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HUMAN ADIPOSE-DERIVED STEM CELLS OBTAINED FROM LIPOASPIRATES ARE HIGHLY SUSCEPTIBLE TO HYDROGEN PEROXIDE MEDIATED CYTOGENOTOXICITY

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Abstract: There is evidence that H_2O_2 can induce the proliferation, migration, and regeneration of stem cells, as well as that of adipose-derived stem cells (ASCs). This could be useful to expand the possible uses of ASCs in therapeutic applications. However, the safety profile of H_2O_2 use in stem cells is not clear yet. Therefore, the present study evaluated the acute cytotoxic, oxidative and genotoxic effects of different concentrations of H_2O_2 on ASCs obtained from human lipoaspirates. The ASCs were treated with 1–1000 µM H_2O_2 for two hours. Cell viability was evaluated by double-strand DNA determination. Apoptosis induction was analyzed measuring active levels of caspases 1, 3 and 8. Biochemical oxidative stress markers were analyzed and genotoxic effects were assessed by DNA comet assay. All H_2O_2 concentrations increased ASC mortality rates with approximately 100% mortality achieved at ≥ 200 µM. Active caspases 1, 3 and 8, oxidative stress, as well as oxidative damage as assessed by lipid peroxidation increased dose-dependently. There was also an approximate 50% increase in catalase levels in cells exposed to all H_2O_2 tested concentrations. H_2O_2 concentrations of ≥ 10 µM were genotoxic. These results suggest that ASCs are highly sensitive to H_2O_2 exposition. In addition, DNA damage in the surviving cells may affect their proliferative and differentiation capacity, as well as their safety profile for therapeutic use.
Keywords: Adult stem cells genotoxicity. Oxidative stress. Lipoaspirates. Cytotoxicity.

1 INTRODUCTION

Embryogenesis occurs by the differentiation of stem cells into several distinct body tissues. However, throughout the lifespan of an organism, the body also maintains special cells with self-renewal capacity as well as the potential to proliferate and differentiate into specialized cells to replace tissues with a high rate or to regenerate damaged areas. Most cells originate from mesenchymal stromal cells called adult mesenchymal stem cells (MSCs).[1]

Some tissues, such as processed lipoaspirates, are composed of cells with significant similarities to MSCs. Such similarities include the capacity to differentiate into bone, fat, muscle, cartilage cells, cardiomyocytes, periodontal tissue, neurogenic, endothelial, and other lineages. These cells are identified as a type of adipose-derived stem cell or ASC.[2] Therefore, the potential of ASCs to differentiate into multiple lineages makes these cells potentially useful for tissue and cell replacement therapies.[3]

However, despite the large quantity of lipoaspirate ASCs, adequate cell concentrations are necessary for the *in vitro* expansion of these cells. Previous evidence

has suggested that reactive oxygen species (ROS) at low levels may play a pivotal role as second messengers, and they can also induce the proliferation, migration, and regenerative potential of ASCs.[4,5]

Theoretically, the addition of an ROS donor may reduce the costs for the expansion of ASCs in culture, while ROS preconditioning may potentially enhance the regenerative capacity of ASCs in clinical applications. Therefore, dosage is a key determinant of the harmful or beneficial effects of ASC exposure to ROS.

Additional studies also showed that H_2O_2 preconditioning treatment is able to enhance the therapeutic efficacy of some stem cells in regenerative processes. This was described by Zhang *et al*.[6] in a study that used Wharton´s Jelly mesenchymal cells in neovascularization after a myocardial infarction in mice using H_2O_2 at a 200 µM concentration. Robaszkiewicz *et al*.[7] described the role of H_2O_2 in the induction of MSC osteogenic programs. The H_2O_2 exposure presented procalcifying effects and inhibitory regulation of the general antioxidant enzymes, catalase and GPX. In this process, it increased the levels of H_2O_2, caused by the upregulation of NADPH oxidase enzymes.

However, in contrast to embryonic cells, adult MSCs exhibit limited proliferative potential *in vitro*; this is the so-called Hayflick limit, and studies described that those high levels of H_2O_2 can induce the senescence process. This is the case in an investigation performed by Burova, Borodkina, Shatrova, and Nikolsky,[8] which exposed endometrium-derived mesenchymal stem cells (hMESCs) to 200 and 900 µM H_2O_2. As a consequence, the surviving cells irreversibly lost their proliferative capacity.

Another limiting factor in the use of H_2O_2 to induce MSCs is related to the potential genotoxic effects of high levels of ROS in these cells. This fact can be considered as a potential contraindication for H_2O_2 therapeutic applications in human procedures. Even though there are no studies about the effects of H_2O_2 in the genotoxic response of MSCs, this type of study is relevant because it is necessary to consider not only the efficacy of a treatment, but also its safety especially when dealing with the use of ROS. Therefore, an *in vitro* protocol was developed in this study in order to test the effect of H_2O_2 at different concentrations on acute cyto-genotoxicity and oxidative stress using ASCs obtained from human lipoaspirates.

2 MATERIALS AND METHODS

2.1 ISOLATION AND STEM CELL CULTURE

Human adipose tissue was collected from patients who were undergoing elective liposuction surgery. The protocol was reviewed and approved by the Federal University of Santa Maria Ethics Committee Board (23081.015838/2011-10), and the participants signed a copy of the written informed consent. The tissue was collected from three female patients aged from 30 to 55, with an average age of 45 ± 7. ASCs were isolated following the protocol described by Buehrer and Cheatham.9 Briefly, the lipoaspirate was initially washed three times with PBS buffer (pH 7.4) to eliminate blood cells, and it was then digested for 20 min in 0.075% collagenase I at 37 °C. The resulting suspension was centrifuged at 2000 rpm 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 it was passed through a 40-µm cell strainer. The remaining cells were plated on standard tissue made of culture plastic and incubated overnight at 37˚C and 5% CO_2. After 24 hours, the non-adherent cells were removed with two rinses with 1x PBS, and then serially passaged at 70% confluence. The growth media was changed every 3–4 days. At the fourth cell passage, blood and fat wastes were no longer present, and only ASCs remained adhered to the bottle (Fig. 1).

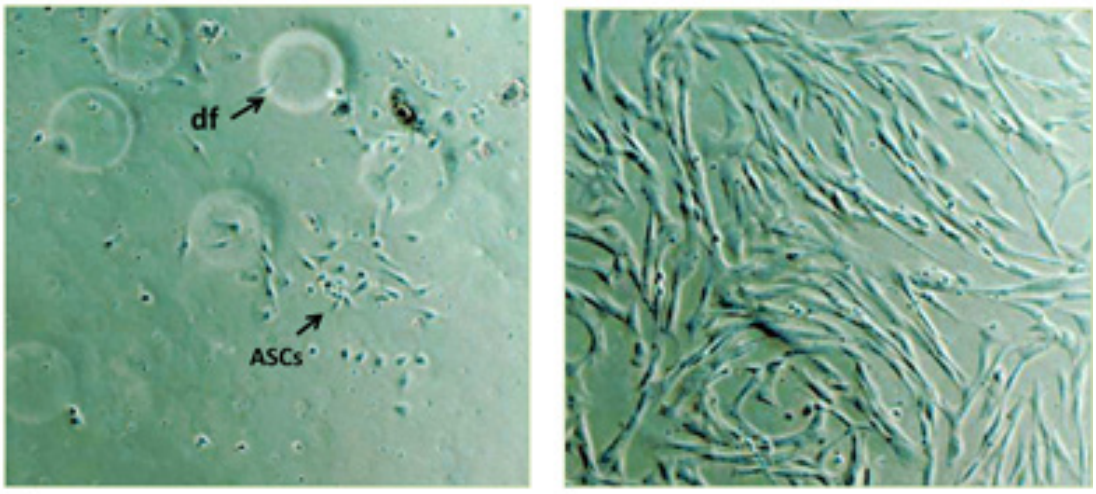

Figure 1 – Adipose-derived stem cells (ASCs) obtained from human lipoaspirates.
Note: Phase-contrast images denoting: (A) ASCs and waste drop fat (dp) are observed in the processed lipoaspirate samples after 2 days of isolation at 40X magnification; (B) ASC monolayer adherent cells with fibroblast-like morphologies after 5 days of isolation at 400X magnification.

2.2 HYDROGEN PEROXIDE EXPOSITION

After 15 days of ASCs isolation, these cells were treated following the procedure of Zhang *et al*.,6 except that eight different H_2O_2 concentrations (1–1000 µM) were used. The samples were treated in 96-well plates (2.5 X 105 cells/mL in each well). After 2h of incubation, the samples were centrifuged for 10 min at 2000 rpm, the supernatants were isolated and the cells were suspended in DMEM culture medium for subsequent analysis. Every group of H_2O_2 concentration was composed by four different wells of treatment in the same 96-well plate and the same experimental design was repeated in three independent experiments.

All subsequent analysis obtained results were expressed as the percentage of the tested variable in relation to untreated control samples.

2.3 CELL CULTURE-FREE DSDNA ASSAY

To evaluate the acute H_2O_2 cytotoxic effect on ASCs, the presence of dsDNA in the supernatants was determined, using the Quant-IT™ PicoGreen® ds DNA kit (Invitrogen -

Life Technologies, SP - Brazil) according to the manufacturer's instructions. When the cell dies, the membrane is disrupted and dsDNA fractions are released into the extracellular medium. The DNA PicoGreen® dye presents a high affinity for the dsDNA and is able to quantify the dsDNA released. The dsDNA was measured by using 50 µL of the sample and 50 µL of the DNA Picogreen® dissolved in TE buffer, 1X (1:1; v.v), following a 5-min incubation in the dark room. The fluorescence was measured at an excitation of 485 nm, and an emission of 520 nm was recorded at room temperature.

2.4 DETERMINATION OF CASPASE LEVELS

The levels of active caspases 1 (cat. # DCA100), 3 (cat. # KM300) and 8 (cat. # K113-100) of ASCs treated with H_2O_2 were determined by ELISA immunoassays and using the Quantikine Human Caspase Immunoassay® kit (R&D Systems) according to the manufacturer's instructions.

2.5 ROS QUANTIDICHLOROFLUORESCEIN DIACETATE ASSAY

The reactive oxygen species (ROS) in the cells was determined by using the dichlorofluorescein diacetate assay (DCFH-DA) as described in Costa *et al*.[10] In this assay, DCFDA 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 obtain the measurements, the sample cells were treated with DCFDA (10 µM) for 60 min at 37 oC, and the fluorescence was measured at an excitation of 488 nm and an emission of 525 nm.

2.6 BIOCHEMICAL OXIDATIVE MARKERS ASSAYS

The lipid peroxidation was performed by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described in Ohkawa, Ohishi, Yagi.[11] Total blood superoxide dismutase (SOD) (E.c.1.14.1.1) activity was measured spectrophotometrically according to Boveris and Cadenas.[12] One unit of activity is defined according to the amount

of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. Catalase activity (EC 1.11.1.6) was determined according to Aebi.[13] One unit of catalase activity was defined as the activity required to degrade 1 µmol of H_2O_2 in 60 s.

2.7 THE ALKALINE SINGLE-CELL GEL ELECTROPHORESIS ASSAY (ALKALINE DNA COMET ASSAY)

To detect the DNA damage, we performed the well-known comet assay, first conducted as Singh, McCoy, Tice, and Schneider[14] with modifications from Nadin, Vargas-Roig, and Ciocca.[15]. Such samples were placed on slides containing low melting agarose and, after that, they were submitted to cellular lysis, for each sample two slides were prepared. Then, electrophoresis was performed, as well as the staining with silver nitrate. After all the process, 50 cells were read by two analysts considering that the larger the size of the DNA drag is, the larger the increase cell damage gets. Thus, the damage index was calculated from the equation: x.(n°0)+x.(n°1)+x.(n°2)+x.(n°3)+x.(n°4).

2.8 CELL MORPHOLOGY EVALUATION

The analysis of ASC morphology after H_2O_2 exposition was evaluated by optical microscopic visualization. The ASC samples were centrifuged at 2000 rpm for 10 min, and the cell pellets were resuspended in fixative solution (ethanol: methanol, 1:1, v:v). Then, the material was transferred to microscope slides and after drying, using Giemsa dye, the cells of each treatment were microscopically analyzed at 40X magnification.

3 **STATISTICAL ANALYSIS**

The statistical variance among treatments were compared by one-way ANOVA, followed by Tukey's post-hoc test using Graphpad Prism 5 software. The results were expressed as mean ± standard deviation. A level of statistical significance at $p \leq 0.05$ was used.

4 RESULTS

The acute effect of H_2O_2 exposition on ASC viability was investigated and the results are presented in Figure 2. After two hours of H_2O_2 exposition, all concentrations increased the cell mortality when compared to the untreated control group. At concentrations of 1–10 µM, 29.7± 9.3% of the cells died, whereas cell mortality increased to 52.9 ± 8.4% after exposure to 30–100 µM H_2O_2. In the present study, 91.1 ± 13.3% of ASC cells exposed to 200 µM H_2O_2 died when compared to the control group, with 100% mortality occurring at concentrations ≤ 300 µM H_2O_2.

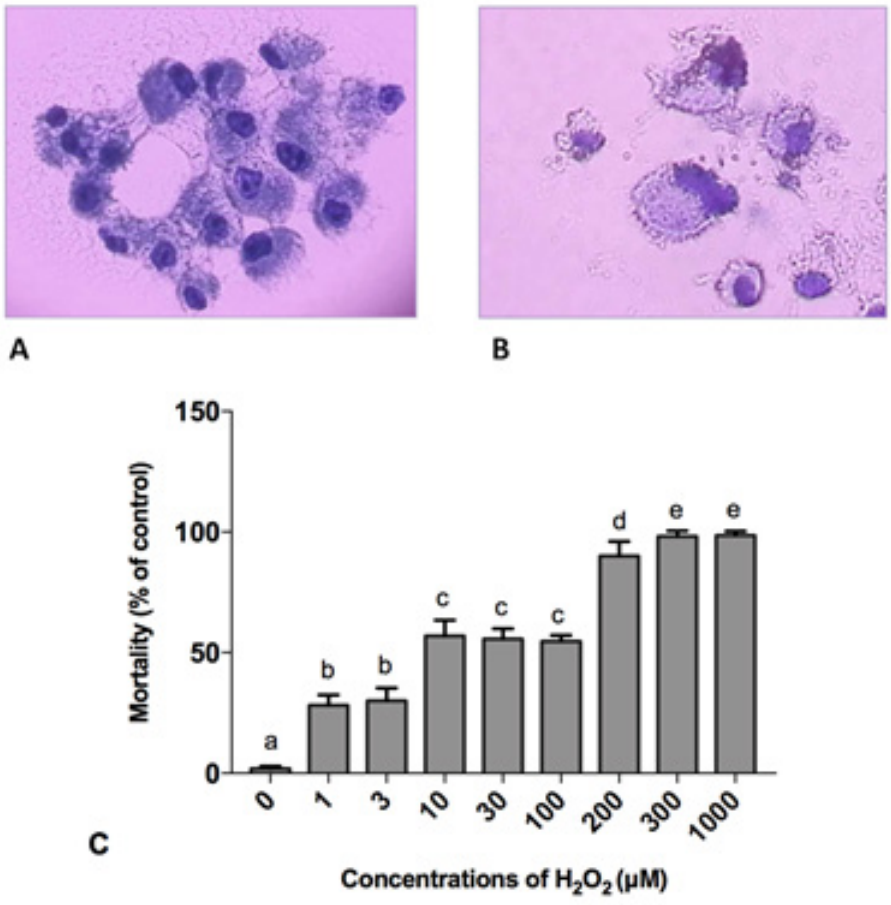

Figure 2 – Cytotoxity of adipose-derived stem cells (ASCs) obtained from human lipoaspirates treated with different H_2O_2 exposition concentrations

Notes: (A) Living cell morphology modification analyzed by optic microscopy (x 400 magnification); (B) Cells exposed to H_2O_2 showing fragmented nuclei and membrane blebbing; (C) Cell mortality was estimated by a direct fluorimetric degredation assay using DNA®Picogreen dye to quantify the cell-free dsDNA from ASCs. Data are presented as the percentage of untreated control group. Different letters indicate significant differences ($p \leq 0.05$) by one-way ANOVA followed by Tukey's post-hoc test.

Cells exposed to ≥ 200 µM H_2O_2 concentrations presented significant membrane disruption and loss of cytoplasmic prolongations that are characteristic of mesenchymal-like cells such as ASCs. Many cells wilted indicating loss of water in the extracellular medium (Figs. 2 A, B).

The levels of caspases 1, 3 and 8 caspase were quantified as an indication of cellular apoptosis induced by H_2O_2. A significant increase in these proteins was observed in the cells exposed to all H_2O_2 concentrations when compared to the untreated control group. The increase in caspase levels tended to be concentration-dependent (Fig. 3).

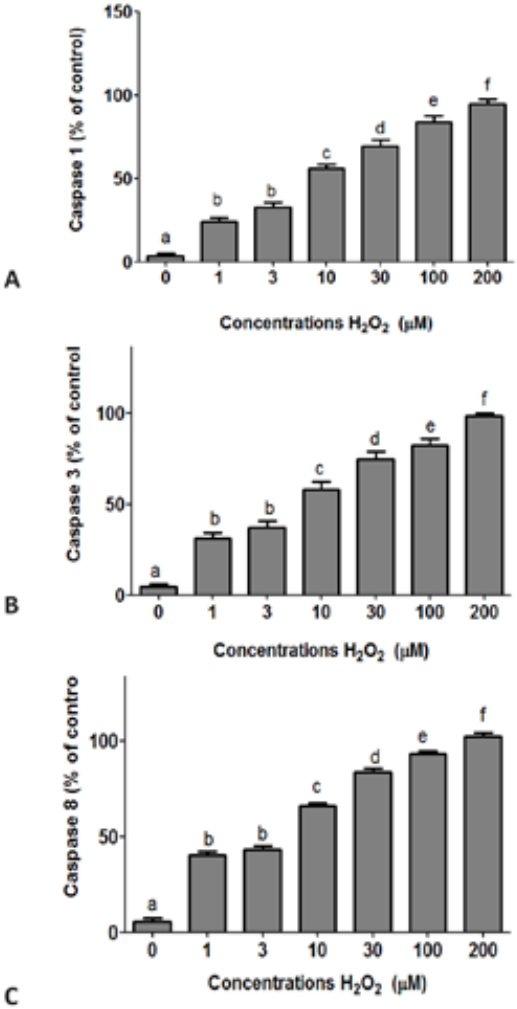

Figure 3 – Caspase levels of adipose-derived stem cells (ASCs) obtained from human lipoaspirates treated with different H_2O_2 exposition concentrations

Notes: (A) Caspase 1; (B) Caspase 3; (C) Caspase 8. Different letters indicate significant differences ($p \leq 0.05$) by one-way ANOVA followed by Tukey's post-hoc test.

As expected, the ROS and TBARS levels increased in a dose-dependent manner when the cells were exposed to H_2O_2 (Table 1). The catalase levels also increased approximately 50% in the cells exposed to H_2O_2 when compared to the control group. However, this effect was not dose-dependent. The cells exposed to 1 µM H_2O_2 presented an intense SOD total activity. Nevertheless, this activity decreased from H_2O_2 concentrations of ≥ 3 µM, mainly in 10, 30 and 100 µM.

	H2O2 Concentration (µM)						
	0	1	3	10	30	100	200
ROS	100.3 ± 7.5[a]	106.0 ± 9.7[b]	108.8 ± 11.9[b]	166.8 ± 23.5[c]	165.1 ± 22.0[c]	185.8 ± 31.3[d]	368.3 ± 11.1[e]
TBARS	100.4 ± 5.3[a]	305.4 ± 18.0[b]	656.3 ± 40.7[c]	776.3 ± 40.7[d]	838.2 ± 28.6[e]	915.0 ± 90.6[f]	1823.0 ± 35.2[g]
CAT	100.1 ± 3.3[a]	152.3 ± 11.5[b]	151.5 ± 10.5[b]	154.1 ± 8.8[b]	159.3 ± 10.8[b]	156.3 ± 7.6[b]	156.3 ± 7.6[b]
SOD	100.0 ± 1.5[a]	211,7 ± 25,8[b]	89.9 ± 0.8[c]	22.1 ± 3.5[d]	24.9 ± 2.8[d]	30.5 ± 6.3[d]	34.12± 4.6[d]

Table 1 – Oxidative metabolism parameters of Mesenchymal Stem Cells (MSCs) obtained from human lipoaspirates exposed to diferent H2O2 concentrations

Notes: Data are presented as percent of control group; ROS= reactive oxygen species determined by DCFH-DA assay; CAT = catalase; SOD= superoxide dismutase. Different letters indicate statistics significant differences using oneway analysis of variance followed by Tukey *post hoc* test at $p<0.05$.

A genotoxic effect was not detected at lower H_2O_2 concentrations (< 3 µM) when compared to the untreated control group. However, this result was completely altered in concentrations of ≥ 10 µM (Fig. 4). In the higher concentrations, the damage index increased by approximately 30% when compared to the control group. The frequency of undamaged nuclei in the untreated group was estimated to be 55.76%. This value decreased to 36.5% at 1 µM and 24.8% at 3 µM H_2O_2. From 10 µM H_2O_2 concentrations, the frequency of cells with no DNA damage (damage 0) was 19.5% of the analyzed samples.

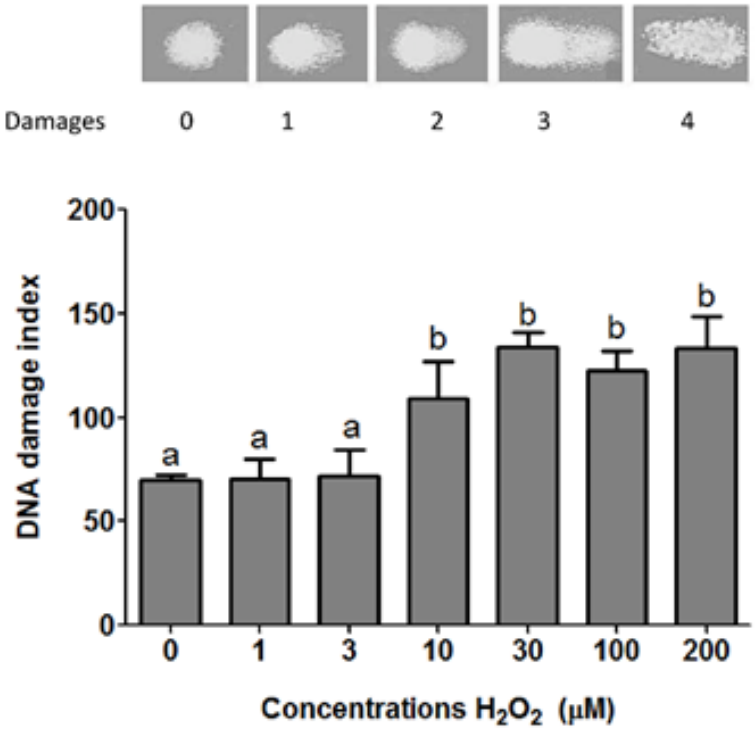

Figure 4 – DNA damage index of adipose-derived stem cells (ASCs) obtained from human lipoaspirates treated with different H_2O_2 expositionconcentrations determined by the DNA Comet Assay (from 0 for all cells with no migration, to 400 for all cells with maximal migration).

Note: Different letters indicated significant differences ($p \leq 0.05$) by one-way ANOVA followed by Tukey's post-hoc test.

5 DISCUSSION

The present study evaluated the cytotoxic and genotoxic effects of different H_2O_2 concentrations on the cyto- and genotoxicity of ASCs obtained from human processed lipoaspirates. Cells exposed to ≥ 200 µM H_2O_2 concentrations showed high mortality index compared to untreated cells. Additionally, ASCs exposed to H_2O_2 ≥ 10 µM also presented increased mortality as well as significant DNA damage indicating induced genotoxic effect. Extensive DNA damage was observed in approximately 80% of cells exposed to H_2O_2 treatments tested in this study. The oxidative stress caused by H_2O_2 exposition in

the concentrations here analyzed was also confirmed from the analysis of biochemical biomarkers (Table 1).

It is already known that in response to genotoxic effects leading to DNA double-strand breaks, differentiating cells activate at least three DNA damage response programs in order to preserve the genome integrity and prevent malignant transformation: DNA repair, apoptosis or senescence. Our obtained results are in concordance with that information sense in addition to the acute cytotoxic effect of H_2O_2, surviving ASCs presented increased caspases 1, 3 and 8 levels in a concentration-dependent manner, which indicates imminent apoptosis induction. On the other hand, recent studies of normal and cancer precursors (or stem cells) have suggested that cell stress with genotoxic effects are able to induce differentiation processes,[16] but in these cases it is necessary to focus on efficacy and safety conditions.

The ability of ROS molecules such as H_2O_2 to induce MSC differentiation raised the possibility of using these molecules in protocols applied to clinical therapies. However, the ROS dosage used to induce cellular differentiation programs is a central issue that needs to be determined for each SC type.[4] Until recently, H_2O_2 was considered a destructive ROS molecule. This view changed when several studies observed the ability of H_2O_2 to act as a cell signal, mainly through oxidation of specific target molecules.[5,17-19]

Therefore, the increase in ROS levels has been described as a key inducer of MSC proliferation and differentiation inductor, which are important steps in the regeneration of tissues and organs.[1] Previous studies demonstrated that MSCs remain in a quiescent state in low levels of endogenous H_2O_2. However, exposure of MSCs to high H_2O_2 concentrations induced cell growth and differentiation including activation of senescence processes, which led to a premature exhaustion of self-renewal characteristic in these cells.[5,20] Furthermore, in high H_2O_2 concentrations, oxidative processes may lead to irreversible damage, followed by cell death,[w] as well as to cellular senescence.[8]

However, cytotoxicity and DNA damage produced by H_2O_2 exposition varies in intensity according to the cell line type.[22] The obtained data here described suggest that ASCs from human processed lipoaspirates are very sensitive to H_2O_2. The present study showed that H_2O_2 exposition causes acute cyto- and genotoxicity in ASCs from 10 µM concentrations. This minimal H_2O_2 dose is 20 times less concentrated than that used by Zhang *et al*.[6] to stimulate SC proliferation and differentiation of Wharton's Jelly mesenchymal stem cells.

Unfortunately, comparative studies to evaluate the effect of H_2O_2 in different MSCs and ASCs in similar experimental conditions are pending. This may be due to the difficulty in obtaining these cells at the same time and at the same sample of subjects.

The results published in the literature, as described in the present investigation, suggest that the cells surviving high H_2O_2 exposition can present an oxidative stress resistance and a poor genome quality. This condition is similar to that observed in cancer cells, and it can be related to some situations in which SCs can be involved in tumor development and not differentiated tissues as expected.[23] In general, cancer cells exhibit an accelerated metabolism, demanding high ROS concentrations to maintain their high proliferation rate.[24]

In this regard, and considering the results already described, the genotoxic stress induced by H_2O_2, even though it is a potential co-activator of cellular differentiation programs, can also produce differentiated cells with deleterious genetic alterations that may impact the safe use of those cells in therapy. Consequently, these genetic alterations may compromise the expected regenerative results of oxidative stress-induced cells. Unfortunately, the number of studies involving the analysis of H_2O_2 genotoxic effects on MSCs remains low. Therefore, we suggest that studies involving the use of H_2O_2 as a potential differentiation inducer, also include concomitant protocols to evaluate the DNA damage effect on surviving cells, mainly considering ASCs obtained from processed human lipoaspirates.

6 CONCLUSIONS

The results obtained here suggest that ASCs obtained from processed human lipoaspirates are highly sensitive to H_2O_2 exposition. In addition, the number of healthy H_2O_2-induced ASCs, which could be potentially used in the regenerative processes, is very low. In addition, the surviving ASCs may suffer important DNA damage that could affect their proliferative and differentiation capacity.

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