ABSTRACT.- Ribeiro G., Massoco C.O. & Lacerda Neto J.C. 2013. Culture of equine bone marrow mononuclear fraction and adipose tissue–derived stromal vascular fraction cells in different media. Pesquisa Veterinária Brasileira 33(Supl.1):20-24. Departamento de Patologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: gesiane@usp.br

The objective of this study was to evaluate the culture of equine bone marrow mononuclear fraction and adipose tissue–derived stromal vascular fraction cells in two different culture media. Five adult horses were submitted to bone marrow aspiration from the sternum, and then from the adipose tissue of the gluteal region near the base of the tail. Mononuclear fraction and stromal vascular fraction were isolated from the samples and cultivated in DMEM medium supplemented with 10% fetal bovine serum or in AIM-V medium. The cultures were observed once a week with an inverted microscope, to perform a qualitative analysis of the morphology of the cells as well as the general appearance of the cell culture. Colony-forming units (CFU) were counted on days 5, 15 and 25 of cell culture. During the first week of culture, differences were observed between the samples from the same source maintained in different culture media. The number of colonies was significantly higher in samples of bone marrow in relation to samples of adipose tissue.

INDEX TERMS: Horses, bone marrow, adipose tissue, culture medium.
with good results (Gutierrez-Nibeiro 2011). Oliveira et al. (2011) utilized bone marrow mononuclear fraction cells in the treatment of induced tendinitis in horses and observed improvement in tissue organization, with greater alignment of fibers and higher percentage of non-lesioned area.

Also, adipose tissue-derived stromal vascular fraction cells have been used to improve tendon and ligament repair, with apparently good results (Gutierrez-Nibeiro 2011). In a Nixon et al. (2008) study that compared intralesional adipose tissue-derived stromal vascular fraction cells and saline solution in a collagenase-induced model of superficial digital flexor tendinitis, treated tendons had significantly better histologic organization and reduced inflammatory cell infiltrate than controls at 6 weeks.

In veterinary medicine, knowledge of these cells is still very limited, and they are utilized based on the hypothesis that their essential characteristics, such as time of multiplication and potential for differentiation, are the same for all species (Vidal et al. 2007). However, according to Fraser et al. (2008), stem cells from adipose tissue and from bone marrow of the same individual are similar but not identical. The advent of stem cells and stem cell-based therapies for specific diseases requires particular knowledge of laboratory procedures, which not only guarantee the continuous production of cells, but also provide them an identity and integrity as close as possible to their origin (Renzi et al. 2012).

The first works with tissue cultures were carried out with organic fluids of animals, such as lymph. When Eagle (1955) formulated a basic culture medium containing amino acids, carbohydrates, vitamins and minerals, he noted that the supplementation of the medium with organic fluids was still necessary, since such fluids contained factors that were undefined but essential for cell growth. The supplementation of basic culture medium with 20% animal serum was widely utilized. Due to the presence of growth factors and a small quantity of gammaglobulin, fetal bovine serum (FBS) is one of the most utilized. FBS is used at a concentration of 10%, but it can be increased or decreased depending on the culture (Gonçalves 2002).

The main advantages of utilizing FBS are the following: presence of the majority of factors necessary for the proliferation and maintenance of cells, its efficacy in different types of cell cultures and its buffering action, preventing adverse effects such as change in pH. On the other hand, the addition of FBS to the medium can have some disadvantages such as: cost, availability, origin and quality, besides the risk of contamination by bacteria, fungi and viruses (Gonçalves 2002).

Many other cell culture media have been developed from Eagle’s basic medium, for example, Dulbecco’s modified medium Eagle’s (DMEM), with increased amounts of vitamins and amino acids. DMEM was utilized for the first time for cultivating rat embryonic cell, and it is currently used in the culture of various mammalian cell strains, mainly those that grow as adherent monolayers. However, cell growth in DMEM still needs supplementation with FBS (Morgan & Darling 1995).

The possibility of interference from unknown factors present in FBS has led to the increasing use of some culture media without the addition of serum. AIM-V from GIBCO is one of these new media. However, often the use of medium without added serum requires the addition of specific supplements that have not been fully determined, and while some types of cells can be grown in medium without serum, some characteristics can be negatively affected (Morgan & Darling 1995).

Thus, the objective of this work was to evaluate the characteristics of equine bone marrow mononuclear fraction and adipose tissue-derived stromal vascular fraction cells cultivated in the presence or absence of FBS.

MATERIALS AND METHODS

This study was approved by the Committee of Animal Ethics and Well-being (CEBEA) of the School of Agricultural and Veterinary Sciences of São Paulo State University (Unesp), Jaboticabal Campus/SP, Brazil, under Protocol No. 013041-5.

Animals. Five male mixed-breed horses with a mean age of 11±3.71 years and weight varying from 400 to 500 kg were used. They were from the Serum Production Farm of Butantan Institute, located in the city of São Roque, SP, Brazil. These horses were healthy animals that were selected after a general clinical examination and that were not being utilized in immunization protocols at the Institute.

Each animal was submitted to two types of tissue collection. First, bone marrow was aspirated and then subcutaneous adipose tissue was obtained. Tissue collection and processing were done on the same day, and samples were transported to the laboratory refrigerated.

Bone marrow. The bone marrow samples were collected from the sternum of each horse by needle aspiration biopsy. The procedure was done with the animal standing, restrained with a halter.

The biopsy site was shaved and prepared for sterile collection. The skin and tissues adjacent to the sternebrae were anesthetized by infiltration of 5ml of 2% lidocaine hydrochloride with vasoconstrictor (Xilestesin®, Cristália, Itapira, SP, Brazil). The puncture was performed with the introduction of a 14G catheter needle (Nipro Medical Ltda, Sorocaba, SP, Brazil) into the fifth sternebra, and the aspiration was carried out with a 20-ml syringe containing 0.2ml sodium heparin (Liquemine®, Roche Brasil, Rio de Janeiro, RJ, Brazil).

Adipose tissue. The samples of adipose tissue were collected from the fat located under the dorsal surface of the gluteus muscle, close to the insertion of the tail. The procedure was carried out with the animal standing and kept in upper torso restraints.

The region was shaved, anesthetized with local infiltration of 2% lidocaine hydrochloride with vasoconstrictor (Xilestesin®, Cristália, Itapira, SP, Brazil), and prepared for sterile tissue collection. An incision was made in the skin of approximately 5.0cm, slightly parting the skin to expose the subcutaneous tissue and to obtain a sample of adipose tissue. Next, the skin was closed with 0 nylon suture (Brasutere, São Sebastião da Grama, SP, Brazil) in separate simple stitches. The material collected was placed in tubes containing RPMI-1640 culture medium (Cultilab, Campinas, SP, Brazil) and kept refrigerated in a thermal container while transported to the laboratory.

Isolation of cells from the bone marrow mononuclear fraction and adipose tissue-derived stromal vascular fraction. The samples of bone marrow aspirate were diluted in HBSS ( Hank’s Balanced Salt Solution; Cultilab, Campinas, SP, Brazil). The samples of adipose tissue were enzymatically treated with collagenase I (Cultilab, Campinas, SP, Brazil) for 20 minutes at 37°C.
In relation to the number of CFU observed in cultures of cells obtained from adipose tissue (AD) and of cells obtained from bone marrow (BM), there were no differences between the samples of the same origin cultivated in different media, indicating that the culture medium had no influence on this characteristic. However, significant differences were found between the samples of AD and BM, where bone marrow samples showed a higher number of CFU than did samples from adipose tissue (Fig. 1), suggesting that bone marrow cells had a greater colony-forming capability.

**Morphological analysis**

At the beginning of morphological evaluations (day zero), AD and MO cultures showed similar characteristics, with rounded cells of different sizes and very heterogeneous shapes. However, on the third day of culture, after removal of the non-adherent cells, AD cultures showed a population of fusiform and firmly adhered cells, while the MO cells were round and fewer in number.

During the first week of culture, it was possible to detect differences between the cultures maintained in DMEM (containing 10% FBS) and those grown in AIM-V. AD cells maintained a predominantly fusiform shape and showed greater cellularity in DMEM compared to cells cultivated in AIM-V (Fig. 2). Meanwhile, in cultures of MO, there was a population of adhered cells with greater numbers and more refringence in AIM-V.

As of the 14th day of culture, differences between the two culture media were no longer found. AD cells maintained a fusiform shape and showed disorganized proliferation. The samples of MO displayed the presence of rounded cells and cells with a fusiform appearance, and they formed colonies or cell aggregates.

At 21 days of culture, the AD samples contained strongly adhered cells with a fusiform appearance, which showed organized proliferation. In MO cultures, there was a greater refringence and presence of rounded cells and fusiform cells.

The last morphological evaluation was done at 32 days of culture, at which time AD cultures showed the same characteristics as previously seen and those of MO had a predominance of adhered fusiform cells.
B cells morphology by the fourth day of culture. Therefore, between 24 and 48 hours of culture, and fibroblast-like adherent cells from bone marrow mononuclear fraction the cells found in the present work. Maia et al. (2012) had 

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Similar results were obtained by Cowan et al. (2004) who 

studied murine mesenchymal stem cells and reported that 

the rate of expansion of cells derived from adipose tissue 

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Besides, it was possible to observe that the cells from 

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cells morphology by the fourth day of culture. Therefore,

in this study, it appears that the non-adherent cells were 

successfully removed from the adipose tissue cultures in 

the first exchange of medium, while the cell cultures from 

bone marrow still showed non-adherent cells, although in 

decreasing amounts, up to the last evaluation, thus expla 

ning the heterogeneous appearance of the culture.

De Vita et al. (2013), studying mesenchymal stem cells 

from equine amniotic fluid, reported an initial difficulty in 

cell isolation, and achieved better cell adhesion on the cul 

ture plate by decreasing the amount of medium in the pri 

mary culture to leave the cells closest to the plate, and by 

increasing the time to the first exchange of medium from 2 

to 7 days, to prevent the removal of cells that were capable 

of adhesion and were still in suspension.

Vidal et al. (2007) also observed that cell populations 

obtained from adipose tissue did not form as many colo 

nies as cells from bone marrow, and that they were distri 

buted more uniformly and adhered more firmly to the cul 

ture plate, as observed in this study.

Mesenchymal stem cell cultures with DMEM medium 

supplemented with fetal bovine serum (FBS) are often used 
today (De Vita et al. 2013, Maia et. al 2012). However, to 

avoid the potential risk of exposing human patients to cells 
cultivated in the presence of FBS, some investigators have 

searched for a substitute supplement (Bieback et al. 2009) 
because, according to the results of Pal et al. (2009), the 
presence of serum is essential for maintaining and expan 
ding mesenchymal stem cell cultures. In the present work, 

despite samples coming from adipose tissue, they showed 
greater cellularity in DMEM supplemented with FBS in the 

first two weeks, but afterwards, there is no longer any diffe 
rence in relation to grown in AIM-V medium without FBS, 
suggesting that this can be an alternative for the culture of 
cells destined for transplantation.

In a current study about the potential of neural diffe 

rentiation of mesenchymal stem cells from equine bone 

marrow, Maia et al. (2012) surprisingly found that control 
cells culture that have not undergone any treatment, also 

expressed neural markers. These researchers suggested 

that FBS present in the culture medium may have influen 
ced the differentiation in the control group, because it is

DISCUSSION

The methods used to collect equine bone marrow and adi 
pose tissue, as well as the techniques used for the isolation 
of cells from the bone marrow mononuclear fraction and 

adipose tissue-derived stromal vascular fraction were con 

sidered simple and easy, and had already been described in 
detail (Ribeiro et al. 2012).

The cell population with the fusiform appearance de 

scribed here in samples from both adipose tissue and bone 

marrow were morphologically similar to mesenchymal 

stem cells from human (Zuk et al. 2001, You et al. 2009), 

sheep (Fadel et al. 2011), horse (Fortier et al. 1998, Smith 


Maia et. al 2012, De Vita et al. 2013), and other animals re 

ported in many studies, in which these cells were described 
as star-shaped or fibroblast-like.

In relation to cell expansion, it was observed in the 
course of culture that AD samples always reached 90% 

confluence before MO samples, and thus, AD cells were 

passaged about every three days, whereas MO cells every 
five days. These observations corroborated the findings 
of Colleoni et al. (2009), indicating that, in horses, cells from 
adipose tissue show a greater capacity for proliferation in 
culture compared to cells obtained from bone marrow. 
Similar results were obtained by Cowan et al. (2004) who 

studied murine mesenchymal stem cells and reported that 
the rate of expansion of cells derived from adipose tissue 

was five times greater in the first seven days compared to 

bone marrow cells.

Besides, it was possible to observe that the cells from 
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between 24 and 48 hours of culture, and fibroblast-like 
cells morphology by the fourth day of culture. Therefore,
known that FBS is rich in steroids, such as glucocorticoids and estrogen, which may have interacted with other growth factors, also present in the serum, driving a cascade of events to cell differentiation. Therefore, if FBS presence in mesenchymal stem cells culture could induce cell differentiation, it is very important more studies about it are carried out and other alternatives of culture media able to support the mesenchymal stem cells culture without FBS are analyzed.

CONCLUSIONS

The AIM-V culture medium is an alternative for the culture of equine bone marrow mononuclear fraction and adipose tissue–derived stromal vascular fraction cells in the absence of FBS.

Equine adipose tissue–derived stromal vascular fraction cells have a greater capacity for proliferation in culture compared to bone marrow mononuclear fraction cells.

Equine bone marrow mononuclear fraction cells have a greater capacity for agglutination and formation of colonies compared to adipose tissue–derived stromal vascular fraction cells.

REFERENCES


