

Development of a Protocol for Human Adipose Stem Cell Culture in CO₂ Independent Medium and Perfusion Bioreactor

Silva ARP^{1,2*}, Paula ACC², Zonari A², Martins TMM², A M Goes² and M M Pereira¹

¹Laboratory of Biomaterials, Department of Metallurgical and Material Engineering, Engineering School, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

²Laboratory of Cellular and Molecular Immunology, Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Abstract

Advances in research on stem cells derived from human Adipose tissue (hASC) may allow its use for bone tissue engineering. In such context, it is important to standardize a methodology to culture cells in high quantity. Bioreactors, in which cells are cultured in three-dimension, mimic the physiological environment *in vitro*, allowing the hASC maintenance, proliferation and differentiation. The aim of the study was to assemble a perfusion bioreactor, and to develop a new protocol for hASC cultivation in a CO₂ independent medium to be used in the bioreactor. The hASC were isolated from human lipoaspirate, and a two-dimensional cell culture was performed in DMEM, supplemented with 10% Fetal Bovine Serum (FBS). The follow step was to adapt the cells to Leibovitz's CO₂ Independent Medium (LEI+10% FBS), and compare the MTT and Alkaline Phosphatase Activity (ALP) assays to cells cultured in DMEM. The cellular adaptation from DMEM to LEI chosen was the most gradual, beginning in the first passage with 10% LEI, continuing with 25%, 50%, 75% and 100% LEI in the subsequent passages. Phenotypic characterization was performed and hASC cultivated in DMEM and LEI expressed the following markers: CD29, CD44, CD73, and HLA-ABC. In a second step, hASC were evaluated in relation to MTT, ALP, and collagen synthesis, when cultivated in LEI and osteogenic LEI (LEI O), compared to DMEM and osteogenic DMEM. And finally, analysis of immunofluorescence were made of hASC cultivated in LEI and LEI O, showing that in induction medium, this cells expressed osteopontin, osteocalcin, and type I collagen, characteristic of osteogenic cell. The results suggest that Leibovitz's CO₂ Independent Medium supplemented with 10% FBS is an adequate model for *in vitro* cultivation of hASC in perfusion bioreactor.

Keywords: Human adipose stem cell; Perfusion bioreactor; Leibovitz's CO₂ independent medium; Osteogenic differentiation

Introduction

The ease of collection, the large number of cells *in vitro*, and rapid expansion are quite attractive advantages of human Adipose Stem Cells (hASC) over Mesenchymal Stem Cells (MSC), when the contemplation of clinical strategies. The patient's own cells can be harvested with minimal discomfort, expanded in culture, and seeded into a scaffold to be implanted in the bone defect site [1-4]. The hASC can be safely and effectively transplanted into an autologous or allogeneic recipient [5], and are able to differentiate into chondrogenic, adipogenic, osteogenic and myogenic lineages, suggesting its applicability in tissue repair [6].

Despite the traditional 2D cell culture have provided the majority of modern knowledge of the biology, the cells reside, proliferate, and differentiate into the body in complex 3D microenvironments. The 3D structure provides an ideal platform for cell-cell communication and cell-material interaction [7].

For tissue engineering applications, large quantities of cells must be kept alive, both *in vitro* and *in vivo*. The development of systems to assure cell survival and rapid proliferation includes bioreactor systems, a strategy to overcome the mass transfer limitations, which require living tissue for proper nutrition and waste disposal cell, in addition to providing mechanical stimuli to the specific tissue development, by hydrodynamic pressure and shear stress [8].

Studies have shown that perfusion bioreactors significantly increased the differentiation and proliferation of osteoblasts, super-regulation of angiogenic and osteogenic factors, and production of mineralized scaffold by osteoblasts [9-12]. While the perfusion system was initially designed to improve the transport to chemical constructs,

three dimensional flows also imparts a fluid shear force on the cells housed in the array, a process very similar to physiological fluid flow observed in native bone [13].

With few exceptions, as Leibovitz's and Hanks balanced salt solution media, most methods require the use of 5% CO₂ with sodium bicarbonate, or other buffers to maintain the appropriate pH [14]. Thus, the effectiveness of Leibovitz CO₂ Independent Medium (LEI), which is able to maintain pH stability in the long run without CO₂ atmosphere, was studied. This medium has been used for maintaining or growing Vero cells-cell line of monkey kidney and MDCK-epithelial cells [15], various tumor [16], embryonic cells [17], and stem cells isolated from brain tissue [18], HepG2-cell line of liver cancer [19], Caco-2-cell line of epithelial colorectal adenocarcinoma-to study drugs [20]. It was not found in the literature tests related with LEI medium and human adipose stem cells.

***Corresponding author:** Alexandra Rodrigues Pereira da Silva, Ph.D., Laboratory of Biomaterials, Department of Metallurgical and Material Engineering, Engineering School, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Av. Antônio Carlos, 6627-Pampulha-Bloco II-sala 3640, 31270-910-Belo Horizonte, MG, Brazil, Tel: +55 31 34091045/+55 31 34092632; E-mail: ale_efoa@yahoo.com.br

Received October 27, 2012; **Accepted** January 23, 2013; **Published** January 25, 2013

Citation: Silva ARP, Paula ACC, Zonari A, Martins TMM, Goes AM, et al. (2013) Development of a Protocol for Human Adipose Stem Cell Culture in CO₂ Independent Medium and Perfusion Bioreactor. J Tissue Sci Eng S11: 006. doi:10.4172/2157-7552.S11-006

Copyright: © 2013 Silva ARP, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Therefore, the purpose of this study was to assemble a perfusion bioreactor and develop a new protocol for human stem cell cultivation in a CO₂ independent medium, to be used in this bioreactor providing a new tool for bone tissue engineering.

Materials and Methods

The assembly of the perfusion bioreactor

The perfusion bioreactor developed in this work is a closed system, able of undergoing sterilization by autoclaving. The culture medium is continuously pumped at a constant flow rate by a peristaltic pump through connection tubes. The scaffold must be appropriately fitted inside the perfusion chamber, forcing the flow to permeate it completely, and return through tubes to the medium reservoiry (Figure 1).

The perfusion chamber was designed with an inlet and an outlet to allow the perfusion of the medium throughout its interior, where the construct (biomaterial and cells seeded) must be properly adjusted. The perfusion chambers, designed to encapsulate scaffolds with 6 mm diameter and 10 mm length, were machined in the machine shop of the Department of Metallurgical Engineering at Universidade Federal de Minas Gerais. The material chosen for the construction of the chambers was polypropylene, a low-density material that offers a good balance of thermal and electrical properties and moderate resistance. The polypropylene of choice is nontoxic, and allowed easy machining and autoclaving.

Selected tubes to connect the bioreactor system were silicon calibrated peristaltic tubes with 2.6 mm internal diameter. These tubes were chosen because they are autoclavable, translucent, and present biocompatibility and high gas permeability.

The culture media reservatories of choice were bottles of stock culture medium, which are glass bottles of Sarstedt brand. In the lids of the bottles, three holes were made, two of them to adapt the connection tubes-one for input and one for output of medium-and one for the adaptation of a Millipore filter breather, in order to avoid the formation of a vacuum inside the bottle during the tests, and more likely to be a gas *via* exchange.

The peristaltic pump used was Miniplus pump 3, which produces a smooth flow, and is pulse-free. It allows working with 8 perfusion chambers at the same time fed by a constant flow of culture medium. This pump meets a wide flow range: from 0.05 mL/min-40 mL/min.

Some physiological conditions in the bioreactor could be controlled such as temperature of 37°C, guaranteed by the maintenance of tubes and reservatories of culture medium in a water bath and monitored daily, and pH, monitored at each exchange of culture medium.

The change of culture medium in the bioreactor should be partial to keep new components produced by cells, such as growth factors, hormones and enzymes, important in regulating their own cellular mechanisms [21,22].

Because the perfusion bioreactor system was adapted for use in a water bath, it was necessary to use a special medium to ensure pH stability of the cell culture. Leibovitz CO₂ Independent Medium (Gibco) is a culture medium able to maintain pH stability in the long run, without CO₂ atmosphere. Thus, some tests with hASC were carried out, since the stability of the culture pH depends on the cell type used, and there is no literature studies involving the cultivation of hASC in LEI.

hASC adaptation to LEI medium

LEI medium was prepared according to the manufacturer's specifications and supplemented as DMEM medium: 10% FBS, 1000 units/ml penicillin, 1 mg/ml streptomycin, 2.5 mg/ml of amphotericin B, and 60 mg/L gentamycin. The pH was adjusted to 7.4, and the medium was filtered to polyvinylidene difluoride membrane of 0.22 micrometers.

Initially, it was tried to isolate and culture the hASC directly with the medium LEI, but without success. The alternative was to develop a new method of adapting hASC to the LEI environment. Therefore, tests were performed on cells using three adaptation groups, compared to cells grown in DMEM medium: (G1) slow adaptation-addition of 10% LEI through first passage, 25% in second, 50% in third, 75% in fourth, and finally 100% in fifth passage; (G2) faster adaptation medium with 25% LEI in first passage, followed by 50% and 75% in the second and third passage, and 100% at the fourth passage, and finally (G3) rapid adaptation with 50% LEI in the first passage, 75% in second passage, and 100% in third passage.

Despite hASC cultivated in 100% LEI medium were maintained in a CO₂ incubator in all experiments, they were grown in closed bottles and 24-well plates, to assure an environment without CO₂.

Evaluation of adaptation of hascs to LEI medium

hASC cultured in DMEM and in the three groups of adaptation to LEI were seeded in 24-well plates (NUNC), at a density of 5×10³ cells/

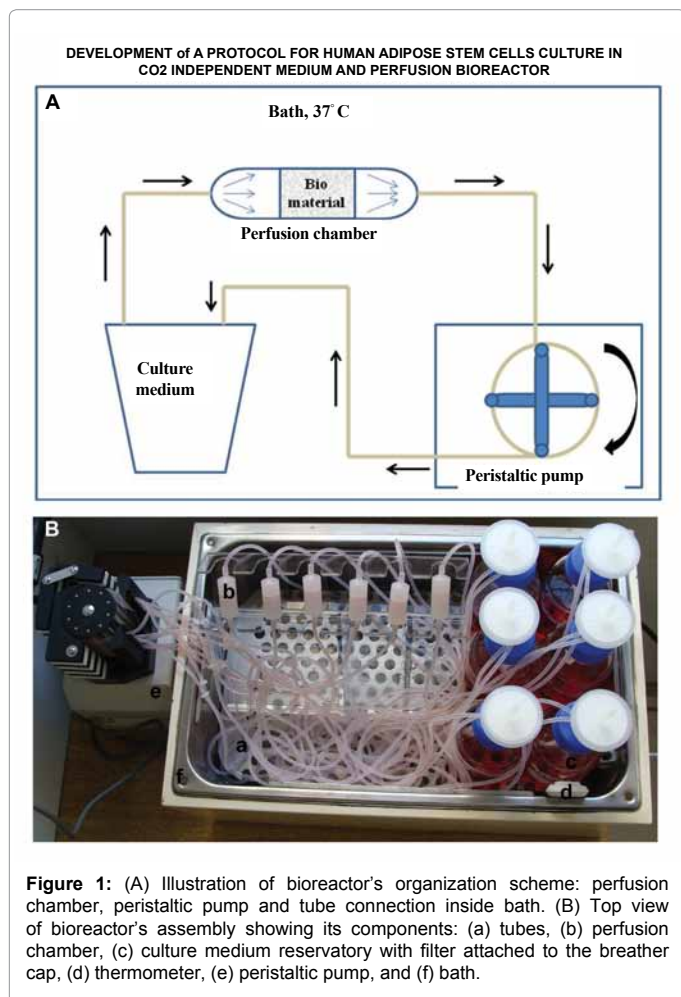


Figure 1: (A) Illustration of bioreactor's organization scheme: perfusion chamber, peristaltic pump and tube connection inside bath. (B) Top view of bioreactor's assembly showing its components: (a) tubes, (b) perfusion chamber, (c) culture medium reservoir with filter attached to the breather cap, (d) thermometer, (e) peristaltic pump, and (f) bath.

well, with 1 mL of the respective culture medium. Cells were incubated at 37°C in humid atmosphere.

Viability and cell proliferation: The proliferation and viability of hASC cultivated were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cat. M-6494, Invitrogen), as previously described [23]. Wells without cells were subjected to the same procedure, as a test control.

Activity of alkaline phosphatase: The Alkaline Phosphatase Activity was evaluated with the BCIP-NBT Kit assay, as described by the manufacturer (Cat. 00-2209, Invitrogen). At the end of each adaptation, the supernatant of each well was removed and the cells were washed twice with PBS. Then, 200 µL of BCIP-NBT solution was added to each well, and incubated. Two hours later, the insoluble purple precipitants were dissolved with SDS-10% HCl. After 18 hours, 100 µL of solution was transferred to a 96-well plate, and the OD was measured at 595 nm. Wells without cells were subjected to the same procedure, as an experiment control.

Cultivation of hASC in DMEM and LEI media

Characterization of the immunophenotype of hASC: Immunophenotypic analyses were performed at fifth passages of hASCs cultured in DMEM and LEI media. The protocol was adapted from a study that has been previously described [24]. The hASCs were incubated with only Alexa Fluor 488 goat anti-mouse IgG (H+L) (Cat. A11001, Invitrogen), to assess the background fluorescence of the secondary antibody as a control. The following unconjugated mouse monoclonal antibodies were used: integrin β1chain-CD29 (Cat. sc-9970, Santa Cruz Biotechnology), HSCAM-CD44 (Cat. sc-7297, Santa Cruz Biotechnology), Hematopoietic Stem Cell (HSC) associated marker-CD34 (Cat. ab8147-500, Abcam), and pan-leukocyte marker CD45 (Cat. 616256, BD biosciences), which were stained with Alexa Fluor 488 goat anti-mouse IgG (H+L), as a secondary antibody. The following conjugated antibodies were used: ecto-5'-nucleotidase-CD73-Phycoerythrin (PE) (Cat. 550257, BD biosciences), HLA-ABC-Fluorescein Isothiocyanate (FITC) (Cat. ab20313-100, Abcam), and HLA-DR-FITC (Cat. ab36775-500, Abcam). The hASCs were analyzed by flow cytometry with a FACSCalibur (Becton Dickinson Immunocytometry System, San Jose, CA), using CELLQuest acquisition software (BD biosciences, USA). A minimum of 20,000 events were acquired for each sample of cells analyzed for each surface marker. Cell marker expression was determined by comparison with control, and the data were analyzed with WinMDI 2.8 software.

Evaluation of osteogenic differentiation: The hASC were induced to differentiate by the addition of osteogenic differentiation factors to DMEM and LEI media: 50 µg/mL ascorbate-2-phosphate (Ecibra), 10 mM β-glycerophosphate (Sigma-Aldrich), and 0.1 µM dexamethasone (Aché) [6]. hASC were seeded in 24-well plates at a density of 5×10³ cells/well, and grown in DMEM and LEI, and their respective osteogenic media DMEM O and LEI O. Cells were incubated at 37°C in humid atmosphere for 7, 14 and 21 days, and evaluated by MTT, Alkaline Phosphatase Activity assays described previously, and Collagen synthesis.

Evaluation of Collagen synthesis: To evaluate collagen synthesis by hASC cultured in DMEM and LEI osteogenic media, a colorimetric assay was performed based on Sirius Red [25]. As a control, the hASC were also cultured in their respective basal culture media, under the same conditions.

At the end of each induction period, cells were washed with PBS

and was added 1 mL/well of Bouin's solution, incubated at 37°C under stirring for 1 hour. Cells were washed in running and distilled water, and subsequent staining reagent Sirius Red at 37°C under stirring for 30 minutes. The wells were washed with 0.01 N HCl and 0.5 M NaOH for 30 minutes under stirring. 100 mL of the solution was transferred to a 96-well plate, and optical density was measured at 540 nm. Wells without cells were subjected to the same procedure, as an experiment control.

Evaluation of the expression of markers of osteogenic lineage cells: Briefly, 1×10⁵ cells were seeded on sterile coverslips in 6 well-plates, and cultivated for 7, 14 and 21 days in LEI and LEI O media. After each period of test, they were fixed in 4% paraformaldehyde for 15 minutes. For permeabilization, the cells were incubated for 10 minutes in PBS containing 0.1% Triton X-100. The cells were blocked for 1 hour at room temperature, with blocking buffer containing 1% Bovine Serum Albumin (BSA) and 5% goat serum in PBS. Subsequently, the cells were incubated for 2 hours in blocking buffer containing the following antibodies: rabbit polyclonal antibody against osteopontin (Cat. ab8448, Abcam), mouse monoclonal antibody against collagen type I (Cat. ab6308-100, Abcam), and mouse monoclonal antibody against osteocalcin (Cat. ab13418, Abcam). Afterwards, the cells were washed with PBS and incubated with the respective Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Cat. A11008, Invitrogen), and Alexa Fluor 488 goat anti-mouse IgG (H+L) (Cat. A11001, Invitrogen), antibodies for 1 hour. The cells were washed with PBS, and the nuclei were stained with 1 µg/mL Hoeschst 33258 pentahydrate (Cat. H3569, Invitrogen), for 30 minutes.

The coverslips were mounted with Hydramount Aqueous media (Cat. HS-106, National diagnostics), and analyzed with a Zeiss LSM 510-Meta confocal microscope. The images were taken at Microscopy Center, Universidade Federal de Minas Gerais, Brazil.

Statistical Analyses

All experiments were repeated three times with triplicate samples. Statistical analyses were performed by using Graph Pad Prism 5.0. The values were represented as the mean ± standard error of the mean (SEM). Statistical differences were calculated by using ANOVA and *Bonferroni's* post tests. Differences were considered significant at p<0.05.

Results and Discussion

Evaluation to adaptation of hASCs to LEI medium

Primary cultures of hASC were obtained after the enzymatic processing of lipoaspirate, showed type fibroblastoid-shaped cells and adherent to plastic surface, according to the literature [6].

Viability and cell proliferation: According to the cell viability and proliferation test, when compared to the control group-hASC cultivated in DMEM-the group that presented the best result was the G1, with a slower and gradual adaptation to LEI, when compared to the groups of faster adjustments (Figure 2A). When the medium contained 100% of LEI in the three groups tested, the result of absorbance of the G1 was the one closest to the absorbance of hASC grown in control DMEM.

Alkaline phosphatase activity: The test to assess the activity of alkaline phosphatase was also used to compare the performance of production of this enzyme by hASC in the three different groups of adaptation to the LEI, when compared to control medium DMEM. As

in the cell proliferation and viability test, the group with the best result when compared to control, was G1 (Figure 2B).

Comparative tests between hASC cultivated in DMEM and LEI

The first comparative test between hASC in DMEM and LEI was performed by Optical Microscopy (OM). Comparing the cells after the second pass, hASC grown in DMEM and adaptation to the LEI presented very similar morphological characteristics as adherence to plastic, colony formation, and growth profile (Figure 3). It was also observed that at the fourth passage, there is a greater density of cell population for culture in LEI, compared to DMEM medium, suggesting excellent adaptation to the new environment proposed.

Characterization of the immunophenotype of hASC: As a cell population can be defined by the types of antigens expressed on the plasma membrane of its cells, in the fifth passage, a phenotypic analysis of cells grown in DMEM and LEI was performed. Cells were incubated with antibodies specific for the following markers: CD29, CD34, CD44, CD45, CD73, HLA-ABC, and HLA-DR.

The population selected for the analysis of surface markers was determined from a chart *versus* size granularity obtained from cells without any marking. The analysis of the histograms has shown

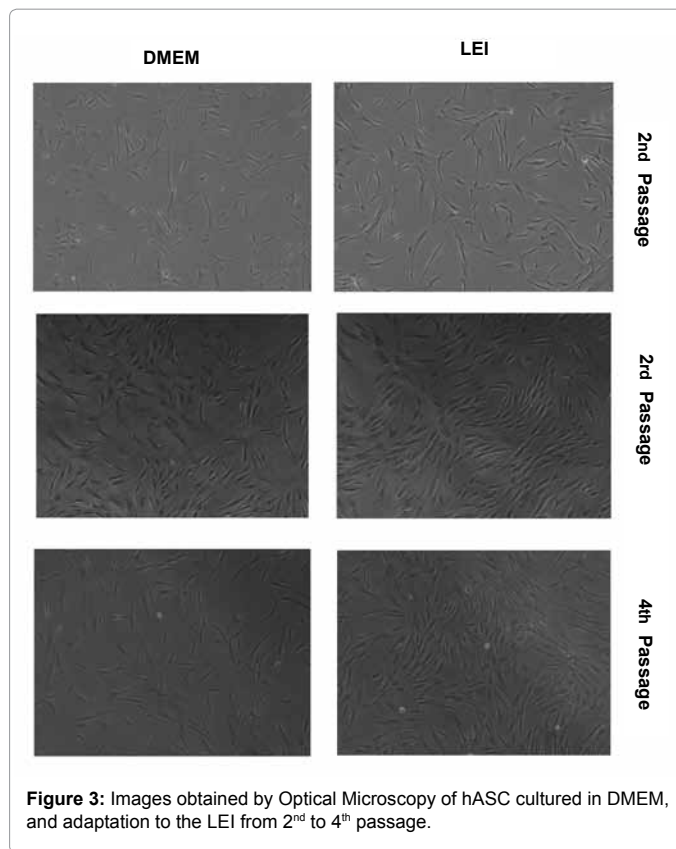


Figure 3: Images obtained by Optical Microscopy of hASC cultured in DMEM, and adaptation to the LEI from 2nd to 4th passage.

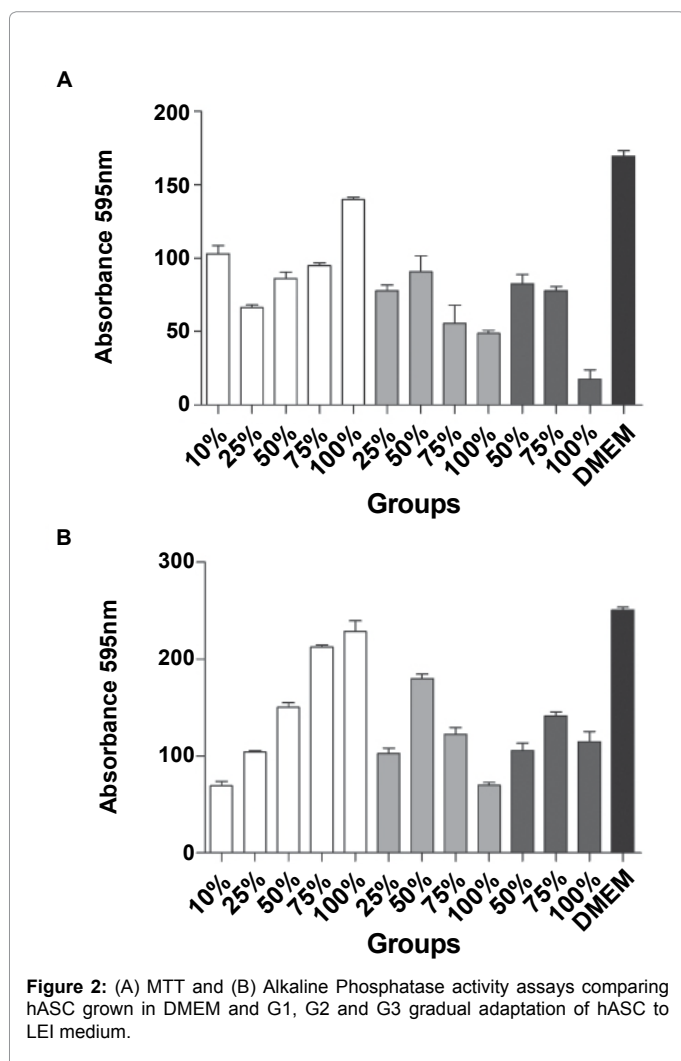


Figure 2: (A) MTT and (B) Alkaline Phosphatase activity assays comparing hASC grown in DMEM and G1, G2 and G3 gradual adaptation of hASC to LEI medium.

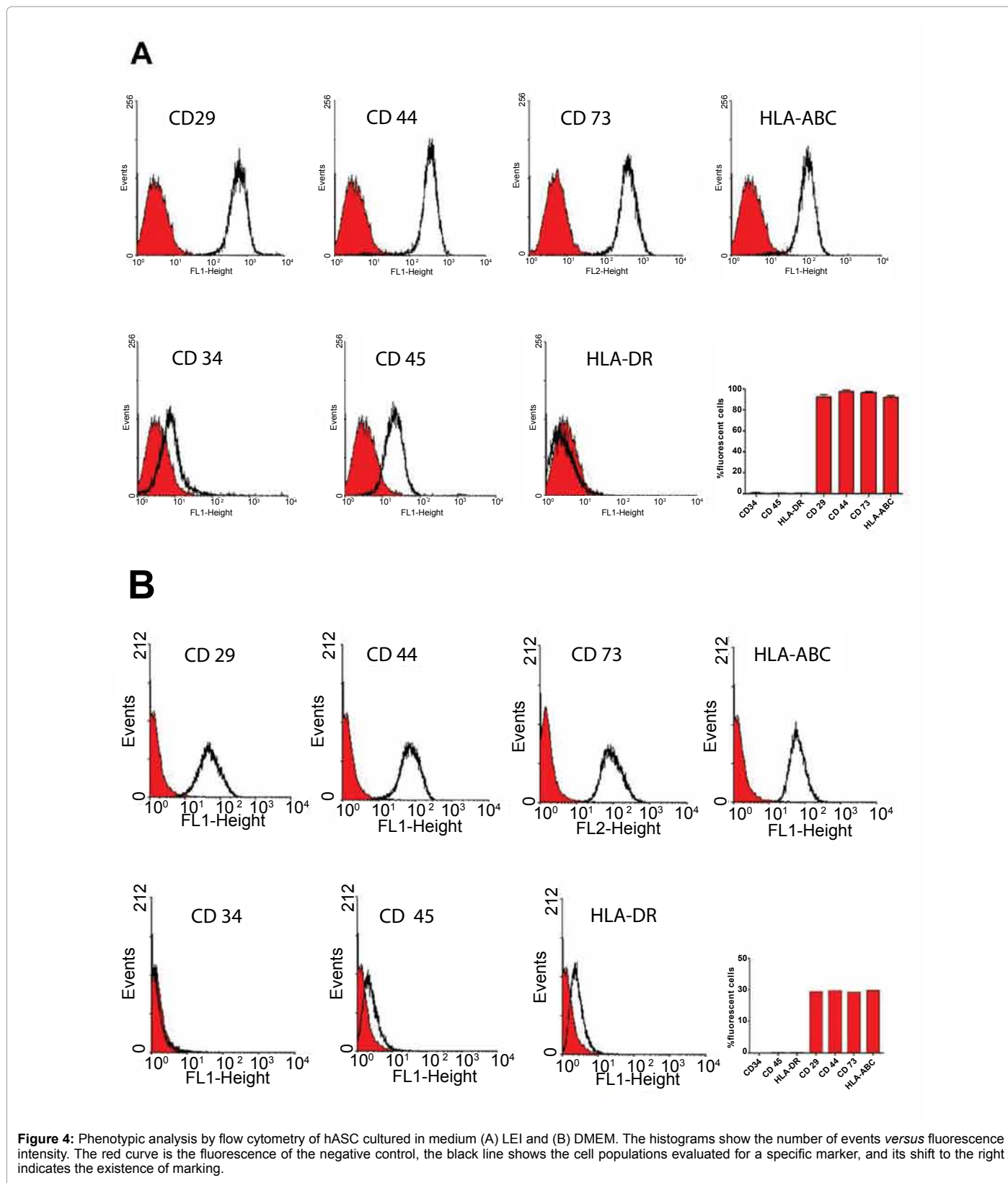
that approximately 96% of cells grown in LEI expressed CD 29, 96% expressed CD 44, 96% expressed CD 73, and 96% expressed HLA-ABC. The CD 34 was expressed as 5%, CD 45 as 2%, and HLA-DR in less than 1% of the cells (Figure 4).

The hASC cultured in DMEM and LEI media formed *in vitro* a homogeneous population of cells that express the surface markers characteristic of the MSC: CD29, CD44, CD73 and HLA-ABC, and does not express markers of hematopoietic lineages: CD34, CD45, and HLA-DR.

In this work, it was found around 5% of expression of CD 34. One of the minimum criteria established for the definition of mesenchymal stem cells is that, less than 2% of cells express CD34 [24]. Besides, CD34 expression decreases with passages throughout the cultivation, with the cellular expansion and medium exchange [26].

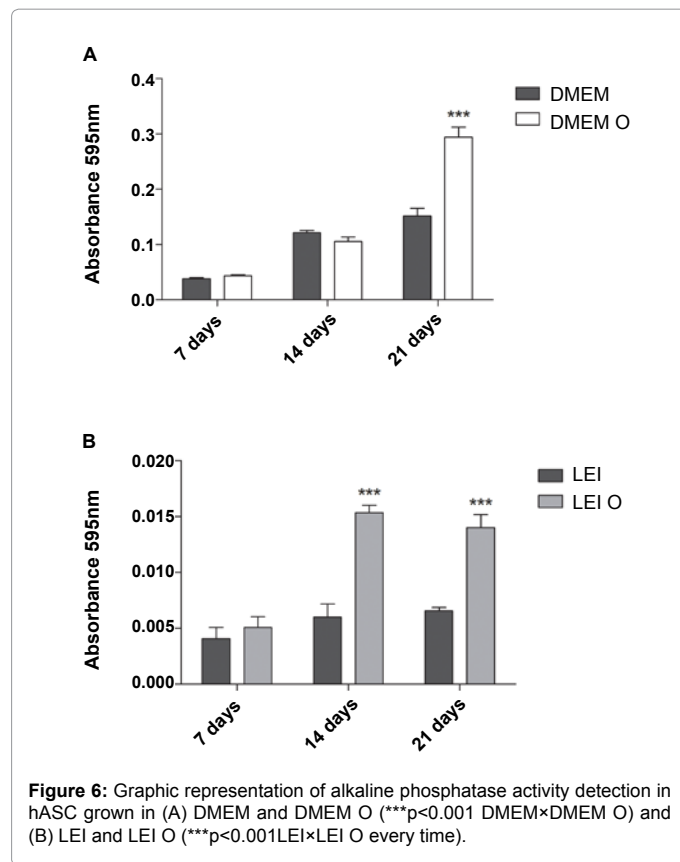
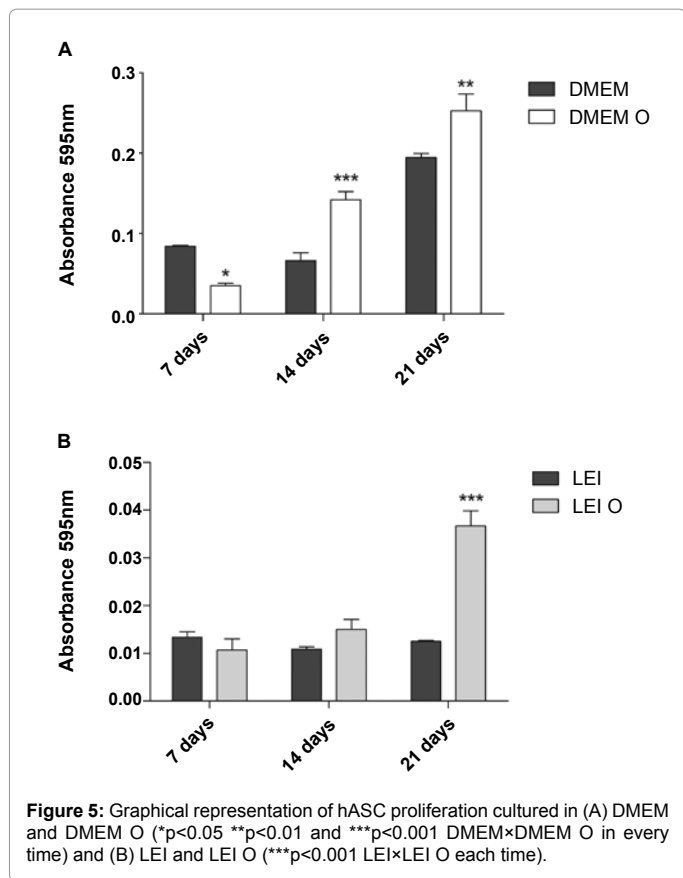
These results showed that there was no interference of culture medium used in the expression of the surface markers. Phenotypically, the hASC isolated are in accordance with the profile proposed by the literature [6,27], and accepted by the International Society for Cell Therapy [26].

Viability and cell proliferation: Cells grown in all media tested were able to metabolize MTT and produce formazan crystals. Initially, the cells in both basal media (Figure 5) had a slightly larger proliferation than those grown in osteogenic media. One may suggest a lower cell proliferation due to a change of composition of media, which were supplemented with osteogenic factors. Then, the larger absorbance observed for hASC cultured in osteogenic media for 14 and 21 days of culture indicate that the supplement used, favored the proliferation of



these cells after an initial period of adaptation. It is also noted that cells grown in LEI followed growth profile similar to that observed in cells grown in DMEM.

Alkaline phosphatase activity: The levels of alkaline phosphatase production by hASC increased over time of cultivation in DMEM, especially when grown in DMEM O, while for cells grown in LEI, the



production levels of alkaline phosphatase increased significantly at all time points when in osteogenic medium (Figure 6).

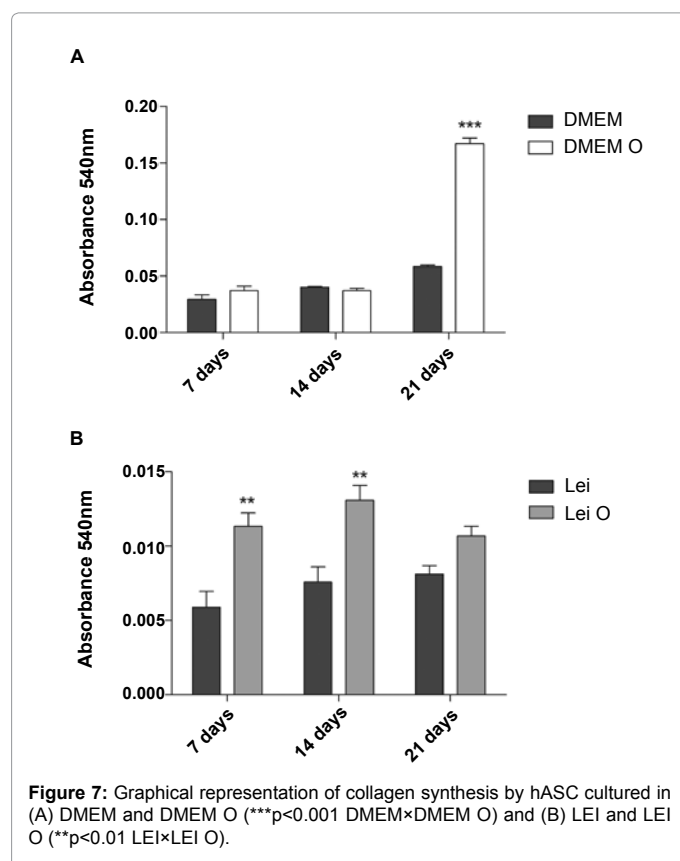
When comparing the graphs of alkaline phosphatase activity, taking into account the graphics of the MTT assay, we observed that the levels of absorbance were higher on 14th day for cells grown in LEI O, maintaining this production until the 21th day, while in DMEM O, the absorbance levels were significantly higher on 21th day of culture.

It was observed a peak of activity of the enzyme alkaline phosphatase in 14 days of culture using LEI O, which is in agreement with the literature [28,29]. Alkaline phosphatase is an enzyme present in undifferentiated cells, so it is used as a marker of stem cells; however, this enzyme is produced at high levels in early osteogenic differentiation, and is considered an early marker of differentiation [28].

Evaluation of collagen synthesis: A significant increase in collagen synthesis from 21th day in hASC grown in DMEM O was observed, while cells grown in LEI O increased collagen synthesis in from 7th day of cultivation, peaking on 14th day (Figure 7).

Although the dosage was not specific for type I collagen, it is known that this type of collagen is a marker for osteoblasts, quickly expressed in cells committed to a lineage, and extracellular matrix deposition as a relatively early event in the pathway differentiation of osteoblasts [30]. Thus, the levels of absorbance produced by the synthesis of collagen by hASC grown in the LEI O had already suggested a commitment to the osteogenic phenotype, starting from 7th day of culture.

Evaluation of expression of markers of osteogenic lineage cells: hASC cells grown in LEI culture medium presented no marking for the three proteins tested, while cells grown in differentiation began to



express the three proteins in small amounts, up to 7th day of induction. The expression of osteopontin (Figure 8), osteocalcin (Figure 9), and type I collagen (Figure 10) remained and intensified after 14th and 21th day of osteogenic induction.

Osteopontin can be expressed in various tissues, including bone, which is produced by osteoblasts in different stages of maturation. In bone formation *in vitro*, there is initial production of osteopontin, and this is found in high levels of expression even after mineralization. The intracellular expression of osteopontin in osteogenic cells, even in proliferation appears to be related to cell migration, while extracellular osteopontin seems to regulate the mineralization process [31].

Osteocalcin, in turn, has a higher expression only in the stage after the proliferation, concomitant with the mineralization of extracellular matrix. Therefore, its expression is delayed in the development of osteoblasts and contributes to the regulation of the mineral phase of bone [32]. Recently, it was demonstrated that prior to their deposition in the extracellular matrix, osteocalcin can be secreted, and thus quantified in the medium before the mineralization stage, representing a good marker for predicting the osteogenic potential of cells [33].

Stem cells can differentiate into osteoblasts depending on conditions of the medium, thus, dexamethasone, ascorbic acid and β -glycerophosphate are indispensable factors for differentiation [28]. Dexamethasone induces the initial stage of differentiation, which is

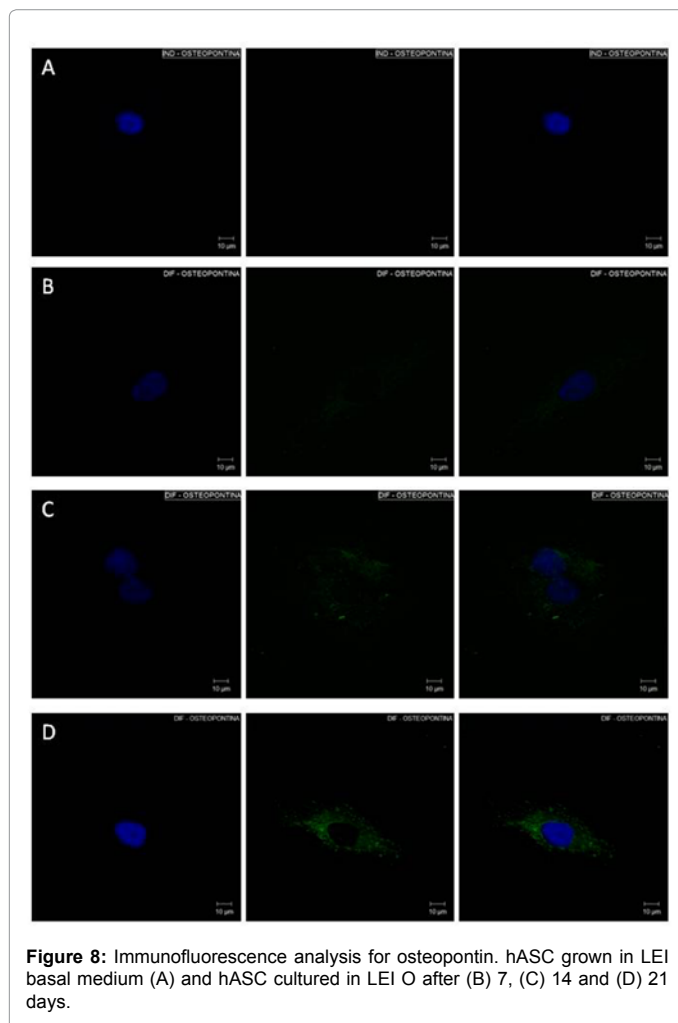


Figure 8: Immunofluorescence analysis for osteopontin. hASC grown in LEI basal medium (A) and hASC cultured in LEI O after (B) 7, (C) 14 and (D) 21 days.

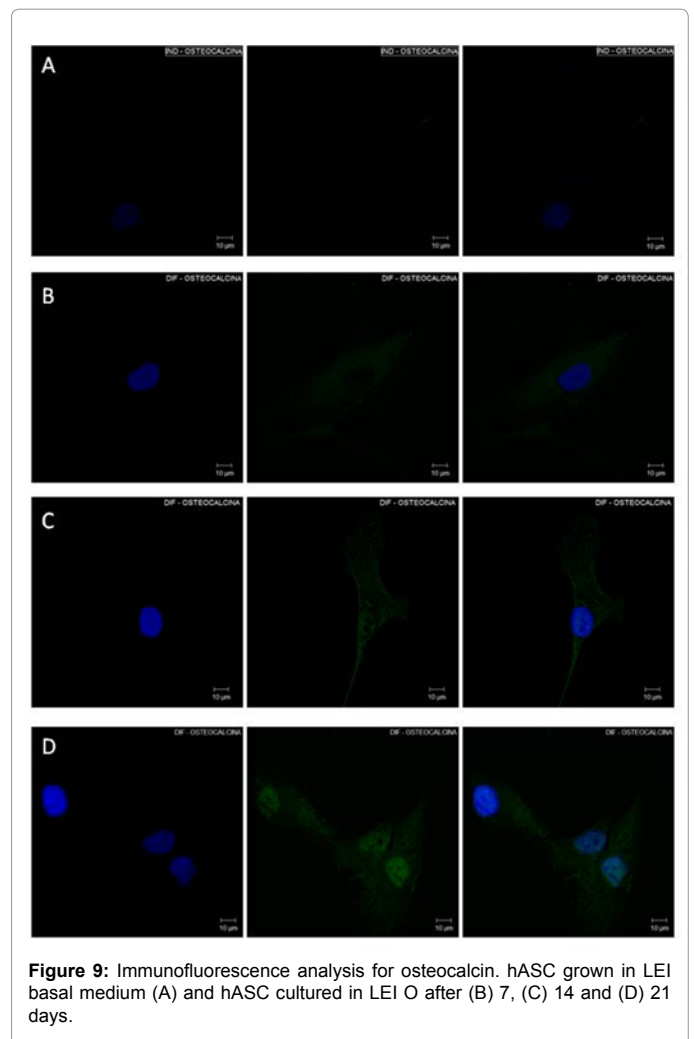


Figure 9: Immunofluorescence analysis for osteocalcin. hASC grown in LEI basal medium (A) and hASC cultured in LEI O after (B) 7, (C) 14 and (D) 21 days.

accompanied by increased expression of alkaline phosphatase. Since ascorbic acid and β -glycerophosphate are essential for the cells reach late stages of differentiation, in which the formation and mineralization take place in the extracellular matrix [29].

The cells in bone differentiation go through three distinct phenotypic phases, starting with a proliferative (which already exists synthesis of type I collagen), then formation of extracellular matrix (high alkaline phosphatase activity), and finally matrix mineralization (increased expression of osteopontin, osteocalcin and mineral deposition) [32].

Therefore, the results obtained in MTT, alkaline phosphatase activity, collagen synthesis and immunofluorescence tests showed that there was a commitment of hASC in osteogenic differentiation, when cultured in LEI osteogenic medium.

Conclusion

The adaptation of hASC to Leibovitz CO₂ Independent Medium is a new methodology developed in this work, for its use in the perfusion bioreactor adapted to the water bath. Analyzing the results of comparative tests between DMEM and LEI media, it was concluded that cells grown in LEI showed morphological and phenotypical similarities with those grown in DMEM, and presented adequate

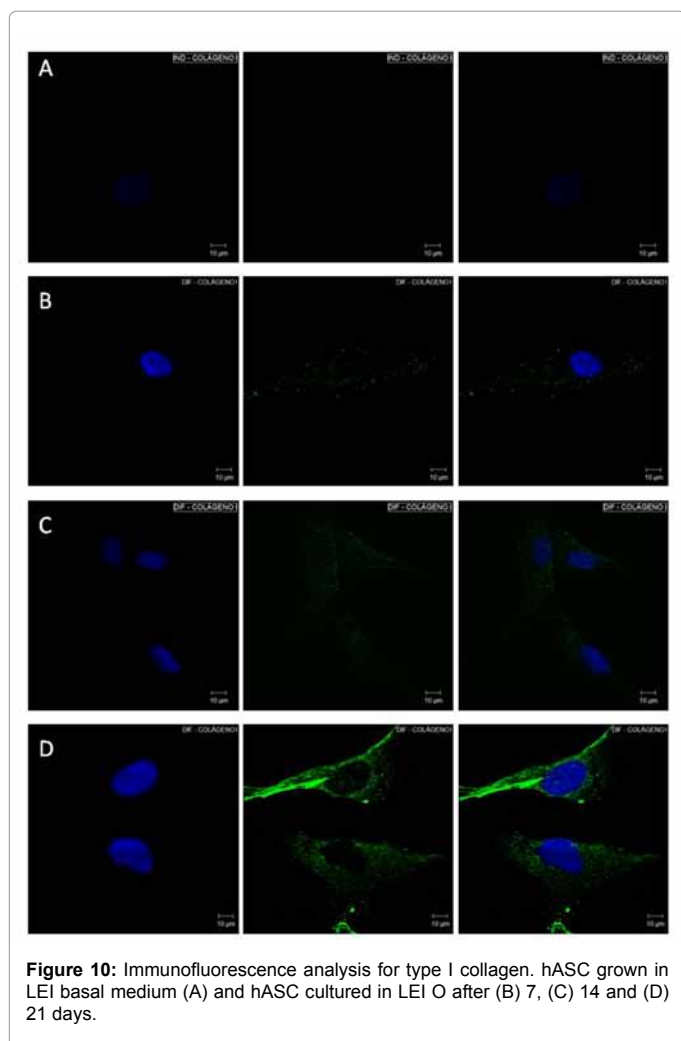


Figure 10: Immunofluorescence analysis for type I collagen. hASC grown in LEI basal medium (A) and hASC cultured in LEI O after (B) 7, (C) 14 and (D) 21 days.

results when evaluated the viability and cell proliferation, alkaline phosphatase activity, collagen synthesis, and expression of osteogenic markers for cells cultivated in LEI O.

Acknowledgment

The authors gratefully acknowledge the financial support from CNPq, CAPES and FAPEMIG/Brazil.

References

- Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, et al. (2004) Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nat Biotechnol* 22: 560-567.
- Fraser JK, Wulur I, Alfonso Z, Hedrick MH (2006) Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol* 24: 150-154.
- Schäffler A, Büchler C (2007) Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells* 25: 818-827.
- Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. *Circ Res* 100: 1249-1260.
- Lindroos B, Suuronen R, Miettinen S (2011) The potential of adipose stem cells in regenerative medicine. *Stem Cell Rev* 7: 269-291.
- Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7: 211-228.
- Dawson JL, Oreffo RO (2008) Bridging the regeneration gap: stem cells,

biomaterials and clinical translation in bone tissue engineering. *Arch Biochem Biophys* 473: 124-131.

- Vacanti J, Vacanti C (2007) The history and scope of tissue engineering. *Principles of Tissue Engineering* 1: 3-6.
- Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, et al. (2002) Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci U S A* 99: 12600-12605.
- Wang Y, Uemura T, Dong J, Kojima H, Tanaka J, et al. (2003) Application of perfusion culture system improves *in vitro* and *in vivo* osteogenesis of bone marrow-derived osteoblastic cells in porous ceramic materials. *Tissue Eng* 9: 1205-1214.
- Wendt D, Marsano A, Jakob M, Heberer M, Martin I (2003) Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. *Biotechnol Bioeng* 84: 205-214.
- Martin Y, Vermette P (2005) Bioreactors for tissue mass culture: design, characterization, and recent advances. *Biomaterials* 26: 7481-7503.
- Allori AC, Sailon AM, Pan JH, Warren SM (2008) Biological basis of bone formation, remodeling, and repair-part III: biomechanical forces. *Tissue Eng Part B Rev* 14: 285-293.
- Lennon DP, Edmison JM, Caplan AI (2001) Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol* 187: 345-355.
- Barngrover D, Thomas J, Thilly WG (1985) High density mammalian cell growth in Leibovitz bicarbonate-free medium: effects of fructose and galactose on culture biochemistry. *J Cell Sci* 78: 173-189.
- Vistica DT, Scudiero D, Skehan P, Monks A, Boyd MR (1990) New carbon dioxide-independent basal growth medium for culture of diverse tumor and nontumor cells of human and nonhuman origin. *J Natl Cancer Inst* 82: 1055-1061.
- Perin L, Giuliani S, Jin D, Sedrakyan S, Carraro G, et al. (2007) Renal differentiation of amniotic fluid stem cells. *Cell Prolif* 40: 936-948.
- Westerlund U, Moe MC, Varghese M, Berg-Johnsen J, Ohlsson M, et al. (2003) Stem cells from the adult human brain develop into functional neurons in culture. *Exp Cell Res* 289: 378-383.
- Talens-Visconti R, Bonora A, Jover R, Mirabet V, Carbonell F, et al (2006) Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World J Gastroenterol* 12: 5834-5845.
- Lind ML, Jacobsen J, Holm R, Mullertz A (2007) Development of simulated intestinal fluids containing nutrients as transport media in the Caco-2 cell culture model: Assessment of cell viability, monolayer integrity and transport of a poorly aqueous soluble drug and a substrate of efflux mechanisms. *Eur J Pharm Sci* 32: 261-270.
- Goldstein AS, Juarez TM, Helmke CD, Gustin MC, Mikos AG (2001) Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. *Biomaterials* 22: 1279-1288.
- Martin Y, Vermette P (2005) Bioreactors for tissue mass culture: design, characterization, and recent advances. *Biomaterials* 26: 7481-7503.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.
- Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, et al. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 24: 376-385.
- Tullberg-Reinert H, Jundt G (1999) *In situ* measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: effects of transforming growth factor beta2 and ascorbic acid 2-phosphate. *Histochem Cell Biol* 112: 271-276.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8: 315-317.
- Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, et al. (2005)

-
- Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 33: 1402-1416.
28. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, et al. (1998) Mesenchymal stem cells in osteobiology and applied bone regeneration. *Clin Orthop Relat Res* S247-S256.
29. Payushina OV, Domaratskaya EI, Starostin VI (2006) Mesenchymal stem cells: sources, phenotype, and differentiation potential. *Cell Biol* 33: 2-18.
30. Aubin JE (1998) Bone stem cells. *J Cell Biochem Suppl* 30: 73-82.
31. Sodek J, Ganss B, McKee MD (2000) Osteopontin. *Crit Rev Oral Biol Med* 11: 279-303.
32. Lian JB, Stein GS (1992) Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med* 3: 269-305.
33. Nakamura A, Dohi Y, Akahane M, Ohgushi H, Nakajima H, et al. (2009) Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. *Tissue Eng Part C Methods* 15: 169-180.