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Porto alegre, junho de 2010.

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Trabalho de Conclusão da Disciplina de Trabalho de Conclusão de Curso

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Comparing two human umbilical cord mesenchymal stem cell isolation protocols

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Abstract

Human umbilical cord is a rich source of mesenchymal stem cells (MSCs) as reported in many studies. Many techniques are used to obtain these cells and their variations are related to the part of the tissue from which the stem cells can be extracted (umbilical vein, arteries, Wharton's jelly or the whole umbilical cord - UC). In this sense, it is really important to compare and optimize isolation methods in order to obtain the greatest number of MSCs to enhance culture success and expansion for further use in cell based therapies. In this study we compared two different isolation protocols in order to improve the MSCs harvesting process in our laboratory. UCs (n=10) were sectioned in two parts during collection and each fraction from this pair was isolated, simultaneously, into the following groups according to each protocol: Group 1 - cells harvested from UC vein and Group 2 - cells harvested from UC vein and arteries with additional mechanical digestion. Cells were maintained in the same culture conditions and after reaching the 5^{th} passage, groups were compared in several aspects: isolation parameters, growth characteristics, immunophenotypic analysis, differentiation potential and proliferation rate. Group 1 demonstrated better results for isolation success and lower cost. Both groups were able to harvest MSCs and the other analysis could not demonstrate significant differences between them. According to the conditions adopted in our laboratory protocol from group 1 it is more advantageous for lower cost and better isolation success rate.

Key words: human umbilical cord, mesenchymal stem cells, umbilical cord vein, Wharton's jelly.

Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic cells which have been first isolated from the bone marrow (BM) but subsequently from other adult connective tissues [1,2]. They exhibit multilineage differentiation capacity being capable of giving rise to diverse cells, such as osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and possibly neural cells [2,3]. Similar to other stem cell types MSC possess the potential for self-renewal and for differentiation into highly specialized cells upon appropriate stimulation [3]. However, MSCs are considered the most pluripotent adult stem cells, and some authors consider them to be the most similar to embryonic stem cells. These cells are being increasingly developed for indications in the growing field of regenerative medicine and also for their ability to modulate the immune response [4,5]. Recent clinical trials have suggested that bone marrow–derived MSCs (BM-MSCs) can aid children born with imperfect osteogenesis [4], improve cardiac function after myocardial stroke [5] and treat acute graft-versus-host disease (aGVHD) after BM transplantation [6,7]. Usually, BM represents the major source of MSCs for cell therapy. However, BM aspiration involves invasive procedures and does not present immunological advantagens such as can be seen using umbilical cord blood (UCB). Both the frequency and differentiation potential of BM-MSCs decrease significantly with age. It is, therefore, important to find an alternative source of MSCs [8]. Because of this, MSCs have been isolated from umbilical cords that would be routinely discarded after birth.

Originating from extraembryonic mesoderm at day 13 of embryonic development [9], umbilical cord is composed of two arteries and one vein, all of which are surrounded by a unique connective tissue stroma rich in proteoglycans and mucopolysaccharides [10], called Wharton's jelly (WJ). These tissues, as well as the primitive germ cells, are differentiated from the proximal epiblast at the time of formation of the primitive line of the embryo, which contain MSCs and even some cells with pluripotent potential [11].

Wharton's jelly cells have been cultured for more than 80 population doublings with no indications of senescence, changes in morphology, increased growth rate, or change in ability to differentiate into neurons [12].

Isolation and characterization of MSCs from the umbilical cord vein (UCV) have been reported. Mesenchymal stem cells derived from the umbilical cord vein are functionally similar to BM-MSCs and since the cells are of fetal origin, their proliferative and differentiation potential could be better than that of MSCs from other sources [13,14]. Thus the whole umbilical cord is thought to be a promising non-invasive source of MSCs.

An important issue of interest in adult stem cell studies is the availability of the source and efficacy of isolation techniques to yield a reasonable number of viable cells for cell proliferation [15]. In this study we compared two human umbilical cord mesenchymal stem cell (HUCMSC) isolation protocols emphasizing their advantages and disadvantages in different aspects such as isolation parameters, growth characteristics, immunophenotyping analysis, proliferation rate and differentiation potential in culture. We intend to optimize our MSC isolation protocol for better culture conditions to improve its use in cell-based therapies.

Materials and Methods

1. Isolation of human umbilical cord MSCs

Two eight-centimeter long fractions of umbilical cords (n=10) from each donor were collected (cesarean section) after informed consent was obtained from the mothers in accordance with the ethical committee of Moinhos de Vento Hospital (mean maternal age 34.3 and gestational age from 38 to 40 weeks).

During collection, cords were transversaly sectioned in two portions of the same size. A total of twenty umbilical cord fractions were collected from ten different donors. The paired

samples were divided into two groups according to each isolation protocol: **Group 1** (n =10) - cells harvested from the UC vein and arteries using gentle massage [16] and **Group 2** (n=10) - cells harvested from the UC vein (modified protocol described by Covas *et al*, 2002) [13].

The samples for group 1 were washed in a hypochlorite solution (1:3) and rinsed by PBS (phosphate-buffered saline) then stored in DMEM Low Glucose (Dulbecco's modified Eagle's medium – Sigma Aldrich) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 2% (v/v) antibiotics (penicillin/streptomycin and gentamycin 112 µL – Schering Plough). For group 2, a cord buffer solution (albumin 1% v/v – Sigma Aldrich, glucose 2g/L, fungizon 250 µg/mL – 200 µL, penicillin/streptomycin – 1,000 µl and PBS – 1L) was used. After the treatment described above, both samples were transported to the laboratory and processed according to each protocol at the same time (never exceeding the period of 24 hours between collection and processing).

Group 1 Cell Isolation

Umbilical cords (UCs) were washed with PBS (30 mL) and rinsed up to three times in order to remove blood clots. Using tweezers, dirt was gently removed. For enzymatic digestion vein and arteries were filled with 0.1% (w/v) collagenase (Gibco) in PBS (3 mL) using injection (needles and syringes) until the cord assumed a tumescent appearance. After 20 minutes of incubation, the enzyme was inactivated using a proliferation medium consisting of DMEM – LG (Sigma Aldrich), 10% fetal bovine serum (Gibco), 2% (v/v) antibiotics (penicillin/streptomycin) and gentamycin 2.24 µl/mL (Schering Plough), by injection in the vein and in the arteries (both ends). The detached cells were then harvested after gentle massage of the cord (friction). This resulting suspension was collected and centrifuged; the pellet was resuspended and seeded to a 25 cm^2 flask and cultured in DMEM, supplemented with 10% FBS, 1% antibiotics in 5% CO_2 in a 37°C incubator. After 24

hours of incubation, nonadherent cells were removed. Density was established using a Neubauer chamber.

Group 2 Cell Isolation

UC veins were cannulated using a site coupler similar to a catheter (Baxter) and washed internally with 10 mL of PBS. The distal end of the cord was clamped. For the digestion step, the vessel was filled with 0.5 % (w/v) collagenase in DMEM – LG 3 mL (Sigma Aldrich) alternating this procedure at both ends. The cord was incubated with approximately 40 mL of a cord buffer solution for 40 min. After incubation the enzyme was inactivated by washing the vein internally with the proliferation medium, DMEM-LG (Sigma Aldrich), 10% fetal bovine serum (Gibco), 2% penicillin/streptomycin and 2.24 µL/mL of gentamycin (Schering Plough). This resulting suspension was centrifuged and the pellet resuspended and seeded onto a 25 cm^2 flask and cultured in DMEM supplemented with 10% FBS, 1% antibiotics in 5% CO_2 in a 37°C incubator. After 24 hours of incubation, nonadherent cells were removed. Density was established using a Neubauer chamber. The culture medium was changed every 3 days for both groups and after reaching 90% confluence, cell passages were carried out using 0.25% trypsin-EDTA (Sigma). For all the following analysis one culture from each group was evaluated (corresponding pair from the same donor).

2. Immunophenotyping

To analyze cell-surface expression of typical protein markers, the cells in the fifth passage (group 1 n=1, group 2 n=1) were labeled with monoclonal antibodies CD29, CD34, CD44, CD45, CD90, HLA-DR (Pharmingen-BD Biosciences) and analysed by flow

cytometry. The antibodies PE-IgG1 and FITC-IgG1 were used as isotype controls. Data acquisition was performed using the FACSCalibur flow cytometer (BD Biosciences) and 10,000 events were analyzed using the CELLQuest software (BD Biosciences).

3. Cell Differentiation Procedures

To evaluate MSC pluripotency, cells in the fifth passage, when they achieved 80-90% confluence (group 1 n=1, group 2 n=2), were seeded in twelve-well plates (10,000 cells/cm^2) and subjected to adipogenic, chondrogenic and osteogenic differentiation. The medium was replaced twice a week for 15 days. A negative control for each differentiation assay was obtained by culturing the same cells with a conventional medium.

Adipogenic Differentiation

Subconfluent cells were cultured in Iscove's medium (Gibco), supplemented with 20% human plasma, 10^{-7} M dexamethasone, 5 µM rosiglitazone, 5 µM indomethacin (Sigma-Aldrich), 2,5 µg/mL bovine insulin and 10 mM/mL heparin. Adipogenic differentiation was confirmed by intracellular accumulation of lipid-rich vacuoles stained with oil red O. For the Oil red O stain, cells were fixed with 4% paraformaldehyde for 20 minutes, washed with deionized water and stained with a working solution of Oil red for 10 minutes [17].

Osteogenic Differentiation

To promote osteogenic differentiation, subconfluent cells were treated with a proliferation medium - DMEM/Hepes, supplemented with 10% fetal bovine serum, 10% b-glycerophosphate (10mM), 1% ascorbate-2 phosphate 5mg/mL and 0.1% dexamethasone

10^{-5}M. After the period of differentiation, the cells were washed and fixed with 4% paraformaldehyde for 20 minutes. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate, assessed by *Alizarin Red* staining [17].

Chondrogenic Differentiation

For chondrogenic differentiation, subconfluent cells were treated with a suplemented medium – HDMEM, ascorbate-2 phosphate 50nM, bovine insulin 6,25ug/mL and 10ng/mL of TGFβ 1 (Millipore, Japan e other reagents from Sigma-Aldrich). After the period of differentiation, the cells were washed and fixed with 4% paraformaldehyde for 20 minutes. Chondrogenesis was demonstrated by staining with *Alcian Blue* [18].

For differentiation analysis all reagents used are from Sigma-Aldrich except those specified in the text.

4. Proliferation assays

In order to compare proliferation characteristics between the groups, the cells in the fifth passage were labeled with Ki-67 monoclonal antibody (DAKO). The cells were seeded in 24-well plates (6.250 cells/cm^2 and 12.500 cells/cm^2) and fixed after two days with 4% paraformaldehyde for 20 minutes. The cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes and to block unspecific binding of the primary antibody, the cells were incubated with 1% BSA in PBS-Triton for 30 min. After this, the cells were incubated in the diluted antibody (1% BSA in PBS) overnight at 4°C. The remaing solution was decanted and the cells were washed three times with PBS (5 min each) before they were incubated with the secondary antibody (FITC diluted in 1% BSA) for 2 hours at room temperature in the

dark. The secondary antibody solution was decanted and the cells washed three times with PBS (5 min each) in the dark. Negative controls were carried out by omitting the primary antibodies. Ki-67 labeling was analyzed by immunofluorescence microscopy. The number of immunopositive cells was counted in five non-overlapping visual fields at a magnification of x400. For the total cell count in each field, stained cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 1 minute. The proliferation rate was defined as the percentage of cells expressing the proliferation marker Ki67 [19].

5. Statistical analysis

Mann-Whitney and Chi-square tests were applied to evaluate the groups (success, isolation density and proliferation assays). Data were analyzed using SPSS 15.0 software, where, for decision criteria significance, a level of 5% was adopted.

Results

Isolation Parameters, establishment of cultures and growth characteristics

Both isolation protocols took approximately one hour to be executed. At least 48 hours after isolation it was possible to observe the presence of blood and debris in all cultures from group 1 while cultures from group 2 were cleaner immediately after isolation. Two cell morphologies could be observed in cultures from both groups; the majority of these cells had a MSC-like phenotype and the others were similar to endhotelial cells, which disappeared after the first passage (Figure 1).

Despite variations in cell density after each isolation procedure, significant differences between the groups could not be observed ($p=0.393$). Although it was possible to obtain cells

from all isolation procedures, UC-MSC isolations were considered successful when it was possible to observe adherent cells, isolated or in colonies over a period of up to 48 hours. From 20 cultured samples (ten pairs), 4 could not follow this parameter and thus, sixteen isolations were considered successful (80%). In group 1, 90% of the isolations were successful (9/10) while in group 2, 70% (7/10). Statistical analysis shows a significant difference (p=0.007) for the isolation success rate (Table 1).

Between the sixteen isolations considered successful, three could not reach the first passage for unknown reasons and two of these were from group 1. Reaching the first passage is an important parameter to consider culture establishment successful. According to figure 2, the time to reach this confluence was 17-46 days (group 1) and 13-84 days (group 2). Although we can observe differences between group 1 and group 2, it could not be considered statistically significant (p=0.756). Success in culture establishment was similar for both groups (p=1.000).

Immunophenotypic Analysis

Cells in the fifth passage from both groups were uniformly positive for mesenchymal stem cell markers, such as CD44, CD90 and CD29. Hematopoietic markers, CD45, CD34 and HLA-DR, were not expressed. As shown in figure 3, no obvious differences between the groups could be observed.

Multilineage Differentiation Potential

After 15 days, the cells submitted to differentiation induction in both groups were able to demonstrate adipogenic, osteogenic and chondrogenic differentiation assessed by the appropriate staining protocol. With adipogenic supplementation, the differentiation was

apparent after one week of incubation, characterized by lipid vacuole deposits (Figure 4a) and in general, achieved phenotypic modifications before the others. Osteogenic differentiation could be identified by calcium deposits (Figure 4b) and chondrogenic differentiation by mucopolysaccharide-rich extracellular matrix (Figure 4c). No morphological differences between the analyzed cultures from group 1 and group 2 could be observed.

Proliferation assays

Proliferation profile was evaluated for both groups in two different densities (6,500 and 12,500 cells/cm^2) considering the total number of Ki67 stained nuclei in relation to the total DAPI stained nuclei (figure 5). The cells in both groups had similar proliferation rates in both analyzed densities. Although we can observe differences between the groups for the proliferation rate in 6,500 and 12,500 cells/cm^2 this could not be detected by statistical analysis (Table 2).

Discussion

The MSCs from the umbilical cord are obtained by different isolation methods depending on the source of cells: connective tissue (Wharton's jelly), umbilical vessels or whole umbilical cord explants. Cell isolation and processing techniques can affect the number and phenotype of isolated cells, both of which are critical for cell-based therapeutics [15]. In this study, we have compared two different protocols for umbilical cord mesenchymal stem cell isolation. Both protocols are based on collagenase digestion, with a varying enzyme concentration. Collagenases are enzymes that are able to cleave the peptide bonds in the triple helical collagen molecule. In this way cells are released from the tissue and can be easily collected by washing and centrifugation [20]. UC fractions (same donor and size) were divided in groups according to each isolation procedure. In group 1, where both vein and arteries were manipulated during the process, collagenase concentration was lower,

incubation time was shorter and cells were detached mechanically as well as using gentle massage. In group 2, where only the cord vein was manipulated, collagenase concentration was higher and incubation time longer. Several aspects relating to both techniques could be observed in this study. In the isolation process in group 1, UC arteries are sometimes difficult to be seen, while in group 2, the vein is easily identified before cannulation by its caliber. The coupler device (group 2) permits safer manipulation conditions and avoids cord damage, although it increases the process cost and can only be used in the cord vein. After isolation, the presence of blood and dirt was observed in cultures from group 1, making it more difficult for identification of adherent cells. This may have occurred because of the massage step and for the absence of cleaning the vessels internally as this step is only described for Group 2 isolations.

The number of isolated cells was similar for both groups ($p > 0.05$) and this could be related to some compensation between the techniques. In group 1, there are more MSC sources (vein and arteries), a lower concentration of collagenase, and a 20 min period of incubation and an additional step of mechanic digestion (massage). On the contrary, in group 2, only the umbilical vein is manipulated for MSC harvest, a higher collagenase concentration than group 1 is used with an incubation time of 40 min, thus, the higher enzyme concentration followed by a longer incubation time may detach a similar number of cells, compensating for the massage step and artery manipulation in group 1.

The success rate was higher in group 1 and this could be related to the technique and its variations, such as the massage step and artery manipulation, as more MSC sources are exploited. The heterogeneity of human umbilical cord mesenchymal stem cells (HUCMSCs) has been reported. Isolated and cultured HUCMSCs, though considered mesenchymal stem cells in nature, contain at least two distinct cell populations, which might have been derived from different tissue sites and/or have an embryological origin [21]. Cells expressing mesenchymal markers CD44, CD105, CD73, and CD90 are present in situ in both the WJ

and UC vessels. Collagenase-based digestion applied to the whole UC will consistently isolate a specific cell population expressing high levels of MSC surface markers and moderate levels of CD146 (40%–50%). This method can generate large numbers of cells at the time of isolation [15].

We have observed that different isolation protocols could not affect the physiological behavior of cells when assessing the proliferation rate using the Ki-67 monoclonal antibody.

Regardless of the isolation method, all established cultures could be considered MSCs because they were adherent to plastic when maintained in standard culture conditions. They also showed pluripotency: evaluated cells differentiated into osteoblasts, adipocytes and chondroblasts in vitro. Flow cytometric analysis revealed high levels of CD44, CD29, CD90, but lack expression of CD-proteins typical for hematopoietic lineages (CD34, CD45 and HLA-DR). Both groups fulfil the described criteria by Dominici et al 2006 [22].

We should consider different conditions from each laboratory related to protocols, culture mediums, reagent concentrations and brands, those related to culture maintenance, isolation method, passage confluence, plating density, etc [23,24]. Therefore, it is important that different techniques are evaluated within the parameters of each laboratory.

Analyzing all data under the conditions used in our laboratory, it was possible to obtain mesenchymal stem cells using both protocols. However, the protocol from group 1 is more advantageous because, besides incurring lower costs and not being inferior in any analysis compared to group 2, it demonstrated a higher success rate in umbilical cord mesenchymal stem cell isolation.

This study aims to demonstrate the most efficient method for collecting MSCs from this new source of cells. The use of cord blood shows certain advantages, such as their facility to be accessed and collected, and because this method is not invasive. Another advantage is that in transplant patients these cells provoke less rejection in comparison to other cells used in such treatment, such as cells from bone marrow. Umbilical cord blood (UCB) has been used

since 1988 for transplants [25]. The reason why the MSCs were extracted from the umbilical cord and not from the UCB is that, dispite this material being a good source of HSC (hematopoietic stem cells), it does not, contrary to what was thought previously , contain a high number of MSCs. It has also been shown that these cells are in much greater numbers in the the umbilical cord itself and not in the UCB [26,27]. Although MSCs cannot be used immediately after collection, as these cells need to be cultivated for some weeks before use, using MSCs is a better alternative, as they are the cells responsible for regenerating damaged tissue in tissue engineering.

It is, therefore, extremely important to identify the best method for extracting MSCs from umbilical cord as the use of this source of stem cells is considered a promising strategy for regenerative medicine for the near future.

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Author Disclosure Statement

No competing financial interests exist.

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Figures

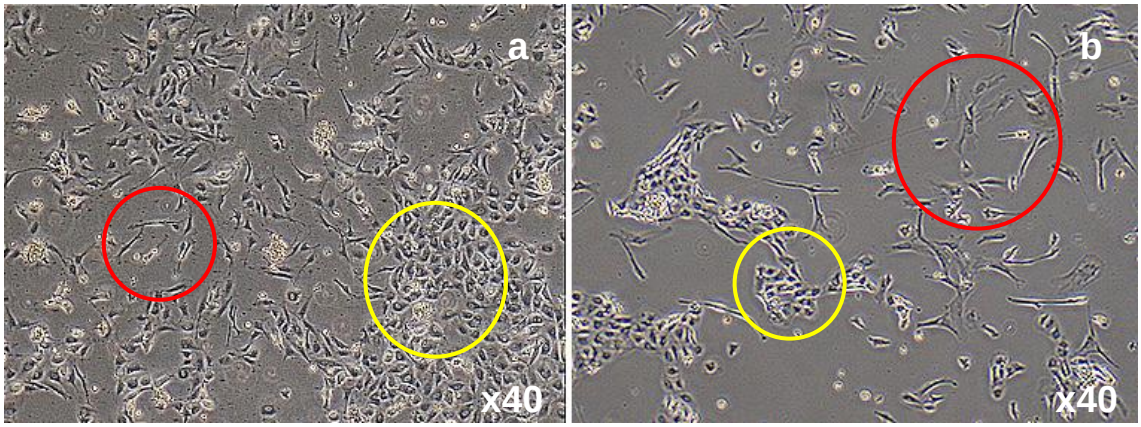

Figure 1 – Adherent cell morphology 12 days (P0) after isolation: (a) group 1 adherent cells x40; (b) group 2 adherent cells x40. Yellow elipses indicate residual cells with endothelial phenotype and red ellipses indicate MSC-like cells.

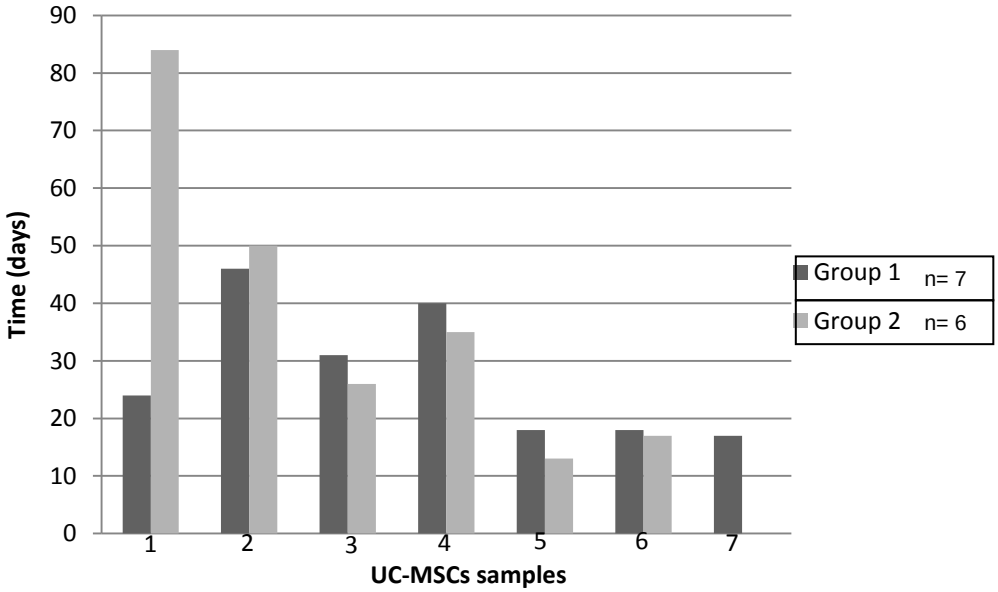

Figure 2 – Comparative Analysis of the groups until the first passage. The average time to reach the first passage (days) was similar between group 1 and 2 (p*=0,756).

*p= minimum level of significance of non parametric Mann-Whitney test.

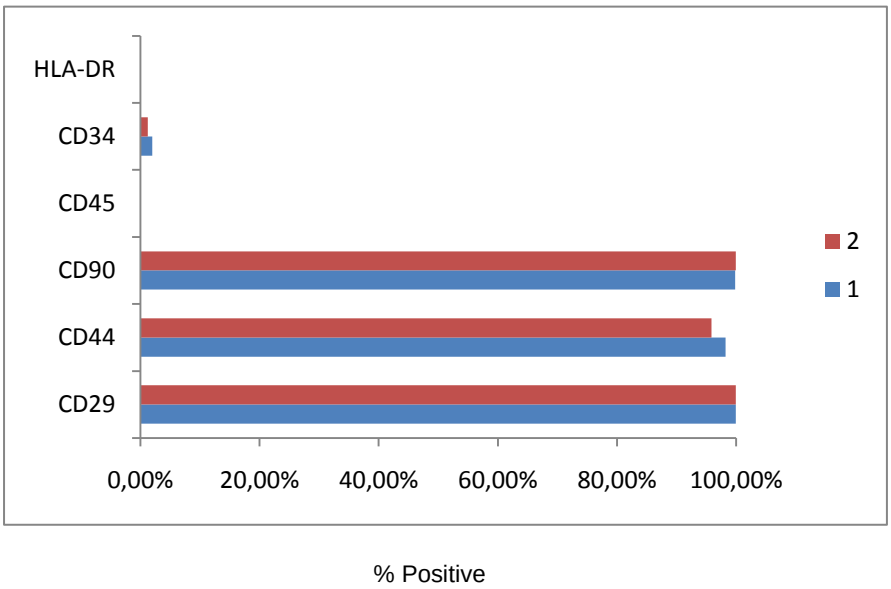

Figure 3 – The graph shows results for flow cytometry analysis. Both groups expressed high positivity for MSC markers CD90, CD44 and CD29. In contrast, there was no relevant expression for hematopoietic lineage markers HLA –DR, CD34 and CD45.

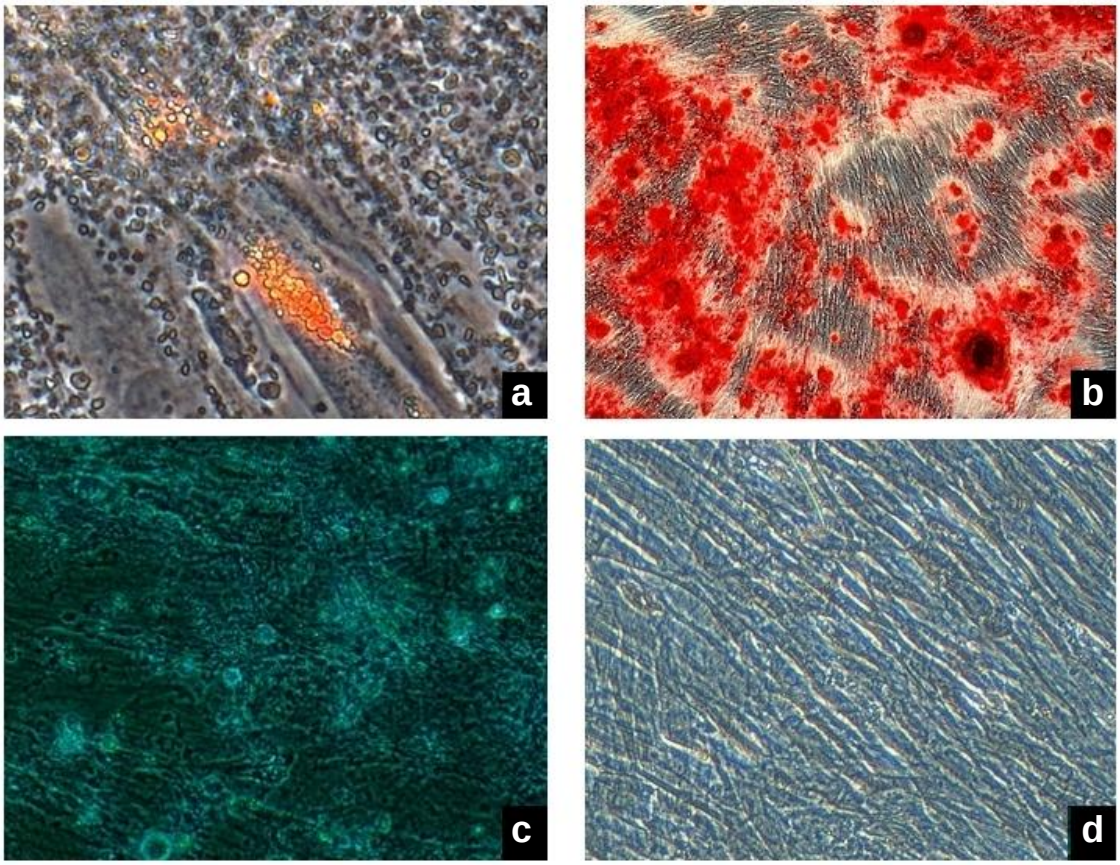

Figure 4 – Differentiation potential of adherent cells isolated from UC: (a) adipogenic differentiation (b) osteogenic differentiation (c) chondrogenic differentiation (d) negative control.

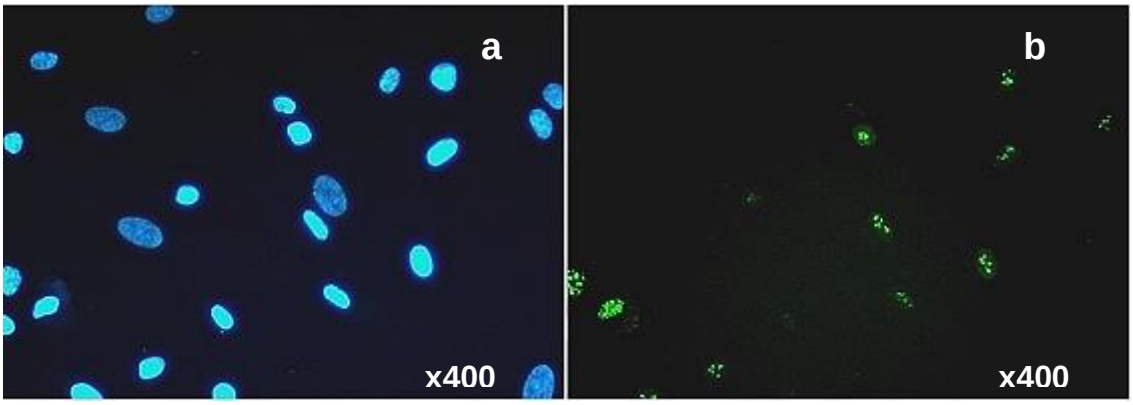

Figure 5 - Immunofluorescence staining of UC-MSC (a) Total number of cells labeled with DAPI in a field (b) Ki-67 immunopositive cells in the same field of figure 5a.

Tables

Table 1 – Density description of seeded cells and percentage of isolation success rate.

	Group 1 (n=10)	Group 2 (n=10)	p
Density (cells/cm^2)			
Mean ± standard deviation	924,784 ± 1,092,810.51	483,467 ± 427,166.90	0.393[a]
Minimum - maximum	41,040 – 3,480,000	44,000 – 1,328,000	
Success rate			
Successful	9 (90%)	7 (70%)	0.007 [b]
Unsuccessful	1 (10%)	3 (30%)	

[a] Mann-Whitney test, [b] chi-square test

Table 2 – Proliferation rate percentage for Group 1 and Group 2 (6,500 and 12,500 cells/cm^2).

Density (cm^2)	**6,500**		**12,500**		
	Mean ± SD	**Minimum and maximum**	**Mean ± SD**	**Minimum and maximum**	**p**
Group 1 (%)	33.00 ± 4.13	28.40 – 36.40	32.47 ± 2.04	30.70 – 34.70	0.658
Group 2 (%)	13.23 ± 5.77	7.00 – 18.40	14.15 ± 0.21	14.00 – 14.30	0.827
	p 0.100		**p** 0.700		

Mann-whitney test

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