

Isolation and characterization of mesenchymal progenitors derived from the bone marrow of goats native from northeastern Brazil¹

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ABSTRACT

PURPOSE: To characterize bone marrow progenitors cells grown *in vitro*, using native goats from northeastern Brazil as animal model.

METHODS: Ten northeastern Brazil native goats of both genders were used from the Piauí Federal University Agricultural Science Center's (UFPI) – Goat Farming Sector. Bone marrow aspirates were taken from the tibial ridge and seeded on culture plates for isolation, expansion and Flow Cytometry (expression markers – Oct-3/4, PCNA, Ck-Pan, Vimentina, Nanog).

RESULTS: Progenitor cells showed colonies characterized by the presence of cell pellets with fibroblastoid morphology. Cell confluence was taken after 14 days culture and the non-adherent mononuclear cell progressive reduction. After the first passage, 94.36% cell viability was observed, starting from 4.6 x 10⁶ cell/mL initially seeded. Cells that went through flow cytometry showed positive expression for Oct-3/4, PCNA, Ck-Pan, Vimentina, and Nanog.

CONCLUSIONS: Bone marrow progenitor isolated of native goats from northeastern Brazil showed expression markers also seen in embryonic stem cells (Oct-3/4, Nanog), markers of cell proliferation (PCNA) and markers for mesenchymal cells (Vimentina and Ck-pan), which associated to morphological and culture growth features, suggest the existence of a mesenchymal stem cell (MSC) population in the goat bone marrow stromal cells studied.

Key words: Cell Culture Techniques. Cell Differentiation. Stem Cells. Flow Cytometry. Goats.

Introduction

At first, studies on stem cells were restricted to progenitor cells committed to tissue renovation and differentiation, from resident cells in the same tissue¹⁻³. However, the progress observed in the last few years, due to the biology of stem cells, has shown high levels of plasticity in certain types of cells⁴, demonstrating that cells from specific tissue^{5,6} have the capacity to give rise to cell lineages different of those of their primary source⁷⁻⁹.

Although studies are preliminary concerning the ability of stem cells to originate cells with a mature phenotype different from that observed in the tissue of origin¹⁰, they probably are the basis to the progress already seen in research associated to stem cell therapy^{11,12}, as an alternative to intermediate mechanisms responsible for the regeneration of diverse tissues.

The search for suitable biological models¹³ has become increasingly important as a way to produce results closer to possible application to human medicine as well as improving the quality of animal life¹⁴. There are numerous animal models, such as rodents^{12,15}, swine¹⁶ and canines¹⁷⁻¹⁹ that have been used in experiments related to cell therapies. Regarding such animals, outcomes were found in studies about neurodegenerative injuries^{12,13}, as well as in attempts to use cell therapy as a way to treat cardiovascular diseases²⁰.

Experiments with sheep were described for obtaining the main uses of stem cells/progenitor cells^{21,22}. However, such potentialities have been little studied in the goat species. The goat herd, in Brazil, represents approximately 7.2 million animals, almost all of them found in Brazil's northeast. This scenario favors the introduction of the goat as an alternative potential model to obtain mesenchymal cells. In addition, the goat model finds a particular acceptance in Orthopedic Medicine fields^{23,24}. Lechner *et al.*²⁵ reported that goat joint injuries showed similarities to those observed in rheumatoid arthritis in humans.

Thus, the characterization of mesenchymal progenitors derived from the bone marrow of goats native to northeastern Brazil is a preliminary stage in the consolidation of a low-cost animal model to acquire cells whose differentiation potential may contribute to the understanding of the study of stem cells in preclinical studies and their role in tissue regeneration.

Methods

The experiment was conducted in accordance to the ethical principles for animal experimentation adopted by the Committee of Ethics in Animal Experimentation of the Federal

University of Piauí (Protocol n. 023/2010).

Ten northeastern Brazil native goats of both genders were used, between 1.0 and 1.5 years old, from the Piauí Federal University Agricultural Science Center's (UFPI) – Goat Farming Sector. The animals were taken for clinical and lab exams, in order to ensure the strict parameters demanded by the experiment, in accordance to Radostits, Gay and Hinchcliffet²⁶. The animals were placed in 6m²/animal covered stalls, under a semi-extensive grazing system.

Anesthetic procedures for bone marrow gathering

The animals were sedated (meperidine hydrochloride, AgribandsTM -5 mg/Kg/IM) and then positioned in lateral recumbence, in order to shave the tibial ridge. The whole left pelvic member (chosen for gathering means) was cleaned (1% iodized alcohol, ImpexTM) – following the anesthetic block on the shaved area (lidocaine hydrochloride without vasoconstrictor; XylestesinTM, Cristália farmaTM -5 mg/Kg/SC. The anesthesia was obtained from an association of Xylazine hydrochloride RompumTM, Bayer do BrasilTM -1 mg/Kg/IM) and ketamine hydrochloride (KetalarTM, Pfizer do BrasilTM -40mg/Kg/IM). The animals were kept under anesthesia (1% Propofol, Fresofol, Fresenius KabiTM -7 mg/Kg/IV) throughout the whole gathering procedure. After the surgical procedure, the puncture area was cleaned with Dakin liquid (6% Sodium Hypochlorite and 5% sodium bicarbonate), followed by oxytetracycline hypochlorite topic application associated to hydrocortisone (Terra-cortilTM, Pfizer do Brazil) for six days.

Bone marrow gathering

Bone marrow aspirates were obtained from punctures in the tibial ridge (9 mL), using a 40 x 12 sterile hypodermic needle (18G), connected to a syringe filled with anticoagulant, ethylenediaminetetra-acetic acid (EDTA, Sigma-Aldrich). The collected sample was diluted in a buffered phosphate solution (PBS), concentration 1:1, and filtered to remove residual bone tissue. The filtered content was carefully transferred to a 15 mL tube (Falcon TubeTM, ACQuímicaTM) filled with ficoll solution (Ficoll HistopaqueTM, SigmaTM) and centrifuged at 2.000 rpm for 25 min. at 20°C, to separate the contents, due to concentration gradient. The mononuclear cell fraction obtained was gently aspirated with an automatic pipette (HoustonTM) and immediately, washed two times with sterile PBS and centrifuged at 2.000 rpm for 10 min at 4°C. The supernatants were discarded, the pellet resuspended in

14mL Dulbecco Medium Eagle Modified (DMEM) – low glucose, filled with 15% fetal bovine serum (SFB, Invitrogen Corporation), 1% non-essential amino acids, 1% L-glutamine, 1% antibiotic (TECMAL TE-399™), then seeded in six-well plates (TPP) at 1.6×10^6 cells/well, at 37°C, in a 5% CO₂ humidified incubator and kept in semi-confluence to avoid differentiation.

Cellular viability

Fifty μ L of the cells obtained in culture were diluted in 50 μ L Trypan Blue (Trypan Blue™, Sigma-Aldrich). The solution was homogenized and counted by hemocytrometer, as previously described by Meirelles and Nardi²⁷.

The features of the isolated cultured cells were preserved by an enzymatic dissociation process by Trypsin-EDTA digestion, washed with PBS and resuspended in freezing environment (40%SFB, 50%DMEM low glucose and 10% dimethylsulfoxide, Sigma-Aldrich). After this process, the cells were transferred to 10 freezing tubes (cryo-tubes, TPP) in 1.0×10^6 cells/mL and placed in -196°C liquid nitrogen.

Flow cytometry

Cells were submitted to flow cytometry in the fourth passage to ensure a sufficiently undifferentiated cell population for the accurate analysis of expression of their membrane proteins. Flow cytometry was performed using the Guava EasyCyte System (Guava Technologies). The cells were trypsinized and centrifuged at 1000 rpm for 5 minutes and resuspended in PBS at a concentration of about 1×10^5 cells/mL. A 10^5 cells/tube aliquot was used, equally diluted and resuspended in 200 μ L PBS, for further addition of each antibody, in the dark, for 45 minutes at room temperature. The cells were washed three times in PBS and resuspended in 0.20 ml ice-cold PBS. PE anti-mouse secondary antibody (Guava Technologies) was used For the samples incubated with non-conjugated antibodies. The samples were incubated for 15 minutes, washed three times with PBS and resuspended in 2.20ml ice-cold PBS.

The flow cytometer was calibrated using non-marked cells. The cells were separated by forward scatter in order to eliminate debris. To eliminate a possible autofluorescence, parameters were adjusted so as to remove any contribution from marked cells. At least 9000 events were counted for each sample. The following markers were analyzed: OCT-3/4, PCNA, CK-PAN, VIMENTINA, NANOG.

Outcome analysis

The cells obtained were analyzed by *in vitro* morphology and growth, photographed in an inverted light microscope (COLEMAN NIB-100™) objective lens x10, x20 and x40, and the images were stored by an image capturing software (MTO Digital Color Camera™). Cells submitted to flow cytometry were evaluated by a graphic that shows the relative fluorescence found from each cell population in the samples studied.

Results

Isolation and expansion of bone marrow cell progenitors

The mononuclear cell fraction obtained was highlighted in an interface between the plasma and Ficoll Histopaque™ solution and centrifugation then showed a 4×10^6 cells/mL concentration. After seeding in culture, these cells showed a heterogeneous pattern in the first 24 hours, characterized by a population of cells adhering to the plastic bottom of the well (Figure 1A). There were also non-adherent cells in suspension, probably lymphocytes and macrophages, which were taken off during the consecutive PBS washes, 72h after the beginning of culture (Figure 1A, B). Adherent progenitors were sequentially selected by the basal medium used. A homogeneous culture was obtained eight days of culture, with a progressively reduced amount of non-adherent cells. Such a process develops to create a cell monolayer, consisting of spindle-shape cells that divided; they kept a cell-to-cell interaction and showed cytoplasmatic extensions parallel to each other (Figure 1C). When the cells reached 80% confluence (14 days culture), the first enzymatic dissociation was performed (P1) (Figure 1D). After 17 days culture, the cells showed homogeneous colonies that were fibroblastoid and confluent (Figure 1E).

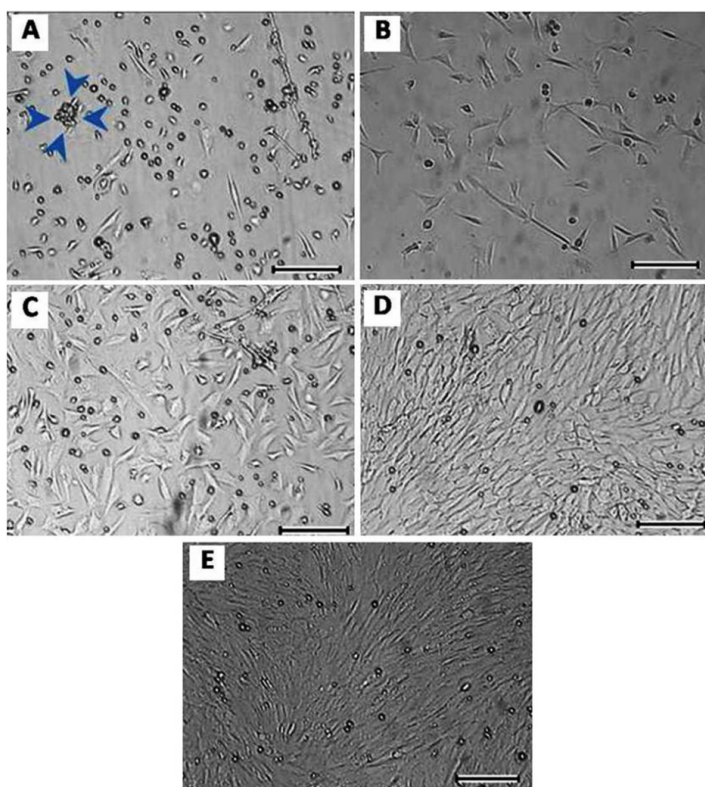


FIGURE 1 - Photomicrography of culture of goat bone marrow adherent cells. (A) Cells after 24 hours in culture. Note the non-adherent mononuclear cell cluster and the presence of cells already bonded to the culture plate, in a spindle shape. (B) Cells after the first PBS wash. Note the decrease in the amount suspended cells and the higher definition of the adherent mononuclear cells. (C) Cells after eight days culture presenting 60% confluence. Note the small amount of suspended non-adherent cells. (D) The 14th culture day and (E) on the 17th culture day, both with 80% confluence. Note the spindle shape pattern and the radial growth adopted during this particular phase. Rod: A-E 100µm.

The average cell viability of the harvested cells was 99.45%, and throughout the 49 days in culture a 97.72% average was maintained throughout all the passages (Figure 2). When expanded, the trypsinization at P2 resulted in 16 culture flasks, frozen in 12 cryotubes a concentration of 1×10^6 cel/mL, placed in liquid nitrogen at -196°C.

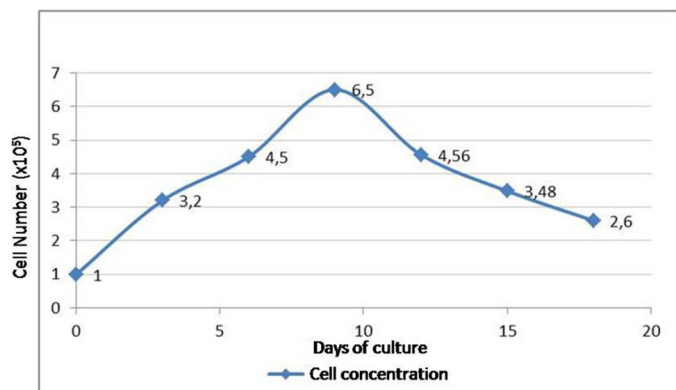


FIGURE 2 - Cell viability (%) of progenitor cells from bone marrow of goat after eight passages in different days of culture.

Flow cytometry

The progenitor cells obtained from goat bone marrow aspirates were characterized for expression of markers Oct-3/4, Nanog, and Ck-Pan Vimentin by flow cytometry, which showed positive expression as observed in Figure 3.

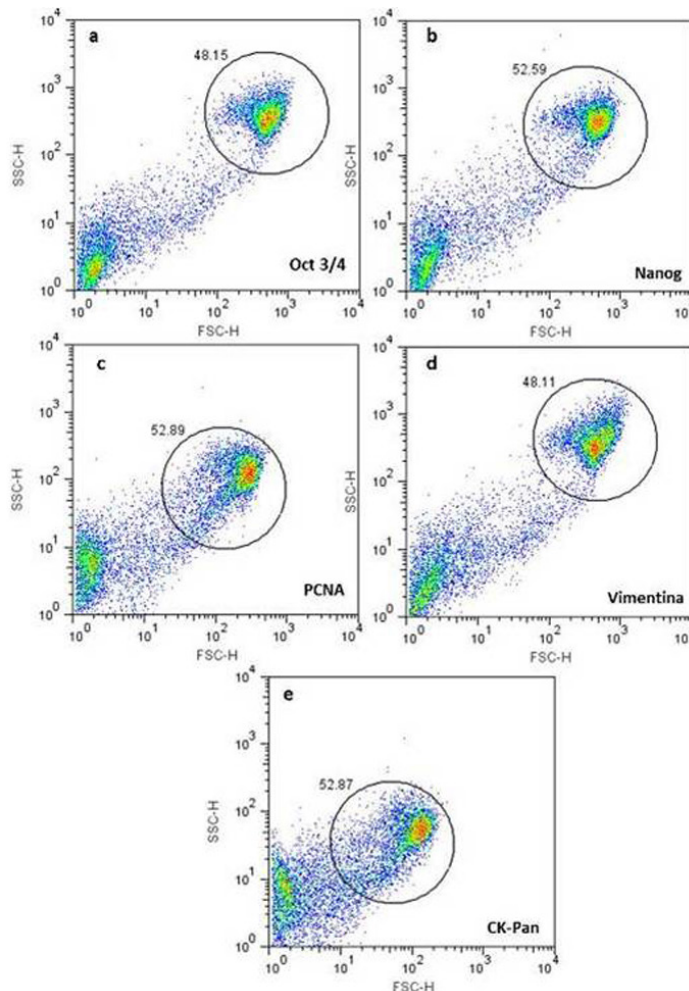


FIGURE 3 - Cell progenitor flow cytometry from goat bone marrow, showing positive expression for undifferentiated cell markers (A) OCT3/4 and (B) NANOG and the positive expression for PCNA cell proliferation (C) and the cytoskeleton for cell morphological identification Vimentine (D) and CK-PAN (E).

Discussion

Cell culture

Throughout our observations we found the presence of adherent cells, forming matrix cells that remained bonded after 72 hours culture, even after undergoing consecutive PBS washes. For the isolation method used to obtain bone marrow stem cells, our results were similar to Friedenstein *et al.*²⁸, when they established the ability to adhere to plastic as a main feature, when suspension cells are washed two to four times a day, after the beginning of the culture.

The primary culture was established by seeding a concentration of a bone marrow aspirate at 4.6×10^6 cells/mL concentration, similar to that described by for MO primary rat cultures, using a cell concentration of 5×10^6 cells/mL²⁹. However, there are still differences in the behavior of MSC *in vitro*, given the wide range of cell concentrations used to obtain primary cultures from 1×10^6 cells/mL³⁰ to 7.5×10^6 cells/mL³¹, in Dulbecco's Modified Eagle Medium, which showed similar results to ours.

A heterogeneous population of cells attached to the bottom of the culture flask began cell growth and this was observed until the eighth day of culture, but was gradually replaced by a more homogeneous population. Phinney *et al.*³² and Sekiya *et al.*³³ demonstrated it with mice, in MSC isolation from bone marrow aspirates that resulted in a heterogeneous cell population with a high degree of hematopoietic contaminants that were subsequently removed by successive washes in PBS.

Otherwise, Tropel *et al.*³⁴ observed a purified population only after 30 days of cultivation, while Meirelles and Nardi²⁷ found similar results to ours. In fact, the time for obtaining homogeneous primary cultures from bone marrow aspirates is still controversial, since, according to the conditions of the medium, the cells lose their proliferative capacity³⁵, which may explain differences in the time of obtaining purified or homogeneous populations in different experimental assays.

The fibroblastoid morphology observed in our *in vitro* studies showed morphology similar to that reported in cats³⁶, as well as to descriptions of rodents and humans³⁷. Tropel *et al.*³⁴ found that such spindle shaped and fibroblastoid featured population became prominent in mice cultures, approximately two weeks after culture.

The potential for division in culture was measured by time in culture, verified by their growth curve, with the possibility of up to 99.36% in 15 passages. Similar assays were performed keeping rat bone marrow MCS cultured for 50 passages²⁷ and in cats for 25 passages³⁶.

Flow cytometry

Cell characterization based on flow cytometry revealed the presence of a cell population that expressed the octameric transcription factors (OCT-3/4), POU domain containing protein encoded by the Pou5f1 gene. Scholer *et al.*³⁸, Nichols *et al.*³⁹ and Niwa *et al.*⁴⁰ described the transcription factor Oct-4 as essential regulator for the formation and/or maintenance of the inner cell mass (ICM) during pre-implantation development in rat embryonic cells. Studies by Loebel *et al.*⁴¹ and Tropel *et al.*³⁴ demonstrated the self-renewal capacity indefinitely in culture, as well as their potential

to differentiate into specific cell types that holds great promise for regenerative medicine.

In our observations, the isolation of a positive OCT-3/4 population demonstrated the purification level achieved during successive passages in culture to MSC from bone marrow of goats native to the state of Piauí. Reports by Baddoo *et al.*⁴² demonstrated high expression of OCT-4 in MSC derived from rat bone marrow and these have shown great potential to differentiate to adipogenic, chondrogenic and osteogenic lineages. Similarly, Kerkis *et al.*⁴³ identified a population of MSCs obtained from human dental pulp, which expressed embryonic stem cell markers such as Oct-4, SSEA-3 and SSEA-4, with high plasticity to generate muscle tissue, neurons, cartilage and bone tissue and demonstrated the potential of obtaining these cells from other body sites.

The characterization essays performed for goat CMT showed that these cells also expressed the Nanog gene. Chambers *et al.*⁴⁴ observed the action of the Nanog gene in the pluripotency regulation in the inner cell mass (ICM) during embryonic development. Pan and Thomsom⁴⁵ showed that only the LIF/OCT-4 pathway is insufficient to prevent cell differentiation events, suggesting that other factors could also be committed in the preservation of this feature. Mitsui *et al.*⁴⁶ found high concentrations of mRNA Nanog within the morula stage during embryonic development, and likewise, Tay *et al.*⁴⁷ found that in the absence of OCT-3/4 and LIF, Nanog is capable of maintaining a pluripotent cell. Riekstina *et al.*⁴⁸ reported the presence of embryonic stem cells markers such as OCT-4, Nanog, SOX 2 and SSEA-4 in the bone marrow as well as in cells isolated from adipose, heart and dermis tissues.

The ability to maintain in culture, beyond the immunophenotypic characteristics established by the International Society for Cellular Therapy, is one of the criteria for qualifying a population of cells as MSCs. The present study showed the positive expression of PCNA in cultures in fourth passage. In studies for cell therapy, the proliferative capacity has shown particular importance⁴⁹. Morigi *et al.*⁵⁰ used the Monoclonal Anti-Proliferating Cell Nuclear Antigen (anti-PCNA) to study renal cell repair in experimental models of acute kidney injury (AKI), and detected high PCNA expression in the renal tissue treated. Similarly, Gong and Niklason⁵¹, in studies with synthetic vascular prostheses, demonstrated positive staining by immunohistochemistry for PCNA. The use of bone marrow MSCs in the treatment of periodontal defects showed the presence of PCNA positive cells in the focus of injured tissue⁴⁹.

Positive staining for both vimentin and CK-PAN helped in the characterization of the cell population found. Kurrey *et*

al.⁵² and Elloul *et al.*⁵³ verified the presence of the expression of mesenchymal cell markers such as CD44, CD29, CD90 and co-expression with high participation for vimentin and Ck-Pan.

Conclusions

The plasticity of cell progenitors obtained from bone marrow aspirate from goats native to Piauí State. The expression of undifferentiated cells markers, typical of embryonic stem-cells, such as OCT-3/4, Nanog, cell proliferation markers (PCNA) and from mesenchymal cells (Vimentina and Ck-pan), associated to morphological and culture growth features, allowed us to suggest the existence of a MSC population in the studied goat bone marrow, which can be potentially explored in further pre-clinical studies.

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