Toxicity and oxidative stress of canine mesenchymal stromal cells from adipose tissue in different culture passages[1]

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ABSTRACT.- Sprada A.G., Rosa M.P., Machado A.K., Pippi N.L., Bayard P. & Cruz I.B.M. 2015. **Toxicity and oxidative stress of canine mesenchymal stromal cells from adipose tissue in different culture passages.** *Pesquisa veterinária Brasileira 35(Supl.1):15-20.* Laboratório de Cirurgia Experimental, Pós-Graduação em Medicina Veterinária, Universidade Federal de Santa Maria, Avenida Roraima 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: aricia.sprada@hotmail.com

Stem cells in regenerative therapy have received attention from researchers in recent decades. The culture of these cells allows studies about their behavior and metabolism. Thus, cell culture is the basis for cell therapy and tissue engineering researches. A major concern regarding the use of cultivated stem cell in human or veterinary clinical routine is the risk of carcinogenesis. Cellular activities require a balanced redox state. However, when there is an imbalance in this state, oxidative stress occurs. Oxidative stress contributes to cytotoxicity, which may result in cell death or genomic alterations, favoring the development of cancer cells. The aim of this study was to determine whether there are differences in the behavior of cultured mesenchymal stem cells from canine adipose tissue according to its site of collection (omentum and subcutaneous) evaluating the rate of proliferation, viability, level of oxidative stress and cytotoxicity over six passages. For this experiment, two samples of adipose tissue from subcutaneous and omentum where taken from a female dog corpse, 13 years old, Pitbull. The results showed greater levels of oxidative stress in the first and last passages of both groups, favoring cytotoxicity and cell death.

INDEX TERMS: Stem cell, culture, passage, cell viability, free radicals.

RESUMO.- [**Toxicidade e estresse oxidativo das células mesenquimais estromais do tecido adiposo de cão em diferentes passagens de cultura.**] O uso de células-tronco como terapia regenerativa tem recebido atenção de pesquisadores nas últimas décadas. A possibilidade de cultivá-las permite o estudo de seu comportamento e metabolismo. Assim, o cultivo celular representa a base para pesquisas de terapia celular e engenharia de tecidos. Uma das principais preocupações relativa ao uso de células-tronco cultivas na rotina clínica humana ou veterinária é a reprogramação dessas células em tumores benignos ou malignos. As atividades celulares necessitam de um estado redox balanceado e quando há algum desequilíbrio nessas reações ocorre o estresse oxidativo. O quadro de estresse oxidativo contribui pra a citotoxicidade podendo resultar em morte celular e até mesmo em alterações genômicas e ocorrência de células cancerígenas. O objetivo deste trabalho foi verificar se há diferenças no comportamento de células-tronco mesenquimais estromais de tecido adiposo de cão de acordo com o seu tecido de coleta (omento e subcutâneo) avaliando o cultivo dessas células quanto a sua taxa de proliferação, viabilidade, estresse oxidativo e citotoxicidade ao longo de seis passagens. Para a execução deste experimento foram utilizadas duas amostras de tecido adiposo coletas do subcutâneo e omento do cadáver de um cão, fêmea, 13 anos de idade, da raça Pitbull. O cadáver era oriundo do Hospital Veterinário Universitário e sofreu eutanásia devido a complicações no seu quadro de cardiomiopatia. As duas amostras foram encaminhadas para o isolamento e cultura celular. Os resultados mostraram que a primeira e última passagem em ambos os grupos são as

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passagens mais submetidas ao estresse oxidativo ficando mais sujeitas à citotoxicidade.

TERMOS DE INDEXAÇÃO: Células-tronco, cultura, passagem, viabilidade celular, radicais livres.

INTRODUCTION

Stem cells (SC) are investigated since 1960 when Ernest A. Mac Culloch and James E. Till observed for the first time a certain undifferentiated bone marrow cell with the ability of self-renewal, self-generation and differentiation (Bonventre & Yang et al. 2003). Since then, stem cells are considered a promise in the field of regenerative medicine and many studies have been developed for their better understanding (Fortier 2005). SC can be classified according to their origin: embryonic stem cells (ESC) are derived from embryos, more specifically in the blastocyst stage, and are able to differentiate themselves in all cell types; adult stem cells (ASC) are found, virtually, in any tissue, however their differentiation is limited to their germ layer origin (Tuan et al. 2003).

The first studies with embryonic stem cells (EST) were performed in mice in 1981. Subsequently in 1998 a study with EST derived from a human embryo was reported and it raised ethical and religious issues (Thomson 1998). Due to the controversy involved in the use of EST, adult stem cells became an alternative to new research in cell therapy (Williams 2007). Within literature, ASC isolation has been reported in bone marrow, adipose tissue, kidney, liver, tendon, synovial membrane, amniotic fluid, placenta, umbilical cord and dental pulp (Fortier 2005, Fadel et al. 2011). Among those, adipose tissue was shown to be a reliable and easily accessible source (Kern et al. 2006).

The expansion and cultivation of adult and embryonic stem cells allowed significant progress in the fields of regenerative medicine and tissue engineering, as well as in the pharmaceutical industry, in the development and evaluation of new drugs (Sareen 2009). Nevertheless, particular issues must be clarified before cultivated stem cells are widely used in clinical routine. The major concern regarding the cultivation of adult and embryonic stem cells is the development of chromosomal abnormalities, possibly leading to loss of function or potentiating the risk of carcinogenesis. These complications may prevent the therapeutic application of stem cells and should be investigated carefully (Furlani 2009, Sareen 2009).

One of the main factors that could be related to chromosomal instability in cultured cells is oxidative stress (OS) (Riley et al. 2008). Some free radicals are physiologically generated during cellular metabolism and often play an important role as messengers and regulators in proliferation, differentiation and apoptosis processes (Dröge 2002, Halliwell 2007). However, when free radical and reactive oxygen species are not regulated by a cascade of antioxidants systems, oxidative stress occurs. OS is detrimental to cells causing ruptures of membranes, protein degradation and DNA damage, which may lead to cell death or genomic abnormality, also favoring the occurrence of carcinogenesis (Pujatlé et al. 2011).

The aim of this study was to evaluate the rate of proliferation, viability, oxidative stress and damage of mesenchymal stem cell from canine subcutaneous and omental adipose tissue over six passages.

MATERIALS AND METHOD

For this study was used the corpse of a 13-year-old female Pitbull from the Veterinary Hospital routine. The patient was euthanized due to complications of dilated cardiomyopathy and its death had no relation with this experiment. The owners donated the corpse for research. At about 30 minutes after euthanasia, the samples of adipose tissue were removed from subcutaneous (SUB) and omentum (OM). For the transport of the material previous to cells' isolation, the adipose tissue was separately in falcon tubes containing Hanks' balanced solution (1% streptomycin, 1% amphotericin B) to avoid contamination.

Inside the laminar flow, the fragments of adipose tissue from SUB and OM were transferred to two Petri plates. Making use of two scalpel blades each fat was sectioned into small fragments. These fragments were placed separately in 50mL falcon tube along with 2mg/mL collagenase type I in order to promote tissue degradation. The tubes remained in water bath at a temperature of 37°C for 40 minutes being manually shaken every 10 minutes. After this process, the tubes were referred to laminar flow again and complete DMEN medium (1% penicillin, 1% amphotericin B, 10% fetal bovine serum) was added to the solution at a ratio of two parts of medium to one part of collagenase to neutralize the collagenase, given its citotoxicity. The tubes were centrifuged at 600 G for ten minutes. The supernatant was discarded and a new complete medium was added to the cells pellet. The latter solution was transferred to a cell culture flask identified with SUB and OM according to the tissue origin. The flasks were maintained in a CO_2 incubator at 37º and 5% concentration of carbon dioxide. The first medium exchange was performed every 24 hours, the following exchanges were realized at every 72 hours. The cells were submitted to six passages and each passage length varied according to the cell growth. When the flask reached 90% of cell confluence, a new passage was made.

Each passage was performed after complete removal of the medium by adding 2.5mg/mL trypsin in the flask to remove cells from the plastic. The bottles were subsequently placed in the co_2 incubator for five minutes. At the end of this process a new complete medium was placed to inactivate trypsin. The solution from the bottle was then transferred to falcon tubes and centrifuged for five minutes at a speed of 1900 rpm. The precipitate obtained was allocated in the cell culture flask along with complete medium. For every passage the rates of viability, proliferation, cytotoxicity and oxidative stress were assessed.

The viability and proliferation rates were estimated by counting viable and non-viable cells in a Neubauer chamber. For this 20 µl of the cell solution was placed in a 1mL micro tube and it was added 20 µl of trypan blue dye at 0.4%. Cells were counted in a Neubauer chamber and the estimation was performed using the following formula: Number of cell per mL = Number of cells counted x dilution coefficient x 10^4. The number of cell counted was divided by the number of quadrants counted. The dilution coefficient was defined by the rate of cell suspension and the amont of dye (i.e., 1:1) which results in two.

Cytotoxicity was measured by means of the DNA fragmentation by fluorimetric Picogreen analyses. A small amount of DNA picogreen was added to the samples, which remained at rest in the dark for five minutes. Soon after, the free-DNA concentration was measured by the fluorometer excited at 485 nm wavelength and fluorescence intensity at 520 nm. Samples were prepared in quadruplicate.

The production of oxidative stress was evaluated using a non--fluorescent cell-permeating compound 2-70-dichlorofluorescein diacetate (DCFH-DA assay). DCFH-DA is hydrolysed by intracellular esterases to dichlorofluorescein (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. The samples of each culture passage were treated with DCFH--DA for 60 minutes at 37°C. The fluorescence was measured at en excitation wavelength of 485nm and emission of 520 nm. The calibration curve was performed with standard DCF and the level of reactive oxide species production was calculated as nmol DCF formed/mg protein.

The lipid peroxidation was determined by measuring thiobarbituric acid reactive species (TBARS). The cell solutions were centrifuged for 10 minutes at 2000rpm, the supernatant was discarded and saline solution (0.9% NaCl) was added, followed by two additional centrifugations at 2000rpm for 10 minutes. After that, the supernatant was discarded and 100ml Butylated hydroxytoluene (BHT 100 mM) and 500µl of trichloroacetic acid (TCA 20%) were added to the sample, followed by final centrifugation at 2000rpm for 5 minutes. Immediately after centrifugation, two samples with 900µl of the supernatant were mixed with a reaction medium containing thiobarbituric acid (TBA 0.8%). Then, the samples were incubated at 95°C for one hour. The absorbance was measured at a wavelength of 532nm in a spectrophotometer. The results were expressed in nmol MDA/10^6 cells.

The results of this experiment were analyzed with one-way analysis of variance followed by Tukey with GraphpadPrism 5.1 software. P-values <0.05 were considered statistically significant.

RESULTS

The adipose tissue samples from both sites studied in this experiment were considered easily accessible and presented sufficient amounts of fat for the isolation of mesenchymal stromal stem cells. The method used for cell isolation and culture was satisfactory in this study and there was no interference, such as contamination or differentiation of cells, during the experiment. From the first to the sixth passage, the subcutaneous and omentum cells remained with fusiform morphology - similar to fibroblast cells - and adhered to the bottom of the culture flask (Fig.1A and B).

Regarding the viability and proliferation of cells in each passage, there was no statistically significant difference (P=0.0283) between the SC and OM group. However, it was observed that cells derived from subcutaneous tissue needed less time to reach a greater number of cells/ml when compared with the omentum group, except for the third passage. The results are shown in Table 1.

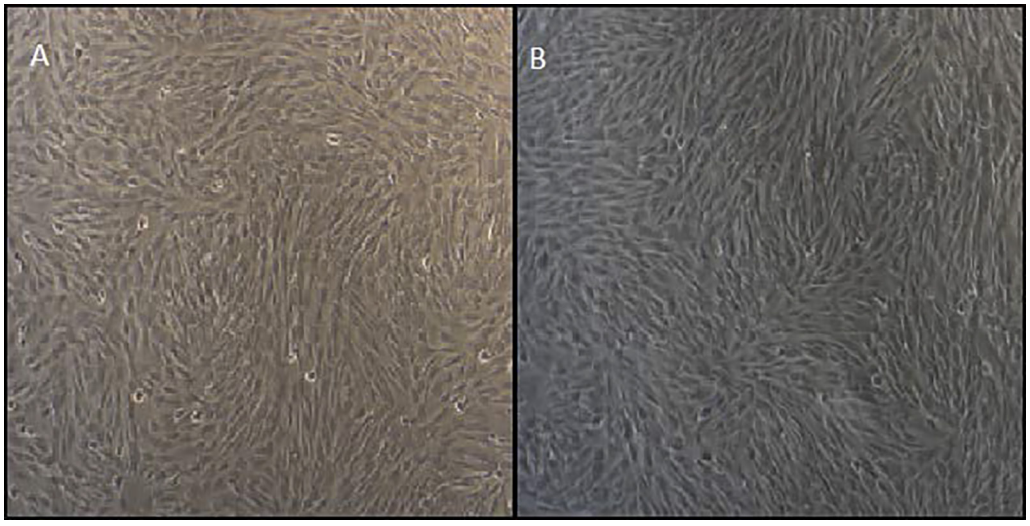

Fig.1. (**A**) Microscopic figure of cultured mesenchymal stromal stem cells from canine subcutaneous at sixth passage. Obj.20x. (**B**) Microscopic figure of cultured mesenchymal stromal stem cells from canine omentum at sixth passage. Obj.20x.

Table 1. Time (day) to reach 90% of cell confluence in culture cell flask (75cm2), number of cells per mL and viability percentage of each passage (P) of cultured mesenchymal stem cell from adipose tissue of canine subcutaneous and omentum

Group	Proliferation	Viability	Time
Subcutaneous	(P1) 2 x 106	95,6%	7 days
	(P2) 2,2 x 106	96,4%	6 days
	(P3) 3 x 106	98,1%	3 days
	(P4) 2,5 x 106	97%	6 days
	(P5) 2,2 x 106	97,8%	7 days
	(P6) 2 x 106	96,3%	5 days
Omentum	(P1) 5 x 105	95,6%	7 days
	(P2) 8 x 105	96,2%	13 days
	(P3) 1,5 x 106	98,9%	5 days
	(P4) 1 x 106	96,2%	5 days
	(P5) 1 x 106	97,5%	7 days
	(P6) 3 x 106	96,7%	7 days

It was possible to observe that the first passage presented the lowest rate of cell proliferation and viability percentage in both groups. The SC group reached its maximum cell number and viability in the third passage, with a slight decreased result in the subsequent passages. It is worth noting that it took 72 hours for the third passage to reach a 90% of cell confluence. The OM group reached its maximum cell proliferation in the last passage, but the best viability was found in the third passage. The shortest time (5 days) between passages in the OM group occurred in the passages number three and four.

The rate of reactive oxygen species (ROS) was determined by testing diclorofluoresceina diacetate (DCFH-DA),

in which it was observed an increase of ROS in the first passage of SC and OM group. The fifth passage of SC group showed lower rate of ROS, whereas in the OM group it was in the sixth passage. There was a statistically significant difference between groups in the fifth and sixth passages for ROS rates. The results are shown in the Table 2.

Lipid peroxidation was determined by the rate of thiobarbituric acid reactive species (TBARS). The assay demonstrated higher levels of peroxidation in the first passage of the SC group. Conversely, the OM group presented a greater percentage of TBARS in the last passage. For subcutaneous cells, the third passage presented the least peroxidation levels, whereas in the omentum group this trend was observed in the forth passage. Additional data is presented in Table 3.

The percentage of free DNA present in each passage of the culture was evaluated by the DNA fragmentation with Picogreen dye assay. Free DNA has presented its lowest results in the first passage of the SC group and in the fifth passage of the OM, as presented in Table 4. The free DNA greatest percentage occurred in the third and first passage in the SC and OM group, respectively.

Table 2. Reactive oxygen species percentage meausered by 2-70-dichlorofluorescein diacetato (DCFH-DA) assay over six passages of cultured mesenchymal stem cells from adipose tissue of canine subcutaneous and omentum

Passage	Subcutaneous	Omentum
P1	94.44427a	91.80975a
P2	80.90575a	73.74637a
P3	88.25431a	75.21389a
P4	87.45013a	75.19928a
P5	46.58894b	87.93534a
P6	81.99699a	5.727817c

Table 3. Lipid peroxidation percentage measured by thiobarbituric acid reactive species (TBARS) over six passages in cultured mesenchymal stem cells from adipose tissue of canine subcutaneous and omentum

Passage	Subcutaneous (%)	Omentum (%)
P1	50.33436a	34.99421a
P2	30.45267a	36.58749a
P3	30.06687a	42.4971a
P4	30.11831a	33.31402a
P5	31.73868a	40.23754a
P6	42.23251a	88.29664b

Where "a" there is no statistically difference, "b" there is statistically difference.

Table 4. Free DNA percentage measured by DNA fragmentation through fluorimetric Picogreen over six passages in cultured mesenchymal stem cells from adipose tissue of canine subcutaneous and omentum

Passage	Subcutaneous (%)	Omentum (%)
P1	22.43864a	81.9571c
P2	35.51861a	71.08434a
P3	92,31987b	51.11666a
P4	40.30087a	62.00411a
P5	33.37292a	49.42698a
P6	32.03484a	61.46048a

Where "a" there is no statistically difference, "b" there is statistically difference.

DISCUSSION

The omentum is an intra-abdominal adipose membrane that actively participates in the repair of injured abdominal organs by promoting neovascularization, lymphatic drainage and enhancement of wound healing (Ruffini 1992). In recent years, the omentum has been studied as a source of mesenchymal stem cells and it is known that these cells are involved in the healing of internal organs and tissue (García-Gómes et al. 2005). In this study, the omentum proved to be a suitable source of stem cells in dogs. This result goes against the findings of Neupane et al. (2008) that did not succeed in the isolation of stem cell from omentum and inguinal fat. The disadvantage of using omentum to isolate stem cells is the need of celiotomy to obtain the material. In this case the samples were collected from a corpse, thus opening the abdominal cavity was not a challenge at present. Moreover, after the abdominal wall incision, the omentum fat was easily located and quickly removed.

Stem cells from subcutaneous adipose tissue are already well discussed in literature. The extraction of subcutaneous fat can be achieved by liposuction or lipectomy and, in both situations, the stem cells are able to be undifferentiated for long periods in culture and have high capacity of differentiation (Danoviz et al. 2011). However, Heimburg et al. (2004) found that subcutaneous fat liposuctioned showed a better performance when compared to those removed by excision. In this present experiment, the fat was excised with Metzembaum scissors from the abdominal middle line, and demonstrated a high proliferation capacity in all passages.

Additionally, there was no statistical difference between OM and SC group. The SC group presented a progressive increase in cell number, reaching its peak in the third passage with a mild reduction in the subsequent passages. This cell growth behavior was also found by Colleoni (2009) and Patrício et al. (2013), who studied the kinetics of mesenchymal stem cells growth of adipose tissue in horses and dogs, respectively. Similarly, Patrício et al. (2013) reported that the highest peak of proliferation also occurred in passage three. The OM group showed the same pattern of cell growth, despite having a reduced proliferation rate. However, during the sixth passage there was a considerable peak of cell proliferation for the OM group, which was not perceived in the SC group.

The viability rates found in both groups did not statistically differ and were considered highly viable in this experiment. The smallest viability percentage in this experiment was 95.6% in the first passage of SC and OM groups. This was also line with former results of Patrício et al. (2013), in which the lowest rate of viability found was 96% in the first two passages. Conversely, a previous study involving subcutaneous fat from rabbits reported viability rates close to 100% in the first passages, decreasing in the third and reaching the lowest levels in eighth (Treichel 2014).

It is noteworthy that the amount of cells and high viability rates do not necessarily mean a safe therapeutic choice. Equally important, oxidative stress has been linked to several diseases and genomic altered cells in culture (Halliwell 2007, Duailibi 2012). The assay of DCFH-DA performed in

this paper suggests that the first passage of both groups presented higher rates of reactive oxygen species (ROS). However, in the subsequent passages the level of ROS did not vary significantly until the fifth passage of the SC and the sixth passage of OM group, which happened to be the lowest rate reported throughout the experiment. The authors correlate this simultaneous significant reduction of ROS in the last passage of the OM group with the highest peak of cell proliferation. This suggests that the low levels of free radicals may have influenced the increase of cells proliferation. This phenomenon did not occur in the SC group, but it is possible to argue that the decrease of ROS in SC group was not sufficient to promote a new peak of cell proliferation. Notice that the decreased level of ROS in the OM group was eight times higher than that observed in the SC group. In a similar study using adipose tissue from rabbits, the increase in ROS percentage was correlated with a lower percentage of cell viability (Treichel 2014). This relationship was also observed in the first passage of both groups in the present investigation, nonetheless it did not remain as a constant pattern throughout the passages.

When the lipid structures are damage by the action of free radicals, there is the ultimate formation of malondialdehyde (MDA) as a subproduct of polyunsaturated acids oxidation. The TBARS analysis identifies the presence of MDA and its increased levels are associated to damage in the cell membranes (Yang et al. 2008). At present, TBARS levels varied between groups. In the SC group the highest rate of lipid peroxidation occurred in the first passage, coinciding with the lowest percentage of viability and high level of reactive oxygen species. This happens due to an alteration in cell permeability caused when the membranes are damaged and, as a result, there is loss of selectivity, thus allowing input and output of nutrients and toxic molecule (Hershko 1989). In the following passages the percentage of lipid peroxidation varied slightly. The OM group presented higher TBARS levels, nevertheless, it was only in the last passage that it was noted a statistical difference when compared to SC group.

Furthermore, through analysis of data presented in Figure 4, where the percentage of free DNA was measured, it may be notice that lipid peroxidation relates to DNA levels. That is mainly because the presence of DNA outside the nucleus implied nuclear membrane damage (Caldecott 2008). At some points, it is also perceived a relationship between the high level of ROS and the presence of free DNA, as observed in the third passage of SC group and first pass of OM.

In this study, according to tests and assessment results the first and last passages are of particular interest in terms of viability, oxidative stress and cytotoxicity. Nikita et al. (2011) also showed similar results when evaluating the quality of mesenchymal cells from human bone marrow. The authors reported damaged cells in the initial and final stages of culture. Taking cell proliferation, viability, oxidative stress and cytotoxicity into account, the fifth passage of subcutaneous group displayed best results for those variables. In the OM group the best observed passages considering the mentioned variables were the fourth and fifth. In contrast with our results another study, in which the authors evaluated oxidative and DNA damages of stem cells from canine dental pulp, the first passages showed lower oxidative stress and genomic damage. From the forth passage, the levels of ROS and DNA damage began to increase considerably (Aramburú Junior 2013). This variation in results may be due to different methods of cultivation and tissue origin, suggesting that each research group should evaluate the quality of their own cultivated cells.

The data obtained here may help researchers in future studies proposed to understand the behavior of mesenchymal stromal stem cells from adipose tissue. This also may apply to conduct veterinarians who want to explore the regenerative potential of stem cells as therapy in clinical practice. Given the importance of discussing the positive and negative factors involved in cell therapy, the authors suggest that more research must be directed to the stability of cultured stem cells before clinical use. Amongst the methodological limitations related to the present study was lack of investigation of some biomarkers of viability, genetic mutation and cellular senescence.

It is important to note that, samples were collected of only one dog in order to avoid variables such as age, health and nutritional status, which may lead to variations during the harvesting of mesenchymal stem cells and oxidative stress levels. The accumulation of senescent cells in aged animals may result in decrease of cells' replicative life in vitro due to the shortening of telomeres. Besides that, the regulatory mechanisms of free radical production become ineffective over time (Teixeira & Guariento 2010). Nevertheless, to obtain sufficient samples to statistics analysis, all the tests were performed in quadruplicate.

CONCLUSIONS

Based on the methodology in which the experiment was conducted and the final data were obtained, it can be concluded that the subcutaneous adipose tissue and the omentum from dogs are a viable source of mesenchymal stem cells, even after 30 minutes postmortem with no significant differences between these tissues.

The first and last passage should be avoided in the treatment of clinical diseases, given the large exposure of these cells to stress and oxidative damage.

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