DNA damage in dental pulp mesenchymal stem cells: An *in vitro* study

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Abstract

The aim of this study was to evaluate the potential use of a DNA comet assay. DNA fragmentation fluorimetric assay and reactive oxygen species levels as potential biomarkers of genome conditions of dental pulp stem cells (DPSCs) isolated from dog canine teeth. Mesenchymal stem cells were isolated from the dental pulp collected from dog teeth. The results obtained suggest the ideal moment for clinical application of cellular therapy for this type of cell. The cell culture was maintained with Dulbecco’s modified Eagle’s medium supplemented with 10.00% fetal bovine serum for eight passages. During each passage, cell proliferation, oxidative stress and level of DNA fragmentation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, testing 2,7 dichlorodihydro-fluorescein-diacetate and PicoGreen®, respectively. There were important differences among the first three DPSC passages compared to passages 4–8 and a large number of nuclei with some levels of DNA damage (30.00 to 40.00% in initial DPSC passages and > 50.00% in late passages), indicating *in vitro* DPSC genomic fragility. Within the limitations of this study, the results suggest these relatively simple and inexpensive approaches - comet and DNA fragmentation assays - could help sort stem cells with less DNA damage for use in research or therapies.

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Introduction

The considerable therapeutic potential of embryonic and adult stem cells has sparked increasing interest in a wide variety of biomedical disciplines, with mesenchymal stem cells being of particular interest. There is also therapeutic potential in adult dental pulp stem cells (DPSCs) isolated from humans and animals including dogs and these cells represent a distinctive population of pluripotent cells.\textsuperscript{1-4} The DPSCs offer a potentially renewable source of cell types that are easily isolated and expanded for use in regenerative therapies as the formation of dentin, odontoblasts, dental pulp, bone, cartilage, muscle, hair follicle, cornea, neuronal cells, melanocytes and endothelial cells.\textsuperscript{5} However, while the therapeutic potential of advanced stem cell modification has been observed in clinical trials with long-term follow-up, potential adverse reactions related to insertional mutagenesis by integrating gene vectors and chromosomal instability in proliferating cells have emerged as a major limitation.\textsuperscript{6,7} The stem cells’ susceptibility to the acquisition of chromosomal anomalies is related to their significant cell proliferation and metabolic activity.\textsuperscript{8-12} The major concern regarding chromosomal instability is whether manipulation during in vitro cell culture expansion increases tumorigenicity risk.\textsuperscript{13-15}

A study performed by Duailibi et al. using a human dental stem cell (DSC) line revealed that approximately 70.00% of the cells exhibited karyotypic abnormalities including polyploidy, aneuploidy and ring chromosomes.\textsuperscript{16} The authors emphasize the need for the careful analysis of genome instability in cultured human dental pulp stem cells (hDPSCs) before they can be clinically used.\textsuperscript{16} Despite the relevance of regenerative therapies to human and veterinarian medicine, studies to determine potential biomarkers of genome damage that can help researchers choose better cell lines to perform regenerative studies or use those cell lines in clinical applications are still incipient.\textsuperscript{17}

Thus, this study aimed to isolate dog DPSCs using methods previously described for isolating hDPSCs and evaluate potential genotoxicity during eight cell passages, DNA fragmentation by fluorometric quantification and reactive oxygen species (ROS) levels as biomarkers of oxidative status of the DPSC culture passages. The results obtained suggest the ideal moment for clinical application of therapy with this type of cells.

Materials and Methods

A total of three maxillary canine obtained from three dogs at the Veterinary Hospital of the Federal University of Santa Maria were used in this study. This study was approved by the research ethics committee of the Federal University of Santa Maria, Rio Grande do Sul, Brazil.

Isolation and cultivation conditions of mesenchymal stem cells. The isolation and cultivation of mesenchymal stem cells were performed as previously described by Bernardi et al.\textsuperscript{18} Briefly, shortly after extraction, the teeth were immersed in Dulbecco’s modified Eagle’s medium supplemented with 10.00% fetal bovine serum, 100 units mL\textsuperscript{-1} penicillin, 100 µg mL\textsuperscript{-1} streptomycin, 0.45 µg mL\textsuperscript{-1} gentamycin and 3.70 mg L\textsuperscript{-1}(4- (2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) with 0.45 µg mL\textsuperscript{-1} of amphotericin B to transport the teeth from the operating room to the cell laboratory.

Upon removal of the pulp tissue, the teeth were clinically evaluated for the presence or absence of the remaining root to assess possible contact zones of the pulp tissue with the external environment. The pulp was isolated from the dental tissue with the aid of an excavator and all of the pulp tissue was removed (crown and root) from the dentin. This procedure was performed in the laminar flow hood under sterile conditions. The pulp was minced and incubated with 5.00% CO\textsubscript{2} at 37 °C for 60 min in a buffer containing 0.20% type I collagenase (Gibco Products International Inc., Los Angeles, USA). The cells were disrupted from the dental pulp and cultured in the same media conditions described before. The culture medium was changed after 24 hr of initial plating and every 3 to 4 days thereafter. When the culture reached 90.00% confluence, a passage using 0.50% trypsin - ethylenediamine tetraacetic acid (Sigma-Aldrich, St. Louis, USA) was performed to loosen the cells from the plate. The density of the cells seeded in each passage was 10\textsuperscript{5} cells per cm\textsuperscript{2}.

Cell proliferation and DPSC morphological analysis. The proliferation rate in the different DPSC culture passages was determined by cell count using a Neubauer chamber as well as by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using DPSCs seeded into 96-well plates at a density of 2 × 10\textsuperscript{3} cells per well. The MTT assay was evaluated according to the manufacturer’s instructions (Sigma, Munich, Germany). The MTT reduction/attenuation values for each well were measured spectrophotometrically at 570 nm. The MTT assay was carried out during each culture cell passage in quadruplicate. The morphological analysis of the DPSCs in different passages was also microscopically evaluated for cell form and structure.

Reactive oxygen species production. The accumulation of intracellular oxidative damage is related to the aging of the cells in vitro and intracellular oxidative damage subsequently decreases the potential for further cellular proliferation and differentiation.\textsuperscript{17} Therefore, biomarkers including intracellular ROS production and lipid peroxidation were evaluated during the DPSC culture passages. The ROS production was evaluated using the non-fluorescent cell-permeating compound 2-70-dichloro-fluorescein diacetate (DCFH-DA; Merck KGaA, Darmstadt,
DNA fragmentation assay. The DNA fragmentation in DPSCs from each culture cell passage was performed using an ultrasensitive protocol for quantification of the level of DNA fragmentation using Quant-it™ PicoGreen® dsDNA (Invitrogen, Carlsbad, USA) and a protocol for quantitative assessment of DNA concentration and damage (fragmentation) employing a protocol similar to that described by Batel et al. and Georgiou et al. but adjusted to fit a 96-well cell culture plate.21,22

The method is based on the ability of the specific fluorochrome dye (PicoGreen; Thermo Fisher Scientific, Massachusetts, USA) to make a very stable complex with double-stranded DNA (dsDNA) under alkaline conditions instead of single-stranded DNA (ssDNA), proteins, sodium dodecyl sulfate and urea. This selective characteristic is used to follow DNA denaturation with decreasing fluorimetric signal intensity proportional to increasing ssDNA and mononucleotide content. The PicoGreen® dye was diluted 1:200 with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.50; Thermo Fisher Scientific) and incubated with cell culture samples (1 × 10⁵ cells well⁻¹) in the dark at room temperature for 5 min. To minimize the photo-bleaching effects, the time for fluorescence measurement was kept constant for all samples. Fluorescence was measured at an excitation of 485 nm and an emission of 520 nm at room temperature. All fluorescence measurements were recorded in a fluorometer (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, USA). A standard curve was generated using double-stranded lambda DNA provided by the manufacturer. The fluorescence emission measurement of each sample of eight DPSC passages was evaluated. The cell sample was an average of four independent measurements and the corresponding standard error is indicated in the data. As during the cell passage, the number of cells was variable due to the potential difference in cell proliferation and the data were initially adjusted to the number of cells in each passage (dsDNA fluorescence number of cells). Additionally, DNA fragmentation in different DPSC culture passages was expressed as a percentage of dsDNA calculated based on the first cell culture passage.23-26

Comet assay. To detect DNA damage, we performed a comet assay, in which samples were placed on slides containing low melting agarose and then subjected to cell lysis, with the preparation of two slides per sample. Electrophoresis and staining with silver nitrate were performed. After that, 50 cells were read by two analysts (JSAjr and SAG) considering that the larger the size of the DNA drag, the larger the increase in cell damage.

Statistical analysis. The results of the experiments were analyzed by one-way analysis of variance followed by Tukey’s post-hoc test. p-values less than 0.05 were considered significant.

Results

Cell proliferation and DPSC morphological analysis. The morphology of DPSCs did not change during the eight passages. It remained similar to the fibroblast cells (Fig. 1). The proliferation of DPSCs increased in the second and third passages (p = 0.001), showing intermediate values between the first, second and third passages (Figs. 2A and 2B).

![Fig. 1. Photographs depicting the morphological appearance of DPSCs isolated from dog canine teeth. No differences were found in morphological appearance between DPSCs in the eight cell passages.](image)

Reactive oxygen species production. The ROS levels were assessed in each passage of dog DPSCs. Considering the first passage as control, the others presented higher rates, especially during the 3rd, 6th and 8th (p = 0.003) passages (Fig. 3).

DNA fragmentation assay. The DNA fragmentation was evaluated in each of the eight passages in which the fluorimetric assay was used. When comparing the other passages with the first one used as reference (representing 100% of dsDNA), the second passage had a higher level of dsDNA (p = 0.0001), while the other DPSCs had intermediate levels between the first and second passages (Fig. 4).

Comet assay. Results of potential DNA damage during DPSC passages were analyzed by the DNA comet assay using the damage score (0-none, 1-low, 2-medium, 3-high and 4-severe), as shown in Figure 5A. Undamaged nuclei were classified as having a damage level of 0 to 1. The DNA damage index was less intense for the first three DPSC passages, with 75.40% of the nuclei without damage or a damage level of 1 (damage 0 or 1). However, DNA damage increased after the 4th passage and showed severe levels of damage in the 7th passage (Fig. 5B).
Discussion

Adult stem cells including DPSCs are important in the long-term maintenance of tissues throughout life because these lineages are responsible for body tissue regeneration when damage has occurred or for the replacement of...
senescent or terminally differentiated cells.\textsuperscript{27} Accordingly, these cells have an effective system to maintain their genomic integrity, both by increased stress defense and DNA repair mechanisms. This maintenance is very important because genomic alterations can potentially compromise the functionality of the entire cell lineage. The dsDNA breaks are considered to be one of the most lethal forms of damage and failure to adequately repair dsDNA breaks can compromise the self-renewal and differentiation of stem cells and lead to genomic instability and disease.\textsuperscript{27}

Therefore, the major challenge concerning the use of DPSCs or other adult stem cells as a source of cells in tissue regeneration therapies is related to the ability of the cells to maintain their genetic integrity during long-term culture and differentiation.\textsuperscript{7,16}

However, evaluating the \textit{in vitro} conditions of the genome quality of stem cells is not easy. This is so because most stem cell cultures do not have morphological biomarkers that help differentiate better cells from those with a compromised genome. Hence, a potential fast screening of the genomic conditions of the cells to be used in regenerative approaches is desirable. Therefore, the present study investigated the quality of eight initial DPSC passages derived from dog dental tissues in relation to the potential DNA damage using two approaches including DNA comet assay and DNA fragmentation.

Before discussing the results of this study, it is important to comment on the use of the DNA fragmentation assay as a complementary quantitative test for the analysis of DNA damage of DPSCs. As characterization of DNA structural integrity is critical for a wide variety of biological applications, the analysis of DNA damage has important clinical and molecular research applications. Several studies have analyzed the precision of repair in pluripotent cells and their differentiated progeny using several biomarkers such as zinc finger nucleases.\textsuperscript{28} However, assays as DNA fragmentation using PicoGreen® dye could help eliminate subjectivity and evaluate the presence of DNA damage easily and quickly. Therefore, this study used a protocol adapted from Georgiou \textit{et al.} to quantitatively evaluate DNA damage in different DPSC passages together with the DNA comet assay, a classic and widely used genotoxicity assay.\textsuperscript{21}

PicoGreen® dye has the ability to measure 25 pg mL\textsuperscript{-1} of dsDNA with a standard spectrophotometer and fluorescein excitation and emission wavelengths and to minimize the fluorescence contribution of RNA and ssDNA. Additionally, PicoGreen®-based DNA quantification is used in different cell lysis protocols and frequently used to quantify the cellularity of three-dimensional (3D) cell-seeded scaffolds to induce \textit{in vivo} tissue formation as well as to quantify the DNA from two-dimensional and 3D cell cultures.\textsuperscript{29,30} Since necrosis, apoptosis and dsDNA breaks cause DNA degradation, cells with DNA damage potentially present a lower level of dsDNA and, therefore, lower fluorescence than cells with genomic integrity.

Alternatively, in the last 20 years, the comet assay has proven to be a useful tool for investigating the induction and repair of DNA in various cell types. Previous studies, as that performed by Nikitina \textit{et al.}, analyzed DNA damage in human bone marrow cells and multipotent mesenchymal stromal cells using the DNA comet assay.\textsuperscript{31}

Our results showed important differences in DNA fragmentation between the first three DPSC passages compared to passages 4 to 8 when the comet assay was performed. These data are in agreement with the results described by Nikitina \textit{et al.}, who assessed DNA damage in human bone marrow cells and multipotent mesenchymal cells from cell culture passages 3 to 11.\textsuperscript{31} These authors observed that, in the early and late passages, mesenchymal cells presented some DNA damages. These results suggest that both tests could be used as a complementary assay to help researchers choose which cell passage contains less damage in genomic studies of cell regeneration and differentiation.

However, the large number of cells with DNA damage found in the comet assay in all DPSC passages (approximately 30.00\%-40.00\% of the nuclei presented some DNA damages) is noteworthy. These results indicate the potential fragility of the DPSCs genome when these cells are cultured \textit{in vitro} and such fragility could lead to further differentiation and regenerative processes.

This assumption is supported by a previous study performed by Duailibi \textit{et al.}\textsuperscript{16} These authors analyzed hDPSC cytogenetic integrity using classical G banding and fluorescent \textit{in situ} hybridization with an X chromosome specific probe in the second cell passage. They found that approximately 70.00\% of the cells had karyotypic abnormalities including polyploidy, aneuploidy and ring chromosomes. These results showed a heterogeneous spectrum of abnormalities indicating a high frequency of chromosomal mutations that continuously arise after extended culture time. Unfortunately, the authors did not study further passages to permit a comparison with our results.

To ensure maintenance of the stem cells as DPSCs and to coordinate differentiation of progeny, all of these cells must protect the genome from genotoxic lesions potentially caused by exogenous and endogenous factors. Among the endogenous factors, ROS play an important role and generate DNA damage by oxidative modification of DNA bases or spontaneous hydrolysis of nucleosides. These molecules lead to the formation of dsDNA breaks. Because DNA damage can originate from oxidative attacks of ROS to DNA, owing to nuclease action during DNA repair or during early or late events in apoptosis or necrosis, the present study investigated whether DPSC ROS production could be different in different DPSC culture passages.\textsuperscript{21} The results showed an increase in ROS...
levels from DPSCs in the second passage and this level of ROS increased significantly in passages 6 to 8.

Considering all results, including the cell proliferation rate, DNA damage as evaluated by DNA PicoGreen® and the comet assay as well as ROS production, the second DPSC passage was better, as it had lower DNA damage and oxidation.

Baum et al. have commented about the management of genotoxicity of stem cells with the potential for therapeutic use and hypothesized that some mutations are not necessarily dangerous. However, this study did not determine where and when bad mutations can affect the success of regeneration or cause adverse effects such as cell malignization.

Considering the results presented here, we suggest that relatively simple and inexpensive approaches such as the comet assay, DNA fragmentation and ROS levels determination, be used for sorting cultured stem cells and cells with less DNA damage should be used for research or determination, be used for sorting cultured stem cells.

This study demonstrated the possibility of analyzing potential DNA damage in different DPSC passages by a DNA fragmentation assay as well as the DNA comet assay. Within the limitations of this study, the results obtained suggest that incorporation of DNA damage assays in stem cell cultures may help us choose the best stem cell passage to conduct regenerative experiments.

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Conflict of interest

The authors declare that there is no conflicts of interest.

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