ISOLATION, EXPANSION AND CHARACTERIZATION OF EQUINE ADIPOSE TISSUE DERIVED STEM CELLS

ISOLAMENTO, EXPANSÃO E CARACTERIZAÇÃO DE CÉLULAS-TRONCO DO TECIDO ADIPOSO DE EQUINOS

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SUMMARY

Due to the excellent results of stem cell research and its promising applicability, the therapy with these cells is undergoing a period of maximum visibility and numerous studies. In order to standardize the culture and to evaluate the viability of mesenchymal stem cells from adipose tissue of horses, aiming at cell therapy in this species, a study of isolation, expansion and characterization was conducted. For this, adipose tissue was collected from five crossbred horses aged approximately five years old. Subsequently, the tissue was immediately refrigerated and transported to the laboratory where the isolation and culture protocol, lasting approximately 14 days, was performed. After the end of this procedure, a cell pellet was obtained and resuspended in PBS. A portion of material obtained by centrifugation was collected and the cells were stained with 1% Trypan Blue for quantification and assessment of cell viability. Another sample was collected to confirm the lineage of stem cells by flow cytometry using monoclonal against the surface markers CD11b, CD45, CD90 and CD105; and by cellular differentiation assays to chondrogenic, adipogenic and osteogenic lineages. The isolated cells differentiated in the three lineages and showed positive staining for CD90 and CD105 and negative staining for CD11b and CD45, confirming the isolation of stem cells of mesenchymal origin. The average cell viability obtained was 92.83% and thus satisfactory. Therefore, the use of this protocol to obtain mesenchymal stem cells from adipose tissue was deemed to be a promising option for cell therapy in horses.

KEY-WORDS: Adherent cells. Cell differentiation. Cell therapy. Flow cytometry. Mesenchymal.

RESUMO

Devido aos excelentes resultados obtidos em pesquisas com células-tronco e suas aplicabilidades promissoras, a terapia com estas células encontra-se em momento de máxima ascensão, estimulando assim, inúmeras pesquisas. A fim de padronizar o cultivo e avaliar a viabilidade de células-tronco mesenquimais do tecido adiposo de equinos visando a terapia celular nesta espécie, conduziu-se um estudo de isolamento, cultivo e caracterização de tais células. Para tal, foi realizada coleta de tecido adiposo de cinco equinos sem raça definida, com aproximadamente cinco anos. Em seguida, o tecido foi refrigerado e transportado ao laboratório, onde foi realizado o protocolo de cultivo celular durante aproximadamente 14 dias. Após o término deste procedimento, uma alíquota de células foi encaminhada para a realização do teste de quantificação e viabilidade celular com Azul de Tripan 1%, sendo outra porção destinada à confirmação da linhagem de célula tronco mesenquimal por meio de imunofenotipagem com os anticorpos monoclonais anti-CD11b, anti-CD45, anti-CD90 e anti-CD105, e de diferenciação celular em linhagem condrogênica, adipogênica e osteogênica. Houve diferenciação celular e marcação positiva dos antígenos de superfície CD44 e MHCI e negativa dos CD45 e CD11b, confirmando-se a origem mesenquimal e não hematopoiética. A viabilidade celular média obtida das células-tronco mesenquimais foi de 92,83% considerada satisfatória. Portanto, a utilização deste protocolo para obtenção de células-tronco mesenquimais do tecido adiposo se mostrou uma opção confiável e viável para utilização na terapia celular em equinos.

PALAVRAS-CHAVE: Células aderentes. Citometria de Fluxo. Diferenciação celular. Mesenquimais. Terapia celular.

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INTRODUCTION

Stem cells (SC) have several therapeutic applications, both with respect to tissue engineering and gene therapy (ZUK et al., 2001). The mesenchymal stem cells (MSC) are non-hematopoietic stem cells that have multipotent differentiation (GIORDANO et al., 2007; KINGHAM et al., 2007), and can create cells of mesenchymal and non-mesenchymal lineage in the body (GIORDANO et al., 2007). These cells can, therefore, give rise to lineage of bone, cartilage, adipose, muscle, liver, endothelial, epithelial and neurogenic cells (BROOKE et al., 2007; PERONI et al., 2008) and so they have been considered an attractive source for tissue regeneration (KINGHAM et al., 2007).

The interest for MSC as an effective treatment for musculoskeletal injuries that occur frequently in horses has been increasing (VIDAL et al., 2007). These cells can be isolated from both the bone marrow and adipose tissue. From the adipose tissue, in addition to the easiness of collection compared to bone marrow, it is possible to find about 2% of MSC in the total cell lipoaspirate (KINGHAM et al., 2007), thus optimizing the harvest and cell expansion (BUNNEL et al., 2008), while the bone marrow has approximately 0.001 to 0.01% of MSC of the total (MIAO et al., 2006). This is interesting for cell therapy applied to horses, mainly because lesions in tissues, such as bones and tendons, require large amounts of cells in order to achieve therapeutic efficacy (VIDAL et al., 2007).

After digestion of the adipose tissue with collagenase, a monolayer culture of fibroblastic shaped cells adherent to the plastic was obtained, thus fulfilling the first criteria for the characterization of mesenchymal stem cells, as considered by Dominici et al., 2006 and other authors, who conducted research in horses (CARVALHO, 2009) and dogs (VIDAL, 2007).

A major difficulty for the characterization of ADSCs in veterinary medicine is the low availability of specific monoclonal antibodies (CARVALHO et al., 2009).

The objective of this study is to establish a protocol for isolation, cultivation and characterization of mesenchymal stem cells from the adipose tissue of horses that will allow satisfactory cell viability and concentration to perform stem cell therapy in this species.

MATERIAL AND METHODS

Horse adipose tissue was collected at Hospital Veterinário de Grandes Animais of Universidade de Brasília (FAV/UnB), Brasília, DF, and both isolation and cultivation of SC were performed at the Laboratório de Reprodução e Biotecnologia Animal (FAV/UnB), following protocol recommended by NARDI & MEIRELLES (2006). The MSC were derived from adipose tissue of the paraxial caudodorsal gluteal region (Figure 1A).

Five healthy horses aged approximately 5 years old of non-defined breed were used. They were kept on station, restrained and sedated with detomidine, 0.02 mg/kg body weight intravenously, which was followed by shaving of an area of approximately 10 x 10 cm on the paraxial caudodorsal gluteal region. A local anesthesia in an inverted "L" with lidocaine 2% was performed and pain assessment was made by observing animal response to painful stimuli. A half-moon, 10-cm incision was made approximately 10 cm abaxial and lateral from the base of the tail, followed by dissection of the subcutaneous tissue, from where 10 g of adipose tissue was collected (Figure 1B). The sample was placed in sterile Petri dish and weighed on a precision scale.

The skin incision was not sutured due to irreducible dead space left after the tissue was collected, which could predispose to contamination. Therefore, daily dressing was performed with povidone-iodine 0.1% until complete healing. After this procedure, the tissue collected was placed in 50 mL conical tube containing the transport solution DMEM (Dulbecco's Modified Eagle's Medium Gibco, Invitrogen, California, USA), antibiotics and antimycotics (AT/AM) (Gibco, Invitrogen, California, USA), immediately refrigerated, kept at 8˚C, and transported to the cell culture room in laminar flow. At this stage, the sample was washed with phosphate-buffered saline (PBS Gibco, Invitrogen, California, USA) and transferred to a Petri dish, where it was fractionated in 40 portions using an anatomical forceps and scalpel. Subsequently, the sample was placed again in 50 mL conical tube containing 10 mL of 0.05% trypsin (Gibco, Invitrogen, California, USA), previously heated to 37ºC, and kept at the same temperature in water bath for 30 minutes. The adipose tissue was then transferred to another 50 mL conical tube containing collagenase type I 0.3% (Gibco, Invitrogen, California, USA) at the 1:3 ratio (tissue volume:solution volume), incubated in water bath at 37 ˚C during 30 minutes, and agitated every 10 minutes. After enzymatic digestion, tissue fragments were distributed in two 25-cm² culture bottles, together with 1 mL of fetal bovine serum (FBS) (Gibco, Invitrogen, California, USA) and 9 mL of collagenase type I 0.06%. The bottles were then incubated in a 5% CO_2 atmosphere humidified oven at 37˚C, overnight.

After 18 hours, the contents were transferred to 50 mL conical tube and kept under agitation in a vortex for several seconds. The cells were filtered using a 70 µm filter and the remaining adipose tissue was discarded. The filtered content was centrifuged at 3000 rpm during 10 minutes using 15 mL conical tubes, and the supernatant was discarded. The pellet was resuspended in 1 mL of DMEM.

An aliquot of 500 µL of the sample was placed in 25-cm² culture bottles, together with 5 mL of complete culture medium (90% DMEM, 10% FBS, antibiotics and antimycotics) and incubated in 5% CO_2 atmosphere oven at 37˚C. After three days, the culture medium was changed and the non-adherent cells were eliminated. The medium was changed every three days until it reached a confluence of approximately 75%,

when the procedure of bottle trypsinization and culture passage was performed. For this, the old medium was discarded, and only the attached cell layer was left. We then added 5 mL of trypsin 0.05% and waited for five to ten minutes. The bottle content was then aspired and placed in a 50 mL conical tube containing 4.5 mL of DMEM and 500 µL of FBS that was previously heated in a water bath at 37ºC, in order to inactivate trypsin with FBS. The content was then centrifuged at 1,500 rpm during 10 minutes and the supernatant discarded. The pellet was resuspended in 1 mL DMEM and placed again in the culture bottle to incubate in the oven. This procedure was repeated every time the culture reached confluence.

In order to confirm that cultured cells belonged to the lineage of mesenchymal stem cells, at the end of the fourth passage, a portion of cell lineage was induced to osteogenic, adipogenic and chondrogenic differentiation. For osteogenic differentiation, cells were placed in six wells containing 3 mL of the osteogenic differentiation medium and cultivated in 5 % CO_2 humidified oven at 37 °C. This medium consisted of DMEM with 15mM HEPES (Invitrogen, California, USA); 10% fetal bovine serum; 20nM dexamethasone (Alfa Aesar, United Kingom); 10mM β-glycerophosphate + 0,05 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, United Kingdom); antibiotics and antimycotics. A portion of cells destined to the negative control were grown in DMEM/ 15mM HEPES, 10% FBS, antibiotics and antimycotics. The culture medium was changed every three days and after 21 days of culture, cells were stained with Alizarin Red S (Alfa Aesar, UK) which stains the mineralized matrix red. For adipogenic differentiation, the medium consisted of DMEM with 15mM HEPES (Invitrogen, California, USA); 10% fetal bovine serum; 1% ITS + Premix (BD, Franklin Lakes, NJ, USA); 1µM dexamethasone (Alfa Aesar, UK); 100µM indomethacin; 500µM 3-isobutyl-1-methyl xanthina (IBMX); antibiotics and antimycotics. The cells were stained with Oil-red O (Sigma-Aldrich, UK), which stains the fat droplets red. For chondrogenic differentiation, the medium consisted of DMEM with 15mM HEPES (Invitrogen, California, USA); 10% fetal bovine serum; 1% ITS + Premix (BD, Franklin Lakes, NJ, USA), 10ng/mL recombinant human transforming growth factor-beta 3 (hTGFβ3); 50nM L-ascorbic acid-2-phosphate (Sigma-Aldrich, UK); 100nM dexamethasone (Alfa Aesar, UK); 100µL antibiotics and antimycotics. The cells were stained with Alcian Blue, which stains the proteoglycans blue.

In the second step, an aliquot of cells was tested in the flow cytometer (FACScalibur; BD; Franklin Lakes, NJ, USA) to identify the key markers of mesenchymal stem cells as the primary antibodies: mouse anti-human CD105 (Invitrogen), mouse anti-human CD90 (ABD Serotec), mouse anti-human CD45 and mouse anti-human CD11b (ABD Serotec). The isotope corresponding antibody was used as negative control and as secondary antibody, it was used the goat anti-mouse IgG (H/L):FITC (ABD Serotec). The data were analyzed by FlowJo software (TreeStar, Ashland, USA).

After culture, the medium was discarded and the cells suspended in PBS and placed in Eppendorf. An 50µL- aliquot was taken to measure and test cell viability according to Brunnel (2008), using a Neubauer chamber and 50 µL of Tripan 1% blue, which crosses the cell membrane and stains only dead cells.

The cell count was performed in all cells of the four lateral squares that make up the Neubauer chamber and they were classified as alive or dead, their sum was the total number of cells. The percentage of viable cells was given by dividing the number of living cells by the total. The statistical design was completely randomized with five replicates and data descriptive statistics was performed.

RESULTS

After isolating the adipose tissue cells and obtaining the monolayer cells adhered to the plastic, the first criterion for MSC was fulfilled. The cells were similar to fibroblasts, typical of stem cells.

After collection, isolation and cultivation for 14 days (Figure 2) of the MSC from the adipose tissue of the five animals, the viability test showed that 92.83% of the cells were viable. The individual data of the five animals are shown in Table 1. An average $3{,}3x10^9$ cells was present in 400 µL related to cell concentration in each sample of the five animals, which was calculated from the data of Table 1.

Cell differentiation was confirmed for the osteogenic, chondrogenic and adipogenic lineages by using the inducing medium specific for each case. In osteogenic differentiation, the cells formed white aggregates similar to nodes. After 14 and 21 days (Figure 3A), these white nodes and other adjacent cells were stained with Alizarin red, thus confirming ADSCs differentiation into the osteogenic lineage. For chondrogenic differentiation, after 14 days of culture, the cells formed a well defined cell mass that was still not stained with Alcian blue (Figure 3B). After 21 days of culture, the micro-mass was then stained with blue, thus showing ADSCs differentiation into the chondrogenic lineage. Adipogenic differentiation was confirmed by staining with Red-O. After 14-day culture with the inductor medium, fat droplets were observed in the cell cytoplasm, and after 21 days there was differentiation into adipocytes (Figure 3C).

Flow cytometry was used to identify the key markers of mesenchymal stem cells and it confirmed positively the marking of the antibodies CD105 and CD90 in all evaluated samples, with averages 96.48% and 94.94%, respectively. The opposite was also observed, there was virtually no marking of the major hematopoietic markers CD45 and CD11b in all analyzed samples, with averages 0.59% and 0.76%, respectively, thus confirming the mesenchymal origin of the cells used for implantation (Figure 4).

Figure 1 - Collection of adipose tissue. A) Antisepsis of the collection area. B) Dissection of the subcutaneous tissue and separation of the adipose tissue.

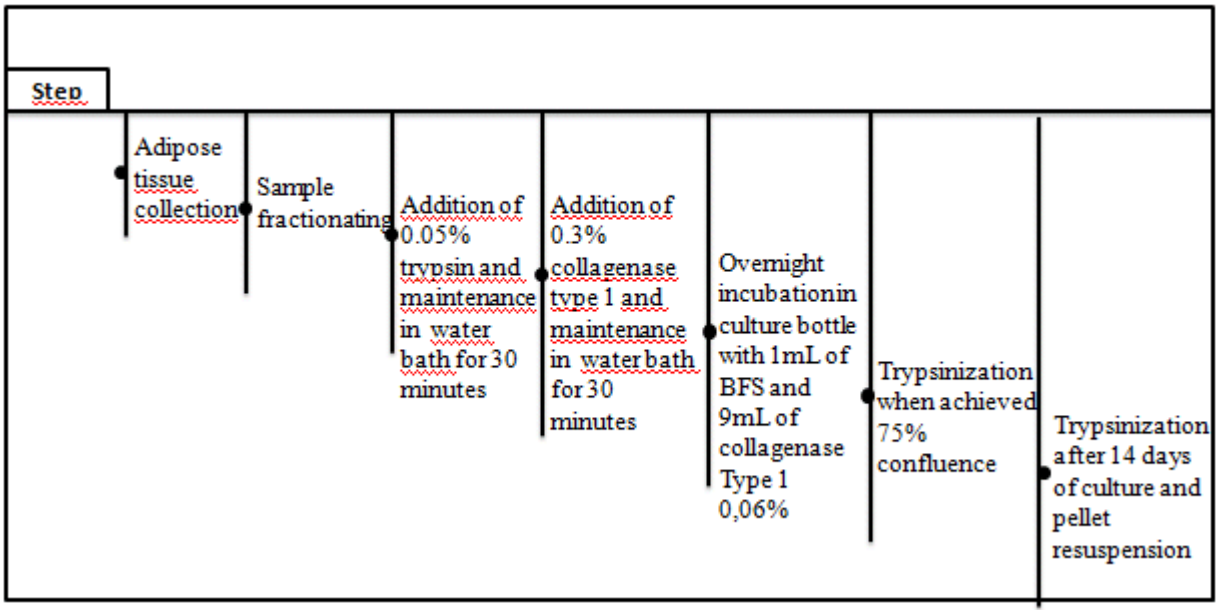

Figure 2 - Steps of the culture protocol followed for the 14-day culture of mesenchymal stem cells from adipose tissue. Brasília, DF, 2010.

Table 1 - Results of counts of total cells (TC), live cells (CV) and dead cells (CM) from the MSC samples of five horses obtained from the viability test expressed as percentage of viable cells (V). Brasília, DF, 2010.

Animal	TC	CV	CM	V (%)
1	817	734	83	89,84
2	890	837	53	94,04
3	1665	1545	120	92,79
4	2416	2368	48	98,01
5	3658	3272	386	89,45
	1889,2	1751,2	138	92,83
DP	±1183,686	±1073,109	±141,5786	±3,489903

– mean;; AM – sample; DP = standard deviation

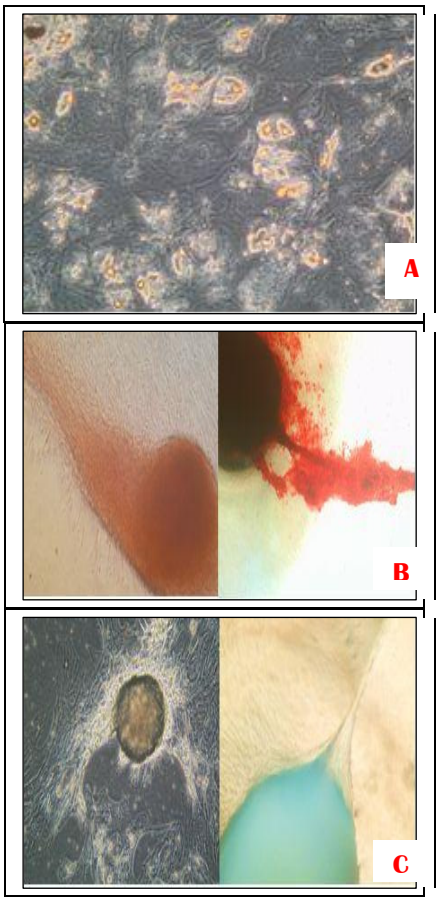

Figure 3 - Confirmation of cell differentiation of ADSC into osteogenic lineage on the 14th day, Alizarin Red staining (A), chondrogenic lineage on the 14th day, formation of the nodular cell aggregate (B) and adipogenic lineage on the 21st day, Oil Red staining (C). Brasília, DF, 2010.

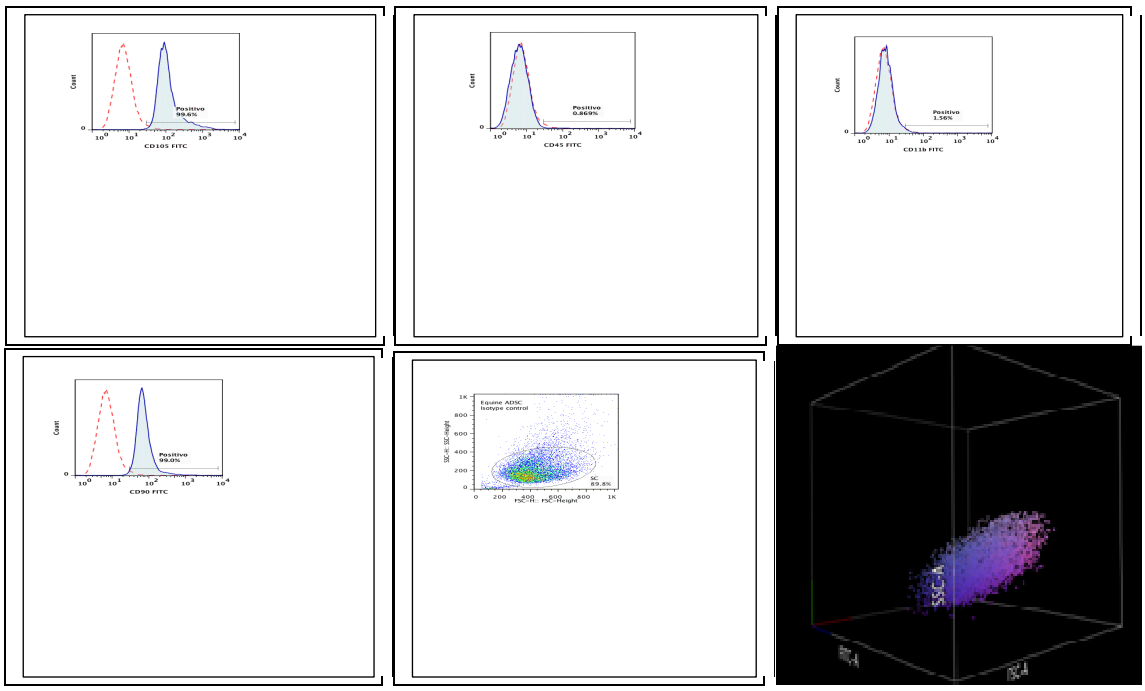

Figure 4 - Cytofluorimetric analysis of stem cell markers from adipose tissue of one of the horses. A: antibody anti-CD105; B: antibody anti-CD45; C: antibody anti-CD11b; D: antibody anti-CD90; E: dot plot of horse stem cells; F: 3D representation of horse stem cell. Blue line: reading of the antibody tested; Dashed red line: negative isotype control. Brasília, DF, 2010.

DISCUSSION

Horse restraint in the chute station associated with sedation and local anesthesia was sufficient to allow the collection of the adipose tissue. General anesthesia was not necessary because during skin incision and dissection of the subcutaneous tissue to perform the collection, none of the five animals showed signs of pain as described by Nixon et al. (2008), even though the surgical technique was not the same of this study. Thus, the costs and risks related to general anesthesia are reduced. A more detailed study of the pain would require other parameters such as measuring the heart rate of the animal before and after the procedure. However, in this case the heart rate becomes a subjective parameter, because changes occur due to the stress of going into the chute and being manipulated, which according to Barreira (2005), hampers its use as an evaluation method.

The incision size and collection site were both adequate. Only one incision per animal was sufficient to collect the adipose tissue, so one local anesthesia and one sedative dose were enough, unlike Nixon et al. (2008) who did not perform cell culture and had to perform adipose tissue collection from two different sites. However, this author used 20g of adipose tissue, twice the amount of the present study. Nevertheless, increasing the amount of fat tissue used does not necessarily increase the efficiency of the process of cell cultivation, since it is not known exactly the percentage of mesenchymal stem cells in the sample.

Kingham et al. (2007) report that about 2% of the human lipoaspirate is MSC. Therefore, from 10 g of adipose tissue, about 0.2 g of MSC could be isolated, which combined with the great capacity for cell expansion in culture would make the amount of collected adipose tissue sufficient to yield the minimum number of cells necessary to perform cell therapy. Moreover, Nixon et al (2008) performed a linear skin incision; however, it was observed in this study that the half-moon incision allows a more effective dissection of the subcutaneous tissue to collect the adipose tissue.

Hand asepsis and sterilization of all materials and solutions used to manipulate tissue and isolated cells in the laboratory were adequate, since during the 14 days of culture there was no sample deterioration or bacterial contamination of the culture medium contained in the culture bottles. Any contamination would be observed as granularity around the core, cytoplasmic vacuolation or cell displacement in the culture bottles, as described by Bunnell et al. (2008). For the same reason, we conclude that the transport solution and the refrigeration method used to transport the tissue to the laboratory were effective.

After digestion with collagenase, we obtained a monolayer cell culture adherent to the plastic and shaped as a fibroblast, thus reaching the first criterion for characterizing the mesenchymal stem cells, as stated by Dominici et al., 2006 and others who

conducted research with horses (CARVALHO, 2009) and dogs (VIDAL, 2007).

Regarding cell viability, the average of more than 90% of viable cells obtained in this study is higher than other sources of MSC in horses. Barreira (2005) who used the separation of the mononuclear fraction of bone marrow in, horses reported 76% of viable cells. Nixon et al. (2008) obtained 87.5% of viable cells from adipose tissue of horses, but this author did not perform cell culture, only isolation of nucleated cells. To this end, the amount of adipose tissue was twice as much as the amount collected in our study from two different sites. Still, the authors obtained nucleated cells that may or may not be stem cells, which confirms the advantages of the cell culture performed in this work, since all cultured cells could be possibly used for cell therapy.

Compared to other species, the results of this study were also satisfactory. For humans, 98.1% of viable cells were obtained after isolation and culture of MSC from adipose tissue (OLIVEIRA et al., 2007).

The cell culture technique used in this study, as well as the trypsinization procedure with 0.25% trypsin during culture, was similar to the technique used by Kingham (2007) with fat from rats. It was also similar to the technique used by Zuk et al. (2001), whose studies are based on human adipose tissue, we can, therefore, infer that the protocol used in other species can be applied to horses, since satisfactory results were obtained in this study. According to Vidal et al. (2007), the MSC from adipose tissue of horses expand quickly in culture in sufficient quantities for tissue engineering, which was also observed in this study, similar to other studied species.

Besides the great expansion capacity of MSC in culture, reported by Keating (2006), the average number of total cells obtained in this study was effective for the realization of cell therapy. Authors such as Gengozian (2000) state the need for a minimum of about $2x10^6$ cells to be inoculated in the animal for the success of cell therapy. One can therefore, conclude that the amount of adipose tissue collected was enough for isolation and culture of MSC, since an average $3,3x10^9$ cells were obtained in 400 µL of sample.

Horse ADSCs in passage 4 showed osteogenic activity on the 14th day of culture in osteogenic medium and activity increase on the 21st day. Dexamethasone, vitamin C and glycerophosphate are required for the ADSCs to differentiate into osteoblasts *in vitro* (CHENG et al., 1994; DEANS; MOSELEY, 2000). According to the authors, desamethasone promotes differentiation and maturation of osteoblasts, increases ALP activity, and promotes collagen synthesis of the extracellular matrix. Vitamin C promotes collagen synthesis and calcification in cell culture, alters ALP and glycerophosphate activity, supplies phosphate ions for the osteoblasts, promotes physiological deposition of calcium and is, therefore, necessary for ADSC mineralization.

The effects of dexamethasone, an inducer of osteogenesis and adipogenic differentiation, depend on the dosage and time. The low concentration leads to differentiation into osteoblasts, while at high concentrations triggers the interaction of the glucocorticoid receptor with insulin. Then, the cells differentiate into adipocytes (HOYNOWSKI et al., 2007). In this study, the hypothesis that ADSCs of horses can differentiate into adipocytes was verified by staining with Oil Red on the 14th day of culture and on. After 21 days, the fat droplets were larger in size and number, as reported by Ladak (2011).

The induced chondrogenic differentiation and ADSCs culture have been extensively researched (NESIC et al., 2006). In this study, the used micromass culture was successful in the induction of chondrogenic differentiation from 21st day of culture in chondrogenic medium and on.

MSC derived from the adipose tissue should have positive staining for the following surface antigens: CD9, CD10, CD13, CD29, CD44, CD49 (d), CD49 (e), CD54 and CD55, CD59, CD73, CD90 and CD105 and CD106 and CD146 and CD165 (SCHÄFFLER; BUCHLER, 2007). The surface markers CD11b, CD18, CD50, CD56, CD62 are not present on the cell surface ADSCs. Such cells do not express the hematopoietic markers CD14, CD31 or CD45. The cells ADSCs are positive for the protein of class I histocompatibility, HLA-ABC and negative for protein of class II, HLA-DR (GIMBLE; GUILAK, 2003). In our study, we confirmed the existence of the markers CD90 and CD105 and the low occurrence of the markers CD11b and CD45, which characterizes these cells as stem cells of mesenchymal origin. The almost non-existing values of the main hematopoietic markers, which confirms the mesenchymal origin of the cells used for implantation, were quite different from the results reported by Ladak (2011), whose results may show more reminiscent such as macrophages, fibroblasts and endothelial cells that also express hematopoietic markers in the mesenchymal stem cell culture in his work with rats.

The protocol used in this study for isolation and culture of mesenchymal stem cells from the adipose tissue of horses was efficient, since cellularity and viability obtained were satisfactory to perform cell therapy in the studied species. After completion of the protocol, it was observed that the number of suspended cells that could be inoculated in the animal to treat various conditions, particularly ligaments and tendon, was higher than the minimum required for successful stem cell therapy according to the literature.

The stem cell therapy in veterinary is becoming more prominent in educational research institutions and laboratories. Despite the more accessible source of MSC being mononuclear fraction from bone marrow at the moment, MSC arising from various tissues has been gaining ground in research and are considered promising due to its features. However, further studies on the methods of expansion in culture and specific markers for tracking stem cells are necessary in order to evaluate therapy success. Despite the satisfactory results reported in several studies, including this, more research is needed using horses to standardize the isolation and cultivation of mesenchymal stem cells coming from the adipose tissue of horses in order to

optimize the time between collection and implementation of cell therapy.

In this study, a monolayer of fibroblast-like cells and adherent to plastic was developed after digestion of adipose tissue by collagenase. The isolated cells were examined in terms of their potential for osteogenic, chondrogenic and adipogenic differentiation. Furthermore, cytofluorimetric analysis of surface cell markers identified cells positive for CD90 and CD105 and negative for CD45 and CD11b, thus confirming the mesenchymal origin of the cells. Therefore, the three criteria proposed to identify the mesenchymal stem cells were fulfilled (DOMINICI et al., 2006). Therefore, the cells isolated from the adipose tissue of horses following the proposed protocol, can be considered as mesenchymal stem cells.

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