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CELLULAR AND MOLECULAR BIOLOGY

Comparison of senescence phenotype of short- and long- term cultured rat mesenchymal stem cells *in vitro*

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Abstract: Mesenchymal stem cells present clinical potential to recover and regenerate injured tissues in diverse pathologies. The *in vitro* expansion and characterization of these cells contribute to elucidation of the mechanisms of senescence and strategies involving cell therapies. This study aimed to compare specific characteristics between initial and advanced passages of mesenchymal stem cells derived from adipose tissue and bone marrow. Both cell types were characterized according to immunophenotype, osteogenic differentiation, genomic instability, migration assay, doubling population time and colony forming ability. Our results demonstrated that both cell types were able to maintain an immunophenotypic profile typical of mesenchymal stem cells during increasing passages. Adipose stem cells at initial passage presented greater migration capacity compared to advanced passage cells, and advanced passage cells proliferated faster than initial passage cells. Bone marrow stem cells at early passages presented higher osteogenic potential than advanced. At advanced passages they presented higher colony forming capacity and genetic damage than those at initial passage. These results suggest that mesenchymal stem cells maintained in culture presented characteristics of senescence that should be monitored prior the use in regenerative medicine and cells derived from bone marrow at initial passage have better potential for therapeutic use in bone tissue engineering.

Key words: osteogenesis, senescence, stem cells, tissue engineering.

INTRODUCTION

Mesenchymal stem cells (MSCs) have great clinical potential to repair injured tissues in diverse pathologies, including osteochondral defects, cardiovascular, neurological and hematopoietic diseases (Salem & Thiemermann 2010). There are many clinical trials underway using MSCs for therapeutic purposes with no reported serious adverse events (Rodríguez-Fuente et al. 2021). MSCs exist in a perivascular niche that shapes their distribution in the body (da Silva Meirelles et al. 2008). Since stem cells are among the cells with greatest longevity within an organism, senescence of these cells is highly relevant to the health and longevity. Despite the variations in mechanisms and phenotypes, all stem cell populations present some degree of function decline with age (Schultz & Sinclair 2016). Transplanted cells can integrate into the site of injury, coordinate wound repair processes, and recover the injured tissue (Hong et al. 2013). Stem cells may accelerate wound healing and affect inflammatory processes (Isakson et al. 2015). A crucial characteristic of MSCs is plasticity. The *in vitro* expansion and characterization of MSCs in

murine models contribute to elucidation of the mechanisms and design of innovative therapeutic strategies involving cell therapies (Meirelles & Nardi 2003). Bone marrow mesenchymal stem cell (BMSC) and adipose-derived stem cell (ASC) share general characteristics, nevertheless, BMSCs present greater osteogenic capacity and bone repair potential *in vivo* (Ena et al. 2015).

The discovery of MSCs challenged cell culture research and therapy. Maintaining or even controlling unique properties of stem cells during the expansion in culture is crucial for creating undifferentiated cell populations that can thereafter be terminally differentiated (Reubinoff et al. 2000). Stem cells can be cultured and maintained *in vitro* for long periods, however the application of these cells in experimental models depends on the preservation of their characteristics. Previous studies show that MSCs isolated from different tissues undergo different changes with increasing culture time (Otte et al. 2013) and defective therapeutic potential of aged progenitor cells is a result of age-related changes in MSC population dynamics (Duscher et al. 2014). The age-dependent changes to DNA fidelity, the epigenetic scenery, metabolic stress, and extrinsic factors contribute and impact stem cell function during aging (Jung & Brack 2014). Age-related modifications in the local or systemic environment implicates in the decline in stem cell function. Understanding mechanisms by which stem cell functionality declines with age is essential to improve tissue repair in the elderly (Liu & Rando 2011). Replicative senescence is an organized process in MSC and is associated with down-regulation of genes involved in DNA replication and repair. These changes increase continuously with every passage and they are not limited to the end of long-term *in vitro* culture (Wagner et al. 2008). Aging is a process that presents loss of tissue homeostasis and progressive deterioration of tissue and organ functions especially due to cellular damage accumulated during life. Senescence is supposed to protect the organism from damage, but when exacerbated or chronic, subvert their purpose and generate further damage (López-Otín et al. 2013). Cellular senescence impacts on various regenerative systems and hold promise for clinical intervention (Yun 2015).

Evidence is still controversial regarding prolonged culture of stem cells. Therefore, it is necessary to further explore aspects of mesenchymal stem cells maintained in prolonged culture periods. This study aimed to compare phenotype characteristics of mesenchymal stem cells between early, also referred as "short-term", and late passages "long-term" in culture of MSC from adipose tissue and bone marrow to assess functional changes resulting from senescence.

MATERIALS AND METHODS

Isolation and culture of ASCs and BMSCs

Six 3-month-old Lewis rats were used in this study. The project was approved by the Ethics Committee on Animal Use of the State Foundation for Health Production and Research (FEPPS) under protocol number 02/2013. The handling of animals obeyed the ethical principles of animal experimentation of the Brazilian Code of Animal Experimentation (COBEA).

Normal culture medium (NCM) consisted of HDMEM (Dulbecco's Modified Eagle's Medium with 25 mM HEPES), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA). Unless otherwise specified, all reagents were provided by Sigma Chemical Co. (St. Louis, MO, USA), and antibodies by Santa Cruz (Santa Cruz, CA, USA).

ASCs were isolated from inguinal adipose tissue. The tissue was washed with saline solution, cut into small pieces, and incubated with 110 U/mL collagenase type I solution for 30

minutes at 37° C. The lipid-containing top layer was discarded, and the pellet was centrifuged at 800 x *g* for 10 min and plated.

BMSCs were isolated from the femur and tibia of the rats. Briefly, after cutting both ends of the femur and tibia, the bone marrow was washed with 1 mL of DMEM (Dulbecco's Modified Eagle's Medium) with 10% fetal bovine serum (FBS), treated with red cell lysis solution by 5 min and cultivated at 37º C and 5% CO_2 atmosphere. The medium was changed 72 h after isolation to remove non-adherent cells.

For culture, cells were resuspended in NCM with 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37ºC with a 5% CO_2 atmosphere. The medium was exchanged 72 h later to remove non-adherent cells. Upon reaching about 80% confluence, the cultures were passaged. Cell cultures are referred to as short-term or long-term cultured when up to passage six or passage 40, respectively. Cultures were periodically examined on a phase-contrast light microscope (Axiovert 25, Carl Zeiss, Hallbergmoos, Germany). All experiments were done in triplicate.

Proliferation assay

Proliferative activity was assessed as population doubling time (PDT), calculated according to the following equation: PDT = (T-T0)*log 2/(log N–log N0). (T-T0) is the duration of the experiment (in hours), N is the number of cells at the end of the experiment, and N0 is the number of cells initially seeded.

Clonogenic assay

Cells were plated at a density of 200 cells/well in 6-well plates in triplicate. After 14 days the cells were fixed with methyl alcohol and stained with Giemsa for counting colonies under an inverted optical microscope. Colony groups with more than 50 cells were considered. The result of the potential of colony formation was expressed by the percentage of colonies formed.

Immunophenotyping

ASCs and BMSCs were removed from plastic using trypsin, then immunophenotyped by flow cytometry. Cells were incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated CD29, CD44, CD90, CD31, CD11b, and CD45 antibodies for 30 min at 4°C. The samples were analyzed in a BD Accuri C6 flow cytometer (Becton Dickinson, USA). At least 10,000 events were collected.

Scratch wound assay

The protocol for evaluating migration capacity was based on the *in vitro* scratch wound test (Walter et al. 2010). ASCs and BMSCs were plated on a 12-well plate and grown to 70-80% confluence. A scratch was made in the cell monolayer with a customized cell scraper, and the plates were washed with phosphate buffer (PBS) to remove cell debris. The lesion area was photographed on a phase-contrast inverted microscope (Axiovert 25, Carl Zeiss, Hallbergmoos, Germany) at marked positions at 0, 24, 48 and 72 hours. Scratched areas were measured with ImageJ software (NIH, Bethesda, USA). The lesion closure was measured in micrometers and expressed by the percentage of space between the resulting ends of the lesion, considering the result of time 0 h as 100% of space and totally repaired lesion as 0%.

Micronucleus assay

The protocol and analyses of micronuclei were performed according to Fenech (2000). Briefly, a total of 10^5 cells were cytocentrifuged onto glass slides at 400 x *g* for 5 minutes. The slides were then stained with Instant Prov (New Prov, Pinhais, PR, BRL), according to the manufacturer's recommendations, and dried

at room temperature. About 1000 cells were counted per slide on an optical microscope (Primo Star, Carl Zeiss, Hallbergmoos, Germany) at 1000 × magnification, for analysis of the presence of micronucleus. The tests were performed in quadruplicates. Micronucleus quantification was expressed by the number of micronuclei per thousand cells counted.

Osteogenic differentiation

For induction of differentiation, the cultures were maintained in a specific medium for 14 days, with medium change every 3–4 days. The osteogenic medium was composed of DMEM supplemented with 15 mM HEPES, 10% FBS, 10^{-8} M dexamethasone, 5 µg/mL ascorbic acid 2-phosphate and 10 mM glycerol phosphate. Cells were maintained in NCM for the same period, as control.

Alkaline phosphatase assay

After 14 days on osteogenic-inducing medium, in a 24-well plate, the medium was removed and the cultures were washed with PBS and then incubated with BCIP/NBT substrate (500 µLof BCIP + 500 µL of NBT + 4 mL of H2O) in a total volume of 400 µL (Invitrogen Co, Carlsbad, CA) for 2 h, at 37°C, protected from light. After incubation, the substrate was removed and 200 µL SDS/10% HCl was added and the cells were left at 37° C with 5% CO2 for 18 hours. Then, 100 µL of each sample were distributed in a 96-well plate. Optical density (O.D.) was determined on a multi-plate reader (Multiskan Ex original, Serial-RS-232c Thermo Fisher Scientific, Vantaa, Finland) at 405 nm. The results were expressed as O.D. ALP activity was calculated according to the formula: U=(C/t)d, where U = ALP activity per µL of solution; C = concentration of product formed; t = incubation time; and d = sample dilution. ALP activity was normalized by the amount of total protein.

Semi-quantitative analysis of mineralization

After staining the cultures maintained in osteogenic medium, the cells were fixed with 70% ethanol for 15 minutes and washed three times with distilled water. Alizarin S red (200 µL) was added in 24-well plates to the cells and incubated for 30 min at room temperature. The excess dye was removed by four washes with distilled water. After the last wash, the cells were incubated with 500 µL of isopropanol for 5 min. Subsequently, the samples were distributed in a 96-well plate for spectrophotometric reading (Multiskan Ex original, serial RS-232C, Thermo Fisher Scietific, Vantaa, Finland) at 540 nm. For quantification of the mineralization, a 1: 2 molar ratio between Alizarin S Red and calcium was applied. The results were expressed = O.D. (differentiated cells) - O.D. (controls).

Statistical analysis

The data were represented as means ± standard deviation (SD) of the means from three or more independent experiments. Statistical comparisons between groups were made using One Way ANOVA. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Population doubling time

After isolation, the ASC and BMSC were maintained in culture and monitored using microscopy during 40 passages. Fibroblastoid morphology, typical of a mesenchymal stem cell, was not altered with passage progression. At each passage progression, the cells were counted, and doubling population time information was available (See Figure 1a). The cell cycle in long-term cultures was faster (ASC - 35 h and BMSC - 36 h) then in short-term cultures (ASC - 47 h and BMSC - 44 h).

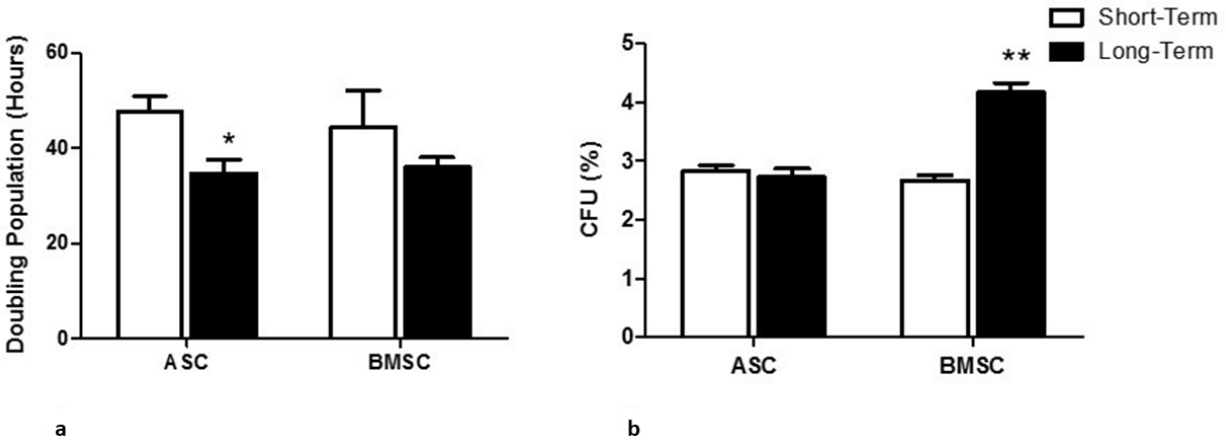

Figure 1. a) Doubling population time comparison of ASC and BMSC; b) and comparison of ASC and BMSC colonies formation units. Data is presented as mean ± S.D. * p <0.05 and ** p <0.01 (n = 3).

Clonogenic assay

Approximately 2.7% of ASC and BMSC in initial passage were able to form colonies and the same was observed in advanced ASCs. On the other hand, BMSC in advanced passage had a significant increase of 1.5 times in the percentage of colony forming cells (Figure 1b).

Immunophenotypic analysis

Both ASC and BMSC were positive for the CD29, CD44, CD90, and negative for the CD31, CD11b and CD45 markers (Figure 2). Values indicating the percentage of positive and negative cells for each marker type are shown in Table I.

Immunophenotypic pattern was similar in both ASC or BMSC cell types. However, we observed variation in percentage of positive cell for the following of some markers. In ASC, CD90 and CD11b were more expressed in the advanced passages ($p = 0.019$ and $p = 0.004$, respectively), while CD44 had decreased expression in advanced passages ($p = 0.016$). In BMSC, there was a decrease in CD44 and CD31 expression ($p = 0.002$ and $p = 0.042$, respectively) and an increase in CD11b expression ($p = 0.013$) (Figure 2).

Migration test

Differences in lesion closure process were observed when we compared ASC in short- and long-term cultures in the 24 h period. No differences were observed for BMSC. In all cells analyzed up to 48 h, closure was partial. Only after 72h, lesion space closed completely (Figure 3).

Micronuclei assay

Greater chromosomal instability was detected in long-term cultured BMSCs when compared to cells short-term cultured (Figure 4). Further, in long-term BMSC the increase in micronuclei was approximately 4.5 times higher than in short-term. The increase in the number of micronuclei may have been generated due to clastogenesis or aneugenesis.

Osteogenic potential

Cells were exposed for 14 days to control medium or osteogenesis inducing medium. When we analyzed ALP activity (Figure 5a), ALP levels were higher in short-term BMSCs compared to long-term BMSCs ($p <0.001$), whereas there was no difference in ALP activity in ASC at different passages.

Alizarin staining was used to detect, by microscopy, the mineralization. Both ASC and BMSC showed differentiation capacity in osteogenic lineage. Mineralization was assessed by indirect analysis of the matrix using red staining, which appears due to calcium phosphate deposition of the differentiated cells. After the cells were stained and processed, alizarin quantification was converted in concentration of calcium deposition. The values obtained were expressed in mM of stained calcium (Figure 5b). There was no difference in the amount of calcium matrix formed by short- and long- term ASCs; however short-term BMSCs produced a higher amount of matrix in relation to long-term ($p < 0.05$).

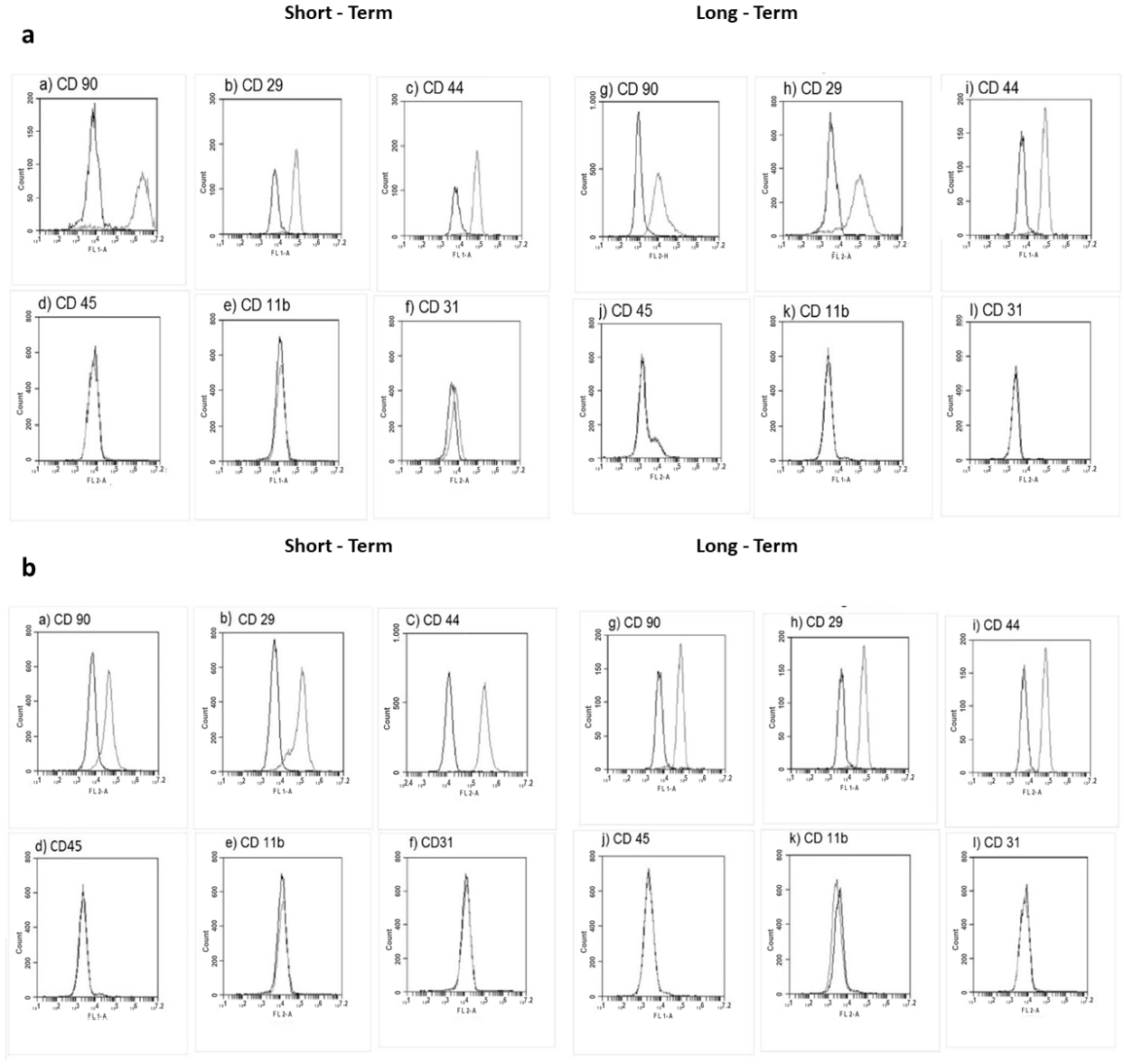

Figure 2. Immunophenotypic profile of short- and long-term cultures ASC (2a) and BMSC (2b), showing fluorescence positivity for CD90 (a, g), CD29 (b, h) and CD44 (c, i) markers (darker color peaks) in relation to histogram control (peak of lighter color). And low fluorescence for CD45 (d, j), CD11b (e, k) and CD31 (f, l) markers. The control histogram corresponds to cells not labeled with antibody.

Table I. Comparison between expression percentage of surface markers at different passages and in each cell type. Short – Short-term culture and Long – Long-term culture. Data is presented as mean ± S.D.* p <0.05 and ** p<0.01 (n=3).

	ASC			BMSC		
Markers	**Short (%)**	**Long (%)**	***p value***	**Short (%)**	**Long (%)**	***p value***
CD90	89.33 ± 2.19	98.00 ± 0.58	0.019 *	82.67 ± 1.20	85.33 ± 1.45	0.230
CD44	98.67 ± 0.33	96.00 ± 0.58	0.016 *	92.33 ± 0.88	80.67 ± 1.45	0.002 **
CD29	94.33 ± 0.33	92.33 ± 2.60	0.489	88.33 ± 1.45	83.33 ± 2.90	0.199
CD45	0.67 ± 0.33	0.67 ± 0.33	1.000	1.33 ± 0.33	3.33 ± 0.33	0.013 *
CD11b	0.67 ± 0.33	3.33 ± 0.33	0.004**	1.33 ± 0.33	1.67 ± 0.67	0.678
CD31	1.33 ± 0.33	1.67 ± 0.33	0.518	5.33 ± 0.88	1.67 ± 0.88	0.042*

Alizarin staining was used to detect, by microscopy, the mineralization. Both ASC and BMSC showed differentiation capacity in osteogenic lineage. Mineralization was assessed by indirect analysis of the matrix using red staining, which appears due to calcium phosphate deposition of the differentiated cells. After the cells were stained and processed, alizarin quantification was converted in concentration of calcium deposition. The values obtained were expressed in mM of stained calcium (Figure 5b). There was no difference in the amount of calcium matrix formed by short- and long- term ASCs; however short-term BMSCs produced a higher amount of matrix in relation to long-term ($p < 0.05$).

DISCUSSION

In Tissue Engineering the use of stem cells is increasingly explored (Meirelles & Nardi 2003). MSCs are present in all organs and tissues (Marx et al. 2015) and are commonly obtained from bone marrow and adipose tissue for later use in research (Shiratsuki et al. 2015). Because they are easy to maintain in vitro, more knowledge about the monitoring of these cells in culture is needed in the short and long term. It is known that long-term expansion may lead to significant changes in morphology and affects proliferation kinetics and the cell cycle (Danisovic et al. 2017). As cells age, some transformations occur, they enter senescence and start to lose their stem cell characteristics as *in vitro* culture begins (Bonab et al. 2006). Therefore, these senescence related transformations in stem cells should be taken into account prior to stem cell-based therapies (Zaim et al. 2012). However, there is no single effective method to monitor *in vitro* senescence (Turinetto et al. 2016).

The results of our study clearly demonstrated a correlation between long-term culture and senescence potential of ASCs and BMSCs. The colony-forming assay can be used to estimate the proliferative and clonogenic potential of ASCs and BMSCs in culture. The potential to form colonies is an important aspect and distinguishes mesenchymal stem cells from other already differentiated cells that can make symmetrical mitoses (Pochampally 2008). In our study we evaluated colony formation during maintenance of the cells in short- and long-term culture. We demonstrated that BMSC had the ability to form more colonies at advanced than at initial passage. Prolonged culture time of BMSC may induce more colony

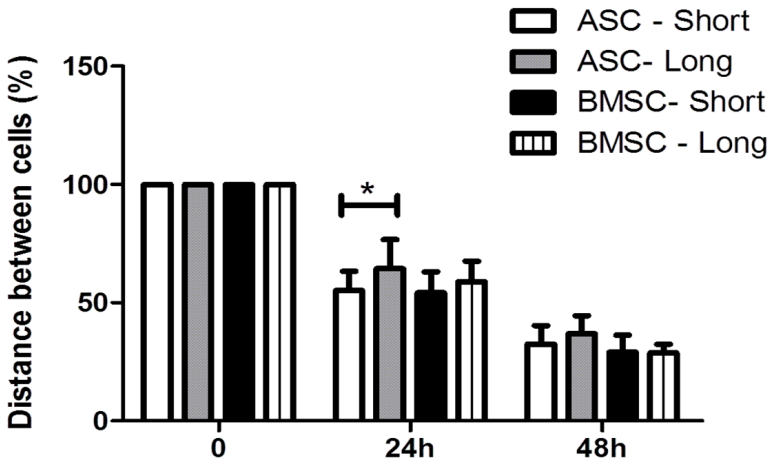

Figure 3. Cell migration after induced injury in the adherent cell layer. The measurement was presented in % of space between the extremities of the lesion. Evaluation at 0, 24, 48 and 72 h. The lesion was fully repaired after 72 hours. Data is presented as mean ± S.D.* p <0.05 (n = 3).

formation, accumulate genetic damage and may be one of the reasons for the loss of its osteogenic potential. Indeed, Kundrotas et al. (2016), indicated that early passage BMSCs were genomically stable and retained identity and high proliferation capacity, while at late passages the cells became senescent. Senescence followed the slower proliferation, changed the morphology and immunophenotype, although it maintained chromosomal stability.

The micronuclei can be defined as a small nucleus-like mass, delimited by membrane, and separated from the main nucleus. It is formed during cell division in the telophase when the nuclear envelope is reconstituted around the chromosomes of the daughter cells (Salvadori et al. 2003). Thus, an increase in the frequency of micronucleated cells may reflect exposure to clastogenic agents or aneugenics (Albertini et al. 2000). Therefore, DNA damage due to exposure to mutagenic agents is detected in micronuclei after a cycle of cell division (Fenech 1997). In this study we found that long-term cultured BMSC accumulated genetic damage, identified through micronuclei assay. This chromosome anomalies might originate from expansion, which could lead to impaired stemness and pluripotency of stem cells (Jiang et al. 2017).

We found that ASC and BMSC maintained in culture showed an immunophenotypic profile typical of mesenchymal stem cells and this characteristic was maintained throughout increasing passages. Danisovic et al. (2017) cultured mesenchymal stem cells for up to 30 passages and have shown that MSCs maintained prototypical immunophenotype profile regardless of passage Danisovic et al. (2017). Another study points out that adult stem cells maintain a constant phenotype profile irrespective of origin and long-term culture (Somasundaram et al. 2015). Although, evidence suggests that MSC enter senescence almost undetectably from the start of in vitro culture (Bonab et al. 2006).

Here we demonstrated that cells at initial passage presented greater migration capacity than when at advanced passage, notably the ASC, which presented a significant difference at 24h. In accordance with our results, another study showed that cells in early passages have better lesion closure than cells in advanced passages (Zhao et al. 2011). Bertolo et al. (2015) compared BMSCs from various donors and passages for their in vitro motility, and the distances were correlated to differentiation potentials, levels of senescence and cell size. They found that slow-moving cells had the higher proportion of

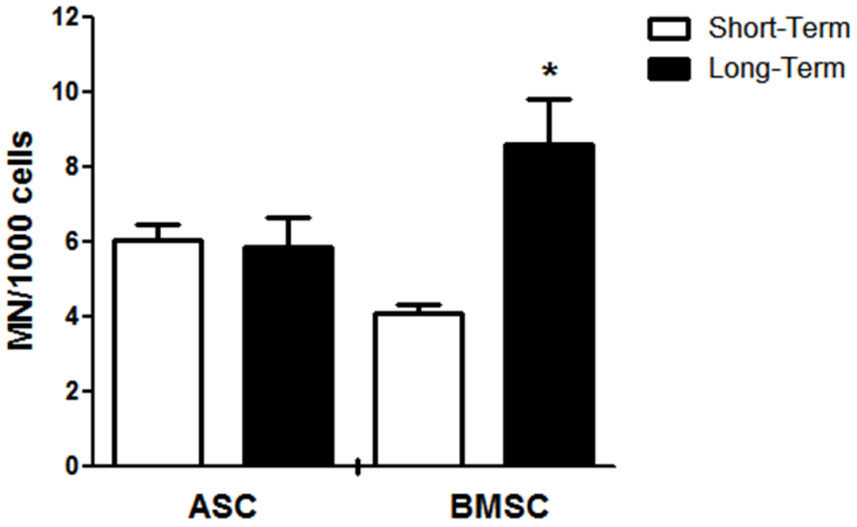

Figure 4. Micronuclei in short- and long-term ASC and BMSC data is presented as mean ± S.D. * p <0.05 (n = 4).

senescent cells compared with fast ones and the larger cells moved less than smaller ones, and spindle-shaped cells had an average speed. Both fast cells and slow cells presented low differentiation potential, and average-moving cells were more effective in undergoing all three lineage differentiations.

Since the aim of this study is to characterize cells according to the culture time to be used in tissue engineering strategies, proliferation rate, cell viability and apoptosis become important after implantation *in vivo*. We found that ASCs at advanced passage decreased their division time in 13 hours. However, BMSC did not present a significant decrease in division time with advancing passages. It has been suggested that senescent cells may be identified based on declining population doubling time (Jeong & Cho 2016). Our results agree with other studies which observed that BMSCs maintain their proliferative potential during extended culture periods (Zuk et al. 2001, Marolt et al. 2014). Diomede et al. (2017) found no difference between early and late passages in proliferation even though senescence markers were considerably increased in late passage.

Our study showed that osteogenic potential is higher in BMSCs in early. The higher osteogenic potential of BMSCs was detected by increased levels of mineralization and alkaline phosphatase activity and indicated that BMSC has greater potential for osteogenic differentiation than ASC. Bertolo et al. (2016), also showed that BMSC in early-passage cells underwent efficient osteogenic differentiation. Late-passage cells preserved only the adipogenic differentiation potential. According to Zhang et al. (2009) the anatomical origin of MSC has a profound influence on its differentiation potential. On the other hand, Fu et al. (2015), demonstrated that MSCs grown up to 20 passages did not show significant changes in phenotype nor did they lose their ability to differentiate into three lineages. However, senescence-associated β-galactosidase expression increased. These results suggest that MSCs maintain their stem cell properties during prolonged culturing, but in fact they undergo senescence. Alt et al. (2012), showed the effect of aging on the self-renewal and differentiation potential of ASCs. Legzdina et al. (2016), found a gradual decline of ASC fitness, altered cell morphology, loss of proliferative, clonogenic and differentiation abilities in

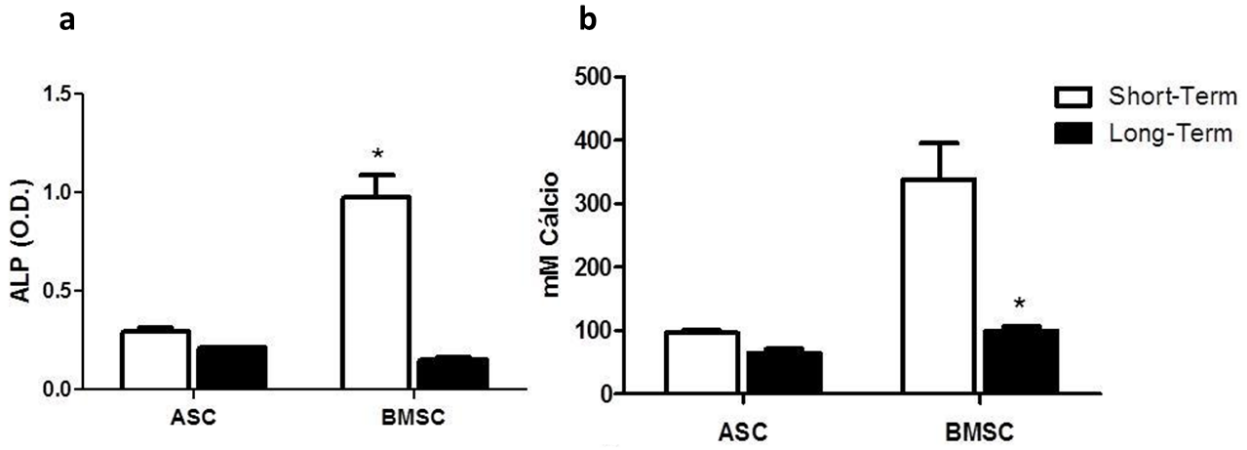

Figure 5. Quantification of the mineralized calcium matrix and indirect analysis of ALP ASC and BMSC in short and long-term. a) Analysis of ALP levels in ASC and BMSC. b) Calcium levels expressed in mM in ASC and BMSC after maintenance in inductive medium for 14 days. Data is presented as mean ± S.D. * p <0.05 (n = 3).

senescent cells. The promotion of the osteogenic differentiation, cell proliferation, and migration potential in short-term stem cells can be related to an important epigenetic mechanism, like the histone demethylase called KDM3B (Zhang et al. 2020). By the way, the epigenetics of aging and senescence indicate that these processes are regulated by similar means. The study of changes associated with senescence *in vitro* has several strategic advantages for research on aging (Wagner 2019).

We cannot rule out the hypothesis that a complete understanding of the senescence characteristics of MSCs can be an alternative to help in the search for methods to rejuvenate senescent MSCs. However, several limitations restrict the application of rejuvenated MSCs. For example, the major pathways that regulate MSC senescence are also important physiological regulators of normal biological functions. Then, the complete suppression or activation of these pathways through interventions may be unacceptable (Zhou et al. 2020). Therefore, to achieve an ideal treatment, it is essential to correctly select MSCs, based on their functional potential, as well as its origin and the result of functional tests after its expansion *in vitro* (Costa et al. 2021). We reinforce the need to analyze biomarkers related to the senescence of cultured cells before clinical application. Our study emphasizes that BMSCs in initial passages are more recommended for bone tissue engineering use due to better differentiation in bone tissue, stable immunophenotypic profile during culture and less damage in their genetic material. More studies are needed to quantify the osteogenic potential of BMSC and ASC along passages to ensure its use in regenerative medicine. In addition, the use of a molecular marker that varies proportionally to the osteogenic potential would be very important to ease the clinical application of mesenchymal stem cells.

Finally, cultured mesenchymal stem cells undergo genetic and differentiation potential changes. It brings to the fore the need for monitoring their functional and senescence biomarkers before use in regenerative medicine. ASC and BMSC isolated from rats and maintained in culture presented an immunophenotypic profile as expected from mesenchymal stem cells and their ability to differentiate were maintained with the increase of the passages. Even though these characteristics were maintained in long-term culture, it does not discard the possibility of senescence. ASC cells at initial passage have a higher migration capacity

and divide faster. It could be an advantage for application in tissue engineering. BMSCs at advanced passage form more colonies than BMSCs at initial passage. On the other hand, they accumulate genetic damages identified by the increase in the number of micronuclei and it may be an evidence of senescence in cells at advanced passages. The osteogenic potential is higher in BMSC at initial passages, an effect assessed by increased levels of mineralization and alkaline phosphatase. Therefore, BMSCs at initial passages are the most recommended for therapeutic use in bone tissue engineering.

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VPAL conceived and wrote the manuscript, MP cultured the cells and provided differentiation, scratch wound and doubling population data, RSC provided data for Table I, BPS contributed to the writing of the discussion, MRS and JS provided data for figure 4, NBN revised it critically for important intellectual content, MC coordinated the experiments and conducted statistical analyses. All authors reviewed the final manuscript.