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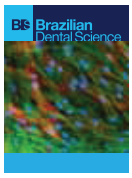

ORIGINAL ARTICLE

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Human umbilical cord vein as a source of osteoblastic cells

Veia de cordão umbilical humano como fonte de células osteoblásticas

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ABSTRACT

Objective: Regenerative medicine and tissue engineering are searching for novel stem cell based therapeutic strategies that will allow for efficient treatment or even potential replacement of damaged organs. The purpose of this work was to study the behavior of human umbilical cord vein cells (UCVs) through osteoblastic differentiation. **Material and Methods:** Cells were isolated, expanded and cultivated in osteogenic medium. After 7, 14 and 21 days of culture, cell morphology, proliferation, viability and alkaline phosphatase (ALP) activity were evaluated. Immunolocalization of ALP was performed after 1, 7 and 14 days of culture and cells were analyzed in a fluorescence microscope. Statistical test utilized was Mann-Whitney ($p < 0.05$). **Results:** The results showed that osteogenic medium induced morphological changes in UCVs. Besides, it permitted cell viability and proliferation, as well as an increase in alkaline phosphatase expression and activity. **Conclusion:** It is concluded that these cells can differentiate into osteoblastic-like cells, contributing to applications for cell therapy and tissue engineering.

KEYWORDS

Cell culture; Differentiation; Osteoblasts; Stem cells; Umbilical cord.

RESUMO

Objetivo: A medicina regenerativa e engenharia de tecidos estão à procura de estratégias terapêuticas baseadas em células-tronco que permitam tratamento eficiente ou até mesmo potencial substituição de órgãos danificados. O objetivo deste trabalho foi estudar o comportamento das células da veia do cordão umbilical humano (UCVs) através da diferenciação osteoblástica. **Material e Métodos:** As células foram isoladas, expandidas e cultivadas em meio osteogênico. Depois de 7, 14 e 21 dias de cultura, foram avaliadas a morfologia celular, a proliferação, a viabilidade e a fosfatase alcalina (ALP). Imunolocalização de ALP foi realizada após 1, 7 e 14 dias de cultura e as células foram analisadas em microscópio de fluorescência. O teste estatístico utilizado foi de Mann-Whitney ($p < 0,05$). **Resultados:** Os resultados mostraram que o meio osteogênico induziu alterações morfológicas nos UCVs. Além disso, foram evidenciadas viabilidade e proliferação celulares, bem como um aumento na expressão e atividade da fosfatase alcalina. **Conclusão:** Conclui-se que as células utilizadas no presente estudo podem diferenciar-se em células semelhantes à osteoblastos, contribuindo para aplicações em terapia celular e engenharia de tecidos.

PALAVRAS-CHAVE

Cultura celular; Diferenciação; Osteoblastos; Células-tronco; Cordão umbilical.

INTRODUCTION

The past two decades have seen significant progress in our understanding of stem cells, which are undifferentiated cells characterized by their ability to self-replicate throughout life and their capacity to differentiate into diverse specialized cell types [1]. These properties make them uniquely situated as a powerful tool for the treatment of a wide spectrum of diseases that are ineffectively treated by traditional approaches [2]. If we can understand the nature and qualities of tissue-specific stem cells as well as the mechanisms by which tissue-specific stem cells differentiate into mature, functional cells, we will be closer to using stem cells in the clinic as a mean for replacing those tissues damaged by disease or injury. There is a consensus that differentiated cells that originate from stem cells should be used directly for transplantation in the clinics [3].

Stem cells are known to exist in post-natal bone marrow [4] as well as synovium [5], lung [6], adipose tissue [7], peripheral blood, dental pulp and periodontal ligament [8]. Umbilical cord blood (CB) is a rich and unlimited source of hematopoietic stem cells for allogeneic stem cell transplant to treat a variety of oncologic, genetic, hematologic, and immunodeficiency disorders [9]. It has been proven to be a valuable source of hematopoietic stem cells, but its therapeutic potential extends beyond the hematopoietic component suggesting regenerative potential in solid organs as well [10]. At this point, however, there is still no ideal stem cell candidate for regenerative medicine [11]. Since the average organ (e.g., heart, liver) contains several types of cells, the most valuable for regeneration would be stem cells that possess high self-renewal and regenerative potential and at the same time contribute to tissues from different germ layers (e.g., organ parenchyma, connective tissue, vessels and nerves). Romanov et al. [12] reported that mesenchymal progenitor cells (MPCs) were present in the subendothelial layer of the human umbilical cord vein (UC). The human UC is embryologically derived at day 26 of gestation, and it grows to form a 30 to 50 cm long helical organ at birth. Other investigations also observed that mesenchymal stem cells (MSCs) exist in human umbilical cord vein that give rise to a population of adherent cells with a typical fibroblast-like morphology [13]. Sarugaser et al. [14] suggested that although the in vivo function of human umbilical cord vein cells still needs to be studied, these cells represent a population of normal, rapidly expandable cells that can potentially generate multiple therapeutic doses of cells for cell-based therapies, and thus they represent a significant alternative to bone marrow in the treatment of pathologies associated with the connective tissues of the human body.

The recent advances brought by tissue engineering can suggest that significant changes in traditional clinical dentistry are beginning to occur [2]. In this context, tissue engineering has tried to establish new therapies to manage dental diseases beyond the traditional approaches that are based solely upon infection control. The future treatment based on the use of stem cells could associate the control of inflammation and infection with the stem cell therapy able to regenerate into new tissues such as periodontal ligament and alveolar bone, providing greater efficiency as well as postoperative stability [8].

The purpose of this investigation was to evaluate human umbilical cord vein cell behavior after osteoblastic differentiation through: 1) cell morphology; 2) cell proliferation and viability; 3) alkaline phosphatase (ALP) activity; 4) ALP expression by immunolocalization.

MATERIAL & METHODS

Cell Culture

The research protocol was approved by the institutional review board, and the samples were obtained after informed consent. The human umbilical cord was collected and processed within 4 h after normal delivery. The cord vein was cannulated on both sides and washed out with phosphate buffered saline (PBS) (Gibco,

Life, USA). The vessel was filled with 0.1% collagenase type I (Gibco) and incubated at 37 °C for 30 min. After gently massaging the cord, the suspension of endothelial and subendothelial cells was collected, and the vein was washed twice again to gather the rest of the cells (15). After centrifugation at 400 g, the pellet was resuspended in growth medium α-MEM (Gibco) and divided in two groups: cells cultured in regular medium (RM), consisted of α-MEM supplemented with 10% FBS (Gibco), 50 μg/mL gentamicin (Gibco), 0.3 μg/mL fungizone (Gibco) and cells cultured in osteogenic medium (OM). Osteogenic differentiation was induced adding 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 5 μg/mL ascorbic acid (Gibco), and 7 mM betaglycerophosphate (Sigma) to the regular medium for 2 weeks (16). Cell morphology was observed during this period using a phase contrast microscope Axiovert 25 (Zeiss, Germany). The second passage was then cultured in the concentration of 10^4 cells per well (n = 5) into 24-well plates to perform the experiments.

Cell proliferation and viability

To evaluate culture growth and viability, cells were plated for 7, 14, and 21 days. After these periods, the medium was removed and cells were enzymatically released from the wells with 1 mM EDTA (Gibco), 1.3 mg/mL collagenase (Gibco), and 0.25 % trypsin (Gibco). After neutralization with new medium, a solution of 100 μl of 0.1 % trypan blue (Sigma) was added to 100 μl of the sample, which helped detecting viable and non-viable cells, as well as cell counting by means of an haemocytometer (Housser Scientific Company, Horsham, PA). Cell proliferation was expressed as absolute number of cells at days 7, 14, and 21. Cell viability was expressed as a percentage of control of the viable cells.

ALP activity

ALP activity was evaluated at 7, 14 and 21 days. The assay was based on the thymolphthalein released from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, MG, Brazil). Samples were assayed for measuring ALP activity according to the kit instructions. Briefly, 50 μl of thymolphthalein monophosphate was mixed with 0.5 ml of 0.3 mmol/ml diethanolamine buffer, pH 10.1, and incubated for 2 min at 37 °C before the addition of the 50 μl of lysates. After 10 min of incubation at 37 °C, 2 ml of 0.09 mmol/ml Na_2CO_3 and 0.25 mmol NaOH was added to allow color development to occur and absorbance was measured at 590 nm. ALP activity was calculated from a standard curve using thymolphthalein, giving a range from 0.012 to 0.40 μmol thymolphthalein/h/ml. Results were calculated and normalized by the total number of cells and expressed as a percentage of control.

Indirect immunofluorescence for alkaline phosphatase localization

At 1, 7, and 14 days, cells were fixed for 10 min at room temperature using 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.2. After washing in PB, cultures were processed for immunofluorescence labeling. Briefly, they were permeabilized with 0.5 % Triton X-100 in PB for 10 min, followed by blocking with 5 % skimmed milk in PB for 30 min. Primary monoclonal antibody to alkaline phosphatase (ALP, 1:100, B4-78, Development Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, USA) was used, followed by Alexa Fluor 594 (red fluorescence)-conjugated goat anti-mouse secondary antibody (1:200, Molecular Probes, Invitrogen, Eugene, OR) and Alexa Fluor 488 (green fluorescence)-conjugated phalloidin (1:200, Molecular Probes), as a marker of the actin cytoskeleton. Replacement of the primary monoclonal antibody with PB was used as control. The incubations were performed in a humidified environment for 60 min at room temperature. Between each incubation step, the samples were washed in PB (3 x 5 min). Before mounting for microscope observation, samples

were briefly washed with dH2O and cell nuclei stained with 300 nM 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes) for 5 min. Discs were placed face up on glass slides and covered with 12 mm-round glass coverslips (Fisher Scientific, Suwanee, GA) mounted with Prolong antifade (Molecular Probes). The samples were then examined under epifluorescence using a Leica DMLB light microscope (Leica), with N Plan (X10/0.25, X20/0.40) and HCX PL Fluotar (X40/0.75, X100/1.3) objectives, outfitted with a Leica DC 300F digital camera, 1.3 Megapixel CCD. The acquired digital images were processed with Adobe Photoshop software (version 7.0.1, Adobe Systems).

Statistical analysis

Quantitative data presented in this work are the representative results of three separate experiments in cell cultures established from three different donors with five different wells for both test and control groups, for each parameter evaluated. Data were compared by Mann-Whitney test. All the results are presented as mean ± SD, and differences at $p \leq 0.05$ were considered statistically significant.

RESULTS

During the period of chemical induction, it was observed that cell adhesion occurred as fast as four hours after the initial culture in the flasks (Figure 1, A-B). A change in the morphology and in the number of cells were evident after seven days, with the control group presenting a cell population that assumed a spindle-shaped morphology in confluent wave-like layers. On the other hand, in the group that received osteogenic medium (OM), there were fewer, larger and more polygonal and flat- shaped cells (Figure 1, C-D). After 14 days, it is clear that the cells in control group had an intense proliferation running to the same direction, creating multilayers, whereas OM group showed well spread osteoblastic cell morphology (Figure 1, E-F).

The quantification of cell proliferation showed that chemical induction promoted a cell proliferation until 14 days in OM group; after this period, there was a significant decrease ($p < 0.05$, Mann-Whitney test) when compared to control group (Figure 2). In spite of this cell decrease after differentiation induction, cell viability was up to 90% in all periods of the experiment, being similar and not statistically different between both groups (Figure 3).

Cells cultured in osteogenic medium showed an increase in ALP activity after 14 and 21 days of culture when compared to control group ($p < 0.05$). In Figure 4, it can be seen that cells cultured in regular medium had almost no positive values for ALP activity. Immunolocalization of this protein was performed in early culture (24 hours), showing that ALP was already present at this moment, especially in OM group. ALP labeling had an increase until the 14 days of experiment, showing a great part of positive cells (Figure 5, A-F).

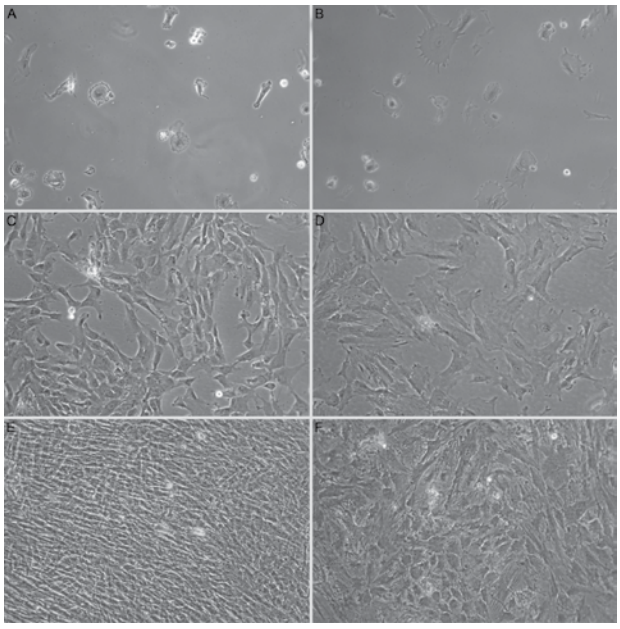

Figure 1 - Phase contrast images of human umbilical cord vein derived-cells cultured in regular growth medium (control group) and in osteogenic medium (treated group). The control group presented a continuous cell proliferation with spindle-shaped morphology after 24 h (A), 7 days (C) and 14 days (E). For the treated group, it was observed a clear change in the morphology, presenting less number of cells which were flat and polygonal in shape (B, D and F after 24 h, 7 and 14 days respectively). Magnification of 100x.

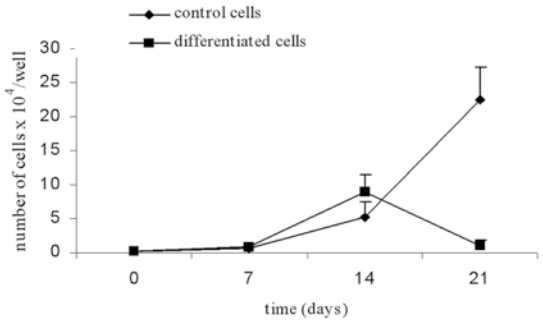

Figure 2 - Growth curve of both control and treated groups cultured for 7, 14 and 21 days. The cell number was greater ($P \leq 0.05$) on control group from day 14. Data are reported as mean ± standard deviation (n = 5).

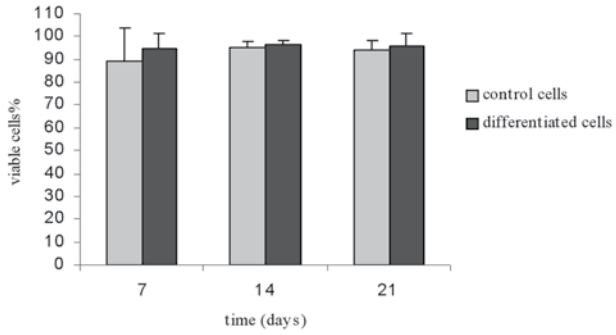

Figure 3 - Cell viability expressed as a percentage of viable cells at 7, 14 and 21 days. Viability was not affected ($P \leq 0.05$) by the osteogenic medium. Data are reported as mean ± standard deviation (n = 5).

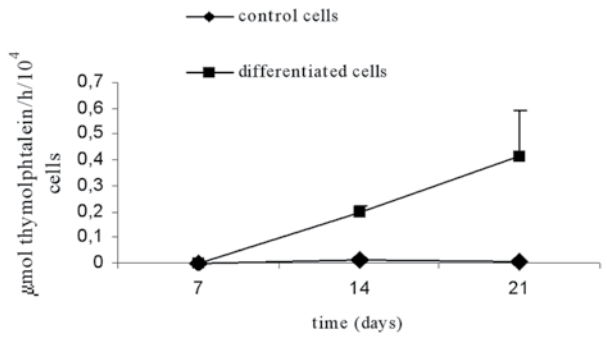

Figure 4 - Alkaline phosphatase (ALP) activity expressed as µmol thymolphthalein/h/mg protein at 7, 14 and 21 days. Cells from the treated group presented higher ($P \leq 0.05$) ALP activity for a period of up to 14 days. Data are reported as mean ± standard deviation (n = 5).

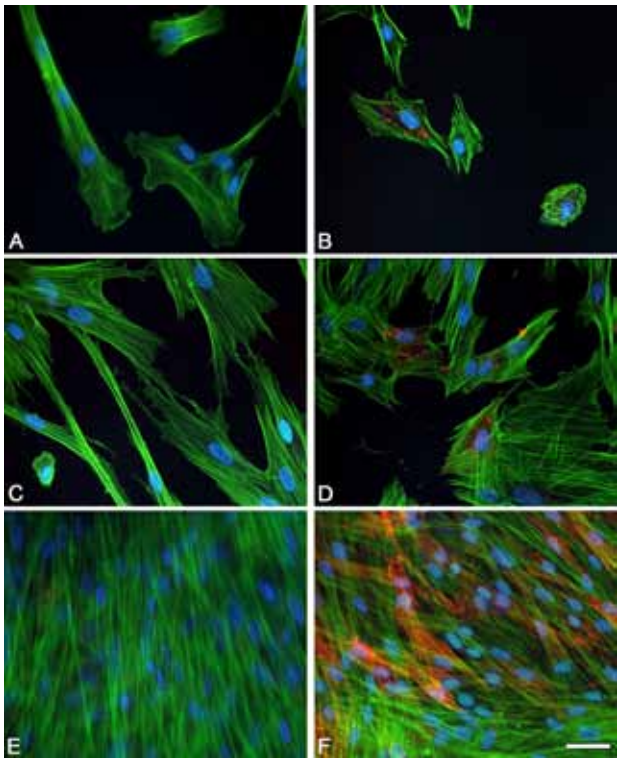

Figure 5 - Fluorescence labeling of human umbilical cord vein derived-cells in contact with glass cover slips cultured in regular growth medium (control) and in contact with osteogenic medium (treated group) after 24 h, 7 and 14 days. Cell-associated green fluorescence reveals actin cytoskeleton (Alexa Fluor 488-conjugated phalloidin). Blue fluorescence indicates cell nuclei (DAPI DNA staining). Cells in the treated group show a change in the morphology and alkaline phosphatase immunolabeling (red fluorescence) showed an increase of expression in the cytoplasm of the treated cells throughout the experiment. The showing bar (50 µm) is valid for all the figures. Immunoflurescence microscopy, magnification of 400x.

DISCUSSION

Self-renewal and multipotentiality are important properties of stem cells, which are divided into two major groups: embryonic and adult stem cells. The latter demonstrate differentiation capacity limited to organs. However, recent studies indicate that tissue-specific stem cells are capable of differentiating into cells of other tissues [3]. Among the different sources of adult stem cells, bone marrow is one of the most studied and utilized nowadays for cell-based therapies. As a matter of fact, adult mesenchymal stem cells (MSCs) from bone marrow and umbilical cord are probably the most realistic sources, in terms of clinical availability, for stem cell-based transplantation therapy [17]. Qiao et al. [18] selected some genes to search for the differences between MSCs derived from bone marrow and those derived from the umbilical cord, finding that both had similar characteristics of gene expression. The same way, Baksh et al. [19] showed through gene array analysis that umbilical cord cells expressed Wnt signaling pathway genes that have been implicated in the regulation of MSCs. Interestingly, these authors also found that osteogenic differentiation of these cells preceded more rapidly than those originated from bone marrow. The similar characteristics pointed above support the applicability of umbilical cord cells for cell-based therapies. Because of that, they have received attention as an alternative to bone marrow for patient-matched stem cell source because it can be noninvasively harvested at birth, and its quality is therefore not influenced by aging and postnatal viral infections [20].

The present investigation proposed to study the behavior of human umbilical cord vein cells after the contact with an osteogenic medium and it was observed that these cells have the potential to differentiate into osteoblastic-like cells. In agreement to this, Sudo et al. [21] proposed that primary fibroblast-like cell populations obtained from various human tissues do not comprise solely fibroblasts, but rather that they also include at least mesenchymal precursor cells (MPCs) and possibly MSCs, to some extent.

We have shown that the contact of human umbilical cord vein cells with osteogenic medium induced changes in their morphology when compared to the control group. Besides, the slower proliferation rate seen in the treated group suggests cell differentiation. This can be stated mainly observing cell viability, which was similar in both groups with up to 90% of viable cells until the end of the experiment. Honsawek et al. [22] also evaluated the osteogenic differentiation of the umbilical cord derived cells showing that osteogenic medium treated cells appeared shortened, flattened and surrounded by extracellular matrix. They have also demonstrated that osteogenic medium inhibited the growth of these cells by 50%, as determined by direct cell counting.

One important finding is that alkaline phosphatase activity, a very early marker of cell differentiation into the osteogenic lineage, was evident by its significant increase after cells were treated with osteogenic supplementation over 2 weeks. This is in agreement with Honsawek et al. [22] that confirmed that osteogenic medium had a strong stimulatory effect on the alkaline phosphatase activities of umbilical cord derived cells. Kang et al. [23] have also demonstrated the osteogenic potential of umbilical cord derived cells through radiographic and histological analysis. On the other hand, Suzdal'tseva et al. [24] stated that umbilical cord fibroblast-like cells can differentiate mainly into adipocytes and chondrocytes, and only few cells in this culture can differentiate into osteoblasts. This was not seen in the present investigation, where ALP expression was evident through immunolabeling and it was demonstrated as early as 24 h in a great part of cells induced with osteogenic medium. This expression increased over time, despite the fact that only a part of the cells were positive. This may be due to the heterogeneous type of the culture,

where not all the cells are MSCs. Panepucci et al. [25] also observed that umbilical cord vein cells undergo osteogenic differentiation demonstrated by alkaline phosphatase expression. The relevance of these results directs toward the possibility of these cells in the future contribute to clinical applications for cell therapy and tissue engineering. It is certainly possible that, once dentist-scientists bring together the new discoveries in material sciences, genetics, molecular and cell biology, new alternatives for regeneration of bone and soft tissues will become available for clinical application.

CONCLUSION

Our data showed that chemical induction of human umbilical cord vein cells allows the differentiation in osteoblastic-like cells, suggesting that they can be used in the future as a source of replacing cells in damaged bone sites, showing that dental clinics could be favored in the near future with the use of stem cells. However, there are serious issues concerning standardization of techniques that should be solved before clinical application in humans.

ACKNOWLEDGEMENTS

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Erratum

In the article: *Nascimento GC, Bighetti RL, Passos Júnior GAS, Bombonato-Prado KF. Human umbilical cord vein as a source of osteoblastic cells. Braz Dental Scien. 2014; 17(3):31-38*, the authors reported that Figure 5c was previously published by the research group whose reference should have been made *Bombonato-Prado KF, Rosa AL, Oliveira PT, Dernowsek JA, Fontana V, Evangelista AF, A. Passos GA. Transcriptome analysis during normal human mesenchymal stem cell differentiation. In: Passos GA (Ed.).Transcriptomics in Health and Disease. New York: Springer;2014. Chapter 6, p 109-119. doi:10.1007/978-3-319-11985-4_6*, to correct this fault, Figure 5c was replaced in the online version of the Journal.

We regret any confusion caused by the error.

Editor

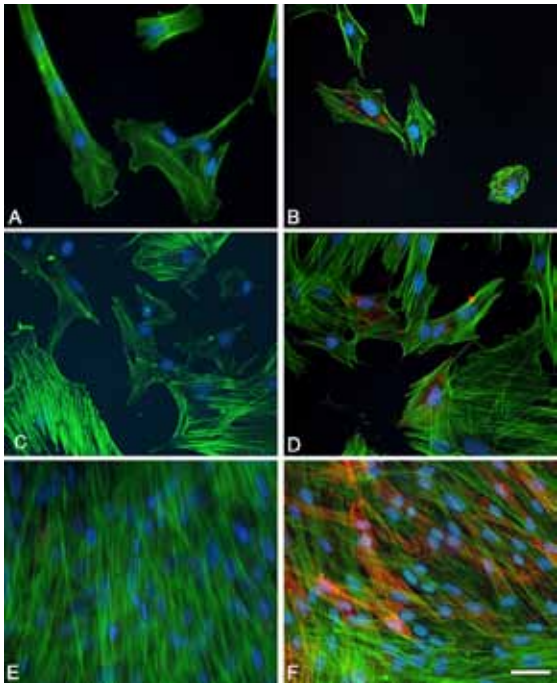

Figure 5 - Fluorescence labeling of human umbilical cord vein derived-cells in contact with glass cover slips cultured in regular growth medium (control) and in contact with osteogenic medium (treated group) after 24 h, 7 and 14 days. Cell-associated green fluorescence reveals actin cytoskeleton (Alexa Fluor 488-conjugated phalloidin). Blue fluorescence indicates cell nuclei (DAPI DNA staining). Cells in the treated group show a change in the morphology and alkaline phosphatase immunolabeling (red fluorescence) showed an increase of expression in the cytoplasm of the treated cells throughout the experiment. The showing bar (50 μm) is valid for all the figures. Immunoflurescence microscopy, magnification of 400x.