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Isolation, Culture and Differentiation Potential of Collared Peccary (*Tayassu tajacu*) Adipose-Derived Stem Cells

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ABSTRACT

***Background*:** The understanding of cell biology and the isolation of mesenchymal stem cells in wild animals show prospects for conducting pre-clinical trials in these unconventional animals. The collared peccary (*Tayassu tajacu*) are suiforms that belong to the Artiodáctyla order, Tayassuidae family and *Tayassu* genus. They adapt easily to captivity conditions that favors their commercial rearing and is an alternative for biodiversity conservation. To evaluate the collared peccary (*Tayassu tajacu*) as a potential animal model for the isolation of mesenchymal progenitor cells, cell culture and cell differentiation protocols.

***Materials, Methods & Results*:** To perform this research we used four collared peccaries (*Tayassu tajacu*) from the Nucleus of Study and Preservation of Wild Animals (IBAMA/PI Nº. 02/08-618) from Federal University of Piauí (UFPI). Adipose tissue fragments were collected from the dorsocervical region and dissociated mechanically in laboratory. The material was placed in an incubator containing CO_2 - 95% at 37°C and the cultures were expanded to fifth passage, evalluating cell concentration and viability. The culture medium alfa-MEM supplemented was changed every three days. The cell kinetics was evaluated in triplicate using growth curve performed during ten days, plating the initial concentration of 5 x 10^4 cells/mL per well in P3 six-well culture plate. For cell differentiation in osteoblasts, adipocytes and chondrocytes were plated 5 x 10^4 cells/mL in P3 cells culture in six wells with the respective medium for inducing differentiation (StemPro Differentiation Kit®) plates. The stem cells isolated from collared peccary adipose tissue were characterized by easy, single layer growth, an extracellular matrix secretion that permitted adhesion to the growth surface and capacity to maintain their multi-potentiality through innumerous passages. The first cells with fusiform adherent morphology were visualized after 5 days of cultivation. On the eleventh day the first colony forming units (CFU), and adherent fibroblastoid morphology were observed. The isolated cells cultured to P5 have always presented characteristic fibroblastoid morphology with basophilic cytoplasm and spherical nuclei proliferation in monolayer with a mean viability of 93.8%. The growth curve showed the lag, log and plateau phases, reaching a maximum value of 14 x 10^4 cells/mL. The osteogenic differentiation showed cytoplasmic calcium deposit and osteoblasts intensely marked by Alizarin Red. After 21 days, the adipogenic differentiation presented cytoplasmic lipid droplets with variable size, stained with Oil Red O. The chondrogenic differentiation performed in monolayer demonstrated the formation of aggregates (nodule-like), confirming its potential for chondrogenic plasticity.

***Discussion*:** The adherent cells isolated from collared peccary adipose tissue were defined as multi-potent and with the ability to differentiate in into mesordermal lines. The use of wild animal models has been very important to research strategies for tissue engineering and regenerative medicine both for perfecting and proving innocuous tissue culture techniques and for the use of adult stem cells in pre-clinical studies. Mesenchymal stem cells from adipose tissue of collared peccaries are a valuable tool for future scientific investigations. We suggest the use of this wild species as an alternative model for preclinical studies in cell therapy.

Keywords: morphology, adipose tissue, mesenchymal stem cell, Tayassuidae, *Tayassu tajacu*.

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INTRODUCTION

MSCs can be obtained from many tissues such as bone marrow, umbilical cord, amniotic membrane, deciduous teeth and can be expanded in *in vitro* adherent culture [12]. Mesenchymal stem cells derived from adipose tissue (ASCs – *Adipose-derived Stem Cells*) are easily isolated and expanded and can differentiate into multiple lines cells [7,22,23].

The isolation and characterization of mesenchymal stem cells from adipose tissue have been studies in various animal species, including pigs [10], goats [16], dogs [3,11,15,21], mice [6] and horses [8] as a strategy to discover a suitable biological model for pre-clinical research.

There are few studies on stem cells in wild animals. In hystricomorphous rodents, the morphology of hematopoetic progenitor constituents and adherent cell progenitor from bone marrow of the black-rumped agouti (*Dasyprocta prymnolopha*) was described previously [17], and preclinical studies were performed in the same species using bone marrow mononuclear cells to treat kidney lesions [2].

The collared peccary (*Tayassu tajacu*) are suiforms that belong to the Artiodactyla order, Tayassuidae family and *Tayassu* genus [13]. They are found from the south of the United States to the South of Argentina [1], inhabiting desert regions, arid steppes and forests [9]. They adapt easily to captivity conditions that favors their commercial rearing and is an alternative for biodiversity conservation [18].

The objective of the present study was to investigate using the collared peccary as a potential model to obtain mesenchymal stem cells from adipose tissue according to innocuous isolation technique, expansion and differentiation into multiple lines.

MATERIALS AND METHODS

Animals

Four male collared peccary (*Tayassu tajacu*) were used aged between one and two years, similar weight (14.56 ± 0.12 kg), reared in the Nucleus for Studies and Preservation of Wild Animals - NEPAS (IBAMA/PI Nº 02/08-618) of the Federal University of Piauí (UFPI), in Teresina, Piauí, Brazil.

Subcutaneous adipose tissue collection

The collared peccary were contained chemically with an intramuscular injection of 15 mg/Kg ketamine hydrochloride[1] and 3 mg/kg midazolam maleate[2]. The area along the right side cervical region was shaved and cleaned with alcohol and povidone-iodine[3] (Figure 1A). A 3 cm incision was made in the skin (Figure 1B) and fragments of subcutaneous adipose tissue were collected and stored in 50 mL conical tubes[4] for histological processing and mesenchymal stem cell isolation. The post-surgery was carried out for two days with daily cleansing of the surgical wound (Figure 1C) with 6% sodium hypochlorite and 5% sodium bicarbonate[5] and antibiotic administration, 40000 UI/kg, IM[6].

Histological processing

Subcutaneous adipose tissue fragments were fixed in 10% buffered formaldehyde solution for a period of 24 h and were later dehydrated by passing in alcohols with increasing concentration, diaphonized in xylol and blocked in paraffin. Cuts 5µm thick were stained with Hematoxylin-Eosin and Sudan Black B[7]. The histological analysis was carried out under a light microscope[8] with lenses of 10x, 20x and 40x magnification, coupled to an image analysis system[9].

ttASCs isolation and expansion

In a air flow chamber[10], subcutaneous adipose tissue samples were washed twice in phosphate buffered saline solution[7] with 10% antibiotic[7] and associated mechanically for 10 min the help of two sterile n°24 scalpel blades, on a sterile 100 x 20 mm petri dish containing 1% collagenase solution[11].

The material was incubated in a CO_2 incubator[12] at 37°C with 5% CO_2 and 95% humidity for 30 min. After this period, the enzymatic reaction was blocked by adding complete basal alfa-MEM culture medium[13] at 37°C, supplemented with 20% bovine fetal serum[14], 1%12, 1% non-essential amino acids[7] and 13 mM HEPES[7] in a volume twice that of the enzymatic digestion solution

The samples in 15 mL conical tubes[4] were transferred to a water bath[15] at 37ºC for 30 min, and every 5 min the tubes were agitated in vortex[16]. The digestion content (Figure 1D) was aspirated, filtered through 70 micra mesh[17] and centrifuged[18] at 20°C in alfa-MEM culture at 205 *g* for 10 min. The supernatant was discarded, the cell sediment re-suspended in 1 mL alfa-MEM culture medium at 37ºC and sown in two 25 cm^2 culture flasks[24] kept in a CO_2 - 95% atmosphere at 37°C.

The culture medium was changed every three days and the culture monitored until the cells reached 70% confluence. The culture flasks were submitted to trypsinization com 1.5 mL trypsin[14] 1x and incubated for 6 min. The trypsin was then de-activated by adding 3 mL supplemented alfa-MEM culture medium. This solution was transferred to a 15 mL conical tube[4] and centrifuged[18] at 20°C and 205 *g* for 10 min. The supernatant was discarded, the cell sediment was re-suspended in 1 mL supplemented alfa-MEM culture medium and the cells counted in a hemocytometer.

The cultures were expanded in 25 cm^2 flasks[4] until the fifth passage, photographed in an inverted microscope[19] and replicated with double the original area after 70% confluence, measuring the cell concentration and viability at each passage.

The cell concentration viability was determined by the Trypan Blue exclusion method. After trypsinization, 50 µL of the cell suspensions measured into 50 µL Trypan blue solution[7] and one 12 µL aliquot was placed in a hemocytometer and observed under an optical microscope (lens 10x). The cells were counted in the four diagonal quadrants, and the cell total multiplied by two (dilution of the stain Trypan Blue) and by 10^4 (chamber depth and correction per mL), divided by the number of quadrants counted. Cell viability was determined by the ratio between the quantity of not stained live cells, and the total quantity of cells.

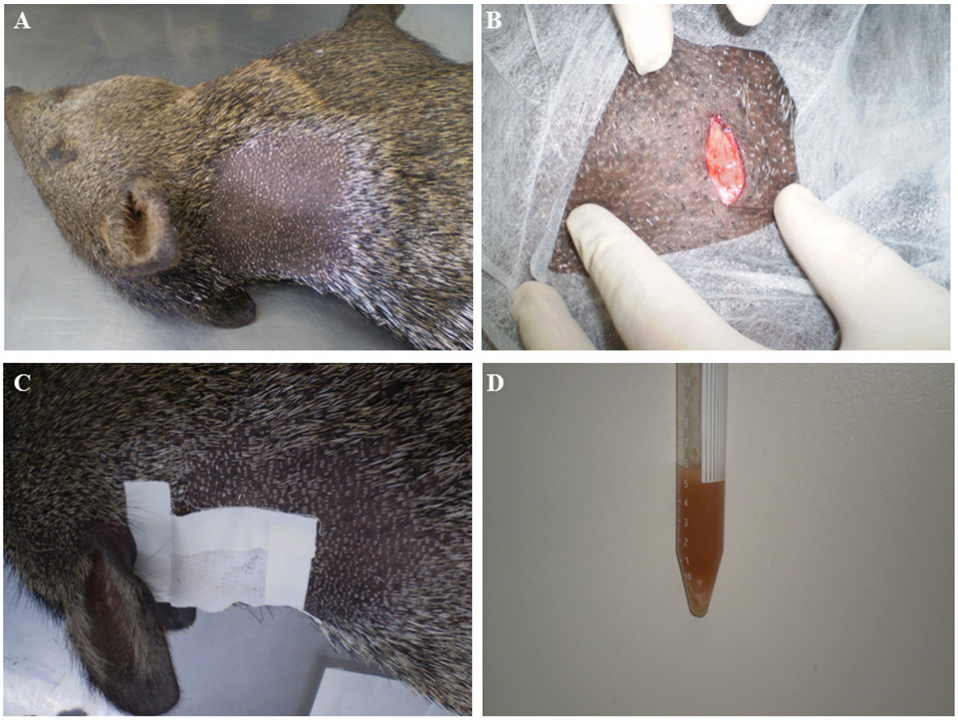

Figure 1. Subcutaneous adipose tissue collection from collared peccary. A - area of the right side cervical region shaved and cleaned. B - 3 cm incision in the skin for the biopsy. C - surgical wound. D - enzymatic digestion of the adipose tissue with 1% type I collagenase.

Fibroblastoid colony forming units experiment (UFC-F)

To assess the potential of the two samples collected for MSCs presence, experiments were carried out to identify the fibroblastoid colony forming units (CFU-F). After enzymatic digestion with type I 1% collagenase the resulting cellular sediment was sown in two 25 cm^2 flasks[4] with alfa-MEM culture medium. The cultures were assessed daily and the culture medium was first changed after five days, when cells adhering to the plastic was observed and then the medium was changed every 72 h, for six days. The cultures were then washed twice in phosphate buffer saline and blocked with 4% paraformaldehyde for 10 min and stained with Giemsa. The staining solution was discarded and the flask washed twice with 3 mL distilled water so that the colonies could be counted.

Cellular kinetics

The cell expansion and replication capacity was assessed by the growth curve, carried out in triplicate. Five six-well plates[4], were plated with 5 x 10^4 cells/mL

in P3, in each well. The plates were incubated in a CO_2 incubator[12] at 37°C with 5% de CO_2 and the alfa-MEM culture medium was changed every three days. The cell yield was assessed at 24 h intervals for 10 days.

ttASCs morphological analysis

The shape and in vitro growth of the ttASCs expanded in culture medium were analyzed by horizontal sweeping with an inverted NIB 100 microscope[19]. Cytometric analysis was performed using the parameters of length, width, area and perimeter with a Leica Qwin[9] in 100 cells randomly selected from each cell passage (P1, P2, P3, P4 and P5) with 70% confluence, of all the samples, totaling 2000 cells. The photomicrographs were taken with the MTC Camera Color Digital system attached to the inverted microscope.

Cell differentiation experiments

Cells from the third passage were used to analyze the cell differentiation, induced to differentiate into osteoblasts, adipocytes and chondrocytes.

Osteogenic differentiation

For osteogenic differentiation 5 x 10^4 cells/mL were plated in P3 in six-well plates28 with alfa-MEM cell culture medium that permitted cell adherence. After 48 h the cells reached 70% confluence and osteogenic differentiation was induced by substituting the alfa-MEM culture medium with the osteogenic differentiation inducing factors Stempro® Osteogenesis Differentiation Kit[11], for 21 days, chanaging the culture medium every 72 h. The culture was fixed in 4% paraformaldehyde and stained with Alizarin Red S[7].

Adipogenic differentiation

To promote adipogenic differentiation, 5 x 10^4 cells/mL in P3 were plated in six-well plates[4] with Alfa-MEM culture medium to permit cell adherence. After 48 h the cells reached 70% confluence and adipogenic differentiation was induced by substituting the alfa-MEM culture medium with culture medium supplemented with the adipogenic differentiation inducing factors Stempro® Adipogenesis Differentiation Kit[11], for 21 h, changing the culture medium every 72 h. After this period the culture was fixed in 4% paraformaldehyde and stained with Oil Red O[7].

Chondrogenic differentiation

For chondrogenic differentiation 5 x 10^4 cells/mL were plated in P3 in six-well plates[4] with alfa-MEM cell culture medium. After 48 h the cells reached 70% confluence and chondrogenic differentiation was induced by substituting the alfa-MEM cell culture medium with culture medium supplemented with the chondrogenic differentiation inducing factors Stempro® Chondrogenesis Differentiation Kit[11], for 21 days changing the culture medium at 72 h intervals.

Statistical analyses

Four samples were compared in five passages (P1, P2, P3, P4 and P5) to analyze the cell parameters (length, width area and perimeter). The ANOVA test was used considering significant statistical difference for $P < 0.05$. The Tukey multiple comparison test was carried out to verify the differences within the variables when $P < 0.05$.

The values of the CFU-F parameters, cell concentration and viability were submitted to descriptive analysis and the data were expressed in the form of mean and standard deviation. The statistical analyses were carried out with the software SAS (Statistical Analysis System, V. 9.3).

RESULTS

Histological analysis of the adipose tissue

The microscopic assessment of the adipose tissue showed large adipose cells with shapes ranging from spherical to polyhedron, cell nuclei located in the adipocyte margin and cytoplasm similar to a peripheral border (Figure 2A and 2B). The histological processing dissolved the lipid content causing an empty appearance.

ttASCs isolation, CFU-F and expansion

The process of isolating the *ttASCs* showed, in all the samples, adhesion to the plastic surface and fibroblastoid cell morphology.

The first plastic-adhering cells, with "fibroblast like" morphology, were observed at 15 days cell culture, and at 11 days the first fibroblastoid colony forming units were observed (Figure 3A), mean 67.2 ± 12.73 CFU-F in the totality of the samples. Basophilic staining was observed and strong cell interaction (Figure 3B). The 70% confluence in the area of the culture flasks was observed at 17 days, and after expansion, the cells were cultured until the fifth passage, always presented characteristic fibroblastoid morphology, with basophilic cytoplasm, spherical nuclei and single layer proliferation.

The mean cell viability of the *ttASCs* was 93% in the first passage and the mean was 93.8% over all the passages (Figure 4).

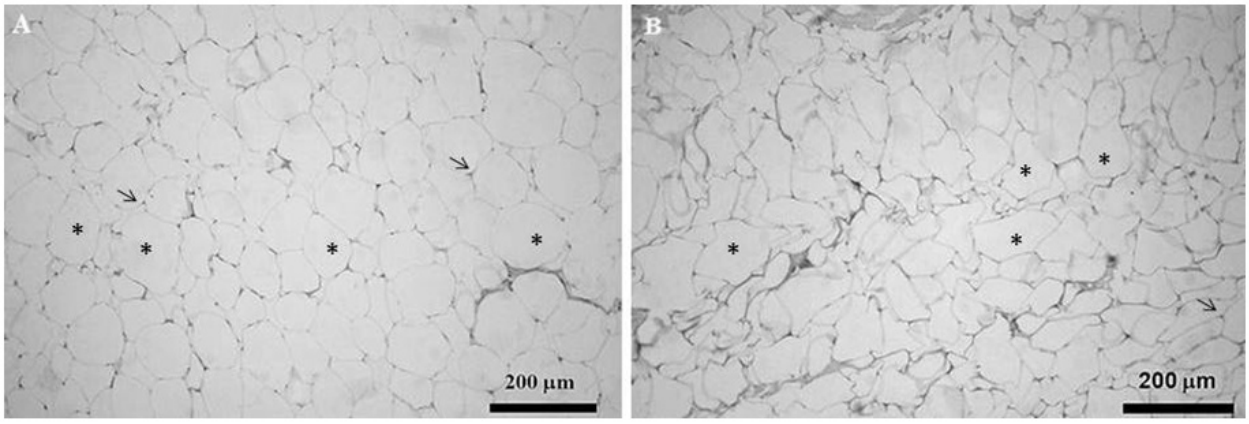

Figure 2. Photomicrograph of subcutaneous unicellular collared peccary adipose tissue (*Tayassu tajacu*). The adipose tissue (*) appears empty because the tissue processing dissolved the lipid material. The nucleus (arrow) is dislocated to the side because of the pressure exercised by the fat droplets. Hematoxilina-Eosina/HE (A) and Sudan Black (B) staining.

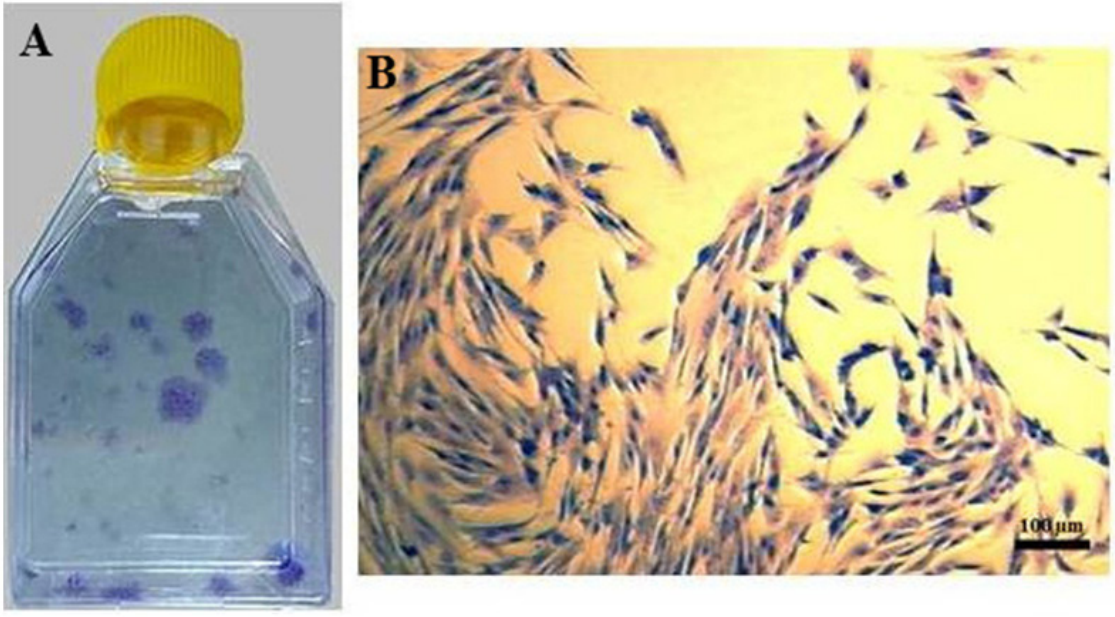

Figure 3. Isolation and SI of *ttASCs* fibroblastoid colony forming units (UFC-F). A - 25 cm^2 cell culture flask with *ttASCs* colonies stained by Giemsa. B - photomicrograph de *ttASCs* showing basophilic staining and intense cell interaction.

Cytometric analysis

Table 1 shows the mean values of the *ttASCs* parameters measured in the five cell passages (P1, P2, P3, P4 and P5). The mean length of the cells ranged from 53.93 to 63.78 µm, the width from 17.57 to 21.16 µm, the mean area from 608.06 to 681.98 µm^2 and the perimeter from 132.16 to 159.18 µm.

There was no significant statistical difference for the length variable among the means P1, P2, P3, P4 and P5 (P = 0. 328) nor for the area variable (P = 0.507) or perimeter (P = 0.235). However, comparison of the widths showed that there was significant difference in at least two of the means (P = 0.016) (Table 2).

Multiple comparison of the means of the width variable showed that there was significant difference between the passages P3 x P4 and P2 x P4. When the other passages were compared, they did not show significant statistical difference (Table 3).

Cell kinetics

The cell kinetics of the ttASCs characterize the LAG, LOG and PLATEAU phases. The initial LAG phase, from the first to the second day, did not present cell proliferation, and maintained the initial cell concentration of 5 x 10^4 cells/mL. In the LOG phase, from the third to the seventh day, there was

exponential cell growth, reaching the maximum value of 14 x 10^4 cells/mL. Starting on the eighth day, the PLATEAU phase, cell proliferation decreased and the cell concentrations declined until the 10th culture day (Figure 5).

Osteogenic differentiation

After 21 days of inducing osteogenic differentiation, the *ttASCs* showed cytoplasmic calcium deposits and osteoblasts intensely marked by Alizarin Red (Figure 6A, 6B and 6C).

Adipogenic differentiation

After 21 days of culture in adipogenic differentiation culture medium, the *ttASCs* presented different sized cytoplasmic lipid droplets, stained with Oil Red O (Figure 6D and 6E).

Chondrogenic differentiation

The chondrogenic differentiation of the *ttASCs* after 21 days *in vitro* single layer culture showed nodule like aggregations that confirmed their chondrogenic plasticity potential (Figure 6F).

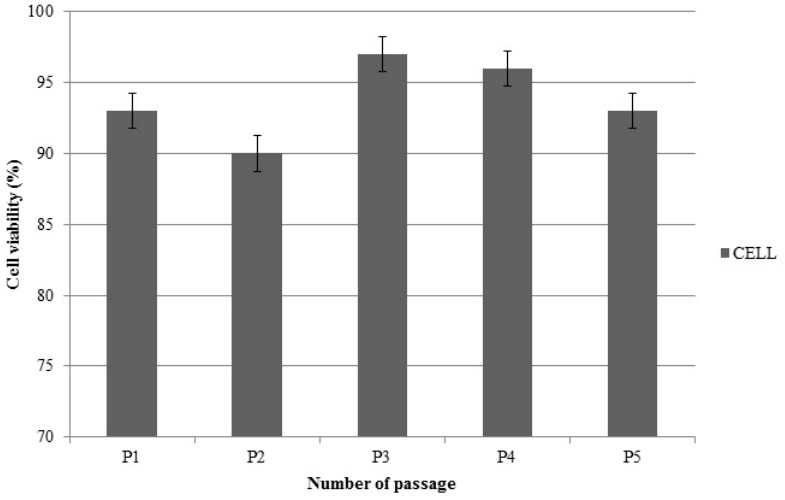

Figure 4. Graphic representation of cell viability (%), during five *ttASCs* passages (P).

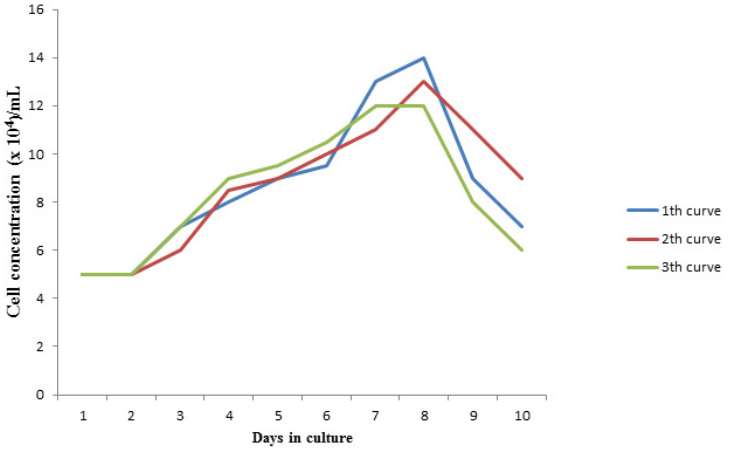

Figure 5. ttASCs growth curve related to the days in culture with the cell concentration (x 10^4)/mL. The LAG, LOG and PLATEAU are evident.

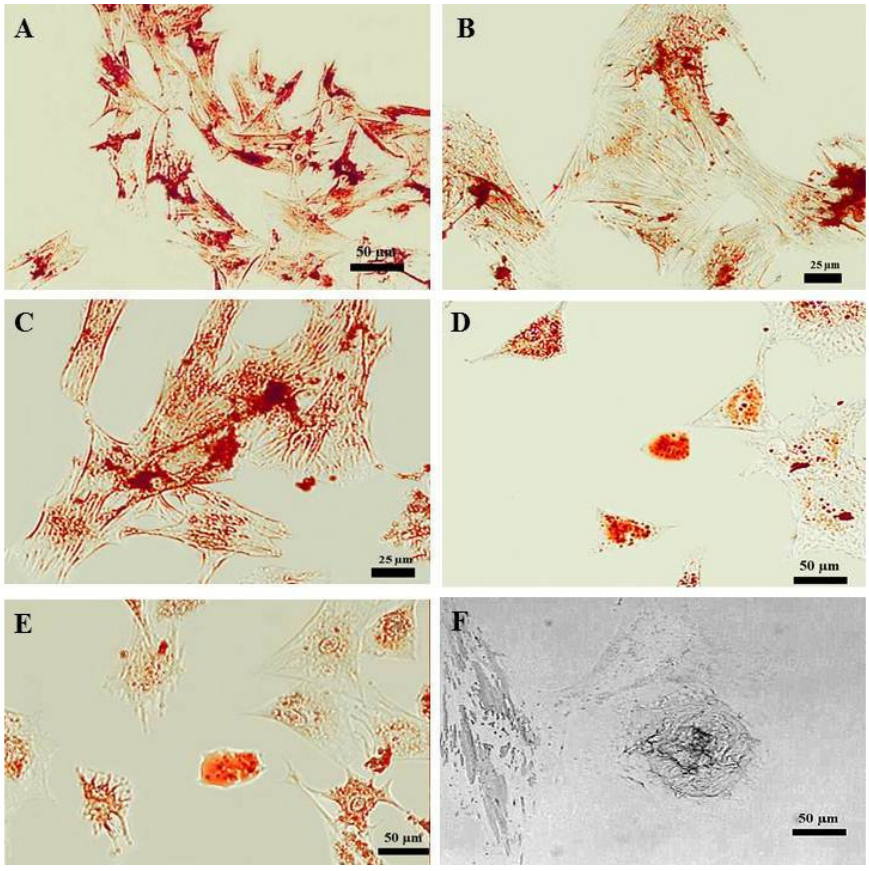

Figure 6. Osteogenic, adipogenic and chondrogenic differentiation of *ttASCs* cultured for 21 days. A, B and C - differentiated osteoblasts stained with Alizarin Red. D and E - lipid granules stained with Oil Red. F - "nodule-like" aggregation resulting from the chondrogenic differentiation.

Table 1. The mean values ± standard deviation of the parameters measured in the *ttASCs* morphological study in the five passages.

Passagen	Length (µm)	Width (µm)	Area (µm^2)	Perimeter (µm)
P1	55,93 ± 5,98	18,74 ± 3,12	633,11 ± 103, 81	132,16 ± 14,53
P2	62,08 ± 10,6	21 ± 3,45	630,39 ± 67,21	147,15 ± 19,70
P3	60,08 ± 5,28	21,16 ± 3,20	681,98 ± 104,28	144 ± 23,43
P4	63,78 ± 4,62	17,57 ± 3,02	608,06 ± 86,76	144,13 ± 14,46
P5	56,17 ± 6,29	20,19 ± 2,05	625,56 ± 76,25	159,18 ± 16,82

Table 2. ANOVA test results among variables (length, width, area and perimeter).

Variable	F calculated	$F_{0,05}$ critical	*P*	Significance
Length	1,263	3,06	0,328	Exist not
Width	4,312	3,06	0,016	Exist
Area	0,507	3,06	0,507	Exist not
Perimeter	1,547	3,06	0,235	Exist not

Table 3. Multiple comparison (Tukey test) of the width variable among the passages, that was statistically different among the means.

Groups compared	q calculated	q(0,05); (15,5)	Hypothesis H_0
P3xP4	4,82	4,367	*Rejects*
P3xP1	3,26	4,367	Accepted
P3xP5	1,29	4,367	Accepted
P3xP2	0,21	4,367	Accepted
P2xP4	4,61	4,367	*Rejects*
P2xP1	3,04	4,367	Accepted
P2xP5	1,07	4,367	Accepted
P5xP4	3,53	4,367	Accepted
P5xP1	1,96	4,367	Accepted
P1xP4	1,56	4,367	Accepted

DISCUSSION

The stem cells isolated from collared peccary adipose tissue were characterized by easy, single layer growth, an extracellular matrix secretion that permitted adhesion to the growth surface and capacity to maintain their multi-potentiality through innumerous passages. These characteristics were also previously described for horses [8], pigs [10], goats [16] and dogs [21], with stem cell cultures of adipose mesenchymal stem cells obtained by enzymatic digestion with collagenase solution. This showed that the cell culture methods established for the *in vitro* study of ASCs in collared peccary are viable and can be used in the future for preclinical protocol tests.

Cell morphology is a consequence of the structural organization of the internal cytoskeleton, because this controls the cell formation, cell junctions and components of adhesive structures [20]. In our assessments, the adipocyte nuclei were visualized displaced to the cell periphery due to the pressure exercised by the lipid vacuoles. The cytometric analysis of the *ttASCs* showed differences for cell width among the cell passages, so that it can be suggested that these variations may result from the pressure exercised cell to cell and from the processes of cell detachment caused by the trypsin proteolytic enzyme.

The cellular kinetics of the *ttASCs* showed high proliferation rates as in other studies [5] that indicated that ASCs have greater proliferative activity and greater cell vascular endothelial growth factor production than bone marrow cells. This characteristic can influence the choice of a MSCs source, when large scale MSCs expansion is needed for clinical applications [14].

The International Cell Therapy Society proposed minimum criteria to define MSC: adherence to plastic when kept under standard culture conditions, expressions of specific surface markers and *in vitro* differentiation in osteoblasts, adipocytes and chondrocytes [4]. However, the characterization of surface markers for MSCs of wild animals, such as the collared peccary, is difficult because commercial antibodies are not available for complex cells in the species. In addition, the surface markers used in murines and humans may not express cross reaction when used in other species [19], so there is no well-defined immunophenotyping that makes comparing studies difficult. Thus, proliferation and *in vitro* differentiation were used to define the mesenchymal stem cells derived from collared peccary adipose tissue.

The *ttACSs* submitted to osteogenic, adipogenic and condrogenic differentiation showed morphological alterations with osteoblastic cells presenting cytoplasmic calcium deposits, presence of cytoplasmic lipid droplets and nodule-like aggregations, respectively. These morphological observations also was observed in equine adipose tissue-derived progenitor cells [8], and goat adipose-derived stem cells when they assessed for the osteogenic, adipogenic and condrogenic cell differentiation [16].

CONCLUSIONS

The adherent cells isolated from collared peccary adipose tissue were defined as multi-potent and with the ability to differentiate in into mesordermal lines. The use of wild animal models has been very important to research strategies for tissue engineering and regenerative medicine both for perfecting and proving innocuous tissue culture techniques and for the use of adult stem cells in pre-clinical studies.

SOURCES AND MANUFACTURES

[1]Vetanarcol, Konig do Brasil LTDA, Santana de Parnaíba, SP, Brazil.
[2]Dormonid, Roche, Basel, Switzerland.
[3]PVP-I, Geyer, Porto Alegre, RS, Brazil.
[4]TPP Techno Plastic Products AG, Trasadingen, Switzerland.
[5]Líquido de Dakin, Asfer, São Caetano do Sul, SP, Brazil.
[6]Penicilina G Benzatina, Provet Produtos Agropecuários, São Paulo, SP, Brazil.
[7]Sigma-Aldrich Co., St. Louis, MO, USA.
[8]Nikon Eclipse E 200, Tokyo, Japan.
[9]Leica Qwin D-1000 versão 4.1, Cambridge, UK.
[10]Veco FUH 2, Campinas, SP, Brazil.
[11]Gibco Invitrogen Cell Culture (Cat. N° 17018-029), Carlsbad, CA, USA.
[12]TECNAL TE-399, Piracicaba, SP, Brazil.
[13]LGC Biotecnologia (Cat. N° 170.83A), Cotia, SP, Brazil.
[14]Cultilab (Cat. N° 009/12), Campinas, SP, Brazil.
[15]Water bath NIT 251, BIOVERA, Rio de Janeiro, RJ, Brazil.
[16]Vortex model 019, EVLAB, Londrina, PR, Brazil.
[17]Cell Strainer, BD Biosciences, São Paulo, SP, Brazil.
[18]Centrifuge model 280 R Excelsa, FANEM, São Paulo, SP, Brazil.
[19]Coleman NIB-100, Santo André, SP, Brazil.

***Ethical approval*.** The protocols used in the present study were approved by the committee of ethics in animal experimentation of the Federal University of Piauí (Nº 018/13) and by the Ministry of the Environment, by the Biodiversity Authorization and Information System SISBIO of the Brazilian Institute of the Environment and Renewable Natural Resources IBAMA (Nº 33058-1).

***Declaration of interest*.** Authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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