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Guaraná (*Paullinia cupana*) improves the proliferation and oxidative metabolism of senescent adipocyte stem cells derived from human lipoaspirates

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ABSTRACT

Cellular senescence is a limiting factor in the proliferative expansion and quality of adult mesenchymal stem cells, often making them unviable in regenerative clinical practice. In *vitro* supplementation by antioxidant food extract of senescent mesenchymal stem cells could reverse these undesirable characteristics. To evaluate this hypothesis, senescent adipocyte-mesenchymal cells (ASCs) obtained from human lipoaspirates were exposed at different concentrations of hydro-alcoholic guaraná (*Paullinia cupana*) extract for 72 h. After the incubation, we performed a proliferative assay. Oxidative stress indicators and antioxidant enzymes (biochemical activity and gene expression by qRT-PCR analysis) in these senescent cells were also evaluated. In senescent cells exposed to guaraná at 5 mg/g concentration increased cellular proliferation occurred compared to untreated senescent cells (79.1 ± 15.7%). Concomitantly, a decrease in several oxidative stress indicators was observed in senescent cells treated with guaraná. A genomic effect of guaraná exposure was observed when the modulation of antioxidant enzymes genes was analyzed. The results described here suggest that the food extract supplementation could reverse the initial senescence processes in ASCs. These results have potential application in regenerative medicine.

1. Introduction

Adult mesenchymal stem cells (MSCs) are capable of trilineage mesodermal differentiation into adipocytes, osteoblasts, and chondrocytes; therefore, they can potentially be used in regenerative medicine.

The low concentration of MSCs in donor tissues makes it necessary to expand these cells *in vitro* conditions before being introduced in injured tissue to be regenerated. In the *in vitro* condition, MSCs display three proliferative capacity phases: phase I corresponds to a period of little proliferation before the first passage, during which the cell culture is established; phase II is characterized by rapid cell proliferation; and in phase III the proliferation rate gradually decreases, indicating cellular senescence (Wagner, Ho, & Zenke, 2010).

Due the mortal phenotype of MSCs cultured *in vitro*, the clinical use of these cells presents some limitations due the necessity to produce MSC lines with high regenerative efficiency and concomitant stable genomes to avoid adverse effects such as tumorogenesis (Casiraghi, Remuzzi, Abbate, & Perico, 2013; Serakinci, Fahrioglu, & Christensen, 2014). Therefore, senescence is a limiting factor in the use of MSCs in regenerative therapies since there is a need to increase the number of MSCs to obtain successful clinical use (Bajek et al., 2012). In clinical terms, it is desirable that MSC expansion occurs over a short period of time. For this reason, several studies have attempted to identify the stimulants of MSC proliferation. Previous evidence has suggested that reactive oxygen species (ROS) at low levels may play a pivotal role as second messenger and can induce MSC proliferation (Wagner et al., 2010). Despite the fast MSC expansion under oxidative stress exposition, undesirable effects appear in cell cultures exposed to prooxidant molecules such as H_2O_2, including intracellular ROS accumulation, which can trigger the senescence of MSCs (Burova, Borodkina, Shatrova, & Nikolsky, 2013; Estrada et al., 2013; Borodkina, Shatrova, Abushik, Nikolsky, & Burova, 2014).

In these terms, there is a paradox: at the first moment a moderate oxidative stress is necessary to induce MSC expansion; however, the

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oxidative stress maintenance generated by a burst of growing can induce a rapid cellular senescence and also produce unsafe cells with chromosomal instability.

Studies developed by Bickford et al. (2006) have demonstrated that green tea can increase the proliferative capacity of stem cells, an effect which has been associated with the functional proprieties of this plant. In addition, Su et al. (2013) showed that caffeine treatment can cause an increase in the differentiation capacity of adult stem cells. In this way, we postulated that supplementation with antioxidant food extracts in a culture medium of senescent MSCs cells (at the beginning of phase III) could help decrease the senescence process, as well as reduce the potential damage caused by oxidative stress – this idea is supported by these previous investigations suggesting an effect of nutritional compounds on *in vitro* MSC senescence.

To test this hypothesis we performed an *in vitro* experiment using senescent adipose-derived stem cells (ASCs) obtained from human lipoaspirates. The culture medium of senescent cells (characterized by a decrease in the proliferative rate and a change in ASCs' morphology) was supplemented with hydro-alcoholic guaraná (*Paullinia cupana*) extract.

Guaraná is an Amazonian food rich in caffeine and catechin that is currently used to produce energetic drinks (Schimpl, da Silva, Gonçalves, & Mazzafera, 2013). The investigation of the potential proliferative reversion of the senescence of ASCs by guaraná supplementation had its basis in previous research suggesting that this fruit performs some important biological roles with antioxidant (Mattei, Dias, Espínola, Carlini, & Barros, 1998; Basile et al., 2005; Bittencourt et al., 2013), antimutagenic, anticarcinogenic (Fukumasu et al., 2006; Fukumasu, Latorre, & Zaidan-Dagli, 2011), and immunomodulatory effects (Costa Krewer et al., 2013).

2. Material and methods

2.1. Materials

All chemicals, solvents, cell culture media, molecular biology assays, reagents, and plastics used in this study were purchased from Gibco® Life Techologies Inc. (Grand Island, NY, USA), Sigma® (St. Louis, MO, USA) and Invitrogen Life Technologies (São Paulo, Brazil). The protocols involving spectrophotometric and fluorimetric analysis were performed in a 96-microplate reader (SpectraMax M2/M2e Multimode Plate Reader, Molecular Devices). A real-time polymerase chain reaction (PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA). The guaraná powder was obtained from Embrapa Western Amazon Co, a non-profit governmental organization located in Maués, Amazonas, Brazil.

2.2. Guaraná hydro-ethanolic extract characterization and treatments

The hydro-ethanolic guaraná extract used in the present study was the same as that prepared and described by Bittencourt et al. (2013), which was lyophilized and stored at −20 °C until used in the experiments described here. The authors obtained the extract using a 70:30 mix of alcohol and water to 100 mL of extraction fluid prepared at a concentration of 300 mg/mL.

After lyophilization, we performed the chromatographic analysis with detection by UV absorbance at 272 nm on a high-performance liquid chromatography (HPLC) system consisting of a Shimadzu Prominence LC-20A, an LC-20AT quaternary pump, a SIL 20 auto sampler– A, a DGU-20A5 on-line degasser, a CBM-20A integrator, and a SPD-20AV DAD detector. For the separation we used a 150 mm × 4.6 mm i.d. ODS-3 column (Phenomenex Prodigy ODS-3 100A, 5-μm particle size; Torrance, CA, USA). We prepared standard solutions of caffeine, theobromine, catechins, and tannin that were maintained at 5 °C. At the moment of use, the standard solutions were diluted in five concentrations.

When the ASCs became senescent they were treated with guaraná extract at the same concentrations of 1, 5, 10, and 20 mg/mL. We used the concentrations based on the results obtained by Bittencourt et al. (2013), which showed an antioxidant effect against oxidative stress caused in embryonic fibroblast cells (NiH-3 T3) by sodium nitroprusside exposition. The modulation of ASCs oxidative metabolism was also evaluated as a potential causal mechanism of the effect of guaraná on ASCs' senescence characteristics. These analyses were performed using the guaraná concentration with the best reversal effect on the ASCs' proliferative state.

2.3. Isolation and ASCs culture

Human adipose tissue was collected from patients undergoing elective liposuction surgery at the Unimed Hospital (Santa Maria, RS, Brazil). The protocol was approved by the Federal University of Santa Maria Ethics Committee Board (23081.015838/2011-10) and donors signed a consent form. Tissue was collected from three female patients ranging in age from 30 to 55, with an average age of 45 ± 7. ASCs were isolated following a protocol described by Buehrer and Cheatham (2013). Briefly, the lipoaspirates were initially washed three times with PBS buffer (pH 7.4) to eliminate blood cells, and then digested for 20 min in 0.075% collagenase 1 at 37 °C. The resulting suspension was centrifuged at 2000 rpm for 10 minutes to obtain an ASC-rich pellet. The new cell pellet was resuspended in growth media (DMEM/F12 plus 10% Fetal Bovine Serum, 100 I.U. penicillin, and 100 μg/mL streptomycin) and passed through a 40 μm cell strainer. The remaining cells were plated on standard tissue culture plastic overnight at 37 °C and with 5% CO_2. After 24 h, the non-adherent cells were removed with two rinses of 1 × PBS, and then serially passaged at 70% confluence. Growth media were changed every 3–4 days.

The cell proliferation was determined for each cellular passage until a significant decrease in growing occurred compared to first passage. The senescent stage (phase III) was determined when the ASCs' proliferation decreased ≥20% compared to the first cellular passage. At this point the experiment was performed to observe the potential guaraná effect on ASCs' proliferative state.

2.4. Guaraná cytotoxic effect

First, we evaluated the potential acute cytotoxic effect on ASC viability by quantifying the dead cells in the concentration of free double-strand DNA (dsDNA) in cell culture supernatant (Souza Filho et al., 2013). When a cell dies, the membrane is disrupted and dsDNA fractions are released into the extracellular medium. As DNA PicoGreen® dye presents a high affinity with the dsDNA and is able to quantify the dsDNA released, the Quant-IT™ PicoGreen® ds DNA kit (Invitrogen – Life Technologies) was used to perform the dsDNA determination following the manufacturer's instructions. The dsDNA was measured using 50 μL of the sample and 50 μL of the DNA Picogreen® dissolved in TE buffer, 1X (1:1; v.v), following incubation for 5 minutes in a dark room. The fluorescence was measured at an excitation of 480 nm and an emission of 520 nm recorded at room temperature.

2.5. Senescence analysis by ASC cell proliferation assay and morphological evaluation

The proliferative rate of all ASCs passages was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Briefly, cells/well treated were incubated for 4 h with MTT reagent. After formazan salt was dissolved with dimethylsulfoxide (DMSO), absorbance was measured at 570 nm in a 96-well micro plate reader. The cell proliferation observed in each passage was expressed as a percentage of the control absorbance value. Since the MTT assay presents some limitations

that can lead to overestimation of the proliferative effects of extracts rich in polyphenols, before the analysis the cells were centrifuged at 3000 rpm for 3 minutes, the supernatant was discarded, and the cells were resuspended in PBS buffer. All procedures were performed in triplicate.

The ASCs' morphology was also evaluated to determine the senescence status. These cells have a similar fibroblast pattern and the loss of their original fibroblastic shape indicates senescence. Aging cells acquire a flattened morphology, characterized by a larger cell size, the occurrence of many vacuoles in the cytoplasm, and a bigger nucleus (Kuilman, Michaloglou, Mooi, & Peeper, 2010).

The potential reversal effect of guaraná supplementation on ASC senescence was evaluated by considering the results of the same tests: MTT assay and optical microscopy of ASC morphology.

2.6. Oxidative stress biomarkers assay

Three assays were performed to determine oxidative stress in senescent ASC cells with and without guaraná supplementation: intracellular ROS, lipoperoxidation, and protein carbonylation.

The quantification of ASCs' intracellular ROS was determined using dichlorofluorescein diacetate assay (DCFH-DA), as described by Ahn, Costa, and Emanuel (1996). In this assay, DCFH-DA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent DFF by cellular oxidants. To perform this measure the sample cells were treated with DCFH-DA (10 μM) for 60 min at 37 °C and the fluorescence was measured at an excitation of 488 nm and an emission of 525 nm.

To evaluate the oxidative state of senescent ASCs, the lipoperoxidation as well as protein carbonylation were spectrophotometrically determined. Lipoperoxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS), as described by Ohkawa, Ohishi, and Yagi (1979), and the protein carbonyls were measured according the method described by Morabito et al. (2004).

2.7. The alkaline single cell gel electrophoresis assay

To detect the possible DNA damage in the samples, we performed the comet assay described by Singh, McCoy, Tice, and Schneider (1988), with a modification in the coloration process, as conducted as Nadin, Vargas-Roig, and Ciocca (2001). In this methodology, two slides are placed for sampling and the blades are passed through processes of cellular lyses, electrophoresis, and staining with silver nitrate. Then, 50 cells are read by two analysts who evaluate the extent of the DNA drag, bearing in mind that the higher the drag, the greater the damage index. The DNA damage index is determined by the equation: x.(n°0) + x.(n°1) + x.(n°2) + x.(n°3) + x.(n°4).

2.8. Antioxidant enzyme analysis by enzymatic and qRT-PCR assays

The activity and gene expression of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione-peroxidase (GPx) was evaluated in the senescent ASC samples with and without guaraná supplementation.

The total SOD activity was determined in the samples treated with guaraná by using the methodology described by Spitz and Oberley (1989). This assay is based on the ability of the SOD to reduce the nitroblue tetrazolium chloride to formazan. Therefore, the greater the activity of the SOD, the higher the absorbance of the sample. The absorbance was determined at 560 nm.

The CAT activity was determined using the methodology described by Aebi (1984). This assay uses hydrogen peroxide as a substrate and a kinetic reading is performed at three points once every fifteen minutes. The results obtained were expressed as k/g of protein present in each sample.

The GPX was indirectly determined from the analysis of thiol groups of each sample treated with the different guaraná concentrations, as described by Ellman (1959), and thee absorbance's were read at 412 nm.

The gene expression of SOD1, SOD2, CAT, and GPx were quantitatively determined by real-time PCR (q-PCR). The determination was performed using the total RNA obtained for each sample in the test. The cells were washed at room temperature with PBS (pH 7.4), and cell lysis was performed using 2.5 mL of Trizol (Invitrogen, Carlsbad, CA). Then, the total RNA obtained was solubilized in 20 μL of water. Using the RNA, we amplified the cDNA using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc. Santa Clara, CA, USA) with an initial step of 95 °C for 5 minutes and 40 cycles of alternating temperatures: 95 °C for 1 minute; 60 °C for 30 seconds; 72 °C for 1 minute; 72 °C for 4 minutes to final extension. The amplification reaction was performed using REDExtract-N-Amp PCR Reaction Mix Kit in an Eppendorf Mastercycler gradient thermocycler. Then, the electrophoresis was conducted in 10% acrylamide gel in 1 × TBE buffer at 220 V for 30 minutes. Using 0.2% ethidium bromide, the bands were revealed in transluminator equipment. The primers used for this technique are listed in Table 1.

Table 1 here

2.9. Statistical analysis

The data were presented as percentages of the control group and statistically analyzed using Graphpad prism software, version 5.0 (Graphpad Prism software company, 2014). All experiments were performed in triplicate. The results were compared by Oneway analysis of variance followed by the Tukey *post hoc* test or Student t test. Data with $p \leq 0.05$ were considered significant.

3. Results

The HPLC assay showed that the hydro-alcoholic guaraná extract presented 12.240 mg/g of caffeine, 6.733 mg/g of theobromine and 4.336 mg/g of total catechins (Fig. 1).

The proliferative capacity of ASCs obtained from human lipoaspirates was analyzed. As Fig. 2 shows, rapid cell growth from the third to the fifth passages was observed where the cells presented a young morphological pattern (Fig. 2A). During the sixth and seventh cell passage a decrease occurred in cell proliferation, which returned to similar levels observed in the first passage. The cell culture also started to change from a young to a senescent morphological pattern (Fig. 2B). The eighth passage presented a significant decrease in cell proliferation when compared to the first passage, and a well characterized senescent pattern (Fig. 2C and D). Therefore, in the eighth passage, the ASCs received guaraná supplementation at different concentrations.

The guaraná supplementation at 5 and 10 mg/mL stimulated ASC proliferation when compared to senescent ASCs in the same cell passage (eitgth) whereas a lower concentration (1 mg/mL) and a higher concentration (10 mg/mL) did not change the cell proliferation pattern when compared to untreated cells. Specifically, at 5 mg/ml concentration a

Table 1
Primers list of antioxidant enzymes genes.

Genes	Primers	
	Forward	Reverse
B-actin	TGTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA
SOD1	GCACACTGGTGGTCCATGAA	ACACCACAAGCCAAACGACTT
SOD2	GCCCTGGAACCTCACATCAA	GGTACTTCTCCTCGGTGACGTT
CAT	GATAGCCTTCGACCCAAGCA	ATGGCGGTGAGTGTCAGGAT
GPX1	GGTTTTCATCTATGAGGGTGTTTCC	GCCTTGGTCTGGCAGAGACT

Primers used to evaluate the gene expression of antioxidants enzymes, including superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT) and glutathione peroxidase 1 (GPx1). The beta-actin gene (B-actin) was used as a house keeping gene.

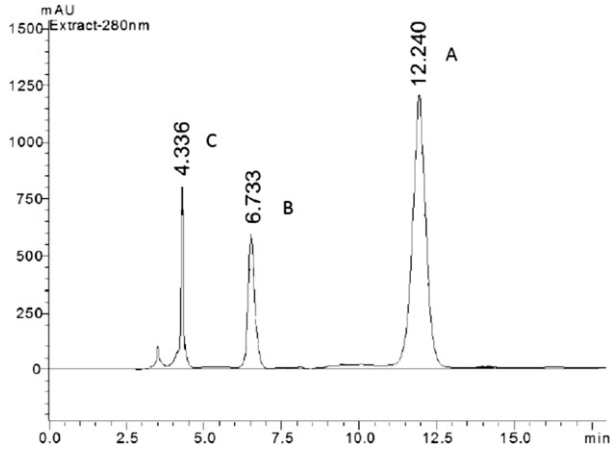

Fig. 1. Characterization and quantification of chemical compounds of hydro-alcoholic guaraná extract. The peaks show that the extract presented 12,240 mg/g of caffeine, 6,733 mg/g of theobromine and 4,336 mg/g of total catechins when compared with the compounds standards.

79.1 ± 15.7% increase in cell proliferation was observed compared to untreated control cells (Fig. 2E).

The ASCs' viability was also evaluated since the results could have been related to the rate of cell mortality and not to the increase in cell proliferation. The results showed a slight and non-significant decrease in the mortality of cells supplemented with guaraná at 5 and 10 mg/mL (84.34 ± 8.06%) determined by free-culture medium dsDNA. In considering these results, and due the limitation of sample cells at the same passage to perform the assays, all further experiments were performed using a lower guaraná concentration, which presented a positive effect on ASC cell proliferative capacity (5 mg/mL).

The senescent ASCs supplemented with guaraná 5 mg/mL showed a significant decrease in the oxidative stress variables analyzed here; that is, of intracellular ROS, lipoperoxidation, and protein carbonylation levels (Fig. 3A–C). The DNA index of damage in cells that received guaraná supplementation was also lower than in untreated cells (Fig. 3D).

The guaraná differentially modulated the ASCs' antioxidant enzyme activity and their gene expression (Figs. 4 and 5). The SOD activity was lower in senescent ASC cells supplemented with guaraná. However, the gene expression analysis showed just SOD1 decreasing, whereas the SOD2 expression was similar to untreated cells. On the other hand, significant increases in CAT activity and gene expression were observed in cells treated with guaraná when compared to a control group. The thiol levels were similar between two ASC cells groups; however, a slight significant decrease in GPx gene expression was also detected in cells supplemented with guaraná.

4. Discussion

The present study analyzed whether guaraná supplementation could act on the senescence of ASCs obtained from human lipoaspirates, and if this action involved the differential regulation of cell oxidative metabolism. When senescent ASCs cells at the eighth passage lost approximately 25% of their proliferative capacity, they received a guaraná 5 mg/mL supplement. These cells showed an improvement in their proliferation as well as a decrease of oxidative stress markers, ROS levels, lipoperoxidation, protein carbonylation, and DNA damage. On the other hand, the guaraná supplementation increased the CAT activity and gene expression significantly, although a lowering in SOD total activity and SOD1 gene expression was detected in the cells exposed to guaraná.

The results described here suggesting that guaraná is able to revert the phenotype of senescent ASCs are highly relevant, especially

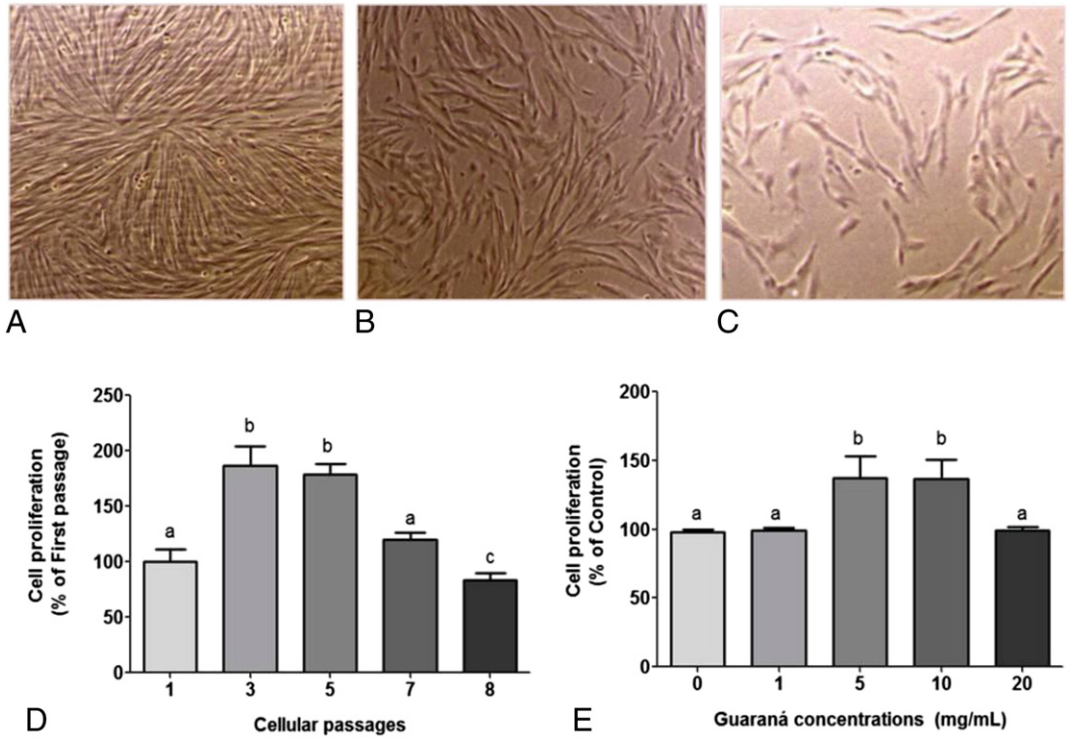

Fig. 2. The process of senescence in adipose-derived stem cells (ASCs) and their proliferative response to guaraná treatments: (A) ASCs between third to fifth passage with intense cell growth and young morphological pattern; (B) ASCs between sixth and seventh passage with decrease in cell proliferation; (C &D) ASCs at eighth passage with significant decrease in cell proliferation, characterizing the senescence process; and (E) guaraná treatments showing a significant increase in proliferation of senescent ASCs mainly at concentration of 5 mg/mL. Data of treatments are presented as % of untreated control group. Different letters indicated significant differences ($p \leq 0.05$) by one-way analysis of variance followed by Tukey post hoc test.

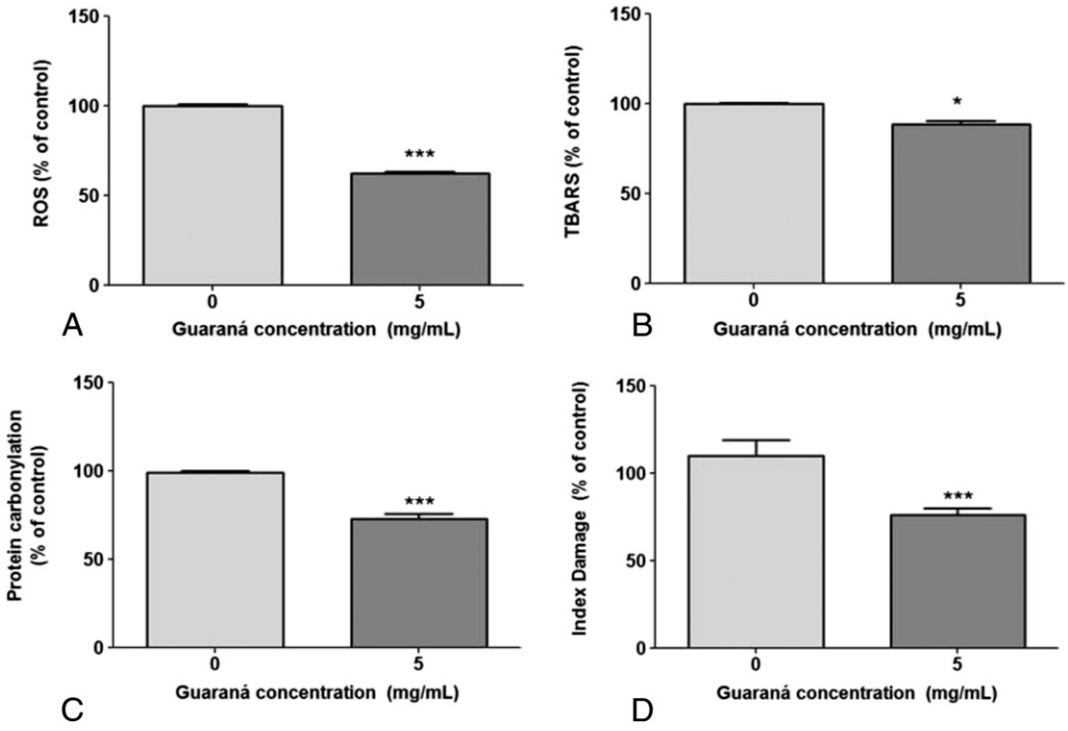

Fig. 3. Senescent ASCs supplemented with guaraná 5 mg/mL: (A, B, C) the treatment decreases the total rate of ROS, the lipoperoxidation level, and the protein carbonylation level, respectively; and (D) the treatment showed lower DNA damage index. Data of treatments are presented as % of untreated control group. The asterisk indicates significant differences ($p \leq 0.05$) by the Student *t* test.

considering that stem cells present in adult tissues are important mediators of tissue maintenance and wound repair. However, similar to differentiated cells, adult ASC stem cells are not immortal and present proliferative senescence when cultured *in vitro*. Their longevity is dependent on a careful control of gene expression, proliferation, and cell cycle, and differentiation signals (Kenyon & Gerson, 2007). The *in vitro* finite lifetime of diploid cell strains may be an expression of aging that denotes long-term loss of proliferative capacity, despite continued viability and metabolic activity (Kuilman et al., 2010; Bajek et al., 2012).

Previous investigations have described how an accumulation of oxidative damage is among the changes related to *in vitro* cell aging (Estrada et al., 2013; Borodkina et al., 2014).

Nowadays, oxygen is considered to be one of the major determinants of stress inducing premature senescence. In fact, singlet oxygen (O_2) did not present cellular toxicity. However, the ROS produced from this molecule affects the cell aging biology. In chemical terms, two groups of ROS can be classified: the first includes ROS as a superoxide and hydroxyl radicals that contain one or more unpaired electrons in their outer molecular orbitals; the second are the molecules such as H_2O_2, ozone, peroxinitrate, and hydroxide that are composed of non-radical ROS, which remains chemically reactive and can be converted to radical ROS (Trachootham, Alexandre, & Huang, 2009).

Among these molecules, H_2O_2 appears to present an important effect on cellular senescence (Chen et al. 2001; Estrada et al., 2013; Borodkina et al., 2014). Therefore, the regulation of H_2O_2 levels could be a mechanism that delays cellular senescence observed in differentiated and adult stem cells as well as ASCs from human lipoaspirates that were studied here. However, fine H_2O_2 cell regulation is difficult to do, since at low physiological levels this molecule is an important cell signaling in several biological pathways.

In these terms, functional foods that present a complex nutritional matrix with several bioactive molecules can play an important role in the ROS modulation of senescent cells, since the concentration of these molecules is in general less than purified substances, and they also create a synergism that produces a different property from that observed in the isolated molecules. Probably, the effect of guaraná on senescent ASCs cells is related to all of its main chemical molecules, which are able to regulate the oxidative metabolism in a harmonic way.

The confirmation of this is based on the results described here that showed a decrease in ROS levels in comparison with untreated cells at the eighth passage of ASCs cells supplemented with guaraná. A previous study by Bittencourt et al. (2013) also showed an increase in the viability and differential oxidative stress modulation of embryonic NH3-T3 fibroblast cells exposed to high NO levels.

Guaraná at 5 and 10 mg/mL concentrations stimulated the ACSs' proliferation when compared to an untreated control group. Investigations have suggested that tissue stem cells in the body have a niche with a specific microenvironment that regulates their self-renewal, proliferation, and differentiation, and the ROS are one such niche regulatory mechanism (Ushio-Fukai & Rehman, 2004). Our results corroborate this hypothesis, indicating that REDOX modulation by a decrease of ROS molecules through the guaraná antioxidant effect could stimulate ACSs' mitotic activity. However, the chemical pathways involved in its effects need to be elucidated.

The reversion of ASC senescent characteristics by guaraná supplementation, with reference to their proliferative state, probably involves the modulation of oxidative metabolism. A possible model of guaraná action on ASC cells is shown in Fig. 5. The expected situation is that ASCs present a senescent phenotype from the seventh to eighth passage, which is mainly detected by a decrease in cell rate proliferation (Fig. 6A).

However, the guaraná supplementation induced a change in this phenotype by decreasing ROS levels, mainly H_2O_2, and as a consequence a decreased cell oxidative state determined by lipoperoxidation, protein carbonylation, and DNA damage. Probably, this effect was obtained by two concomitant pathways: first, by the non-genomic action of antioxidant scavenger molecules present in guaraná's composition, and second, by genomic action involving an increase in CAT activity with the

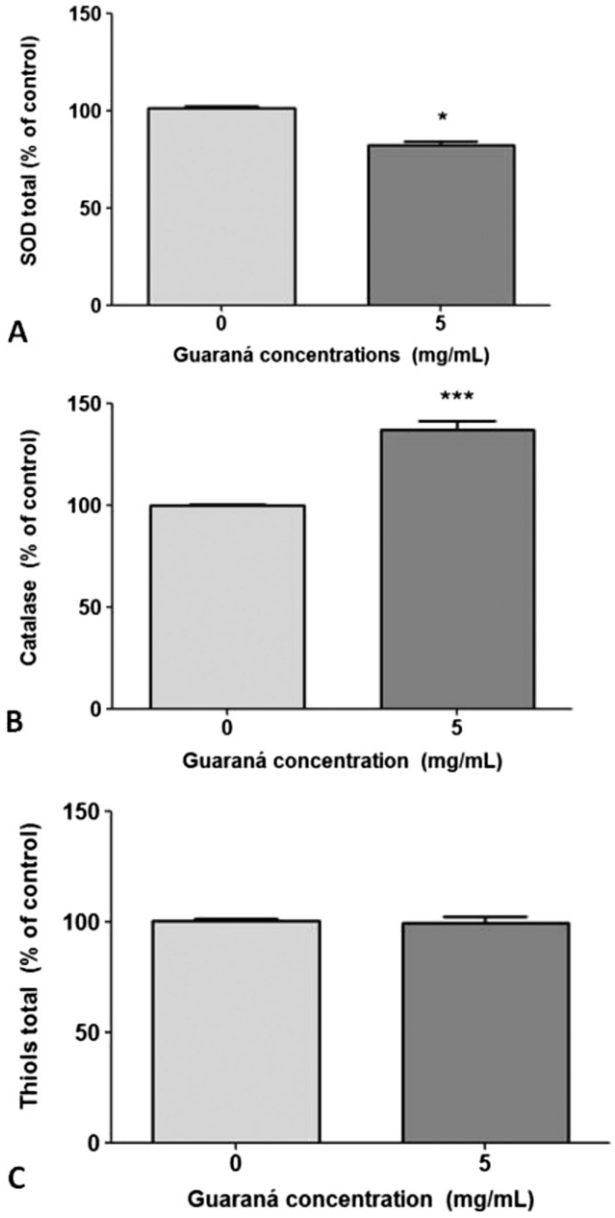

Fig. 4. Guaraná modulates the senescent ASCs antioxidant enzymes activity differentially: (A) the senescent ASCs treated with guaraná 5 mg/mL presented a lower SOD activity; (B) the senescent ASCs treated with guaraná 5 mg/mL presented a increase in CAT activity; and (C) the senescent ASCs treated with guaraná 5 mg/Ml showed a similar response between the groups. Data of treatments are presented as % of untreated control group. The asterisk indicates significant differences ($p \leq 0.05$) by the Student *t* test.

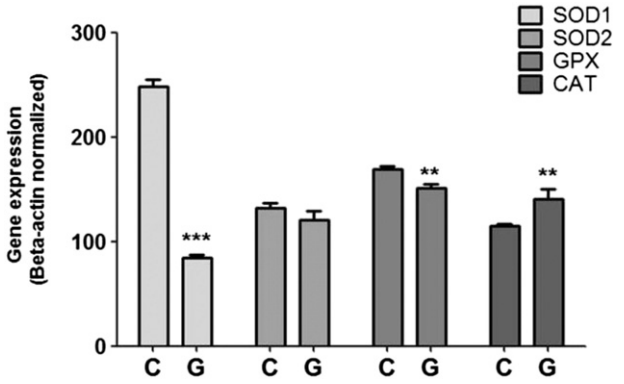

Fig. 5. Guaraná modulate the senescent ASCs antioxidant enzymes gene expression differentially. The senescent ASCs treated with guaraná 5 mg/mL showed a significant decrease in SOD1 expression and a similar expression of SOD2. The treatment also induces increase in CAT expression and a slight significant decrease in GPx gene expression. Data were normalized by beta-actin expression. The asterisk indicates significant differences ($p \leq 0.05$) by the Student *t* test.

up-regulation of this gene and a decrease of SOD activity with down-regulation of the SOD1 gene. The lowering effect on the SOD enzyme decreases the dismutation rate of superoxide ion in H_2O_2 as a consequence. However, this genomic effect seems to occur just in cytoplasm, since an influence of guaraná on SOD2 gene expression that acts only on the mitochondrial level was not detected. This hypothesis is also corroborated by the fact that GPx activity and gene expression is slightly influenced by guaraná supplementation.

One speculation about the relevance of maintaining cellular H_2O_2 at low levels could be that it is related to chemical interactions, as in the Fenton reaction that occurs in cytoplasmic levels involving H_2O_2 and Zn/Cu transition metals that originate hydroxyl (OH^+) molecules. The OH^+ is a high DNA affinity ROS molecule that causes genotoxic effects leading to DNA double-strand breaks and mutation accumulation (Kenyon & Gerson, 2007). Three possible programs were activated to preserve the genome integrity and prevent malignant transformation: DNA repair, apoptosis, or senescence. It is realistic to postulate that control of the H_2O_2 level within the cytoplasm can prevent or control OH^+ production, avoiding the harmful consequences including senescence induction. Both guaraná antioxidant non-genomic and genomic action contribute to a decrease in the ASCs' oxidative state, including reducing the effect on DNA damage, with the final consequence being an increase in cell proliferation (Fig. 6B). However, whether guaraná supplementation can extend the period of ASCs proliferative state (cell longevity?), delaying the senescence period is an open question, which unfortunately the present study did not clarify (Fig. 6C).

The modulatory effect of guaraná on ASCs' cell proliferation obtained from human lipoaspirates is new information, despite the present protocol presenting many methodological constraints such as: (1) guaraná's effect on the prolongation of the proliferative state from supplementation since the first ASCs passage was not determined; (2) the analysis of other senescent characteristics such as telomere shortening, and some aging-related gene modulations were not performed; (3) the analysis of guaraná's regulation of antioxidant metabolism was limited to some biomarkers such as SOD total; (4) as this was an *in vitro* study, the results obtained cannot extrapolated to *in vivo* situations; and (5) this *in vitro* protocol did not use commercial ASC lines or those obtained from animal experimental model as rodents. Therefore, there are great difficulties in obtaining human lipoaspirate samples and performing a prospective analysis involving the analysis of functional food effects on stem cell senescence. However, it is very relevant to consider the potential for the use of adult stem cells in the investigation of regenerative clinical therapies using human MSCs from tissues as lipoaspirates.

Unfortunately, most of the methodological constraints presented in this study cannot to be avoided due to the limitations of obtaining large enough ASC cell concentrations in each passage to permit additional biochemical and molecular analysis. In these terms, whether guaraná in higher concentrations than 5 mg/mL could have harmful effects on ASCs is not known.

A previous study performed by Zeidán-Chuliá et al. (2013) described a negative effect of the main compounds present in energy drinks, including guaraná, on the human neuronal SH-SY5Y cell line due "antioxidant stress." However, a guaraná, taurine, and ginseng exposition was performed on cells with homeostasis integrity, which these compounds disrupted. Therefore, we cannot discard the possibility that at higher concentrations guaraná presents a contrary effect on ASCs. Bittencourt et al. (2013) found that guaraná's effect on cell oxidative stress caused by high NO exposition had not reverted at 20 mg/mL concentration, such as observed here. These

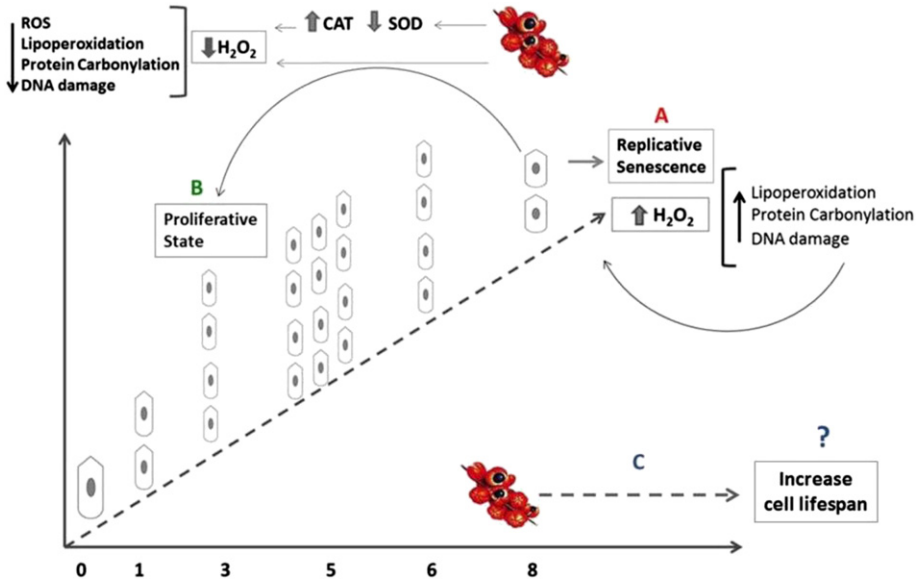

Fig. 6. Schematic illustration of how supplementation with guaraná acts on senescent ASCs: (A) ASCs seventh and eighth passage pass to present decrease in cell proliferation characterizing the senescence process; (B) supplementation with guaraná presents positive effects on senescent ASCs contributing to decrease the ASCs' oxidative states; (C) despite the data obtained, whether the guaraná is able to extend the period of ASC proliferation is an open question.

results suggest a hormetic effect of guaraná on cells, which needs to be considered in the future studies involving this functional food.

For the same reason, by involving a limited cell number to perform the experiments it was not possible to evaluate the isolated effect of the main chemical molecules present in guaraná extract (caffeine, theobromine, total catechins) on senescent ASCs. For this reason, the present study can be considered an exploratory investigation where additional future investigations need to be performed.

Complementary investigations need to evaluate if ASCs' senescent reversion is maintained during future cellular passages. Guaraná's interference in the other important ASC properties such as the potential differentiation of osteoblasts, adipocytes, and chondrocytes also needs to be determined. Both are important issues since previous investigations have considered that an increase in H_2O_2 levels appear to be a good inductor of differentiation of some MSC cells (Robaszkiewicz et al., 2012; Zhang et al., 2012;), whereas other investigations have showed that an increase in H_2O_2 levels has triggered premature exhaustion of MSCs self-renewal (Jang & Sharkis, 2007; Urao & Ushio-Fukai, 2013).

Finally, it is important to comment that epidemiological studies suggest that a high consumption of fruit is beneficial for the prevention of chronic diseases such as cardiovascular morbidities (Hartley et al., 2013), cancer (Kontou, Psaltopoulou, Panagiotakos, Dimopoulos, & Linos, 2011), and neurodegenerative disorders (Joseph, Cole, Head, & Ingram, 2009). There are a large number of studies describing the anti-tumoral properties of several fruits and plant extracts, indicating a positive effect on consumption of fruit and other functional foods and human health (Martin, Zhang, Tonelli, & Petroni, 2013). However, at the present moment, the effect of foods on adult stem cells, mainly of senescence characteristics, is less well studied. These effects are relevant, for at least two reasons: first, to evaluate the potential causal mechanism involved in the preventive effects related to these dietary foods and second, to determine if dietetic supplementation could as successful with regenerative therapies using MSCs as with ASCs from human lipoaspirates.

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