UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Avaliação da resistência de células-tronco derivadas de tecido adiposo humano a quimioterápicos

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Lista de abreviaturas

ABC - *ATP-binding cassete*

ADSCs – *adipose-derived stem cells* (células-tronco adiposo-derivadas)

CBMN - *cytokinesis-blocked micronuclei assay*

CCM – *cell culture medium*

CIS – cisplatina

EMS - *ethylmethane sulphonate*

FBS – *fetal bovine serum* (soro fetal bovino)

FISH - *fluorescent in situ hybridization*

FITC - *fluorescein isothiocyanate*

hADSCs – *human adipose-derived stem cells* (células-tronco adiposo-derivadas humanas)

hBM-MSCs – *human bone marrow mesenchymal stem cells* (células-tronco derivadas da medula óssea humana)

MSCs – *mesenchymal stem cells* (células-tronco mesenquimais)

MTT - brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio

NC – *negative control*

PAC – paclitaxel

PBS - *phosphate buffered saline* (solução salina tamponada de fosfato)

PC – *positive control*

PE –*phycoerythrin*

RT - *room temperature*

SMART – *somatic mutation and recombination test* (teste de mutação recombinação somática em *Drosophila melanogaster*)

TI – *tail intensity*

TL – *tail length*

TM – *tail moment*

Sumário

Resumo

As células-tronco mesenquimais (MSCs) são consideradas o tipo de célula-tronco adulta mais plástico, além de possuírem uma localização perivascular e residirem em todos órgãos e tecidos adultos. Nos últimos anos, as MSCs isoladas do tecido adiposo (ADSCs) têm recebido grande atenção por parte da medicina regenerativa, por serem facilmente isoladas e compartilharem várias características com as MSCs de medula óssea. Vários aspectos básicos da biologia das ADSCs têm sido estudados, mas pouco ainda se sabe sobre os mecanismos envolvidos na resistência destas células à exposição a agentes citotóxicos e genotóxicos, como quimioterápicos utilizados no tratamento do câncer. Desta forma, este trabalho teve como objetivo investigar o potencial citotóxico e genotóxico de duas drogas comumente utilizadas em terapias anti-câncer, cisplatina (CIS) e paclitaxel (PAC) sobre ADSCs humanas, através dos ensaios MTT e cometa. Através do ensaio MTT confirmamos a já descrita resistência das ADSCs humanas aos agentes quimioterápicos e utilizando o ensaio cometa, mostramos que CIS e PAC não são capazes de induzir danos significativos ao DNA destas células. Assim, estes dados mostram que as ADSCs humanas não são sensíveis ao tratamento com os agentes testados.

Abstract

Mesenchymal stem cells (MSCs),which have a perivascular site and reside in all adult organs and tissues, are considered the most plastic type of adult stem cells. In the past few years, MSCs from the adipose tissue (adipose-derived stem cells, ADSCs), have recieved from the field of regenerative medicine great attention, as they are easily isolated and share many characteristics with bone marrow MSCs. Several basic aspects from the ADSCs biology have been studied, but we have little knowledge about the mechanisms involved in the resistance of these cells to the exposition to cytotoxic and genotoxic agents, like chemotherapeutic drugs used in the treatment of cancer. Thus, this work had as objective to investigate the cytotoxic and genotoxic potential of two drugs commonly used in anti-cancer therapies, cisplatin (CIS) and paclitaxel (PAC), on human ADSCs, through the MTT and comet assay. Using the MTT assay we confirmed the already described resistance of human ADSCs to chemotherapeutic agents and through the comet assay we showed that both CIS and PAC are not capable to induce significant DNA damage to these cells. Thus, these data show the lack of sensitivity of human ADSCs to the exposure to tested agents.

1 Introdução

As células-tronco são definidas por sua capacidade de auto-renovação e diferenciação em células funcionalmente competentes (Watt e Hogan, 2000). As células-tronco embrionárias podem dar origem a qualquer tipo celular, enquanto as células-tronco encontradas em indivíduos adultos possuem uma plasticidade mais limitada e têm papel muito importante na homeostase do organismo e regeneração de possíveis lesões. Exemplos incluem células-tronco epiteliais na epiderme e cripta intestinal (Slack, 2000), células-tronco neuronais no sistema nervoso central (McKay, 1997) e células satélites nos músculos (Charge e Rudnicki, 2004). Na medula óssea do adulto, são encontradas as células-tronco hematopoiéticas (Weissman, 2000) e mesenquimais (Prockop, 1997; Beyer Nardi e da Silva Meirelles, 2006), além de precursores endoteliais e possivelmente outros tipos de células primitivas (Kucia et al., 2005).

As células-tronco mesenquimais (*mesenchymal stem cells,* MSCs) (Friedenstein et al., 1976; Huss, 2000) são capazes de se diferenciar *in vitro* e *in vivo* em diversos tipos celulares (Kopen et al., 1999;Woodbury et al., 2000; Sato et al., 2005), e são atualmente consideradas as células-tronco adultas de maior plasticidade (Beyer Nardi e da Silva Meirelles, 2006).

As MSCs podem adquirir características tecido-específicas quando co-cultivadas com tipos celulares especializados ou expostas a extratos de tecido *in vitro* (Houghton et al., 2004; Choi et al., 2005; Lange et al., 2005). Parecem ainda capazes de se diferenciar em tipos celulares que vão além dos de origem mesodérmica, incluindo células de origem ectodérmica (Kopen et al., 1999) e endodérmica (Sato et al., 2005). Dessa forma, as MSCs podem ser caracterizadas como multipotentes, e talvez o termo “mesenquimal” pode não ser apropriado para descrever este tipo particular de célula-tronco (da Silva Meirelles et al., 2006).

O principal método de isolamento das MSCs explora sua capacidade de adesão a diferentes substratos, o que possibilita a remoção de células não aderentes contaminantes (Banfi et al., 2000). Nenhum marcador de superfície se mostrou exclusivo para MSCs, embora alguns deles sejam usados para obter uma fração enriquecida dessas células. Pode-se ainda fazer uma seleção

negativa, ao eliminar as células com marcadores exclusivos de células hematopoiéticas. Como parte de um conjunto mínimo de critérios para definição das MSCs humanas proposto pelo *Mesenchymal and Tissue Stem Cell Committee da International Society for Cellular Therapy*, as células devem ser positivas para as moléculas CD105, CD73 e CD90, e negativas para CD45, CD34, CD14 ou CD11b, CD79a ou CD19 e HLA-DR (Dominici et al., 2006).

As MSCs têm sido isoladas a partir de diferentes órgãos e tecidos, como tecido adiposo (Zuk et al., 2001), tendão (Salingcarnboriboon et al., 2003), membrana sinovial (De Bari et al., 2003) e pulmão (Sabatini et al., 2005). Recentemente, nosso grupo demonstrou a distribuição das MSCs murinas no organismo como um todo, com evidências de uma localização perivascular destas células (da Silva Meirelles et al., 2006).

Entre os diversos tipos tecido-específicos de MSCs, as células-tronco derivadas de tecido adiposo humano são alvo de inúmeros estudos atualmente. Sua fácil obtenção e grande plasticidade descrita tanto *in vitro*, quanto *in vivo*, as credenciam como um importante alvo para possíveis aplicações na medicina regenerativa (Tobita et al., 2011).

1.1 Células-tronco adiposo-derivadas humanas

O tecido adiposo é um tecido altamente complexo, formado por adipócitos maduros, pré-adipócitos, fibroblastos, células de músculo liso vascular, células endoteliais, monócitos/macrófagos residentes e linfócitos (Xu et al., 2003; Weisberg et al., 2003; Caspar-Bauguil et al., 2005). A fração estromal-vascular deste tecido vem ganhando uma atenção especial da área de pesquisa em células-tronco, desde que foi descoberta uma fonte rica em células multipotentes neste compartimento (Zuk et al., 2002; Casteilla et al., 2005). Dessa forma, o tecido adiposo humano descartado durante procedimentos cirúrgicos tem sido considerado uma fonte importante de MSCs, por ser de fácil acesso, necessitar de procedimento cirúrgico simples e ser suscetível a um isolamento enzimático sem maiores complicações (Schäffler e Büchler, 2007).

O termo células-tronco derivadas de tecido adiposo (*adipose-derived stem cells*, ADSCs) passou a ser utilizado então para designar as células multipotentes encontradas na fração estromal-vascular do tecido adiposo, isoladas imediatamente após digestão enzimática com colagenase (Zuk et al., 2002; Prunet-Marcassus et al., 2006). Ao comparar estas células com MSCs de medula óssea e de cordão umbilical, Kern e cols. (2006) não observaram diferenças quanto à morfologia, imunofenótipo, número de MSCs isoladas, freqüência de colônias e capacidade de diferenciação. Assim, as ADSCs representam uma fonte alternativa de células-tronco adultas autólogas que podem ser obtidas repetidamente e em largas quantidades sob anestesia local e com o mínimo de desconforto para o paciente.

As ADSCs, além de serem facilmente isoladas do tecido adiposo humano, têm o potencial de se diferenciar em células ósseas, de cartilagem, tendões, músculo esquelético e adipócitos quando cultivadas em condições linhagem-específicas (Zuk et al., 2001; Lee et al., 2004; Dicker et al., 2005; Wagner et al., 2005). A utilização destas células na engenharia tecidual é de grande interesse em doenças humanas, tais como doenças hereditárias, traumáticas, ou ósseo-degenerativas, articulares e defeitos em tecidos leves (regeneração esquelética e reparo de cartilagem). A reconstrução plástica de tecidos após remoção cirúrgica de tumores e a reconstrução cirúrgica de músculos e tecido adiposo após queimaduras, representam necessidades adicionais para o uso de terapias baseadas em células (Schäffler e Büchler, 2007).

1.2 Resistência celular a múltiplas drogas

O interesse crescente na utilização de ADSCs e outras MSCs na medicina regenerativa, leva à reflexão sobre as condições de preservação nas quais se encontram as células em questão. Sabe-se que o cultivo celular prolongado pode resultar em inúmeras alterações celulares, além da instabilidade cromossômica ocasionada por mudanças nos padrões de metilação do DNA (Schellenberg et al.,

2011) ou aneuploidia, poliploidia e quebras de dupla-fita de DNA (Ahmadbeigi et al., 2011; Ross et al., 2011; Rübe et al., 2011).

A ocorrência de tais alterações deve ser considerada também no contexto do compartimento tronco de cada tecido que compõe o organismo, já que a função primordial das células-tronco adultas, de forma geral, é renovar e auxiliar no reparo tecidual.

Em um organismo multicelular, além das sucessivas divisões celulares – que virtualmente não ocorrem em um compartimento tronco normal, o qual permanece quiescente -, agentes químicos e físicos podem causar danos às células. Tal exposição pode levar à morte celular por apoptose ou a mutações no DNA, que, em última análise, levam ao surgimento do câncer.

Um exemplo de agente físico ao qual as MSCs humanas podem ser expostas é a radiação ionizante, que não mostrou ser danosa à MSCs de medula óssea (Chen et al., 2006; Jin et al., 2008; Mussano et al., 2010).

Entre os agentes químicos que podem causar danos em células-tronco humanas estão os quimioterápicos usados no tratamento do câncer. A cisplatina (CIS) e o paclitaxel (PAC) são dois quimioterápicos amplamente utilizados no tratamento de vários tipos de câncer (Adamo et al., 2004; Zhang e Tuckett, 2008). CIS é um composto derivado de platina e seu principal mecanismo de ação é baseado na formação de adutos com os nucleotídeos do DNA que bloqueiam a replicação e a transcrição do DNA e, finalmente, a divisão celular (García et al., 2008). Essas ligações cruzadas são responsáveis por cerca de 90% dos danos no DNA induzidos por CIS e podem, provavelmente, ser a principal causa dos efeitos citotóxicos atribuídos a esta droga (Bhana et al., 2008). O taxano PAC age através da estabilização dos microtúbulos durante a divisão celular, e assim bloqueia a mitose na fase G2/M, com subsequente indução de apoptose em células sensíveis (Adamo et al., 2004; Specenier e Vermorken, 2009).

Quando estas e várias outras drogas foram testadas em MSCs de medula óssea humana e em ADSCs humanas, esses quimioterápicos, incluindo CIS e PAC, não foram capazes de exercer atividade citotóxica, seja *in vitro* (Li et al., 2004; Liang et al., 2011), ou em pacientes submetidos à quimioterapia (Mueller et al., 2006).

Apesar de as MSCs e as ADSCs serem resistentes aos quimioterápicos testados, nenhuma hipótese foi discutida a respeito dos mecanismos envolvidos na manutenção da integridade celular durante os tratamentos.

Vários mecanismos podem estar envolvidos na resistência celular à múltiplas drogas. Entre estes mecanismos, destaca-se o recrutamento de mecanismos de reparo a danos no DNA, que auxiliam na manutenção da integridade genômica das células (Zhu et al., 2009; Frosina, 2010).

Entre os mecanismos de reparo a danos no DNA mais atuantes em células-tronco adultas estão a recombinação homóloga (Francis e Richardson, 2007; Mohrin, et al., 2010; Rübe et al., 2011) e a junção de extremidades não homólogas (Rossi et al., 2007; Naka e Hirao, 2011), que reparam quebras de dupla-fita de DNA. Além destes, outros mecanismos auxiliam na manutenção da integridade genômica destas células, como o reparo de mau pareamento de bases e a excisão de nucleotídeos (Frosina, 2010), que atuam durante a síntese do DNA.

Vários estudos têm relacionado os mecanismos de reparo com resistência adquirida por tumores contra agentes quimioterápicos (Parker et al., 1991; Fink et al., 1998; Xu et al., 2002; Dizdaroglu, 2012). Além disso, há grandes evidências que demonstram o papel destes mecanismos na manutenção de células-tronco tumorais dentro do seu microambiente, o que pode levar à resistência do tumor à quimioterapia (Gangemi et al., 2009; Honoki, 2010; Monteiro e Fodde, 2010; Maugeri-Saccà et al., 2011).

Como já citado, diversos estudos mostram a importância da manutenção da integridade genômica na suscetibilidade celular a toxinas e agentes químicos, tanto em condições normais quanto em células tumorais. Porém, a literatura ainda é pobre em evidências que demonstrem um possível envolvimento destes mecanismos de tolerância na resistência, já comprovada, de ADSCs humanas a quimioterápicos.

Desta forma, este trabalho buscou avaliar um possível envolvimento da tolerância a danos no DNA na resistência de ADSCs humanas aos quimioterápicos cisplatina e paclitaxel.

2 Objetivos

O presente estudo teve como objetivo geral avaliar o envolvimento da tolerância a danos no DNA na resistência de ADSCs humanas aos quimioterápicos cisplatina e paclitaxel. Para investigar de maneira aprofundada as metas propostas, os objetivos específicos a serem alcançados foram:

1. Confirmar a resistência de ADSCs humanas aos quimioterápicos cisplatina e paclitaxel, através da avaliação dos tratamentos de 6 concentrações de cada quimioterápico pelo ensaio MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio);
2. Avaliar a capacidade genotóxica dos quimioterápicos sobre as ADSCs, através do ensaio cometa.

3 Artigo

Evaluation of the cytotoxic and genotoxic potential of cisplatin and paclitaxel in human adipose-derived stem cells *in vitro*.

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Evaluation of the cytotoxic and genotoxic potential of cisplatin and paclitaxel in human adipose-derived stem cells *in vitro*.

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Abstract

In the past few years, human adipose-derived stem cells (hADSCs) have been subject of many investigations in the regenerative medicine field. Basic mechanisms involved in the stem cells response to damaging agents have been elucidated, but the response of hADSCs to cytotoxic and genotoxic drugs is still poorly understood. Here we investigate the cytotoxic and genotoxic potential of two drugs commonly used in cancer therapies, cisplatin and paclitaxel, using the MTT and the comet assay, respectively, in hADSCs and the sensitive cell line H460. Our results confirmed the resistance of hADSCs chemotherapeutic agents. Regarding the genotoxic potential of the chemical agents, no DNA damage was observed in hADSCs after treatment with both drugs, after 1, 3 and 24 h of exposure. These data show the great potential of hADSCs in resist to genotoxic agents, but we were not able to confirme the possible involvement of DNA repair mechanisms in the maintenance of hADSCs integrity.

Keywords: human adipose-derived stem cells, cisplatin, paclitaxel, cytotoxicity, genotoxicity

1. Introduction

Mesenchymal stem cells reside in all adult organs and tissues (da Silva Meirelles et al., 2006) and are considered the most plastic type of adult stem cells due to their capacity to differentiate into cells of endodermal and mesodermal origin (Kopen et al., 1999; Sato et al., 2005). Adipose-derived stem cells (ADSCs) have emerged as a promising alternative for cell therapy and tissue engineering, because they are easily isolated and manipulated and have similar characteristics to bone-marrow mesenchymal stem cells (Lee et al., 2004; Kern et al., 2006; Schäffler and Büchler, 2007; Tobita et al., 2011).

For the application of these cells in medicine, it is often of interest to expand them by *in vitro* culture. Long-term culture may be harmful and cause DNA double-strand breaks, polyploidy and/or aneuploidy (Ahmadbeigi et al., 2011; Ross et al., 2011; Rübe et al., 2011). Furthermore, stem cells may be exposed *in vivo* to physical and chemical agents, such as ionizing radiation and chemotherapeutic agents. Previous studies have shown that these agents have no effect on the viability of human mesenchymal stem cells from the bone-marrow, during *in vitro* (Li et al., 2004; Chen et al., 2006; Jin et al., 2008; Mussano et al., 2010) or *in vivo* experiments (Mueller et al., 2006), as well as on ADSCs *in vitro* (Liang et al., 2011).

Cisplatin (CIS) and paclitaxel (PAC) are two of the most widely used chemotherapy drugs for the treatment of several types of cancer (Adamo et al., 2004; Zhang and Tuckett, 2008). CIS is a platinum compound and its main mechanism of action is based in the formation of adducts with DNA nucleobases that block DNA replication and transcription and, ultimately, cell division (García et al., 2008). These crosslinks account for about 90% of the total DNA damage induced by CIS and are thought to be a major contributing factor to the cytotoxic effects of this drug (Bhana et al., 2008). The taxane PAC acts through stabilization of microtubules during cell division, blocking mitosis at the G2/M phase with subsequent apoptosis in sensitive cells (Adamo et al., 2004; Specenier and Vermorken, 2009).

Despite the effectiveness of current anti-cancer agents, several studies have shown their mutagenic, teratogenic and carcinogenic potential in experimental

systems (Bhatia and Sklar, 2002; Joshi, 2007). However, the literature is still poor in data regarding the susceptibility of human ADSCs to genotoxic damage induced by chemotherapeutic agents. The present study aimed at evaluating the DNA damage induction potential of these two drugs, commonly used in anti-cancer therapies, on hADSCs during *in vitro* cultivation.

2. Materials and Methods

2.1 Isolation and culture of human adipose-derived stem cells (hADSCs)

Human adipose-derived stem cells were obtained from adipose tissue of four patients undergoing elective liposuction surgery. All patients signed an informed consent form, and the study was approved by the Research Ethics Committee of Irmandade Santa Casa de Misericórdia de Porto AlegreHospital Group.The stromal vascular fraction was isolated as described by Zuk et al. (2001). Briefly, the liposuction material was extensively washed with phosphate buffered saline (PBS) and incubated with type I collagenase for 30 minutes at 37º C.The digested lipoaspirate was centrifuged for 10 min at 800 xg, the supernatant discarded and the cell pellet resuspended in 160mM NH_4Cl to eliminate red blood cells; after 10 min at 37º C, the cells were again centrifuged, resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with HEPES (free acid, 2.5–3.7 g/l), 10% fetal bovine serum (FBS, Cultilab, Sao Paulo, Brazil) and 1% penicillin/streptomycin (Cell Culture Medium – CCM). Cells were seeded at 2 x 10^4 cells/cm^2 onto tissue culture flasks and expanded at 37^oC in a humidified culture chamber with 5% CO_2, changing the culture medium every 2–3 days. Cells were passaged to a new flask when confluence reached 90%. Cells between passages 6 and 9 were used in all experiments. All reagents used here were from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. Plasticware was from TPP (Trasadingen, Switzerland).

2.2 Immunophenotyping

For analysis of surface antigens the cells were trypsinized, centrifuged, and incubated for 30 minutes at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD13, CD34, CD44, CD69, CD73, CD90, CD105, CD117 and HLA-DR (Pharmingen BD, San Diego, CA). Excess antibody was removed by washing. The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected. The WinMDI 2.8 software was used for building histograms.

2.3 hADSCs differentiation induction

Osteogenic differentiation was induced by cultivating hADSCs for up to 15 days in CCM supplemented with 10^{-8} M dexamethasone, 5 µg/ml ascorbic acid 2-phosphate and 10 mM 2-glycerophosphate (da Silva Meirelles et al., 2006). To observe calcium deposition, cultures were washed once with PBS, fixed with 4% paraformaldehyde in PBS for 15-30 minutes at room temperature (RT), and stained for 5 minutes at RT with Alizarin Red S stain at pH 4.2. Excess stain was removed by several washes with distilled water.

To induce adipogenic differentiation, cells were cultured for up to 15 days in CCM supplemented with 10^{-8} M dexamethasone, 2.5 µg/ml insulin and 100 µM indomethacin (da Silva Meirelles et al., 2006). Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 hour at RT, and stained with Oil Red O solution for 5 minutes at RT.

hADSCs differentiation was observed with an inverted phase-contrast microscope (Axiovert 25; Zeiss, Hallbergmoos, Germany) and photomicrographs were taken with a digital camera (AxioCam MRc, Zeiss), using AxioVision 3.1 software (Zeiss).

2.4 NCI-H460 cells culture

The NCI-H460 large cell lung cancer cell line (American Type Culture Collection) was used as a control for sensitivity to the chemotherapeutic agents. Cells were cultured in RPMI1640 medium supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged according to their individual growth profiles in order to ensure exponential growth throughout the experiments.

2.5 Chemotherapeutic agents

Cisplatin (CIS) (CAS No.15663-27-1) and paclitaxel (PAC) (CAS No. 33069-62-4) were obtained as the clinical preparations Platistine® (Pfizer Ltda., São Paulo, Brazil) and Taxol® (Bristol-Myers Squibb Ltda, São Paulo, Brazil). Solutions of these compounds were made with CCM immediately before use.

2.6 MTT assay

Cells were seeded in 96-well plates at a density of $3x10^3$ cells/well for hADSCs and $3x10^4$ cells/well for H460 cells, and treated the following day with cisplatin at 0.5, 1, 3, 5, 10 and 50 µM dosages, and paclitaxel at 0.001, 0.005, 0.01, 0.1, 1 and 10 µM dosages. CCM was used as negative control (NC). After 72 hours, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated at 37°C for 2 h. The formazan crystals resulting from the cleavage of MTT were dissolved in 100 µl DMSO for 5 min with shaking. Each plate was read immediately on a microplate reader (Thermo Scientific,Waltham, MA) at a wavelength of 540 nm. Three independent experiments were performed in triplicate for each type of cell culture. Cell viability is expressed as percentage of the viability of untreated cells.

2.7 Comet assay

To detect DNA strand breaks and alkali labile as well as incomplete excision repair sites, we used the alkaline single-cell microgel electrophoresis (comet) assay as described previously (Tice et al., 2000). hADSCs were treated for 1, 3 and 24 h with three concentrations of CIS (3, 5 and 10 µM) and PAC (0.01, 0.1 and 1 µM). We used ethylmethane sulphonate (EMS) 5 mM as positive control (PC). After the treatments, the cells were trypsinized, resuspended in 0.5% low melting agarose and distributed onto slides (Knittel Glaser, Braunschweig, Germany) coated with 1.5% normal melting agarose (Invitrogen Co, Carlsbad, CA). After cell lysis for 24 h in alkaline lysis buffer (10% DMSO, 1% Triton-X, 2.5M NaCl, 10mM Tris, 100mM EDTA, pH 10), slides were placed in a horizontal gel electrophoresis chamber and covered with alkaline buffer (5mM NaOH and 200mM EDTA) at pH> 13. After a 20-min period for DNA denaturation, electrophoresis was performed under standard conditions (1 V/cm, 300 mA, distance between electrodes 36 cm) for 20 min. Following neutralization at pH 7.5 (0.4M Tris, Invitrogen), cells were stored until analysis. All preparation steps were performed under red or yellow light to avoid DNA damage by UV light.

For analysis of slides an Olympus System Microscope (Model BX41) equipped with an Olympus Reflected Fluorescence System (Model U-RFL-T) and an

Olympus U-TV0.35XC-2 Camera (Tokyo, Japan) was used. After coding and blinding of the slides, they were stained with an ethidium bromide solution and the comets were determined by an image analysis system (Comet Assay IV, Perceptive Instruments, UK). Two slides with 50 cells (total of 100 cells) for every test sample were counted and analyzed for the following parameters to quantify the induced DNA damage: Tail Length (TL), Tail Intensity (TI) and Tail Moment (TM), product of the median migration distance and the percentage of DNA in tail. Data of TI, TL and TM are given in Table 1. However, the final statistical evaluation was based on TM. Results are given as mean±standard deviation (S.D.)

2.8 Statistical analysis

Statistical analysis of the obtained data was performed using the SPSS software, version 13.0. To analyze differences, One Way ANOVA with Dunett post hoc test was applied, where the drug treatments were compared against the Negative Control. Differences were considered statistically significant when the P-value was less than 0.05.

3. Results

3.1 Characterization of hADSCs

hADSCs showed a fibroblast-like morphology when cultured in CCM (Fig. 1A and B). The morphology was maintained through repeated passages under non-stimulating conditions. When subjected to the adipogenic and osteogenic differentiation-inducing medium, hADSCs displayed a typical morphological change, with intracellular lipid droplets stained by Oil Red O (Fig. 1F) and extracellular calcium deposition by Alizarin Red S staining (Fig. 1D), respectively.

Flow cytometry showed that hADSCs were positive for CD13, CD44, CD73, CD90 and CD105 but did not express CD34, CD45, CD69, CD117 and HLA-DR (Fig. 2).

3.2 Cell sensitivity to the chemotherapeutic agents

The sensitivity of hADSCs to CIS and PAC was evaluated through the MTT assay, in comparison with the H460 cells treatment with these two drugs. After 72 h of treatment, hADSCs but not H460 cells showed resistance to the increasing concentrations of CIS and PAC, since the drugs were not able to reduce the cell percentage of hADSCs above 50% in comparison with the corresponding NC (Fig. 3A and B). As we expected, the cell percentage of H460 cells was highly reduced, in comparison with the NC, confirming their sensitivity to the drugs (Fig 3C and D.).

3.3 Genotoxicity

To evaluate the susceptibility of hADSCs to the genotoxic damage induced by CIS and PAC, we used the comet assay. After 1, 3 and 24 h of exposure to the drugs, no DNA damage was observed in hADSCs in all tested dosages, with exception of PC (Table 1).

4. **Discussion**

The resistance of human stem cells to chemical and physical agents has been demonstrated by several studies. Li et al. (2004) investigated the chemosensitivity of human mesenchymal stem cells from bone marrow (hBM-MSCs), showing their variable response to drugs commonly used in anti-cancer therapies, including PAC. Similarly, Mueller et al. (2006) evaluated the viability and characteristics of hBM-MSCs from chemotherapeutically treated patients, showing no effects of the treatment on these cells. Further, these authors demonstrated an elevated apoptotic threshold of the hBM-MSCs through their exposure to drugs at apoptosis-inducing dosages. In a more recent study, Liang et al. (2011) demonstrated the resistance and recovery of hADSCs exposed to several drugs. Our results confirm the great potential of mesenchymal stem cells for resist to agents commonly used in anti-cancer therapies, which are known to be cytotoxic for normal and tumor cells.

Anti-cancer drugs are also known by their potential to cause DNA damage in normal and tumor cells, due to their ability to interact with DNA and/or with proteins involved in cell cycle (O'Connor and Fan, 1996; Ashwell and Zabludoff, 2008; McClendon and Osheroff, 2008; Smart, 2008). CIS is known, as well as others platinum-derived drugs, to form crosslinked DNA adducts involving purine residues (Woynarowski et al., 1998; Nowosielska and Marinus, 2005; García Sar et al., 2008). CIS-DNA adducts can be converted to single and double strand breaks during the repair process (Nowosielska and Marinus, 2005; García Sar et al., 2008), which explains the observed genotoxicity of this drug when evaluated in different bioassays (Katz, 1987, 1998; Silva et al., 2005; Miyamoto et al., 2007; García Sar et al., 2008; Oliveira et al., 2009). Here we demonstrated no genotoxic effects of CIS on hADSCs. This lack of genotoxicity of CIS could be explained by the low concentrations of this drug used in this study, since DNA damage-response pathways are activated in hBM-MSCs exposed to higher concentrations of this drug (Prendergast et al., 2011). Despite the absence of genotoxicity observed in this study, CIS is able to induce DNA damage in other types of human cells *in vitro* at similar concentrations as used here (Gebel et al., 1997; Kosmider et al., 2004; Kosmider et al., 2005).

The taxane PAC stabilizes the microtubules promoting assembly rather than disassembly, leading to the formation of non-functional microtubule bundles (Kumar, 1981; Ringel and Horwitz, 1991). This drug does not directly interact with DNA, although it exerts aneugenic, clastogenic and recombinogenic activities (Digue et al., 1999; Rodríguez-Arnaiz et al., 2004).Here we demonstrated no DNA damage induced by PAC in hADSCs, at clinically relevant concentrations. This data agree with results obtained in other studies using the AMES test, in which PAC did not induce point mutations (Lee et al., 2003). On the other hand, this drug showed high potential to induce centromere-positive micronucleus, when evaluated through the cytokinesis-blocked micronuclei assay (CBMN) in combination with fluorescent *in situ* hybridization (FISH) (Digue et al., 1999) and produced both structural chromosome aberrationsand mitotic recombinationin in *Drosophila melanogaster* (Rodríguez-Arnaiz et al., 2004). Furthermore, PAC at concentrations similar to those used here, was able to induce DNA damage in other types of human cells *in vitro* (Preisler et al., 1999; Steiblen, et al., 2005; Turkez et al., 2010).

The resistance of hADSCs to genotoxic agents could be explained, in part, by the high potential of stem cells to repair DNA damage (Frosina, 2010). Several studies have demonstrated that ageing and cell maturation decrease the ability of stem cells to recruit the repair machinery (Nouspikel and Hanawalt, 2000; Bracker et al., 2006; Prall et al., 2007; Nouspikel, 2007). In contrast, embryonic stem cells, which can generate germ cells within the developing embryo, are hypersensitive to DNA damage and, after exposure to a DNA damaging agent, undergo p53-dependent apoptosis without cell cycle arrest, presumably to avoid a high rate of embryonic malformations (Heyer et al., 2000). Since adult stem cells are responsible for the support of tissues and organs, their capacity to resist to harmful situations, including exposure to genotoxic agents, is crucial for the maintenance of genome integrity, which is required for a viable and healthy progeny.

It should be noted that hADSCs, like all adult stem cell types, are a quiescent population *in vivo* and the response of these rapidly dividing cells *in vitro* may not represent their actual behavior *in vivo*. Previous studies suggested induction of differentiation, cell migration and proliferation (Gimble et al. 1996; Almohamad et

al. 2003) as the probable response of the *in vivo* stroma to acute injury, including chemotherapy. Therefore, the results obtained by our study are limited and experiments exploring other mechanisms involving this issue should be conducted, as a comparative form to determine what is the crucial point for the resistance of hADSCs to genotoxic drugs.

In conclusion, this work confirmed the resistance of hADSCs to commonly used chemotherapeutic agents and showed that these cells are not susceptible to DNA damage induced by these drugs. These conclusions give some light on the basic mechanisms, but further investigations are needed to elucidate the main cause – or causes - of stem cell drug resistance, since the data obtained here are not sufficient to support the hypotesis of the DNA repair pathways involvement in drug resistance.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1 Results from image analysis using the Comet Assay IV software (mean ± standard deviation) for hADSCs treatments.

	hADSCs	1h treatment	3h treatment	24h treatment
	Drug Concentrations	Mean ± Standard Deviation	Mean ± Standard Deviation	Mean ± Standard Deviation
TL	NC	35.43 ± 5.94	40.43 ± 8.57	35.51 ± 8.66
	CIS 3uM	35.32 ± 8.49	37.69 ± 10.48	23.59 ± 9.88
	CIS 5uM	40.05 ± 15.58	40.82 ± 17.70	23.05 ± 7.90
	CIS 10uM	39.11 ± 7.86	27.47 ± 3.99	22.17 ± 7.93
	PAC 0,01 uM	36.20 ± 6.62	40.95 ± 12.58	30.55 ± 7.30
	PAC 0,1 uM	28.98 ± 4.76	35.66 ± 10.45	38.75 ± 8.90
	PAC 1uM	34.04 ± 11.11	48.11 ± 17.06	45.47 ± 7.66
	PC	75.92 ± 48.39*	88.17 ± 27.34*	158.45 ± 22.13*
TI	NC	4.53 ± 0.23	4.82 ± 3.93	5.81 ± 0.83
	CIS 3uM	4.86 ± 0.80	5.29 ± 2.99	5.06 ± 2.00
	CIS 5uM	5.56 ± 2.14	8.14 ± 2.34	4.73 ± 0.81
	CIS 10uM	4.00 ± 1.28	6.50 ± 2.32	6.04 ± 1.82
	PAC 0,01 uM	5.93 ± 1.58	7.38 ± 1.25	5.56 ± 1.25
	PAC 0,1 uM	6.19 ± 2.97	6.32 ± 3.55	5.55 ± 2.05
	PAC 1uM	8.52 ± 3.88	6.04 ± 1.48	10.94 ± 5.10
	PC	21.82 ± 7.84*	50.29 ± 10.92*	89.52 ± 9.90*
TM**	NC	0.90 ± 0.20	0.96 ± 0.77	1.09 ± 0.07
	CIS 3uM	0.89 ± 0.22	1.31 ± 0.81	0.68 ± 0.21
	CIS 5uM	1.40 ± 0.72	2.35 ± 1.37	0.65 ± 0.18
	CIS 10uM	0.83 ± 0.38	1.03 ± 0.35	0.78 ± 0.20
	PAC 0,01 uM	1.07 ± 0.26	1.91 ± 0.90	1.00 ± 0.24
	PAC 0,1 uM	1.03 ± 0.54	1.29 ± 0.65	1.74 ± 0.61
	PAC 1uM	1.71 ± 0.50	1.56 ±0.46	2.93 ± 1.53
	PC	6.55 ± 2.35*	23.01 ± 10.75*	79.57 ± 17.05*

TL: Tail Length; TI: Tail Intensity; TM: Tail Moment; *P<0.05, One Way ANOVA with Dunett post hoc test; **Only TM values were used for final diagnosis.

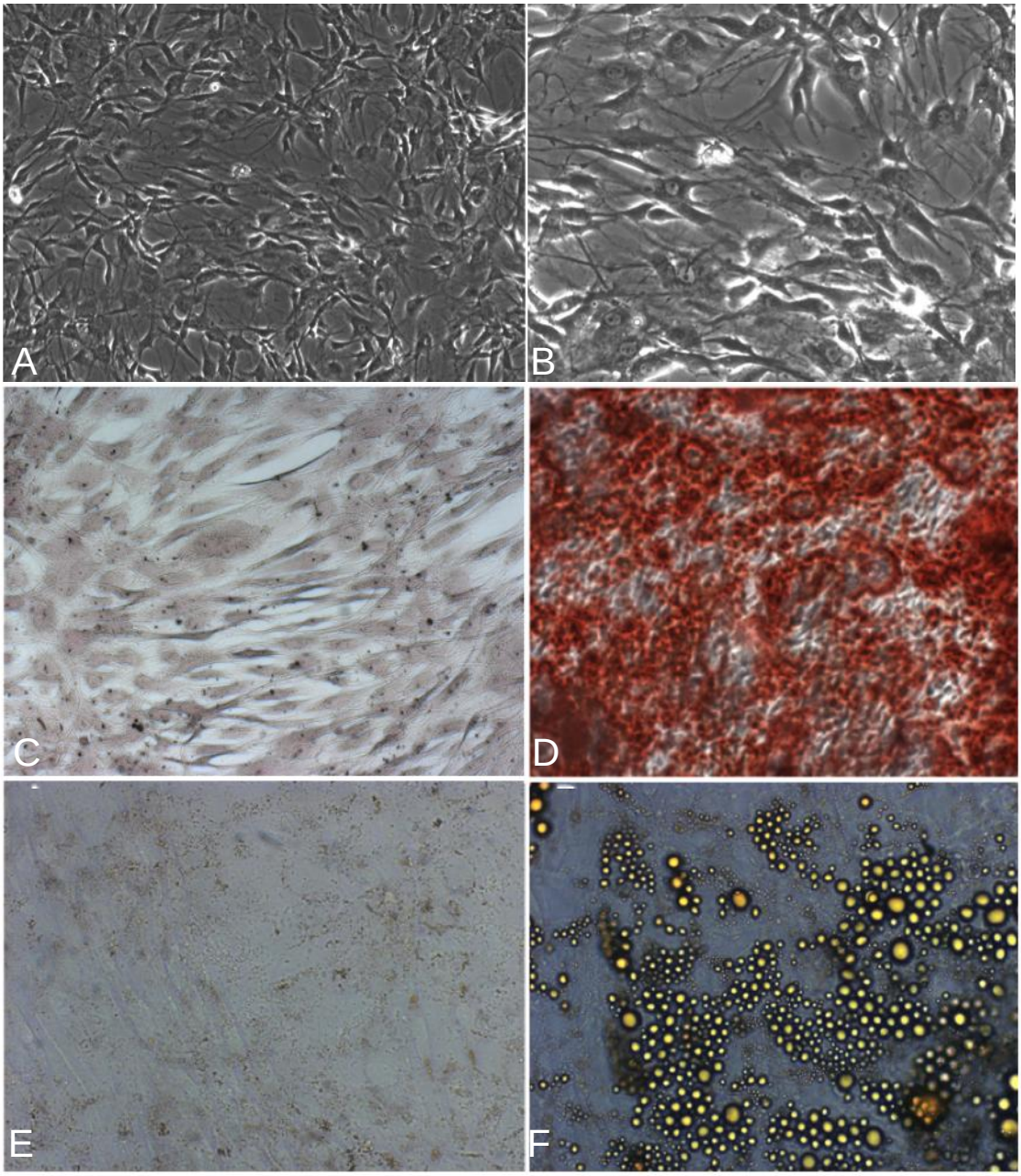

Fig. 1 - A, B: Human adipose-derived stem cells in normal culture conditions, in passage 6 (original magnification x200 and x400, respectively). C, D: hADSCs stained with Alizarin Red S. E, F: hADSCs stained with Oil Red O. Normal cultures are not stained (C, E), but when cultivated with osteogenic or adipogenic media, hADSC show positive staining (D, F). Original magnification x200.

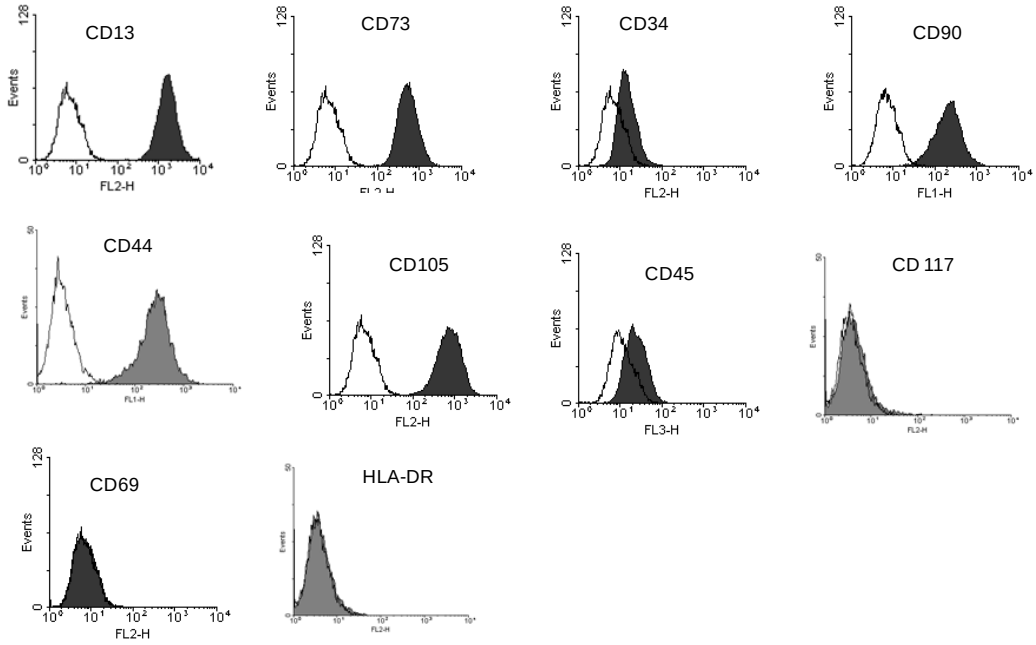

Fig. 2 - Immunophenotypic profile of *in vitro* cultured hADSCs. Flow cytometry histograms show the expression (shaded) of selected cell surface antigens compared with controls (unshaded peaks).

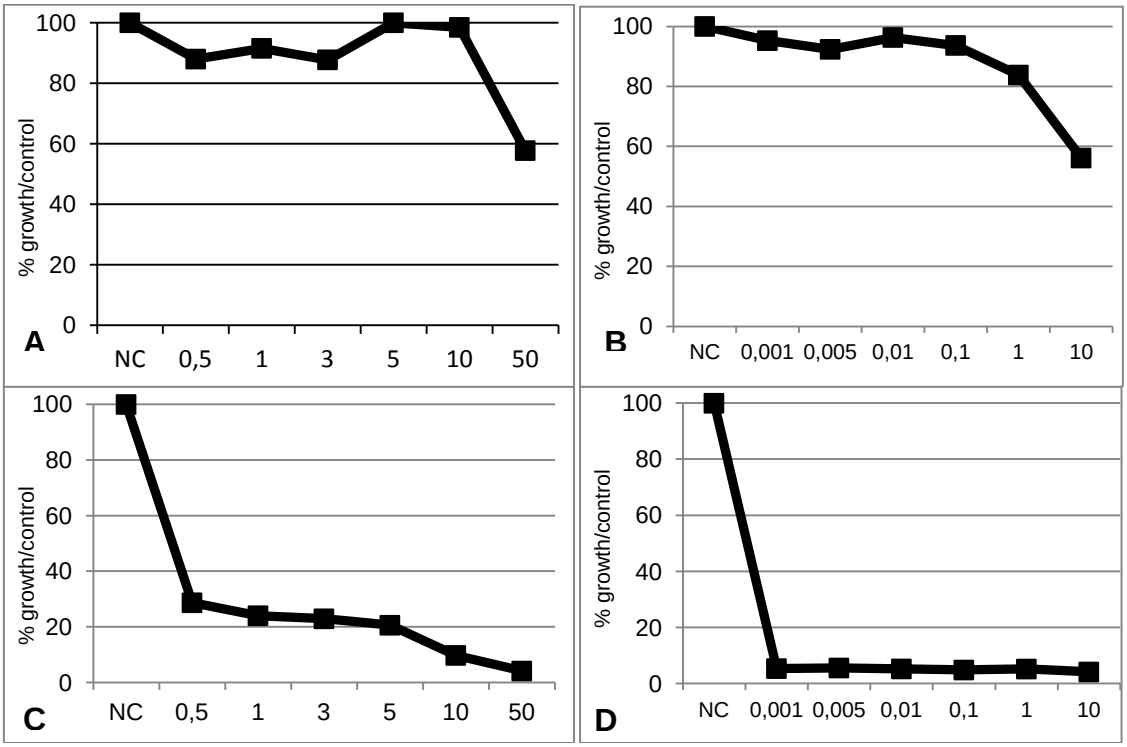

Fig. 3 - Sensitivity of cell cultures treated with cisplatin and paclitaxel for 72 h at the indicated doses (μM). Cell viability was determined using the MTT assay, and is expressed as a percentage of the viability of untreated cells. Data represent the mean of three independent experiments. A: hADSCs treated with cisplatin. B: hADSCs treated with paclitaxel. C: H460 cells treated with cisplatin. D: H460 cells treated with paclitaxel.

4 Discussão

No presente estudo buscamos compreender um dos mecanismos básicos responsáveis pela já descrita resistência de células-tronco mesenquimais a agentes químicos. Para tanto, utilizamos o ensaio MTT, que é baseado na redução metabólica do brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio (MTT) por desidrogenases mitocondriais, e o ensaio Cometa, que detecta danos no DNA pela eletroforese em gel de agarose de células individualizadas, tendo em vista obter dados quanto à atividade citotóxica e genotóxica dos quimioterápicos utilizados em tratamentos contra o câncer, cisplatina (CIS) e paclitaxel (PAC).

Através do ensaio MTT, pudemos confirmar a ausência de citotoxicidade provocada pelos agentes químicos em células-tronco derivadas de tecido adiposo humano (hADSCs), já demonstrada por Liang et al. (2011) e em células-tronco derivadas da medula óssea humana (hBM-MSCs) (Li et al., 2004), *in vitro*. Em outro estudo, Mueller e cols. (2006) avaliaram a viabilidade e as características básicas de hBM-MSCs de pacientes sob tratamento quimioterápico, e demonstraram que este tratamento não causou alterações nas células quanto aos parâmetros avaliados. Além disso, neste estudo os autores demonstraram que as hBM-MSCs possuem um alto limiar de tolerância à apoptose, através de sua exposição aos fármacos em dosagens capazes de tal efeito. Levando-se em conta este e os trabalhos anteriores, podemos concluir que as hADSCs são, de fato, resistentes aos efeitos citotóxicos conhecidos de quimioterápicos amplamente utilizados no tratamento do câncer, visto que estas drogas foram capazes de reduzir significativamente o número de células da linhagem H460 utilizadas neste estudo como um controle de sensibilidade para a avaliação citotóxica.

A partir da confirmação da resistência das hADSCs aos quimioterápicos, utilizamos o ensaio cometa para verificar a sensibilidade destas células em relação a lesões quimicamente induzidas no material genético. Vários estudos já demonstraram o potencial genotóxico de agentes quimioterápicos, seja em células normais, seja em células tumorais, através da interação destas drogas

com a molécula de DNA e/ou com proteínas que regulam o ciclo celular (O'Connor e Fan, 1996; McClendon e Osheroff, 2007; Ashwell e Zabludoff, 2008; Smart, 2008). CIS tem a capacidade de formar adutos ligados de forma cruzada ao DNA (Woynarowski et al., 1998; Nowosielska e Marinus, 2005; García Sar et al., 2008), que podem ser convertidos em quebras de fita simples ou dupla de DNA durante o processo de reparo (Nowosielska e Marinus, 2005; García Sar et al., 2008). Essas quebras na molécula de DNA podem explicar a genotoxicidade causada por esta droga que foi observada em diversos bioensaios (Katz, 1987, 1998; Silva et al., 2005; Miyamoto et al., 2007; García Sar et al., 2008; Oliveira et al., 2009). O taxano PAC age através da estabilização dos microtúbulos durante a divisão celular, levando à formação de um complexo não funcional (Kumar, 1981; Ringel e Horwitz, 1991). Esta droga não age diretamente na molécula de DNA, mas exerce atividade aneugênica, clastogênica e recombinogênica (Digue et al., 1999; Rodríguez-Arnaiz et al., 2004), o que pode explicar seus efeitos genotóxicos.

Neste estudo observamos que, tanto CIS, quanto PAC, não foram capazes de induzir danos significativos no DNA das hADSCs, quando utilizados em concentrações de relevância clínica. De fato, estudos disponíveis na literatura têm demonstrado que CIS e PAC são genotóxicos em diferentes linhagens celulares, em concentrações similares às utilizadas neste estudo (Gebel et al., 1997; Preisler et al., 1999; Kosmider et al., 2004; Kosmider et al., 2005; Steiblen, et al., 2005; Turkez et al., 2010). Por outro lado, a ausência de genotoxicidade de CIS nas hADSCs pode ser resultado das baixas concentrações deste fármaco utilizadas neste estudo, já que CIS é capaz de ativar mecanismos de reparo a danos no DNA em hBM-MSCs quando utilizada em dosagens mais elevadas (Prendergast et al.,2011).

Com relação aos resultados obtidos pelo tratamento com PAC, Lee et al. (2003) mostraram que este fármaco não induziu mutações pontuais no teste de AMES em *Salmonella typhimurium*. Entretanto, PAC induziu mutações cromossômicas quando avaliado no teste de micronúcleos com bloqueio de citocinese (CBMN) combinado com hibridização fluorescente *in situ* (FISH) em linfócitos humanos (Digue et al., 1999), além de produzir aberrações

cromossômicas estruturais e recombinação mitótica em células somáticas de *D. melanogaster* (Rodríguez-Arnaiz et al., 2004).

Ao analisarmos os dados obtidos por este estudo e levarmos em consideração resultados de estudos prévios com os quimioterápicos em diferentes bioensaios de curta duração, podemos constatar que CIS e PAC, quando utilizados em concentrações não citotóxicas, apresentam-se destituídos de efeito genotóxico em hADSCs. Diferentes mecanismos podem estar relacionados com a resistência das hADSCs às lesões induzidas pelos fármacos CIS e PAC. Neste sentido, o grande potencial para ativar mecanismos de reparo de DNA em células-tronco adultas, pode exercer um papel importante na resistência destas células à exposição a diferentes agentes químicos (Frosina, 2010).

Vários estudos já demonstraram a capacidade decrescente das células-tronco adultas em recrutar a maquinaria de reparo durante o envelhecimento ou de acordo com o grau de maturação da célula (Nouspikel e Hanawalt, 2000; Bracker et al., 2006; Prall et al., 2007; Nouspikel, 2007). Em contraste, células-tronco embrionárias, que tem a capacidade de gerar células germinativas dentro do embrião em desenvolvimento, são hipersensíveis a danos no DNA e, após exposição a um agente genotóxico, sofrem apoptose dependente de p53 e sem parada no ciclo celular, presumivelmente para evitar altas taxas de más formações embrionárias (Heyer et al., 2000). Já que células-tronco adultas auxiliam no suporte de órgãos e tecidos, sua capacidade para resistir à situações desfavoráveis, incluindo exposição a agentes citotóxicos e genotóxicos, é de grande importância para a manutenção da integridade genômica, o que é fundamental para gerar uma progênie viável e saudável.

Além da ativação da maquinaria de reparo, outros mecanismos podem estar associados à resistência de células-tronco adultas a agentes químicos, como a ativação de enzimas de detoxificação e expressão de genes da família ABC (*ATP-binding cassete*). Tais mecanismos têm sido amplamente relacionados à resistência de células tumorais aos tratamentos quimioterápicos. A família de enzimas de detoxificação glutationa S-transferases, por serem altamente polimórficas e conjugarem a glutationa com uma grande variedade de compostos

endógenos e exógenos, além de interagirem com diversas moléculas que participam da sobrevivência e morte celular (Townsend e Tew, 2003), tem sido alvo de diversos estudos que às relacionam com a suscetibilidade de células tumorais a diversos quimioterápicos (O'Brien et al., 2000; Harkey et al., 2005; Tsuboi et al., 2011). Porém, ainda pouco se sabe sobre a expressão destas enzimas em células-tronco adultas e não há uma relação evidente entre estas enzimas e a resistência das hADSCs a quimioterápicos.

Outro artefato celular que vem sendo estudado é a atuação de proteínas transmembrana capazes de expulsar ativamente vários tipos de drogas, toxinas e agentes químicos (Sarkadi et al., 1996). A expressão de genes da família ABC tem sido considerada um mecanismo importante na resistência de células tumorais a quimioterápicos, já que estes genes são altamente expressos em diversas linhagens tumorais (Gottesman et al., 2002; Szakács et al., 2004; Auner et al., 2010). Atualmente, o grande foco das investigações sobre a função destes transportadores se concentra na elucidação do seu papel na resistência de células-tronco tumorais aos tratamentos quimioterápicos (Chen et al., 2009; Ding et al., 2010; Elliott et al., 2010) e pouco se sabe sobre suas funções em células-tronco mesenquimais.

Do ponto de vista clínico, a grande capacidade destas células para resistir a agentes citotóxicos e genotóxicos serve para confirmar seu potencial como alternativa segura e promissora para a medicina regenerativa, onde é crescente o interesse na aplicação das hADSCs na reconstrução de áreas afetadas pela quimio- e radioterapia (Rigotti et al., 2007; Sterodimas et al., 2010).

Finalmente, constatamos que as hADSCs não são suscetíveis a danos no DNA que poderiam ser causados pela exposição a agentes comumente utilizados na terapia anti-câncer, o que nos permite hipotetizar o envolvimento da tolerância a danos no DNA na manutenção da integridade destas células. Assim, concluímos que o grande potencial de resitência das hADSCs é uma caracteristica importante e que deve ser levada em consideração na realização de qualquer ensaio que vise a aplicação clínica destas células ou a compreensão de mecanismos básicos de sua biologia.

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