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CELL THERAPY WITH ADIPOSE TISSUE MESENCHYMAL STEM CELLS IMPROVES THE QUALITY OF THE GERMINAL EPITHELIUM IN *SWISS* MICE

[1,2]Juliano Rodrigues de Oliveira, [1,2]Silvia Cordeiro das Neves, [1,2]Giovana Corbucci Danti Rezende, [3]Luciana Nakao Odashiro Miiji, [1,4]Bruno Ivo Pelizaro, [1,2]Diego Duarte Marques de Oliveira, [1,2,4,5]Rodrigo Juliano Oliveira and *[1,2]Andréia Conceição Milan Brochado Antoniolli-Silva

[1]Centro de Estudos em Células-Tronco, Terapia Celular e Genética Toxicológica - CeTroGen, Hospital Universitário Maria Aparecida Pedrossian - HUMAP, Universidade Federal de Mato Grosso do Sul - UFMS, Campo Grande, Mato Grosso do Sul, Brasil

[2]Programa de Pós-Graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina Dr. Hélio Mandetta - FAMED, Universidade Federal de Mato Grosso do Sul - UFMS, Campo Grande, Mato Grosso do Sul, Brasil

[3]Lac Laboratório de Anatomia Patológica e Citopatologia, Campo Grande, Mato Grosso do Sul, Brasil.

[4]Programa de Pós-graduação em Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição - FACFAN, Universidade Federal de Mato Grosso do Sul - UFMS, Campo Grande, Mato Grosso do Sul, Brasil

[5]Programa de Pós-graduação em Genética e Biologia Molecular, Centro de Ciências Biológicas - CCB, Universidade Estadual de Londrina - UEL, Londrina, Paraná, Brasil

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ABSTRACT

Cancer is a disease that affects a large number of people and these are usually undergoing chemotherapy. One of the adverse effects of this therapy is testicular lesions with reduced fertility and one of the alternatives for the treatment of these cases is cell therapy. Thus, the present study evaluated a protocol for explant of mesenchymal stem cells from adipose tissue as well as evaluated the effects of cell therapy with mesenchymal stem cells from adipose tissue under the germinal epithelium of *Swiss* mice treated with cyclophosphamide. The experimental groups were: (I) Negative Control (NC) - the animals were treated with injection water; Positive Control (PC) - animals were treated with cyclophosphamide at a dose of 150 mg / kg; and positive control + mesenchymal stem cells (PC + MSC) - the animals were treated with cyclophosphamide at the dose of 150 mg / kg and transplanted with $1.0x10^6$ mesenchymal stem cells. The results indicated that transplantation of MSC does not alter the frequency of micronuclei in the peripheral blood. However, it improves the quality of the germinal epithelium and the quality score of the PC + MSC group is equal to that of the NC group.

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INTRODUCTION

Approximately 18 Millions cases of cancer are diagnosed annually (Bray *et al*., 2018). This is a public health problem that aggravate in young adults, who still want to start a family and want to have children, since chemotherapy treatments cause genetic damages in different cell lines and reproductive ones (Choy and Brannigan, 2013). Demage to DNA can lead to reduced fertility (Pinar *et al*., 2018) And / or predispose offspring to embryolethality (Torchinsk *et al*., 1995), abortion (Dong *et al*., 2), and embryo-fetal developmental abnormalities such as congenital malformations and delayed neuropsychomotor development (Dornelas *et al*., 2015; Rengasamy, 2017).

***Corresponding author:* Andréia Conceição Milan Brochado Antoniolli-Silva**

However, when considering cost-effectiveness, chemotherapy is still one of the most important ways to treat tumors (Oveissi *et al*., 2019). But, in general, chemotherapeutic agents have many adverse effects (Wolf *et al*., 2008; Oveissi *et al*., 2019). Cyclophosphamide is one of the most usually used chemotherapeutics (Vacchelli *et al*., 2014). And is indicated for the treatment of chronic lymphocytic leukemias, lymphomas and solid tumors (Iarc, 2012; Vacchelli *et al*., 2014). When patients are exposed to this chemotherapy, may occur nephrotoxicity (Pontes *et al*., 2014) and gonadotoxicity (Leroy *et al*., 2015). In this last case, the reduction in spermatic count is included (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Rezvanfar *et al*., 2008; Tripathi; Jena, 2008; Abiodun *et al*., 2016; Onaolapo *et al*., 2017), morphological changes (Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Delbès *et al*., 2010) damage to the genetic material of sperms. (Vaisheva *et al*., 2007; Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Delbès *et al*., 2010; Liu *et al*., 2014), increased number of aneuploidies (Barton *et al*., 2003), telomeric dysfunction (Liu *et al*., 2014), alteration in the protein profile of the nuclear matrix (Condrigton *et al*., 2007) and gene expression (Aguilar-Mahecha *et al*., 2002), the weight loss of the testicles, the epididymis (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Delbès *et al*., 2010) and prostate (Rezvanfar *et al*., 2008; Delbès *et al*., 2010), histopathological changes, with formation of vacuoles (Vaisheva *et al*., 2007; Tripathi and Jena, 2008; Delbès *et al*., 2010; Abiodun *et al*., 2016), the narrowing of the seminiferous tubules, loss of germ cells (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Tripathi and Jena, 2008; Delbès *et al*., 2010; Abiodun *et al*., 2016; Onaolopo *et al*., 2017), the edema (Rezvanfar *et al*., 2008) and increased interstitial space (Rezvanfar *et al*., 2008; Onaolopo *et al*., 2017), decrease at plasmatic level of luteinizing hormone (LH) (Elangovan *et al*., 2006; Abiodun *et al*., 2016), of follicle-stimulating hormone (FSH) (Abiodun *et al*., 2016) and testosterone (Elangovan *et al*., 2006; Rezvanfar *et al*., 2008; Abiodun *et al*., 2016; Onaolapo *et al*., 2017). It is also worth highlight that many times even after suspension of treatment, the testicles do not return to their normal cellular architecture and production. Thus, new therapies are studied in an attempt to improve the infertility caused by this anticancer agent. Among the different therapeutic proposals, cell therapy with mesenchymal stem cells, regardless of their origin (bone marrow, umbilical cord or adipose tissue), has shown positive effects on the recovery of structural damage of the germinal epithelium, testicular weight and fertility (Leu *et al*., 2007; Monfesi *et al*., 2013; Zhang *et al*., 2014; Yang *et al*., 2014; Chen *et al*., 2015; Cakici *et al*., 2013; Mehrabani *et al*., 2015). In front of the view, the present study evaluated an explant protocol of mesenchymal stem cells from adipose tissue as well as evaluated the effects of cell therapy with mesenchymal stem cells from adipose tissue under the germinal epithelium of Swiss mice treated with cyclophosphamide.

MATERIAL AND METHODS

Animals: Were used 40 *Mus musculus* mice of the *Swiss* species, with an average weight of 30 g, reproductive age provided by the Central Animal House of the Institute of Biosciences of the Federal University of Mato Grosso do Sul (INBIO / UFMS). The animals undergo an adaptation period of 7 days before starting the experiments. The animals were kept isolated in polypropylene boxes lined with brush under standard climatic conditions (with temperature maintaining around 22 ± 2ºC and relative humidity of 55 ± 10%) in ventilated rack Alesco®. The animals were fed commercial feed (Nuvital®) and filtered water ad libitum. The study was carried out in accordance with the Directives of the Universal Declaration of the Rights of the Animals and with the approval of the Ethics Committee on the Use of Animals of UFMS under protocol # 920/2017.

Experimental Design: The animals were divided into two lots: (I) composed of 10 donor females to obtain the mesenchymal stem cells(MSC) and (II) composed of 30 males randomly distributed in three experimental groups (n = 10): Negative Control (NC) - the animals were treated with injection water, in the proportion of 0.1mL / 10g body weight (b.w) intraperitoneally (ip) for 30 days, with intervals of five days between administrations; and two administrations of phosphate buffer solution (PBS), free of Ca^{+2} and Mag^{+2} at pH 7.4, intravenously (iv), with 10 day interval between them, starting 24 hours after the last application chemotherapy; Positive Control (PC) - animals were treated with cyclophosphamide (Genuxal - Baxter ®, CAS 2638B5063, Lot 6/138) at a dose of 150 mg / kg (wc; ip) for 30 days, with 5 - day interval between administrations (Drumond *et al*., 2011) and two administrations of PBS (iv), with 10-day interval, starting 24 hours after the last chemotherapy application; and Positive Control + Mesenchymal Stem Cells (PC + MSC) - the animals were treated with cyclophosphamide at a dose of 150 mg / kg (w / w) for 30 days, with 5-day interval between administrations, and two transplants of 1x106 MSC (iv), according to CAKICI *et al*. (2013), with modifications, with interval of 10 days and, starting 24 hours after the last application of the chemotherapeutic. After 60 days of experimentation, the males were submitted to weighing, euthanasia by cervical displacement and removal of the organs (heart, lungs, liver, spleen, kidneys and testicles).

Explant and Cultivation of Mesenchymal Stem Cells: The MSC were explanted according to the protocol of Hermeto *et al*. (2016), with modifications. Adipose tissue samples were obtained from the abdominal / inguinal region of adult female mice after euthanasia by cervical displacement. Briefly,was added half DMEM Low Glucose (DMEMLG) (Sigma-Aldrich®, batch SLBS0097V, catalog number D5523) was added in the proportion of 2 ml of medium for each gram of adipose tissue. Colagenase type A1 medium (Gibco™, batch 1879368, catalog number 17100017) was supplemented in the ratio of 2 mg of collagenase to each ml of DMEMLG medium. The biological material was incubated at 37°C and 5% CO_2 overnight. Inactivation of enzyme activity the following morning was done by addition of DMEMLG culture medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich®, batch SPBB2353V, catalog number F2561) in the same amount of medium used with previously mentioned collagenase. The material was then centrifuged at 300 g for 10 minutes. The cell pellet was resuspended and the material was then plated in 25 cm^2 cell culture bottles with DMEMLG medium supplemented with 10% FBS. The MSC were grown in a greenhouse at 37 ° C with 5% CO_2 until they reached about 80% confluency, when trypsinization was performed with 0.025% trypsin solution (Trypsin-EDTA, 0.025%, LCG Biotechnology, lot 11014AB, catalog number BR30042-01). Subsequently, the number of cells in the Neubauer chamber was counted and the flasks re fl ected with 75 cm^2 of culture area. This culture procedure was repeated until the 4th cell

passage, when the transplantation of the MSC was carried out at the rate of 1.0 x 10^6 cells / animal (Cakici *et al*., 2013).

Osteogenic, Adipogenic and Chondrogenic Differentiation: The flasks destined for the differentiation experiments, when they reached 80% confluency, were again trypsinized and the cells were seeded in 6 flasks of 25cm^2, at the concentration of 2x10^5 cells/flask (Urt-Filho *et al*., 2016). Three flasks of 25cm^2, used as controls, were maintained in DMEMLG medium and suplemented with 10% of FBS. For adipogenic, osteogenic and chondrogenic differentiation, cells were maintained for 24 hours in 10% FBS supplemented culture medium. Then, this was replaced by culture medium STEMPRO Adipogenic, Osteogenesis and Chondrogenesis Differentiation Kit (Invitrogen®) and maintained in culture for 14 days for adipogenic differentiation and for 21 days for osteogenic and chondrogenic cultures, with changes twice a week (Urt-Filho *et al*., 2016). For the confirmation of the adipogenic differentiation, after discarding the differentiating medium, the cells were fixed for 60 minutes at room temperature with 10% formaldehyde. The cells were then washed with 60% isopropanol and then incubated with *Oil Red O* (Sigma®) for 20 minutes at room temperature. The excess dye was removed by washing with distilled water. The differentiation was confirmed by accumulation of intracellular lipids on the 14th day (Pesarini *et al*., 2017; Schweich *et al*., 2017; Pesarini *et al*., 2018). For osteogenic differentiation, after discarding of the differentiating medium, the cells were fixed for 10 minutes at room temperature with 10% formaldehyde.

The cells were then washed 2 times with PBS and stained with *Alizarin Red* (Sigma®) for 5 minutes at room temperature. Excess dye was removed by distilled water washes. Osteogenic differentiation was demonstrated by the visualization of calcium deposits on day 21 (Pesarini *et al*., 2017; Schweich *et al*., 2017; Pesarini *et al*., 2018). During the cell culture process of the chondrogenic differentiation the cells were grouped forming a spheroid. At the end of the process this was collected by aspiration with a Pasteur pipette. The spheroid was fixed in 10% buffered formalin at room temperature and then subjected to the histological routine in the automated tissue processor TP09 TS Lupetec® according to manufacturer's instructions. Subsequently, the spheroid was cut into the Leica® RM2235 microtome in cuts with thickness 3 µm. The slides were stained with *Alcian Blue* using the EasyPath kit according to the manufacturer's specifications. The differentiation was confirmed by the presence of rich extracellular matrix of glycosaminoglycans on day 21 (Pesarini *et al*., 2017; Schweich *et al*., 2017; Pesarini *et al*., 2018).

Preparation and transplantation of MSC: In the 4th weighing the MSC were trypsinized, then the MSC was washed with PBS at exhaustion (homogenization in 5 mL of PBS followed by centrifugation at 300 g for 10 min, until the pellet was completely clear and the supernatant transparent) to the transplant. The animals of the MSC group were submmited inhalation anesthesia (Isoflurane - BioChimico®, Brazil, CAS / 401 4238-3, Lot / 007070). Then,1x10 ^{6} MSC were transplanted in PBS solution (pH 7.4) (i.v)., in a maximum volume of 100 µL, with the aid of a 24 G intravenous catheter (Solidor®, Brazil, Lot / 011606G). Repeated this procedure once more, with a 10-day interval between two applications (Cakici *et al*., 2013).

Peripheral Blood Micronucleus Assay: Was collected 20 µL of peripheral blood by caudal vein puncture and the sample was deposited on a slide previously stained with 20 µL Acridine Orange (1 mg / mL). The sample was then covered by a cover slip. Blood samples were collected 24 hours after Mesenchymal Stem Cell application. The material was stored in a freezer (-20 °C) for a period of 15 days. The presence of micronuclei inside erythrocytes was evaluated by fluorescence microscopy in the 40x magnification, with excitation filter 420-490nm and 520nm barrier filter. 2000 cells / animal were analyzed (Hayashi *et al*., 1990; Oliveira *et al*., 2009).

Histology: The testicles were fixed in Bouin's solution (75.0 mL of Pricular Acid, 25.0 mL of 40% formaldehyde and 5.0 mL of Acetic Acid) for 24 hours and then preserved in 70% ethyl alcohol. The organs were sectioned and included in paraffin and cut into a 5 micrometer microtome and stained with hematoxylin and eosin (HE). Histological analysis was performed according to Johnsen (1970) as well as the calculation of the total scores (can reach 1,000 points). Thus, all the seminiferous tubules of the histological slides were systematically evaluated and each of them received a score of 1 to 10 according to the following criteria: (I) Score 1 – no cells in tubular section; (II) Score 2 – no germ cells but Sertoli cells are presente; (III) Score 3 – spermatogonia are the only germ cells present; (IV) Score 4 – only few spermatocytes (<5) and no spermatids or spermatozoa present; (V) Score 5 – no spermatozoa, no spermatids but several or many spermatocytes present; (VI) Score 6 – no spermatozoa and only few spermatids (<5-10) present; (VII) Score 7 – no spermatozoa but many spermatids present; (VIII) Score 8 – only few spermatozoa (<5-10) present in section; (IX) Score 9 – many spermatozoa present but germinal epithelium disorganized with marled sloughing or obliteration of lumen; (X) Score 10 – complete spermatogenesis with many spermatozoa (spermatozoa are here defined as cells having achieved the small head form of the spermatozoon) and germinal epithelium organized in a regular thickness leaving an open lumen). In order to calculate a mean score the number of tubuli recorded at each score is multiplied with the score and the sum of all 10 multiplications is divided by the total number of tubuli recorded. For the sake of clarity the numbers of tubuli found at each step are in this paper shown in per cent of the number of tubuli. This is not done in the routine work. For some purpose the cumulative percentagens (10 + 9, 10 + 9 + 8 etc.) are formed and these may be multiplied by total testis volume to yield figures for spermatogenesis in absolute terms. In order to calculate a mean score (MS) the number of tubuli recorded at each score is multiplied with the score and the sum of all 10 multiplications is divided by the total number of tubuli recorded. For the sake of clarity the numbers of tubuli found at each step are in this paper shown in per cent of the number of tubuli. This is not done in the routine work. For some purpose the cumulative percentagens (10 + 9, 10 + 9 + 8 etc.) are formed and these may be multiplied by total testis volume to yield figures for spermatogenesis in absolute terms (Johnsen, 1970).

Statistical analysis: Data were presented on average ± Standard Error of Mean. Statistical analysis was performed according to the data distribution. For data with parametric distribution, the ANOVA / Tukey test was used and for the non-parametric data the Kruskal-Wallis / Dunn test was used. The level of significance was stablish $p < 0.05$.

RESULTS

Expansion of Mesenchymal Stem Cells: After isolation of the MSC, they were expanded in culture flasks with successive tests.

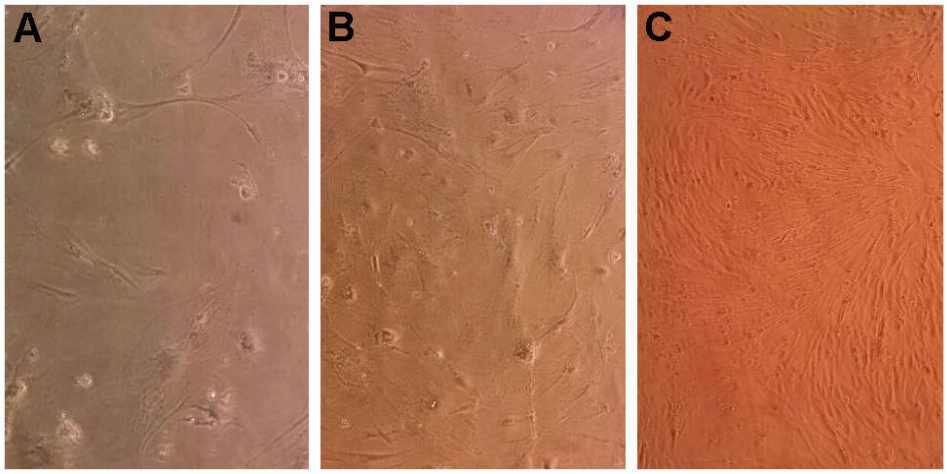

Figure 1. Aspects of growing crops: A - Initial phase cultivation (20 days), 400x increase; B - Culture with confluence close to 50% (7 days after the first passage - 1 peal), increase of 400x; C - Culture at confluence greater than 80%, increase of 100x

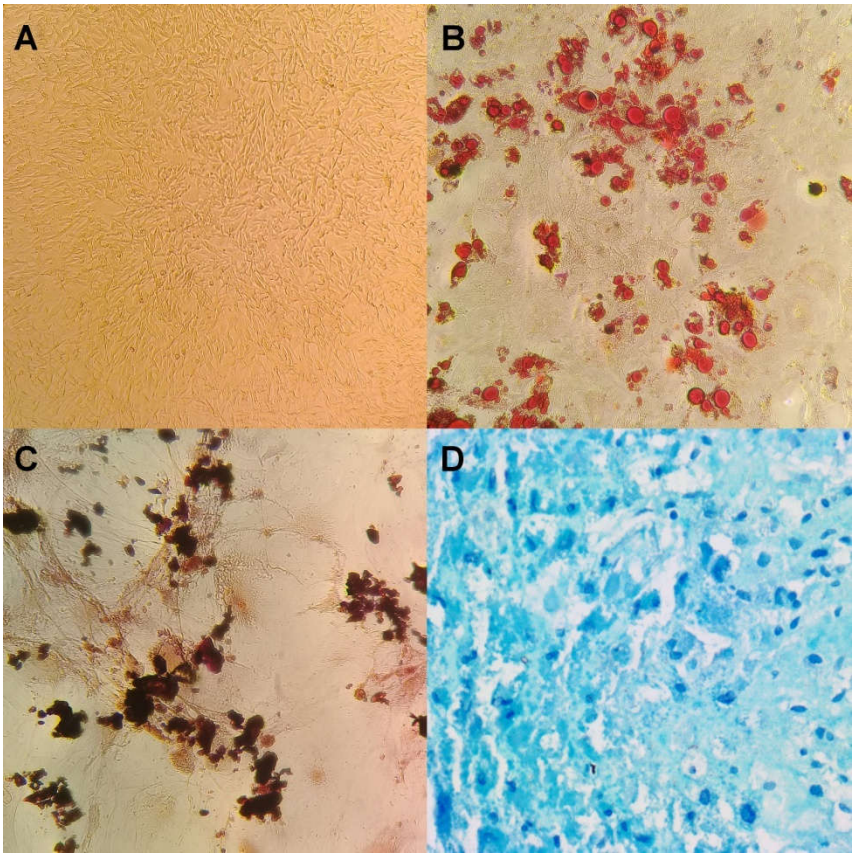

Figure 2. MSC photomicrographs: (A) undifferentiated culture, (B) culture in adipogenic differentiation with lipid vacuoles stained by *Oil red O*, (C) culture in osteogenic differentiation with calcium deposits stained by *Alizarin Red*, (D) Culture in chondrogenic differentiation with rich extracellular matrix of glycosaminoglycans stained by Alcian Blue. 400x magnification

Viability was observed in all passages and only cultures with viability greater than 95% were maintained. In Figure 1 the aspects of the culture used in the experiments can be observed.

Mesenchymal Stem Cells Differentiate in Osteogenic, Adipogenic and Chondrogenic Cells in vitro: The confirmation that the cells in culture really were MSC was through the adipogenic, osteogenic and chondrogenic differentiations where it was possible to identify the accumulation of lipids stained with *Oil red O*, calcium deposits stained with *Alizarin Red* and the rich extracellular matrix of glycosaminoglycans stained with Alcian Blue, respectively (Figure 2).

Biometric Parameters: The initial weight of the different experimental groups did not present statistical difference ($p > 0.05$). However, the animals in the NC group presented higher final weight and lower weight loss when compared to the animals of the CP and CP + MSC groups ($p < 0.05$) (Figure 3) The absolute weights of heart, lung and spleen did not show statistical difference ($p > 0.05$). The weights of the liver and kidneys of the animals of the PC + MSC group were lower ($p < 0.05$) than those of the NC and PC groups. The absolute

weight of the tests of the mice of the NC group was higher (p <0.05) than those of the other experimental groups (PC and PC + MSC) (Figure 4A).

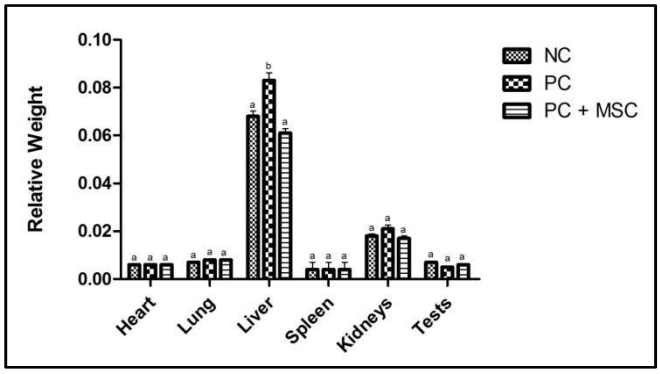

Figure 3. Biometric parameters of Swiss male mice treated with the chemotherapeutic cyclophosphamide and whether or not submitted to mesenchymal stem cell transplantation. Negative Control (NC) - the animals were treated with injection water; Positive Control (PC) - animals were treated with cyclophosphamide at a dose of 150 mg / kg; and positive control + mesenchymal stem cells (PC + MSC) - the animals were treated with cyclophosphamide at the dose of 150 mg / kg and transplanted with 1.0x106 mesenchymal stem cells. Different letters indicate statistically significant differences (Test: 1ANOVA / Tukey; 2Kruskall-Wallis/Dunn, p<0,05)

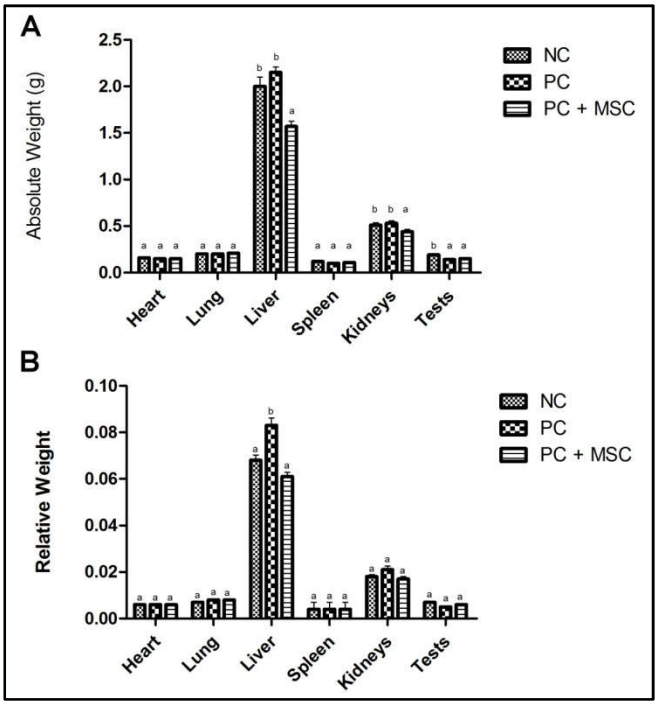

Figure 4. Biometric parameters of *Swiss* male mice treated with the chemotherapeutic cyclophosphamide and with or without mesenchymal stem cell transplantation. Negative Control (NC) - the animals were treated with injected with water; Positive Control (PC) - animals were treated with cyclophosphamide at a dose of 150 mg / kg; and positive control + mesenchymal stem cells (PC + MSC) - the animals were treated with cyclophosphamide at the dose of 150 mg / kg and transplanted with $1.0x10^6$ mesenchymal stem cells. Different letters of statistically significant differences (Test: ANOVA / Tukey; p0.05).

The relative weights of heart, lung, kidneys, spleen and testes did not present statistical difference between groups (p> 0.05). Liver weight increased (p <0.05) in the PC group in relation to the others (Figure 4B).

Evaluation of genetic material integrity: The peripheral blood micronucleus frequency was higher (p> 0.05) in the PC and PC + MSC groups, both treated with cyclophosphamide, compared to the NC group (Figure 5).

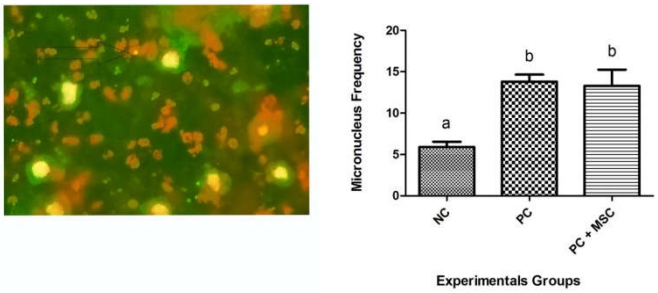

Figure 5. Micronucleus frequency of *Swiss* male mice treated with the chemotherapy cyclophosphamide and with or without mesenchymal stem cell transplantation. Negative Control (NC) - the fish were injected with water; Positive Control (PC) - animals were treated with cyclophosphamide at a dose of 150 mg / kg; and positive control + mesenchymal stem cells (PC + MSC) - the animals were treated with cyclophosphamide at the dose of 150 mg / kg and transplanted with $1.0x10^6$ mesenchymal stem cells. Different letters of statistically significant differences (Test: ANOVA / Tukey; p0,05)

Table 1. Testicular quality score of *Swiss* male mice treated with the chemotherapeutic cyclophosphamide and whether or not submitted to mesenchymal stem cell transplantation

Experimental Group	Testicular Lesion Score
NC	$981,73±3,84^a$
PC	$831,90±50,22^b$
PC + MSC	$953,33±11,34^a$

Legend: Negative Control (NC) - the animals were treated with injection water; Positive Control (PC) - animals were treated with cyclophosphamide at a dose of 150 mg / kg; and positive control + mesenchymal stem cells (PC + MSC) - the animals were treated with cyclophosphamide at the dose of 150 mg / kg and transplanted with $1.0x10^6$ mesenchymal stem cells. Different letters indicate statistically significant differences (Test: ANOVA / Tukey; p0.05).

Histopathological Analysis: Histopathological analysis showed that the administration of cyclophosphamide reduced (p <0.05) the quality score of the seminiferous tubules from 981.73 ± 3.84 in NC to 831.90 ± 50.22 in PC. This shows a loss of 15.26 percentage points in the quality of the germinal epithelium. When MSC was transplanted with cyclophosphamide, the score increased from 831.90 ± 50.22 in PC to 953.33 ± 11.34 in the PC + MSC group. This shows an improvement of 14.60 percentage points and thus NC and PC + MSC are statistically similar. In Figure 6 it is possible to observe seminiferous tubules of the animals of the experimental groups. In the NC and PC + MSC groups normal seminiferous tubules are observed and the presence of secondary spermatids (Figures A, D and C, F). In the group treated with cyclophosphamide alone the seminiferous tubules are altered and not sperm are observed (Figure B). Figure E shows the presence of primary and secondary spermatocytes, but no sperm are observed (Figure E).

DISCUSSION

Traditional medicine undergoes major transformations and one of the promises of medicine of the future is regenerative medicine. This is the possibility of providing for the

regeneration of the organism at the cellular and / or tissue level (Mason; Dunnill, 2008; Acero, 2015). This new paradigm of medicine will provoke transformations in science, therapeutics and public health (Morrison, 2012; Acero, 2015) and revolutions will be generated in the search for homeostasis. For this to materialize, there is a need to accelerate the transfer of knowledge from laboratories (from basic research) to clinical applications; deepen the clinical observations in search of a better understanding of the processes of disease (pathophysiology), therapeutics and cure; in addition to applying biotechnological innovations to the population with responsibility. These are the precepts that define translational medicine, according to Luz (2018), with modifications. Based on these concepts the present work has proposed to test a protocol of explant and culture of adipose tissue stem cells in a preclinical model with the intention of translating it later for clinical studies. The protocol described in this study was adapted from the literature and showed to be efficient in isolating the MSC from small fragments of adipose tissue, at a low cost, in a short time, without many steps (simplified protocol), without the need for large equipment and therefore has potential for clinical application. Another important fact to be informed is that the cells used in the present study were actually MSC because they differed in adipocytes, osteocytes and chondrocytes. According to the specialized literature, these three differentiations indicate that the cells are MSC (Nargesi *et al*., 2017; Patschan *et al*., 2018).

In relation to CTM transplantation, the endovenous route was chosen. This is easily accessible in both preclinical and clinical models and, so, would be easily adhered to by the medical class. There is an extensive discussion in the literature about the different pathways of transplantation of MSC and some authors cite and make the option of placing the cells *in situ* (in the lesion to be repaired). This occurs in kidney injury (Monteiro *et al*., 2018), heart lesions (Woudstra *et al*., 2016; Yamamoto *et al*., 2018), hepatic (Eom *et al*., 2015). However, other authors to treat these same types of lesions, use the endovenous way. (Nargesi *et al*., 2017; Sun *et al*., 2018; Zheng *et al*., 2019). In relation to the testis, in particular, there are reports of used in endovenous way (Yang *et al*., 2014) and intratesticular route (Kadam *et al*., 2018; Meligy *et al*., 2019) both for preclinical (Yang *et al*., 2014; Kadam *et al*., 2018; Meligy *et al*., 2019) and clinical models (Smith *et al*., 2014). However, our preference for the intravenous route is due to the ease of transplantation and, possibly, less resistance by the patient to undergo the procedure. Our studies evaluated the frequency of DNA damages induced by cyclophosphamide in peripheral blood. This is an indirect measure of DNA damage that can also occur in germ cells since cyclophosphamide is an indirectly acting chemotherapeutic and, after metabolization in the liver, by hydroxylation by CYP2B enzyme, releases 4-hydroxycyclophosphamide and its tautomer, aldofosfamide (Colvin *et al*., 1976; Fenselau *et al*., 1977; Sladek, 1988; Zhang *et al*., 2006; Veal *et al*., 2016).

Once in contact with the cells, aldophosphamide undergoes cleavage and releases phosphoramide mustard and acrolein mustard being the first responsible for the antineoplastic action and, therefore, causing DNA damage (Colvin *et al*., 1976; Fenselau *et al*., 1977; Sladek, 1988; Zhang *et al*., 2006; Veal *et al*., 2016). Our results indicated that MSC transplantation does not modify the pattern of DNA damage (chromosomal damage) accounted for by the micronucleus assay. The increase of frequency of micronuclei in general is associated with worse prognosis and development of chronic diseases, (Roth *et al*., 2008), tissue damage (Wultsch *et al*., 2014) and cancer involvement (Ravegnini *et al*., 2015). As a major contribution of this study, the results demonstrated that cell therapy with MSC was able to improve the quality of the germinal epithelium. As expected, cyclophosphamide reduced the quality of the germinal epithelium, which is consistent with reduced fertility and this is due to the histopathological changes with the formation of vacuoles (Vaisheva *et al*., 2007; Tripathi and Jena, 2008; Delbès *et al*., 2010; Abiodun *et al*., 2016), narrowing of the seminiferous tubules, loss of germ cells (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Tripathi and Jena, 2008; Delbès *et al*., 2010; Abiodun *et al*., 2016; Onaolopo *et al*., 2017), edema (Rezvanfar *et al*., 2008) and increase of interstitial space (Rezvanfar *et al*., 2008; Onaolopo *et al*., 2017). In addition, the literature cites a decrease in sperm count (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Abiodun *et al*., 2016; Onaolapo *et al*., 2017), morphological changes of spermatozoa (Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Delbès *et al*., 2010) and decreased weight of the testicles and epididymis (Elangovan *et al*., 2006; Vaisheva *et al*., 2007; Rezvanfar *et al*., 2008; Tripathi and Jena, 2008; Delbès *et al*., 2010). In our studies, we emphasize the reduction of the quality of the germinal epithelium according to Johnsen (1970), highlighting the reduction of cell depletion of the seminiferous tubules and absence of spermatozoa.

On the other hand, treated mice that had cyclophosphamide but received cell therapy with MSC had the germinal epithelium and the seminiferous tubules reestablished to patterns very close to those observed in the negative control group. This assertion is confirmed by the absence of significant differences between the NC and PC + MSC groups. Thus, it can be inferred that the MSC were able to reestablish the quality of the germinal epithelium and this may correspond to the reestablishment of fertility. Our results corroborate the studies of Lue *et al*. (2007), Monfesi *et al*. (2013), Zhang *et al*. (2014), Yang *et al*. (2014), Chen *et al*. (2015), Cakici *et al*. (2013) and Mehrabani *et al*. (2015) who reported recovery of testicular tissue. It is believed that these improvements in the germinal epithelium can occur by two mechanisms: (I) regeneration and (II) immunomodulation by paracrine effect (Vieira *et al*., 2019). In the first case it is believed that the MSC administered, either directly in the lesion or by the intravenous route, has the capacity to recognize the lesion and adhere in the place to be regeneration. In this case MSC undergo transdifferentiation and begin to compose the tissue of the organ assisting in tissue recovery (Little *et al*, (2018)) and physiological to achieve homeostasis. In the second case, MSC is believed to migrate to the injury site and produce endogenous repair factors, anti-inflammatory and antiapoptotic factors that aid in the recovery of the injured organ / tissue. However, without integrating MSC into the tissue matrix (Souza *et al*., 2010).

Regarding the biometric parameters, it was observed that the cyclophosphamide, regardless of the transplantation of the MSC caused a reduction of the final weight and, therefore, was responsible for greater weight loss. This fact was already expected due to the toxicity of this chemotherapeutic and this fact was also reported by Michael *et al*. (2007). Regarding the variations of the absolute weights of the organs that can indicate toxicity (Michael *et al*., 2007), changes were observed in liver, kidneys and testicles. However, in the evaluation of

the relative weight (where the weight of the organ is corrected by the weight of the animal) only differences between the PC group and the NC and PC + MSC groups were observed (the latter two being statistically similar). The increase of the liver observed in the PC group may have occurred due to the need to metabolize cyclophosphamide that is activated in this organ (Colvin *et al*., 1976; Michael *et al*., 2007; Veal *et al*., 2016). This metabolism may require hypertrophy and organ hyperplasia (Michael *et al*., 2007). In addition, the organ may present an inflammatory process characteristic of the great activity and hepatomegaly induced by cyclophosphamide (Faro *et al*., 2009). In view of the above, it is considered that the greatest contribution of this article to the area is to present a protocol of explant of mesenchymal stem cells of viable adipose tissue fragment to be translated for clinical researches due to its low cost and simplicity, and to prove, histologically, that cell therapy improves the quality of the germinal epithelium in a preclinical model.

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