Isolation, culture and characterization of multipotent mesenchymal stem cells from goat umbilical cord blood[1]

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ABSTRACT.- Martins G.R., Marinho R.C., Bezerra-Junior R.Q., Câmara L.M.C, Albuquerque-Pinto L.C. & Teixeira M.F.S. 2017. **Isolation, culture and characterization of multipotent mesenchymal stem cells from goat umbilical cord blood.** *Pesquisa Veterinária Brasileira 37(6):643-649.* Laboratório de Virologia, Núcleo de Pesquisa em Sanidade Animal, Programa de Pós-Graduação em Ciências Veterinárias, Universidade Estadual do Ceará, Avenida Dr. Silas Munguba 1700, Fortaleza, CE 60714-903, Brazil. E-mail: rmgabrielle@hotmail.com

Mesenchymal stem cells (MSC) reside in small numbers in many adult tissues and organs, and play an active role in the homeostasis of these sites. Goat derived multipotent MSC have been established from bone marrow, adipose tissues and amniotic fluid. Umbilical cord blood (UCB) is considered an important source of these cells. However, the MSC isolation from the goat UCB has not been demonstrated. Therefore, the aim of the present study was to isolate, culture and characterize goat umbilical cord blood derived mesenchymal stem cells. MSC were isolated from UCB by Ficoll-Paque density centrifugation and cultured in DMEM supplemented with 10% or 20% FBS. FACS analysis was performed and induction lineage differentiation was made to characterize these cells. They exhibited two different populations in flow cytometry, and revealed the positive expression of CD90, CD44 and CD105, but negative staining for CD34 in larger cells, and positive stained for CD90 and CD105, but negative for CD44 and CD34 in the smaller cells. MSC from goat UCB showed capability to differentiate into chondrocytes and osteoblasts when incubated with specific differentiation medium. Present study established that goat mesenchymal stem cells can be derived successfully from umbilical cord blood.

INDEX TERMS: MSC, caprine, flow cytometry, stem cell, lineage differentiation.

RESUMO.- [**Isolamento, cultura e caracterização de células tronco mesenquimais multipotentes provenientes do sangue do cordão umbilical caprino.**] As células tronco mesenquimais (MSC) residem em pequenas quantidades em muitos tecidos e órgãos adultos, desempenhando um papel ativo na homeostase destes locais. O isolamento de MSC já foi demonstrado em amostras de medula óssea, tecido adiposo e fluido amniótico de cabras. O sangue de cordão umbilical é considerado uma fonte importante desse tipo de células. No entanto, até o presente momento, não foi demonstrado o isolamento de MSC provenientes do sangue de cordão umbilical de cabras. Dessa forma, o objetivo do presente estudo foi isolar, cultivar e caracterizar células tronco mesenquimais provenientes do sangue do cordão umbilical caprino. As MSC foram isoladas utilizando o gradiente de densidade Ficoll-Paque e cultivadas em DMEM suplementado com 10% ou 20% de FBS. A caracterização desse tipo celular foi realizada através de análise por citometria de fluxo e diferenciação em linhagens celulares mesodermais. A analise no citômetro de fluxo demonstrou a presença de duas populações distintas, um grupo com células maiores e outro com células menores; observando expressão positiva de CD90, CD44 e CD105, e negativa para CD34 nas células maiores; enquanto que as menores foram positivas para CD90 e CD105, mas negativas para CD44 e CD34. As células isoladas demonstraram capacidade de se diferenciar em condrócitos e osteoblastos quando incubadas com meio de diferenciação específico. O

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presente estudo demonstrou que células tronco mesenquimais podem ser obtidas com sucesso do sangue do cordão umbilical caprino.

TERMOS DE INDEXAÇÃO: MSC, caprino, citometria de fluxo, células tronco, diferenciação em linhagens.

INTRODUCTION

Mesenchymal stem cells (MSC), first obtained from bone marrow aspirate, have a great plasticity and are able to differentiate into bone, cartilage, fat tissue and even muscle (Pountos & Giannoudis 2005). MSC was originally described for Friedenstein et al. (1968) in bone marrow as a component of the marrow stromal cell population that collectively supports hematopoietic stem cell persistence and differentiation. However, since this initial description, it is now recognized that MSC reside in small numbers in many adult tissues and organs, and play an active role in the homeostasis of these sites. These cells can be isolated from others different tissues, such as the umbilical cord blood and matrix, adipose tissue, synovial membranes, embryonic tissue, and amniotic fluid (Chang et al. 2006, Orbay et al. 2012, Reed & Jonhson 2012, Ren et al. 2012, Lee et al. 2013, Pratheesh et al. 2013,).

Umbilical cord blood (UCB) is considered an important source of many types of stem cells, including hematopoietic stem cells (HSC), endothelial progenitors, mesenchymal stem cells (MSC), very small embryonic/epiblast-like (VSEL) stem cells, and unrestricted somatic stem cells (USSC), potentially suitable for use in regenerative medicine (Pelosi et al. 2012). Researches with UCB stem cells still face some difficulties, mainly during the isolation process (Mareschi et al. 2001, Wexler et al. 2003). MSC was isolated from umbilical cord of human (Lee et al. 2004, Chang et al. 2006), sheep (Fadel et al. 2011), equine (Reed & Johnson 2012) and canine (Lee et al. 2013).

Minimum criteria for the characterization of human MSC were created. These should present adherence to the plastic, positive expression for the surface markers CD105, CD73 and CD90, negative expression for the markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and the HLA-DR surface molecules, and present the capability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici et al. 2006). However, one major difficulty in characterizing MSC in veterinary medicine is due to the low number of specific monoclonal antibodies available and evidence that certain markers from human and mouse do not present a cross-reaction with others species (Taylor et al. 2007). There is a large number of a positive marker described, but each group of investigators using different markers, none specific or single. Perhaps the differences between studies may be attributed to variations in culture methods or stage of cells differentiation (Bydlowski et al. 2009).

Mesenchymal stem cells have low immunogenicity, thus allowing their potential use in regenerative medicine. Human MSC (hMSC) are characterized by the low expression of the major histocompatibility complex class I and are described by their immunomodulatory effect on immune system cells. Additionally, hMSC fail to induce proliferation of allogeneic or xenogeneic lymphocytes, causing effects on B lymphocyte function, inhibiting its differentiation into plasma cells, and showed to interfere with dendritic cell differentiation, maturation, and function (Krampera et al. 2003, Jiang et al. 2005). In equines, preliminary studies examining the use of umbilical cord and placentally derived MSC have suggested that such cells do not incite a cellular immune response, even after repeated injections (Carrade et al. 2011a, 2011b). It is important to recognize that MSC from neonatal sources are likely more immune-privileged than those derived from adult sources. MSC are remarkable potential as a cell mediate repair and regenerative medicine for fatal or incurable diseases, such as spinal cord injury, arthritis, and ischemic heart injury, has been demonstrated in the field of stem cell therapy (Pittenger & Martin 2004, Hatami et al. 2009, Sharp et al. 2010).

Goat derived multipotent MSC have been established from bone marrow (Zhang et al. 2012), adipose tissues (Ren et al. 2012), and amniotic fluid (Pratheesh et al. 2013). However, it has not demonstrated the isolation of mesenchymal stem cells from the goat umbilical cord blood. Therefore, the aims of the experiments in this study were to isolate, phenotypically characterize and investigate the differentiation potentials of goat umbilical cord blood derived mesenchymal stem cells.

MATERIALS AND METHODS

Animals and samples. All experiments were approved by the Ethics Committee for Animal Use at the State University of Ceará, protocol number 127.769.79-0. For this study, mongrel pregnant goats were used, aged between two and three years and free of infection by SRLV. Blood samples were collected during calving. After birth, the goat umbilical cord was clamped in the end next to the fetus, and the umbilical cord blood (UCB) was harvested by syringe aspiration and packed in sterile tube with EDTA (Ethylenediamine tetraacetic acid). The samples were transported within 2h to the laboratory, packed in isothermal box with ice.

Isolation and culture. Mesenchymal stem cells were isolated from goat UCB by Ficoll-Paque density centrifugation (600g for 10 min) and cultured in low glucose DMEM (Dulbecco's Modified Eagle Medium) (Gibco®, Grand Island, NY, USA) supplemented with 300 U/mL penicillin, 300 µg/mL streptomycin, 0,5 mg/mL amphotericin and 10% FBS (Fetal bovine serum, Gibco®, Grand Island, NY, USA). The cells were seeded in A25 cell culture flasks and cultured at 37°C in a CO_2 incubator (5% CO_2). After 4-5 days growth, fibroblast-like plastic-adherent cells were observed. The media was changed after 5 days to avoid any mechanical stress, and thereafter, it was replenished every third day until obtain confluent cell monolayer. It was observed slow growth, due to this; it was increased FBS concentration for 20%, promoting a better cell growth. At 80% confluence, cells were trypsinized (0,25% Trypsin-EDTA, Gibco®, Grand Island, NY, USA) and reseeded in new cell culture flask for expand the cell stock. For all the experiments, mesenchymal stem cells were taken from third passage, whereas rest of the cells was allowed to grow further.

Chondrogenic differentiation. Chondrogenic differentiation was induced in micromass cultures by seeding 5 µL droplets of cell solution (1.6×10^7 viable cells/mL) in the center of 24-well plate. The cells were incubated with DMEM supplemented with 300 U/mL penicillin, 300µg/mL streptomycin, 0.5mg/mL amphotericin and 10% FBS for 2 hours. Afterward, the cells were fed with a specific chondrogenesis differentiation medium (StemPro®, Gibco®,

Grand Island, NY, USA). The medium was replaced every 2-3 days. After 14 days, chondrogenic differentiation was confirmed by staining for Alcian Blue (Sigma-Aldrich®, St Louis, MO, USA).

Osteogenic differentiation. To induced osteogenic differentiation, the cells from the confluent monolayer of the third passage ($5x10^3$ cells/cm^2) were seeded in to a 24-well plate and incubated with DMEM supplemented with 300 U/mL penicillin, 300µg/mL streptomycin, 0.5 mg/mL amphotericin and 10% FBS. After 24 hours, the medium was discarded, and cells were cultured in StemPro® Osteocyte Differentiation Basal Medium (StemPro®, Gibco®, Grand Island, NY, USA). The medium was replaced every 2-3 days. After 14 days of culture, osteogenic differentiation of stem cells was confirmed by Alizarin Red S staining (Sigma-Aldrich®, St Louis, MO, USA).

Adipogenic differentiation. For adipogenic differentiation, trypsinized cells (1x104 cells/cm^2) were seeded into a 24-well plate and incubated with DMEM supplemented with 300 U/mL penicillin, 300µg/mL streptomycin, 0.5mg/mL amphotericin and 10% FBS. After 24 hours, the medium was discarded, and the cells were incubated with StemPro® Adipogenesis Differentiation Basal Medium (Gibco®, Grand Island, NY, USA). The differentiation medium was replaced every 3-4 days. After 14 days of culture, the cells were fixed with 10% formalin at room temperature, washed with PBS, and stained with 0,5% Oil-Red O (Sigma-Aldrich®, St Louis, MO, USA) for 15 min and Mayer's Hematoxylin (Sigma-Aldrich®, St Louis, MO, USA) for 5 min to visualize lipid droplets.

FACS analysis. FACS analysis was performed to investigate the expression of the surface markers CD90 FITC, CD105 APC, CD44 PE and CD34 APC in goat umbilical cord blood derived MSC after the third passage. The cells were harvested and aliquoted at a density of $3.2x10^6$ cells/mL for each marker assay. These cells were incubated with antibodies against surface markers [mouse anti-Human CD90 FITC, CD105 APC, CD44 PE and CD34 APC (BD Biosciences®, USA)] for 30 min at 4°C in the dark. Thereafter, the cells were washed twice with FACS buffer washes, fixed with formaldehyde buffer (PBS with formaldehyde 1%) and analyzed using a flow cytometer (FACS Calibur BD, BD Biosciences®). The negative control was processed in similar manner but without an-

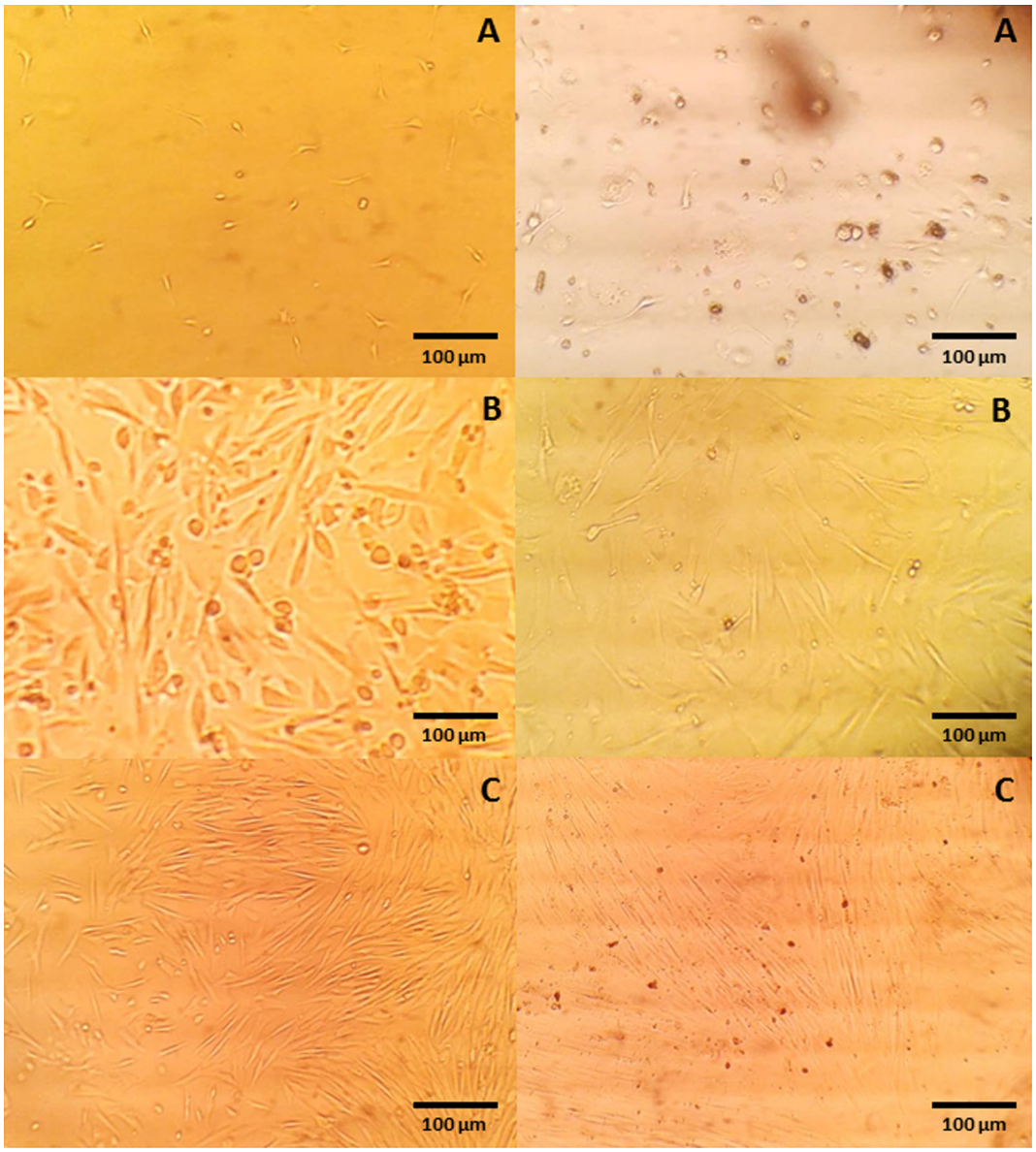

Fig.1. Morphological features of MSC derived from goat UCB. (**A**) Primary culture after 4-5 days. (**B**) Short or long spindles, with uniform fibroblast-like morphology. (**C**) Typical fibroblast-like morphology with 80-85% confluence.

tibodies. The data obtained was analyzed using Flowjo® software and plotted as single parameter histogram.

RESULTS

Growth and culture characteristics of MSC

MSC from goat umbilical cord blood began to adhere after 4-5 days of culture, first with a small, round, polygons and fibroblast-like cells, with some others types of blood cells (Fig.1A). Gradually, they extended into small or large spindles, with uniform fibroblast-like morphology (Fig.1B). Initially, the cells were being grown in DMEM supplemented with 10% FBS, however, the cells showed slow growth, not reached confluence, even after 15 days of incubation. In order to provide the best growth of these cells, the FBS concentration was changed for 20%. Cells culture in 20% FBS reached to 80-85% confluence faster about 8 days than those in 10% FBS in the same inoculation condition (Fig.1C). MSC showed a typical fibroblast-like morphology after passage, with mix of short and long spindles. Cells at passage 3 were used in the differentiation and FACS analysis tests, whereas rest of the cells were allowed to grow further, and it observed that the growth and proliferation activity reduced with aging.

Chondrogenic differentiation

The chondrogenic potential of MSC from goat umbilical cord blood was evaluated by *in vitro* micromass cultures of these cells in a specific differentiation medium. After 14 days of incubation, the accumulation of sulfated proteoglycans was visualized by positive Alcian Blue staining (Fig.2A).

Osteogenic differentiation

In osteogenic differentiation, cells proliferated and reached to complete confluence after 8-10 days of incubation with differentiation medium. The cellular aggregates were then observed and were characterized by calcium deposits, which demonstrated by positive Alizarin Red stain (Fig.2B).

Adipogenic differentiation

Adipogenic differentiation of goat umbilical cord blood derived mesenchymal stem cells was not confirmed. After incubated these cells with adipogenic-inducing medium for 14 days, sparsely small lipid droplets or even no lipid droplets were observed in the cytoplasm by Oil Red-O staining (Fig.2C).

Surface antigen profile by FACS analysis

The study of cells by flow cytometry revealed the presence of two distinct populations of cells. The populations differed in size, being characterized as small or large cells according to the characteristics of size and granularity observed. It evaluated the immunophenotypic characteristics through presence of surface molecules comparing both groups to negative control group.

FACS analysis revealed the positive expression of CD90 (52%), CD44 (19%) and CD105 (15%), but negative staining for CD34 (2%) in larger cells. The smaller cells were positive for CD90 (32%) and CD105 (12%), but negative for CD44 (3%) and CD34 (1%). It observed difference about the fluorescent intensity in the groups. The larger cells showed major fluorescent intensity to CD90 than small cells. Negative control group of cells, which was processed in similar man-

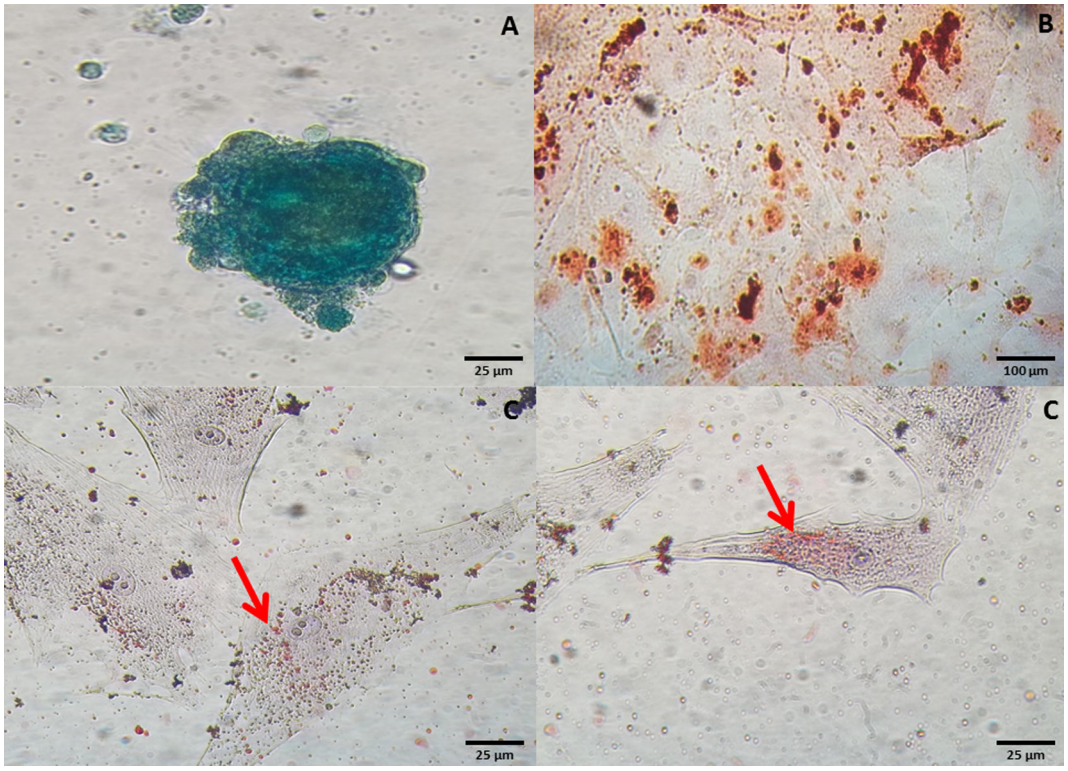

Fig.2. (**A**) Positive staining of Alcian Blue showed the chondrogenic differentiation. (**B**) Positive Alizarin Red stain showed osteogenic differentiation. (**C**) Sparsely small lipid droplets or even no lipid droplets in the cytoplasm, showed lower adipogenic potential.

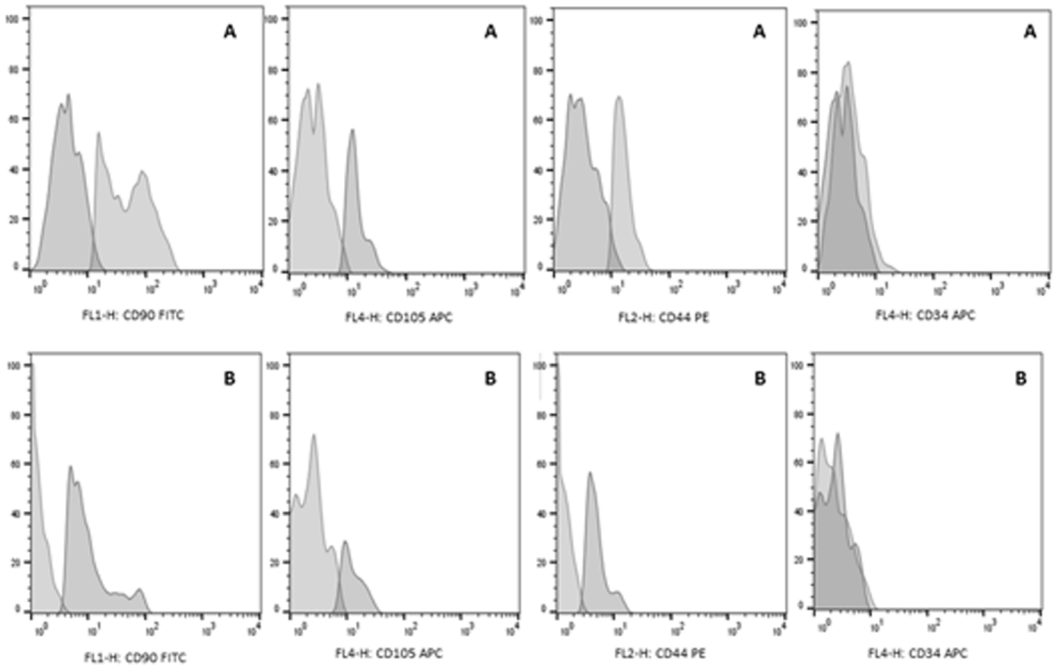

Fig.3. Comparison of cell surface marker profiles between two types of MSC. (**A**) Large fibroblastic MSC. (**B**) Small fibroblastic MSC. Both types of MSC at passage 3 were analyzed by flow cytometry with antibodies against the indicated antigens. Calibrated histogram representing standard number of events in the Y-axis; and fluorescent intensity on X-axis. Negative control was shown in the graph area with fluorescent intensity less than 10^1.

ner but without antibodies in the incubation, didn't show any expression of surface molecules analyzed. Negative control wasn't shown expression, It was represented in the graph area with fluorescent intensity less than 10^1 (Fig.3).

DISCUSSION

Umbilical cord blood represents a potentially important source of mesenchymal stem cells. These cells can be defined as multipotent stem cells capable of differentiating into different tissue cell types, including adipocytes, osteocytes, chondrocytes, myocytes and neuron-like cells. The main source of MSC is represented by marrow; however, UCB represents an important alternative source for generation of MSC (Lee et al. 2004). The umbilical cord is composed of several different parts (amniotic membrane, umbilical cord matrix, umbilical cord vein, and umbilical cord blood) that are available as source of MSC (Lee et al. 2013). In this study, MSC was isolated from goat umbilical cord blood and first, cultured in DMEM supplemented with 10% FBS, however, the cells showed slow growth. In order to provide the best growth of these cells, the FBS concentration was changed for 20% that demonstrated high efficiency to maintenance the culture, and contributed for quick amplification of cell stock. Several culture media have been tested for maintenance of MSC as MEM, DMEM, RPMI-1640 and Basal Medium Eagle (BME), supplemented with FBS, generally at concentration of 10-20%. The choice of the medium culture is important to success of MSC growth in the in vitro cell culture (Tapp et al. 2009). MSC were highly dependent on serum for growth. Cells cultured in 20% FBS reached to 80–85% confluence faster than those in 10% FBS in the same inoculation density (Im et al. 2005).

This is a first study that isolated MSC from goat UCB. It was observed different morphologic phenotypes in primary culture of MSC from goat UCB, but, gradually the cells exhibited uniform fibroblast-like morphology. Goat derived multipotent MSC have been established from bone marrow (Zhang et al. 2012), adipose tissues (Ren et al. 2012), and amniotic fluid (Pratheesh et al. 2013) that observed similar morphology in the same culture conditions.

Phenotypic characterization was performed by flow cytometry. The general strategy for identifying in vitro cultivated mesenchymal stem cells as per ISCT (International Society for Cytotherapy) is to analyze the expressions of cell-surface markers such as CD-73, CD-44, CD-90 and CD-105 (Dominici et al. 2006, Donzelli et al. 2007). During the FACS analysis was observed two different populations, small and large MSC. Large MSC are positive for CD90, CD105 and CD44, whereas negative for CD34 surface marker. Small MSC are positive for CD90, CD105, but negative for CD44 and CD34. Differences in fluorescent intensity were observed, and large cells showed more intensity than small cells, mainly in CD90 marker expression. This result can be compared with others studies, Ren et al. (2012) described MSC from goat adipose stained positively for vimentin, CD49d and CD13, and negatively for CD34 and CD106. Caprine mesenchymal stem cells derived from amniotic fluid were positive for CD90, CD105, CD73, and negative for CD34 (Pratheesh et al. 2013). Fadel et al. (2011) isolated MSC of ovine umbilical cord that were positive for CD44 and negative for CD38, CD41/61 and CD45. Lee et al. (2013) demonstrated by FACS analysis that canine umbilical cord matrix derived MSC were positive for CD44, CD90, CD 105, and CD184, whereas negative for the other CD markers such as CD29, CD33, CD34 and CD45.

CD90 has been known as a negative regulator for hematopoietic proliferation (Mayani & Lansdrop 1994), its expression associate CD34 negative staining confirm that there was no evidence of hematopoietic precursors in this culture. The levels expression of surface markers are also different in others studies. Chang et al. (2006) isolated cells from human UCB, and observed two different morphologic phenotypes: flattened fibroblastic clones and spindle-shaped fibroblastic clones. CD90 was differently expressed by these two cell populations. Spindle-shaped clonogenic MSC expressed a high level of CD90, while flattened clonogenic MSC showed negative expression of CD90.

CD105 is usually express in mesenchymal stem cells. CD34 is a surface marker of hematopoietic stem cells (HSC) and is expressed in lymph nodes, bone marrow HSC, and various endothelial cells. CD34 negative staining demonstrated that cells were not derived from circulating stem cells (Pittenger et al. 1999, Peng & Huard 2004). CD44 is a hyaluronate receptor. The positive staining for CD44 antibody in flow cytometry argued with that published by Barry et al. (1999) and Martínez et al. (2009). These studies showed that CD44 is an antibody that characterizes mesenchymal cells for human and sheep fat tissue derived cells. In case of rabbits, CD44 is negative, according to researches published by Martínez et al. (2009).

The differentiation potentials of MSC from goat UCB were investigated. These cells showed capability to differentiate in chondrocytes and osteoblasts when incubated with specific differentiation medium. Pratheesh et al. (2013) demonstrated that MSC from goat amniotic fluid, like their marrow counterparts, were able to differentiate into adipose cells in addition to osteogenic and chondrogenic lineages. Goat adipose-derived stem cells showed the same differentiation potential (Ren et al. 2012). Chang et al. (2006) investigated the same capability in MSC from human UCB and results showed that both types of clonogenic MSC (flattened fibroblastic clones and spindle-shaped fibroblastic clones) could differentiate into osteogenic and chondrogenic lineages under appropriate conditions. However, in adipogenic induction, the spindle shaped MSC exhibited many typical neutral lipid vacuoles within the cells as mature adipocytes, while the flattened MSC only contained sparsely small lipid droplets or even no lipid droplets at all. It was reported that UCB-derived MSC showed a reduced capability to undergo adipogenesis (Bieback et al. 2004). Recently, we have also found that UCB-derived MSC have lower adipogenic potential than bone morrow-derived MSC in vitro (Chang et al. 2006). In this study, it was observed similar results, the MSC from goat UCB have lower adipogenic potential. However, the cell showed high capability to undergo chondrogenesis and osteogenesis.

CONCLUSIONS

Present studies establishes that goat mesenchymal stem cells can be derived successfully from umbilical cord blood, which exhibit morphology, growth characters, immunophenotype and lineage differentiation potential similar with MSC of other origins.

These results collaborate to advances in research and treatments using stem cells, demonstrating the potential and isolation as protocol of this cell type in goats.

MSC have low immunogenicity, thus allowing their potential use in regenerative medicine.

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Conflict of interest statement.- The authors have no competing interests.

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