days. At

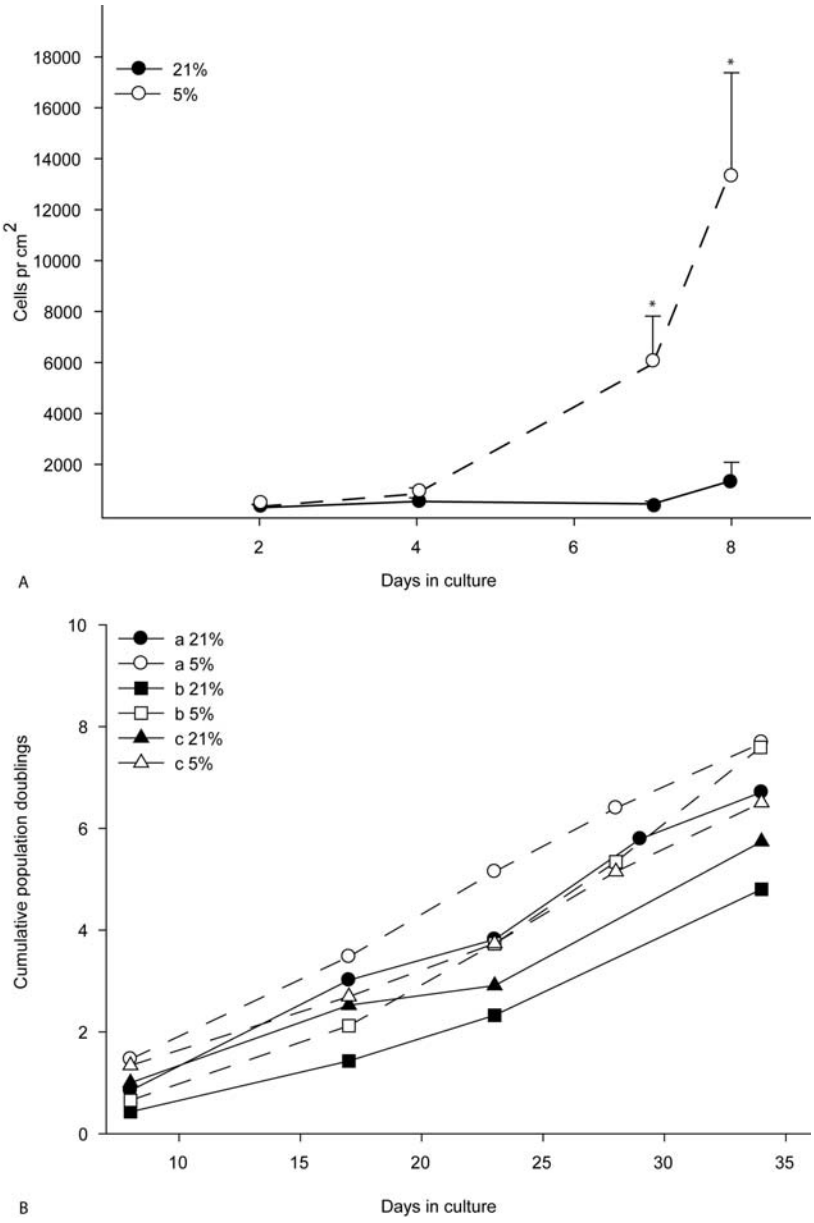

Figure 3. The effect of reduced oxygen tension on the growth rate of ASCs. (A) Cell density was determined for short term cultures. The cells were seeded in 24-well plates, and half the cultures incubated in 5% oxygen (open circles), the other half at ambient oxygen tensions (closed circles). Cells were counted using picogreen. The experiments were performed in duplicate with cells from 5 donors. Error bars represent standard error of mean and asterisks denote a significant difference ($p<0.05$) (B) Cumulative population doublings were calculated for long term cultures cells from three different donors (donor a: circles; donor b: squares; donor c: triangles). The cells were cultured in T25 flasks for 35 days at either 21% oxygen (filled symbols) or 5% oxygen (open symbols). Cultures were continuously monitored, subcultured and counted when 90% confluent.

day 8, although the hypoxic cultures almost doubles, so does the cultures at ambient tension. The hypoxic conditions thus seem to give the cells an initial boost. For the long term cultures, the same trend was seen (Figure 3B). For the cells from all three donors, the hypoxic cultures appear to grow faster than the cultures at 21% ambient oxygen tension, however, these differences were not statistically significant, possibly do to the interdonor variability.

5. DISCUSSION

In the current study, our aim was to identify a standardized method of isolation and propagation of ASCs to render them more amenable for use in tissue engineering and cell based therapies, while still retaining the properties characteristic of mesenchymal stem cells. As defined by the International Society for Cellular Therapy, one of the defining characteristics of mesenchymal stem cells is their

capacity to differentiate into adipocytes, osteocytes and chondrocytes (12). We have previously reported that of these lineages, the adipogenic is the least affected by culture medium and sera (13). The osteogenic differentiation potential of the cells in this study thus functions as a surrogate marker of stem cell characteristics.

Previous investigations have shown that there are significant differences in yield and colony forming potential between both donors and sites of harvest (14). This study confirms these previous investigations of large interdonor variability in that cell yield was more dependent on donor than on the enzymatic activity of the collagenases. We did, however, observe the trend that Liberase 1 and Blendzymes 1 and 2 yielded in general the highest cell numbers and Liberase 3 the lowest numbers, independently of donor characteristics. Contrary to results from the enzyme comparison, the evaluation of different media, sera, and sera replacers yielded results that were consistent between donors. Our results demonstrate, that the use of serum replacers is problematic in that two of the serum replacers SR1 and SR3 did not support cell growth at all, and the third replacer KOSR supported cell growth and maintained the differentiation potential of the cells only at high concentrations of 20%. For the expansion of ASCs prior to clinical use, it would be beneficial to avoid the use of animal sera, however, at this point the quality of the serum replacers is far below that of FCS with respect to ability to support cell growth and preserve differentiation capacity. The evaluation of the media revealed the alpha-MEM was by far the best in the promotion of cell proliferation and subsequent osteogenic differentiation. In addition to the combination of alpha-MEM and FCS, also cell plating densities of 100-200 cells/cm^2 and incubation under hypoxic condition promoted cell growth.

In spite of a plethora of expansion protocols, there is still a need for developing protocols conforming to current Good Manufacturing Practises (cGMP) prior to the use of ASCs as a standard clinical modality. In this aspect, it is interesting to point out, that the alpha-MEM can now be purchased in preparations adhering to cGMP guidelines, and that although xeno-free sera replacers would be preferable, also cGMP-approved FCS can be purchased.

The relevance of osteogenic differentiation potential regarding the potential use of stem cells in cardiac regeneration may not seem obvious. However, recent evidence support the hypothesis, that the cardiac regeneration observed after stem cell treatment, is not due to differentiation of the mesenchymal cells into cardiomycytes, but rather due to paracrine effects of the stem cells leading to either activation of resident endogenous cardiac stem cells or homing of circulating stem cells to the area of the infused mesenchymal stem cells (15-17). Thus, the preservation of the stem cell characteristics of the ASCs prior to their use in cardiovascular regeneration may be highly significant.

In conclusion, we have found that, regardless of donor characteristics, cell yield can be optimized through careful selection of enzymes, and that cells can be propagated efficiently without loss of differentiation potential through manipulation of cell microenvironment, comprising cell density, media composition and gaseous environment.

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