



Cell culture and variations in the comet assay do not affect the genomic integrity of adipose-derived stem cells

Luana Bernardi¹, Laynna de Carvalho Schweich-Adami, Edwin José Torres de Oliveira, Adivânio Baranoski, Rodrigo Juliano Oliveira and Andreia Conceição Milan Brochado Antonioli da Silva

Faculdade de Medicina, Universidade Federal do Mato Grosso do Sul, Av. Costa e Silva, s/n, 79070-900, Bairro Universitário, Campo Grande, Mato Grosso do Sul, Brazil. *Author for correspondence. E-mail: bernardi1284@gmail.com

ABSTRACT. The application of quality control tests, such as the comet assay, are essential when adipose-derived stem cells are cultured for therapeutic purposes. However, the steps involved in the development of this assay should be investigated, in order to reduce their influence on genomic damage in cells. The study aimed to evaluate if the cell culture process causes DNA damage, and if variations in the lysis time and pH of the electrophoresis buffer interfere in the genotoxicity results. Four different comet assay protocols were evaluated, and the effects of lysis time and pH conditions of the electrophoresis buffer solution were stated as follows: 2 hours and pH 12; 24 hours and pH 12; 2 hours and pH ≥ 13 and 24 hours and pH ≥ 13 . The tail moment was analyzed, and results indicated that at the time cells were detached from the flasks, there was little damage to the DNA in the adipose-derived stem cells, which was confirmed by evaluation of the expression of mRNA genes involved in damage and repair processes of genetic material. Also, the tail moment values did not show significant differences among the four evaluated protocols ($p < 0.05$), with no indication of damage when compared to the positive control ($p < 0.05$). Thus, any of the tested protocols can be applied in genotoxicity tests with adipose-derived stem cells, without causing damage to them.

Keywords: DNA damage; gene expression; polymerase chain reaction.

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Introduction

Regenerative medicine and cell therapy are the new promises to treat different diseases for which individuals do not respond adequately to conservative treatments of conventional medicine (Nawab, Bhare, Bommarito, Mufti, & Naeem, 2019). Therapies based on adipose-derived stem cells (ASCs) are increasingly documented in the literature and present results that indicate their use with efficiency and safety (Ntege, Sunami, & Shimizu, 2020). Crohn's disease (Panés et al., 2018), osteoarthritis (Lu et al., 2019), stress urinary incontinence (Choi, Kim, Yang, Suh, & Kwon, 2016), and spinal cord injury (Sahara, Santoro, & Chien, 2015) are among the diseases that can be treated by ACSs.

A *sine qua non* condition for the use of mesenchymal stem cells is that they must be subjected to a cell culture process (*in vitro* expansion) to achieve sufficient quantity for transplantation (Wu et al., 2017). The cell culture process require extensive manipulation, which can alter the biological characteristics of the cell, such as the differentiation state, the potential for proliferation and metabolic activities, for example (Agência Nacional de Vigilância Sanitária [ANVISA], 2020). Expansion safety remains a major concern when it precedes clinical application (Neri et al., 2013). It is already proven that until the 10th passage the ADSCs maintain their quality (Adami et al., 2021), however in longer cultures (passage ≥ 30), changes at the molecular level can be observed as the culture time is prolonged (Geißler et al., 2012). In addition, there is a decline in the potential for differentiation, morphological alteration, increased susceptibility to senescence, reduced migration potential and reduced antioxidant defenses (Geißler et al., 2012). According to Alves et al. (2010), the increase of oxidative stress levels generates progressive accumulation of DNA damage. Consequently, quality control tests such as genetic stability tests are necessary when ADSCs are cultured for therapeutic purposes (Sensebé, Gadelorge, & Cappellesso, 2013).

One of the most sensitive assays for detecting genomic instability is the comet assay, because it is capable of detecting low levels of DNA damage (Tice et al., 2000). According to Hermeto et al. (2015), the pH of the electrophoresis buffer can alter the frequency of damage (tail intensity and tail moment) in ADSCs cultured from rabbit. Regarding the pH, the values may vary among different protocols, with solutions with pH equal to 12 and pH ≥ 13 . Likewise, the time that cells remain lysed during the assay also varies among protocols (Tice et al., 2000; Villela et al., 2007). Thus, in front of all this variation in the application of the comet assay protocol among

the laboratories (Møller et al., 2020), Møller (2018) raised the need to investigate the steps involved in the development of this assay, in order to reduce their influence on the genetic damage observed in cells.

Therefore, the present study aimed to assess if the cell culture process of ADSCs causes DNA damage, and if the variations in the lysis time and pH of the electrophoresis buffer could interfere in the results of genotoxicity.

Material and methods

Sampling data

Three healthy female individuals, between 20 and 50 years, participated in this study, with written informed consent. They underwent elective liposuction procedures at the Plastic Surgery outpatient clinic in *Hospital da Plástica de Mato Grosso do Sul*, Campo Grande, MS. The study was submitted to the Research Ethics Committee Involving Human Beings at the *Universidade Federal do Mato Grosso do Sul* and approved under process number 3.070.576.

Liposuction procedures, isolation and cultivation of adipose-derived stem cells

The adipose tissue was isolated by the abdominal liposuction procedure performed according to Pesarini et al. (2017). For the processing of each of the three liposuction samples and isolation of ADSCs, collagenase was used for enzymatic digestion, as described by Markarian et al. (2014) with modifications. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM Sigma®; catalog number D5523-10L) - low glucose, 10 mM HEPES supplemented with 10% fetal bovine serum (Gibco™ catalog number 10091148) and 1% antibiotic (Penicillin/Streptomycin, Sigma®, A5955). ADSCs until 5th passage were used in the experiments, because according to Adami et al. (2021), this is the limit passage to use the ADSCs to human application, since cells do not suffer any morphological change until this moment. Thus, this conception was also adopted in our laboratory routine.

Characterization of adipose-derived stem cells

The cell surface markers of the extracted cells were examined using a flow cytometer (FACScalibur - Becton Dickinson, San Diego, CA). A total of 2.5×10^5 ADSCs in 5th passage were dissociated in trypsin, centrifuged and incubated during 30 min. at 4°C using the following antibody cell markers: CD105, CD90, MHC II and CD45 (Pharmingen BD, San DIEGO, CA. USA). The cells were analyzed in 10^4 events using the 488nm laser FACScalibur (Becton Dickinson, San Diego, CA) in the CellQuest software. The MDI 2.8 software was used to generate the histograms.

Adipogenic, osteogenic and chondrogenic differentiation assays

For these assays, 1×10^5 cells/well (3rd passage) were seeded in 6-well plates. After cell adhesion (24 hours), the DMEM was discarded, and specific media (adipogenic, chondrogenic and osteogenic) were added for differentiation, as described by Schweich et al. (2017). Their multipotency was also analyzed. To confirm the differentiations, Oil Red (adipogenic), Red Alizarin (osteogenic) and Alcian Blue (chondrogenic) dyes were used. The procedures were performed in triplicates.

Comet assay

The ADSCs (5th passage) were trypsinized as previously described (Markarian et al., 2014), and the comet test was performed according to Singh, McCoy, Tice, and Schneider (1988), with modifications proposed by Navarro et al. (2014). An aliquot (40 µL) was resuspended in low-melting 0.75% (120 µL) agarose (Invitrogen Co, Carlsbad, CA) at 37°C, and distributed on slides previously coated with normal melting point agarose 1.5% (Invitrogen Co, Carlsbad, CA), covered with a coverslip. The slides were immediately placed in the refrigerator (4°C) for 20 minutes. After the solidification of the agarose, the coverslips were removed and the slides were immersed in a lysis solution (2.25 M NaCl, 90 mM EDTA, 9 mM Tris-HCl, 10% DMSO and 1% Triton X-100), being kept in the dark at 4°C, for 2 or 24 hours, according to each protocol. After cell lysis, the slides remained in an electrophoresis vat, covered with alkaline buffer (pH 12 or ≥ 13 , specific to each protocol), for 20 minutes (NaOH 10N, EDTA titriplex 200 mM) for DNA denaturation. Electrophoresis (300 mA and 25 V) was performed with the same buffer for 20 minutes more. Subsequently, the slides were neutralized with 0.4 M Tris, pH 7.5, for 15 minutes (3 times of 5 minutes each), and fixed in absolute ethanol for 10 minutes, then, left to dry overnight at room temperature and stored until analysis.

The material was stained (100 µl of ethidium bromide, 20 mg mL⁻¹) and the nucleoids were photographed under a fluorescence microscope (Leica, DMi8), with 200X magnification. Subsequently, more than 200 nucleoids were analyzed per repetition by the CometScore 2.0.0.38 TriTek program (CometScore Comet

Scoring Software, 2004), and the tail moment parameter (% DNA in the tail multiplied by the length of the tail) was evaluated (Livak & Schmittgen, 2001).

For the different study groups, the lysis solution was the same. However, the lysis time varied (2 hours or 24 hours). To detect DNA damage, for each of these times, two types of electrophoresis buffer were evaluated: one with pH 12 (Vilela et al., 2007) and the other with pH ≥ 13 (Tice et al., 2000). Thus, the analyzed groups were: Protocol 1 - lysis time of 2 hours and electrophoresis buffer solution with pH 12; Protocol 2 - 24-hour lysis time and pH 12 electrophoresis buffer solution; Protocol 3 - lysis time of 2 hours and electrophoresis buffer solution with pH ≥ 13 ; and Protocol 4 - 24-hour lysis time and electrophoresis buffer solution with pH ≥ 13 (Figure 1).

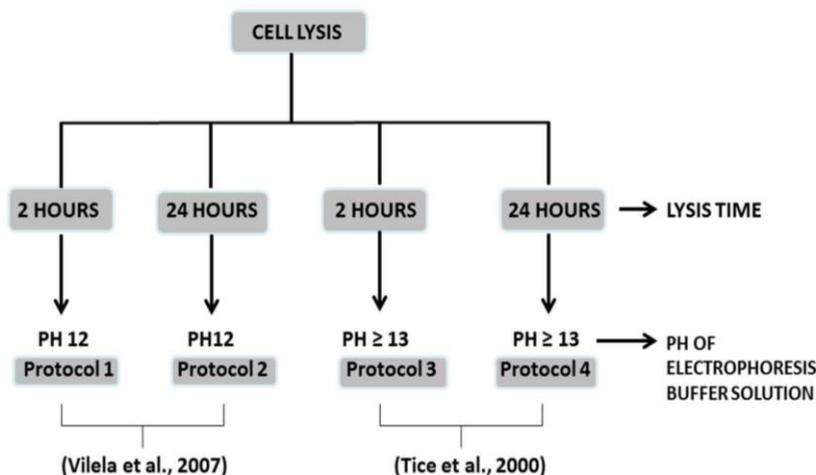


Figure 1. Tested protocols for detecting DNA damage in adipose-derived stem cells.

For validation of the test, three independent samples of ADSCs were treated with doxorubicin (DXR) (Libbs®, catalog number 734079) at a concentration of 5 μM and pH 13 for 3 hours. The slides of this positive control were submitted to lysis and electrophoresis together with the slides of the study protocols.

Gene expression

For the evaluation of gene expression, 2×10^5 ADSCs/well (5th passage), were seeded in 6-well cultivation plates, and freshly extracted ADSCs (passage 0) were used as a negative control. The cells were collected and the total RNA was extracted by an in-house method. For this purpose, it was used a lysis buffer based on guanidine thiocyanate (Guanidine thiocyanate 5 moles L^{-1} , Tris Hydrochloride (HCL) 11.2 g L^{-1} (pH 6.4), ethylenediaminetetraacetic acid (EDTA) NaOH 7, 43 g L^{-1} , Triton X-100 7.8 mL). For each 200 μL of cell suspension, 200 μL of lysis buffer (1: 1) was added. After homogenization during 10 minutes, 50 μL of magnetic bead (Nuclisens®, easyMag®, bioMérieux®, catalog number 280133) was added, with subsequent rest for 10 minutes. Impurities (cell debris) were removed by two washes with lysis buffer (2.5M NaCl, 100 mM EDTA titriplex, 10 mM Tris (pH 10), 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)). The excess of guanidine thiocyanate and salts were removed with two washes of 70% alcohol. The beads were washed with PA acetone, heated for one minute at 60°C in Dry Bath Heat & Cool Control™ dry bath equipment (DB-HC, Loccus®), and the genetic material was eluted with 30 μL of TE solution (NucliSENS®, catalog number 280132). After extraction, the treatment was performed with RQ1 RNase-Free DNase (Promega®, catalog number M6101,) according to the manufacturer's specifications. After the DNA degradation step, the quality of the extracted material was analyzed using: (I) the A260/A280 and A230/A260 quotient in a NanoVue™ Plus spectrophotometer (GE Healthcare - Life Sciences®); and (II) the denaturing agarose gel integrity (1%). Only samples with a quotient between 1.8 to 2.1 were used for the synthesis of complementary DNA (cDNA).

The cDNA synthesis was performed in a T100™ thermocycler (Thermal Cycler, Bio-Rad™) using 250 ng of RNA in the GoScript™ Reverse Transcriptase kit (Promega®, catalog number A5001) following the manufacturer's protocol, in which 5 μL GoScript™ 5X Buffer, 3.8 mM MgCl_2 , 0.5 mM dNTPs, 20 RNase inhibitor units and 1 μL of GoScript™ Reverse were used. The mixture was incubated for 5 minutes at 25°C, followed by 60 minutes at 42°C and 15 minutes at 70°C.

The quality of the reverse transcription step was analyzed by the quotient A260/A280 and A230/A260 in a spectrophotometer NanoVue™ Plus (GE Healthcare - Life Sciences®), and only the samples with a quotient between 1.8 and 2.1 were admitted. Quantification of the cDNA was performed on the same equipment.

The qPCR reactions were performed in triplicates in the Rotor Gene® device (Qiagen). The analyzed genes are described in Table 1. The genes involved in the DNA damage and repair process were tested. For the reaction, it was added 10 µL of GoTaq® master mix, 2 pmol of each oligonucleotide, and 500 ng of cDNA and ribonuclease-free water q.s.p. (Promega®, catalog number A6002), totaling 20 µL. The mixture was submitted to 95°C for 5 minutes and 40 cycles of 95°C for 2 seconds and 60°C for 30 seconds. At the end, the melting curve was performed to assess the specificity of the formed products. The β-actin (ACTB) gene was used as a normalizer (housekeeping), and the results were analyzed using Rotor Gene® (Qiagen) v2.3.1.

Table 1. Sequence of primers (5'– 3') used in real-time PCR reactions.

Gene	Forward	Reverse	Size (pb)	Genbank
ACTB	GGAAATCGTGC GTGACAT	AGGAAGGAAGGCTGGAAG	183	NM_001101.3
ATM	ACCATTGTAGAGGTCCTTC	GTCTCATTAAGACACCGTTCAG	148	NM_002519.2
ATR	CCTTCAGATTTCCCTTGAATAC	GCAGTTCATGTTTTGATGAG	137	NM_001184.3
GADD45	TCAGCGCAGGATCACTGTC	CCAGCAGGCACAACACCAC	82	NM_001924.3
P21	TAGCAGCGGAACAAGGAG	AAACGGGAACCAGGACAC	249	NM_001220778.1
TP53	TACCACCATCCACTACAACCT	GACAGGCACAAACACGCAC	145	NM_001126112.2

Legend - ACTB: β-actin gene (ACTB); ATM: serine/threonine kinase gene; ATR: serine/threonine-protein kinase gene; GADD45: damage-inducible gene; P21: P21 protein; TP53: Transformation-related protein 53.

Statistical analysis

The statistical analysis of comets was performed by ANOVA/Tukey, and results $p \leq 0.05$ were considered statistically significant (GraphPad InStat). The statistical analysis of the data obtained in the RT-qPCR was performed using the REST program, and the difference was considered significant when the level of relative expression was equal to/or less than -2, or equal to/or greater 2.

Results

Number of cells, characterization and evidence of multipotency of adipose-derived stem cells

Undifferentiated cells (control) (Figure 1A) and confirmation of adipogenic, osteogenic and chondrogenic differentiation were observed by the morphological changes of the ADSCs (Figure 2). Calcium deposits stained with Alizarin red confirmed the osteogenic differentiation (Figure 2B). The lipid vacuoles stained with Oil Red O confirmed the adipogenic differentiation (Figure 2C). The extracellular matrix rich in glycosaminoglycans stained with Alcian Blue proved the chondrogenic differentiation (Figure 2D).

ADSCs expressed CD105 and CD90 markers, and did not express CD34 and CD133. Thus, the analysis of this immunophenotypic profile demonstrates that these are mesenchymal stem cells (Figure 2E).

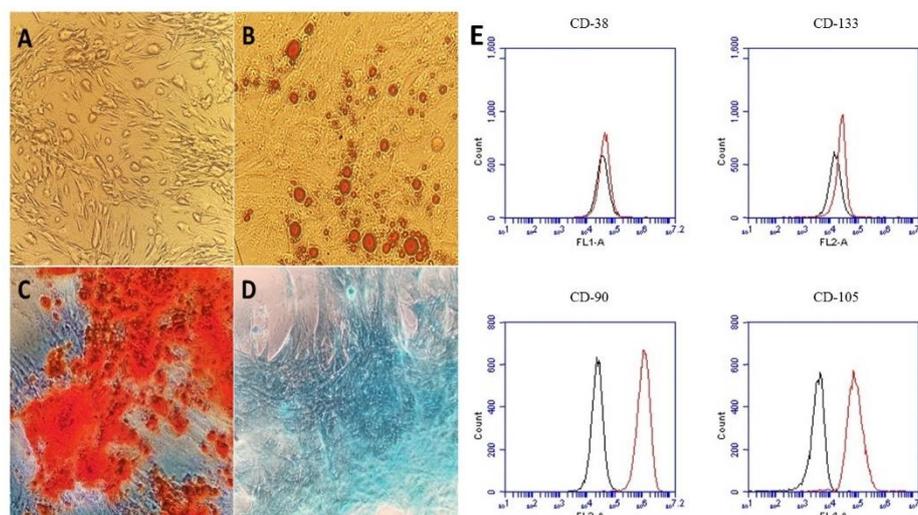


Figure 2. Characterization of multipotency and immunophenotyping of ADSCs. Photomicrographs of cultures of undifferentiated ADSCs with fibroblastoid aspect (A); adipogenic differentiation confirmed by the coloring of Oil Red (B); osteogenic differentiation confirmed by Alizarin Red staining (C); and chondrogenic differentiation confirmed by the coloration of Alcian Blue (D) (400x magnification). Immunophenotypic profile of ADSCs that expressed the CD90 and CD105 markers and did not express the CD38 and CD133 markers (E).

Evaluation of genotoxicity in ADSCs

The comet assay demonstrated that, regardless of the lysis time and pH of the electrophoresis buffer solution, the MSC did not show any DNA damage ($p > 0.05$) (Figure 3A-E). Treatment with DXR demonstrated that the comet assay was properly performed and there was damage to the DNA ($p < 0.001$) of the ADSCs (Figure 3A and 3F).

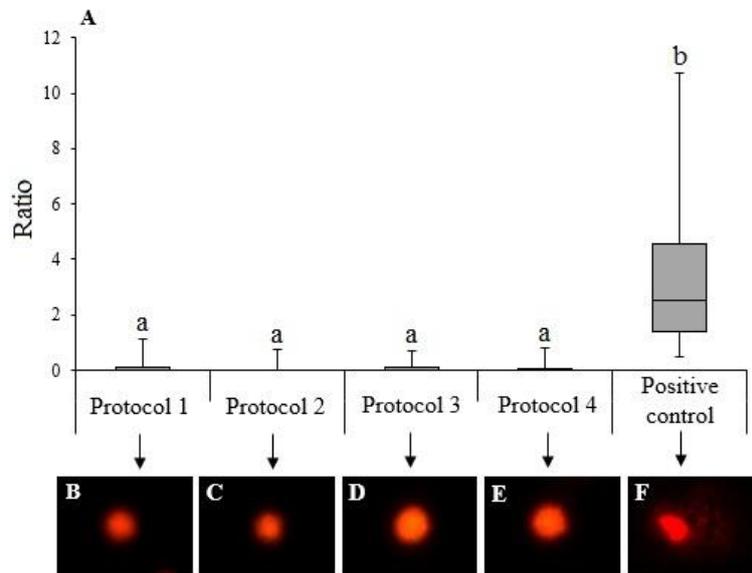


Figure 3. Evaluation of genotoxicity (tail moment) in ADSCs using 4 comet assay protocols. (A) Box plot showing the mean and standard deviation of the frequency of DNA damage for each of the comet protocols and for the positive control (Doxorubicin - 5 μ M); (B) nucleoid of ADSCs submitted to protocol 1 (lysis time of 2 hours and electrophoresis in pH 12 buffer), (C) nucleoid of ADSCs submitted to protocol 2 (lysis time of 24 hours and electrophoresis in pH 12 buffer), (D) nucleoid of ADSCs submitted to protocol 3 (lysis time of 2 hours and electrophoresis in buffer pH \geq 13); (E) nucleoid of ADSCs submitted to protocol 4 (lysis time of 24 hours and electrophoresis in pH \geq 13 buffer); (F) Nucleoid of ADSCs treated with Doxorubicin and submitted to electrophoresis at pH \geq 13. Different letters indicate statistically significant differences ($p < 0.05$, ANOVA/Tukey).

Gene expression

The results of the gene expression assay, performed by qPCR, demonstrated that there was no significant increase ($p > 0.05$) in the expression of the analyzed genes. The ATM gene was expressed at 1.54x, ATR at 1.32x, CDKN1A at 1.48x, TP53 at 1.71 and GADD45A at 1.97x (Figure 4).

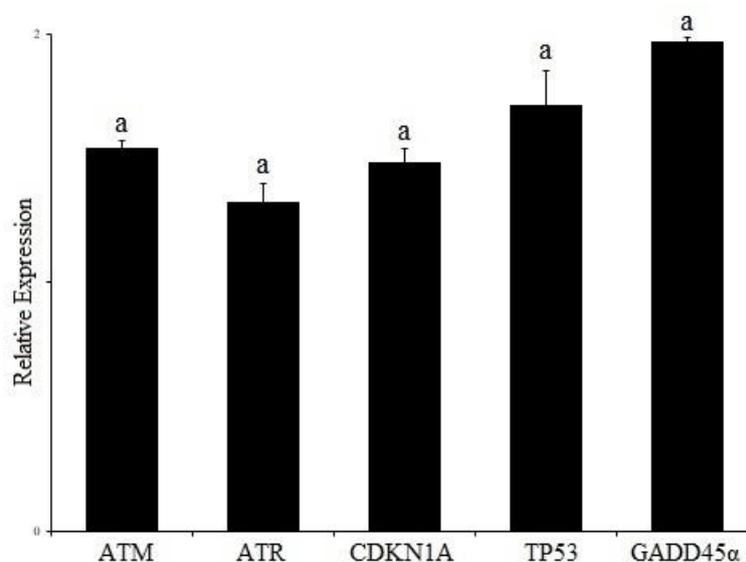


Figure 4. Gene expression assay by qPCR. The analyzed genes are involved in the DNA damage and repair process. As control, ADSCs obtained immediately after the extraction process were used (cells that were not subjected to the cell culture process). The test sample, on the other hand, was composed of ADSCs, which were in the fifth cultivation pass. The ACTB gene was used as a normalizer. There was no significant difference in the expression of the analyzed genes ($p < 0.05$, REST).

Discussion

Some countries, such as China, Japan, India, United States of America (USA), Canada, Italy, Australia, Slovenia, Singapore, among others, there is already a legislation that allows the use of mesenchymal stem cell treatments (Faulkner et al., 2016). Others, such as Brazil, Saudi Arabia, Egypt, Tunisia and Algeria, the legislation still remains in the regulation for research purposes (United Nations Educational, Scientific and Cultural Organization [UNESCO], 2011; Zorzanelli, Speroni, Menezes, & Leibing, 2017). However, it should not be denied that there are still many difficulties that countries with few resources must overcome in order to adapt to the 'global' standards imposed by countries with advanced scientific institutions, such as the USA and member countries of the European Union (Faulkner et al., 2016).

Recent literature reviews indicate promising treatments of several clinical conditions from mesenchymal stem cells (Xu & Yang, 2019; Thompson et al., 2020; Cheng, Huang, & Li, 2020), which demonstrate that the use of these cells will not only help doctors and scientists increase their knowledge, but can also help saving many lives around the world (Jayaraj et al., 2019). Literature indicates that therapy with mesenchymal stem cells is safe for both autologous and allogenic use, since these cells have low immunogenic properties (Ankrum, Ong, & Karp, 2014). However, there is still a great doubt as to whether their extensive manipulation does not induce changes that may correlate with the development of diseases in the individuals who receive the transplant (Mani, Reddy, & Palle, 2020).

The consulted literature demonstrates that there is no standardization on how to evaluate the safety of use, and if the transplanted cells have the required quality. In an attempt to contribute to the knowledge of the area, it was demonstrated that the extraction and cultivation protocol, standardized at the Center for Studies and Stem Cells, Cell Therapy and Toxicological Genetics (CeTroGen), does not induce DNA damage and does not cause increased instability genomics when cells are grown to the tenth passage.

The comet test was the first test used to demonstrate the absence of damage. This is considered a great screening tool to detect DNA damage, which is of great importance, since lesions in the genetic material can be used as a senescence parameter in human ADSCs grown in vitro (Fuchs et al., 2012). Despite good applicability, there is great variability (use of different protocols) and low reliability of results among studies and laboratories (Møller, Möller, Godschalk, & Jones, 2010; Enciso et al., 2018), which makes it difficult to compare studies. However, multicenter studies have already been carried out to standardize this protocol and increase the reliability of results (Bauch, Böcker, Mallek, Müller, & Streffer, 1999). Thus, it was decided to use protocols that are already well established in the literature and in our center.

It is known that the result of the comet may be influenced by the concentration of agarose, the density of the comets formed, the alkaline incubation period of the cells before electrophoresis, the pH of the electrophoresis buffer solution, and by the lysis time, among others (Ersson & Möller, 2011; Azqueta, Gutzkow, Brunborg, & Collins, 2011; Hermeto et al., 2015). Thus, we opted to test different protocols that varied the lysis time and the pH of the electrophoresis buffer, and it was verified that the tested variations did not interfere with the results of ADSCs genotoxicity.

The results of this study demonstrated that the lysis time, being 2 or 24 hours, did not influence the induction of comets. In addition, the two buffers with pH = 12 and ≥ 13 , after 2 or 24 hours of lysis, also did not change the results. Consequently, it was considered that lysis up to 24 hours and pH buffer = 12 and ≥ 13 can be used in comet assays to infer DNA lesions from human ADSCs. This result differs from the conclusions of Hermeto et al. (2015), who reported the need to use the buffer with pH = 12, since pH ≥ 13 induced damage in the ADSCs of rabbits. Aware of the interspecific differences (rabbit vs man), we suggest that the study by Hermeto et al. (2015) has two limitations: the absence of a positive control of the test and the biological relevance between the statistical differences of the Tail Moment. It is understood that a difference lower than 0.04 in this parameter does not indicate the need to choose one of the protocols (pH = 12 or pH ≥ 13). In addition, we understand that the inclusion of a positive control is not mandatory for this type of study. However, this internal control, in addition to validate the test, can correct discrepancies and assist in understanding the biological effects of the test (Møller, 2018; Møller, Stopper, & Collins, 2020). Moreover, we suggest that a positive control must be carried out and included in the statistical analysis.

It is important to highlight that, at the time the cells were detached from the flasks, there was little DNA damage in the ADSCs, which was proven by evaluating the expression of repair and damage mRNA genes (Nash et al., 2001; Massagué, 2004; Bertoli, Skotheim, & De Bruin, 2013; Ueda, Kohama, Kuge, Kido, & Sakurai, 2017). These results indicate that any DNA damage observed in the comet assay occurred during the

performance of the test technique itself. In fact, the process of repairing damage to the DNA of mesenchymal stem cells is associated with increased expression of genes, such as those described in this study, which contributes to preserving their integrity, because otherwise, when these damages go undetected, they can lead to various disorders in an organism in which these cells are transplanted (Mani et al., 2020). Thus, regarding the changes in the genetic material, the activation of the tumor suppressor genes confers stability to the cell, since it interrupts the cell cycle progression until the DNA damage is repaired, or in cases of fatal damage, induces its apoptosis (Khanna, Lavin, Jackson, & Mulhern, 2001; Goodarzi, Block, & Lees-Miller, 2003; Stivala, Cazzalini, & Prospero, 2012; Salvador, Clay, & Fornace Jr., 2013; Chen, 2016).

Conclusion

From the obtained results, it is considered that any protocol tested in this study can be applied in genotoxicity assays with human ADSCs, without causing damage to them. However, more extensive investigations are recommended to the specific conditions of the various stages of the comet assay.

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