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Imbalance between nitric oxide and superoxide anion induced by uncoupled nitric oxide synthase contributes to human melanoma development

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ABSTRACT

Melanoma is the most aggressive type of cutaneous tumors due to its metastatic potential and high mortality. Increased levels of reactive oxygen species, including superoxide anion ($O_2^{\cdot -}$), and the consequent installation of a pro-oxidant environment are associated with melanoma development. The enzyme nitric oxide synthase (NOS), responsible for the production of nitric oxide (NO), when uncoupled is as a source of $O_2^{\cdot -}$, for example by the absence of its cofactor tetrahydrobiopterin (BH4). Western blot analysis showed increased expression of endothelial and inducible NOS in human melanoma cells, altering the stoichiometry between NOS levels and BH4 concentration and together with decreased BH4:BH2 ratio are contributing to NOS uncoupling. The treatment of melanoma cells with exogenous BH4 increased NO concentration and decreased $O_2^{\cdot -}$ levels, leading to NOS coupling, which in turn reduced cell viability, cell proliferation and the ability of melanoma cells to form melanoma spheroids. Moreover, BH4 level restoration rendered melanoma cells more sensitive to apoptosis, demonstrating the role of dysfunctional NOS in melanoma genesis.

1. Introduction

Cutaneous melanoma is the most aggressive type of skin cancers, since it is associated with high metastatic potential and mortality. Melanomas arise from malignant transformation of melanocytes, the melanin-producing cells that are primarily located in the basal layer of epidermis in human skin along with Langerhans cells and keratinocytes. Melanocytic transformation is induced by sequential accumulation of genetic and epigenetic modifications that drive abnormal signaling transduction leading to increased proliferation of melanocytes. The incidence of melanoma is increasing around the world due increased life expectancy and ultraviolet radiation (UV) exposure. UV is associated with abnormal reactive oxygen species (ROS) accumulation in intracellular milieu, which in turn, contributes to melanoma genesis (Emri et al., 2018).

Reactive oxygen species are physiologically generated from cellular aerobic metabolism and modulate signaling pathways that regulate a variety of cellular processes, such as proliferation, metabolism, survival, and differentiation (Ray et al., 2012). However, disturbance of ROS homeostasis seems to be a key event in melanoma development, affecting these important signaling pathways, as well genetic and epigenetic mechanisms (Campos et al., 2007; Ziech et al., 2011). Several studies have shown the relationship between increased superoxide anion production ($O_2^{\cdot -}$) and melanocyte malignant transformation and melanoma genesis (Pervaiz and Clement, 2007; Melo et al., 2011; Molognoni et al., 2013).

In melanoma, dysfunctional mitochondria and upregulated NADPH oxidase have been described as the main sources of ROS (Govindarajan

Abbreviations: NOS, nitric oxide synthase; ROS, reactive oxygen species; BH4, tetrahydrobiopterin; BH3, trihydropterin; BH2, dihydrobipterin; GTPCHI, GTP-cyclohydrolase I; UV, ultraviolet radiation

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et al., 2007; Yamaura et al., 2009; Barbi de Moura et al., 2012; Rodrigues et al., 2016). Nonetheless, dysregulated nitric oxide synthase (NOS) can also generate $O_2^{\cdot -}$ under certain pathophysiological conditions, contributing to melanocyte malignant transformation and melanoma development (Campos et al., 2007; Melo et al., 2011).

In mammals, three different isoforms of the enzyme NOS have already been described: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). To produce NO, all NOS isoforms are dependent on the substrates L-arginine, NADPH and oxygen, and the cofactors tetrahydrobiopterin (BH4), thiol, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Fleming and Busse, 2003; Förstermann and Sessa, 2012). Abnormal NOS regulation is accompanied by increased $O_2^{\cdot -}$ production and decreased NO generation (Rabelink and Luscher, 2006; Cardounel et al., 2005; Pou et al., 1999).

The BH4 cofactor plays a direct and indispensable role in NOS function through binding to the heme active site at the interface between the two monomers, participating in arginine oxidation and subsequent generation of NO. In addition, BH4 promotes the dimerization and stability of NOS, protecting against enzyme inactivation and enhancing L-arginine binding (Alderton et al., 2001; Flinspach et al., 2004). When BH4 is limiting or absent, NOS dimerization is destabilized triggering the loss of its activity. This state is referred as uncoupling because the oxidation of NADPH and the reduction of oxygen are uncoupled from arginine hydroxylation and NO formation. However, the electron transfer from NADPH through the flavin domains to molecular oxygen is not inhibited, resulting in the generation of $O_2^{\cdot -}$ and H_2O_2 and decrease of NO production (Rabelink and Luscher, 2006; Cardounel et al., 2005; Pou et al., 1999; Alp and Channon, 2004; Werner et al., 2011).

There are few studies in the literature demonstrating the participation of BH4 in cancer progression. Additionally, these data are not conclusive, since these studies addressed BH4 as a pro or anti-tumorigenic molecule (Chen et al., 2010; Cho et al., 2011; Pickert et al., 2013; Cardnell et al., 2013; Yco et al., 2015). However, the level and participation of BH4 in human melanomas have not been previously determined, at least to our knowledge. Therefore, the purpose of our study was to evaluate the contribution of uncoupled NOS and BH4 to the development of human metastatic melanoma.

2. Material and methods

2.1. Cell culture

The human melanocyte cell line was grown in 254CF medium (GIBCO, Carlsbad, CA) supplemented with 0.2 mM $CaCl_2$ and human melanocyte growth supplement (HMGS) (GIBCO, Carlsbad, CA) and used as a control. The SK-MEL-28 (ATCC® HTB-72™) human metastatic melanoma cell line was cultured in RPMI 1640 medium (GIBCO, Carlsbad, CA) at pH 7.2 and supplemented with 10% fetal bovine serum (Invitrogen, Scotland, UK). The patient-derived metastatic melanoma cells Mel14 and Mel25 kindly provided by Dr. Débora C.P. Silva (Ludwig Institute for Cancer Research, São Paulo) were also cultured in RPMI 1640 medium at pH 7.2 and supplemented with 10% FBS, 100 μM glutamine and 100 μM sodium pyruvate (Toricelli et al., 2013). The cultures were kept in incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Nitric oxide quantification

2.2.1. DAF-2DA

Cells were grown in 6-well plates and, upon reaching 80% confluence, were treated or not for two hours with 40 μM BH4 or 40 μM L-sepiapterin (Cayman, Ann Arbor, MI). Following the two-hour period, cells were washed with phosphate-buffered saline (PBS) and incubated with 1 μM 4-5-diaminofluorescein diacetate (DAF-2DA), a non-fluorescent cell permeable indicator for nitric oxide, in PBS (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C in the dark. After being washed, cells were analyzed by flow cytometry (FACSCalibur; Becton–Dickinson, Franklin Lakes, NJ, USA) (excitation wavelength 495 nm; emission wavelength 515 nm).

2.2.2. Griess staining

Alternatively, nitrite levels were measured by using the Griess reaction, where a 540 nm-absorbance chromophore results from the reaction between nitrites and a mixture containing naphthylethylenediamine (0.1%) and sulfanilamide (1%) . A standard curve was established using serial dilutions of sodium nitrite (10^{-8}–10^{-3} mol/L).

2.2.3. NO analyzer

Extracellular NO levels were determined after a gas-phase chemiluminescence reaction of NO with ozone by a NO analyzer (NOA 280; Sievers Instruments, Boulder, CO, USA). A standard curve was established with a set of serial dilutions (0.1–100 μM) of sodium nitrate. The concentrations of NO metabolites in samples were determined by comparison with the standard curve and expressed as micromoles per milligram of protein. Data collection and analysis were performed using the NOANalysis software (version 3.21; Sievers Instruments).

2.3. Superoxide anion quantification

Intracellular superoxide amount was measured using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA), a non-fluorescent cell permeable indicator for superoxide anion and analyzed by flow cytometry, fluorometric assay or high-performance liquid chromatography (HPLC) after two hours of treatment or not with 40 μM BH4 or 40 μM L-sepiapterin. For flow cytometry analysis, cells were washed and incubated in PBS for 30 min at 37 °C before being incubated with 2,5 μM DHE in PBS for an additional 40 min at 37 °C in the dark. After washing, cells were analyzed by flow cytometry (FACSCalibur; Becton–Dickinson, Franklin Lakes, NJ, USA) (excitation wavelength 480 nm; emission wavelength 567 nm). For HPLC analysis, cells were washed three times with PBS and incubated in PBS containing 100 μM DTPA (Sigma, St. Louis, MO) and 50 μM DHE for 30 min at 37 °C. After washing, acetonitrile extraction was performed and simultaneous fluorescent detection of 2-E + OH and ethidium was done. DHE-derived product E + OH ere expressed as ratios of generated E + OH over consumed DHE (initial DHE concentration minus remaining DHE) (Fernandes et al., 2007). For fluorometric assay, cells were washed with PBS and incubated with 25 μM DHE for 40 min at 37 °C in the dark. After washing, fluorescence was evaluated in the Spectromaxi3 (Molecular Devices).

2.4. High-performance liquid chromatography analysis of the cellular biopterin content

To evaluate the cellular concentration of tetrahydrobiopterin (BH4), 7,8-dihydrobipterin (BH2), total biopterin, and the BH4:BH2 ratio, reversed-phase high-performance liquid chromatography was used as previously reported (Marinos et al., 2001). SK-MEL-28, Mel14 and Mel25 cell lines were washed twice with cold PBS (pH 7.4). After centrifugation, cells were resuspended in 0.5 mL 0.1 M phosphoric acid containing 5 mM dithioerythritol and sonicated for 40 s, to which 35 μL 2 M trichloroacetic acid (TCA) were added. The solution was centrifuged at 12,000 *g* for 1 min and the supernatant was used immediately for the quantification of all biopterins. The total biopterin amount was measured following oxidation in acidic conditions, whereas BH2 quantification was conducted after its oxidation in alkaline conditions. BH4 was calculated from the difference between the amount of biopterin formed by oxidation in acidic conditions and the amount of biopterin formed by oxidation in alkaline conditions. For oxidation reaction under acidic conditions, 100 μL cell extract were mixed with 15 μL 0.2 M TCA and 15 μL 1% I_2/2% KI in 0.2 M TCA. For oxidation

under alkaline conditions, 100 µL cell extract were mixed with 15 µL 1 M NaOH and 15 µL 1% I_2/2% KI in 3 M NaOH. The oxidation reaction was carried out for 1 h in the dark at room temperature. The next step was to inactivate the excess of iodine by the addition of 25 µL 0.114 M ascorbic acid. The assay mixture was centrifuged at 4 °C for 12 min, and 100 µL of the supernatant were injected into a HPLC system (LCMS-2020, Shimadzu Co., Kyoto, Japan) on a C18 Vydac reversed-phase column (5 µm, 4.6 mm id × 205) and detected by fluorescence (lex = 350 nm; lem = 450 nm). Biopterin was eluted by an isocratic mobile phase solution (5% methanol and 7.5 mM sodium phosphate buffer, pH 6.35) at a flow rate of 1.0 mL/min. Data were collected and analyzed by LC solution software (Shimadzu Co., Kyoto, Japan) and normalized against protein concentration.

2.5. Western blot

Subconfluent cell cultures were trypsinized, washed in PBS and SK-MEL-28, Mel14 and Mel25 whole-cell lysates were prepared using Pierce® IP Lysis Buffer (25 mM Tris·HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) added with the protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) kept for 15 min on ice, followed by centrifugation at 13.000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentration was measured by Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA). Equivalent amounts of protein (40 µg) were denaturated in SDS sample buffer (240 mM Tris − HCl pH 6.8, 0.8% SDS, 200 mM beta-mercaptoethanol, 40% glycerol and 0.02% bromophenol blue) for 5 min. Protein lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Specific antibodies used were rabbit anti-iNOS (Santa Cruz Biotechnology, INC) and rabbit anti-eNOS (Cell Signaling Technology) followed by secondary antibody incubations using anti-rabbit conjugated to peroxidase. The signal was visualized by chemoluminescence using Immobilon Forte Western HRP substrate (Merck KGaA, Darmstadt, Germany). The bands intensity were measured using Processing and Analysis in Java, ImageJ 1.38b (Wayne Rasband, National Institute of Health, USA, http://rsb.info.nih.gov/ij/).

2.6. MTT assay

Cell viability in the presence of BH4 or L-sepiapterin was determined using a standard MTT assay. After treatment or not with 40 µM BH4 or 40 µM L-sepiapterin for two hours, human melanocytes, Mel14, Mel25 and SK-MEL-28 melanoma cells were harvest with trypsin and cultured for 96 h on 96-well plates in the presence of the same treatment which was replaced every 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. As soon as the cells adhered to the substrate and attained their characteristic morphology, 5 mg/mL MTT (Calbiochem, Hesse, Germany) was added to the culture, being considered as time T = 0. Cells were kept in an incubator at 37 °C and 5% CO_2 with MTT for one hour. After medium withdrawn, isopropanol (Merck, Hesse, Germany) was added to all wells for 15 min and absorbance was measured on a spectrophotometer at 620 nm (Multiskan EX, Thermo Electron, Ohio, USA). The procedure was repeated 24, 48, 72 and 96 h after the time zero.

2.7. Trypan blue dye exclusion assay

Cell viability of SK-MEL-28 melanoma cells was evaluated using the Trypan Blue dye exclusion assay. After treatment or not with 40 µM BH4 or 40 µM L-sepiapterin for two hours, cells were harvest with trypsin and cultured for 96 h on 24-well plates and subjected to the same treatment. For T = 0, the same number of cells plated in each well was considered. At the subsequent times (T = 24, 48, 72 and 96 h), the cell culture medium was changed daily, and the same initial plating treatment was employed. The cells were then counted by using 0.4% Trypan Blue solution (Life Technologies, Calif., USA) in a Neubauer chamber.

2.8. Clonogenic assay

After treatment or not with 40 µM BH4 for two hours, Mel14, Mel25 and SK-MEL-28 melanoma cells were harvested with mild trypsin treatment and seeded on 60-mm dishes and grown for nine days in the presence of the same treatment. The cell culture medium was changed daily, and the same treatment as that of the initial plating was used. At the end of this period, the plates were washed in PBS, fixed in 3.7% (v/v) formaldehyde for 15 min, washed with PBS, stained with 1% Toluidine Blue in 1% sodium tetraborate (borax) for five mins and washed with water. For the quantification of surviving cells, the dye was solubilized in 1% SDS under agitation for one h and the absorbance at 620 nm was measured using a spectrophotometer.

2.9. Spheroid-forming assay

After treatment or not with 40 µM BH4 for two h, SK-MEL-28 melanoma cells were harvested with mild trypsin treatment and seeded on 96-well plate previously prepared with 1.2% agarose per well in order to avoid cell adhesion in the presence of the same treatment (1000 cells per well in final volume of 100 µL culture medium). At 24 h, 48 h, 72 h and 96 h, 50 µL medium were added to each well receiving the appropriate treatment. After 14 days, the wells were photographed, and the structures formed were measured with the aid of the Zen software from Zeiss.

2.10. Apoptosis assessment

Apoptosis was evaluated using the CellEvent™ Caspase 3/7 Green Detection Reagent (Invitrogen, Eugene, OR, USA) as manufacturer specifications. After treatment or not with 40 µM BH4 for two, SK-MEL-28 melanoma cells were harvested with mild trypsin and seeded in adherent conditions or submitted to anchorage blockade in the presence of the same treatment for 96 h in humidified atmosphere of 5% CO_2 and 95% air. Every 24 h, the medium corresponding to the treatment indicated was replaced. After 96 h, the cells were washed in PBS and the cells in adherent conditions were trypsinized, resuspended in RPMI medium with 10% FBS and washed in PBS. The cells maintained in deadhesion were collected and washed in PBS. All the cells were incubated with the CellEvent™ Caspase 3/7 Green Detection Reagent for 30 min at 37 °C and immediately analyzed by flow cytometry (excitation wavelength 480 nm; emission wavelength 567 nm) (FACSCalibur; Becton-Dickinson).

2.11. Statistical analysis

To evaluate the results, we relied on Student's *t*-test for two-group experiments and analysis of variance (factorial ANOVA) with Dunnett's post-test for experiments with three or more groups using the Graphpad Prism 7.0® statistical software (GraphPad, San Diego, CA). The significance level was established at $p < 0.05$.

3. Results

3.1. Biopterins and BH4:BH2 ratio are lower in metastatic melanoma cell lines compared to melanocytes

BH4 and total biopterin amount found in melanocytes and SK-MEL-28 melanomas cell lines are not different (Fig. 1A and D), however, in Mel14 and Mel25 melanoma cells, BH4 and total biopterins decrease (Fig. S1A and D). Furthermore, although we also found reduced expression of the enzymes from recycling and recovery BH4 pathways in melanoma cell lines (data not shown), BH2 concentration is increased,

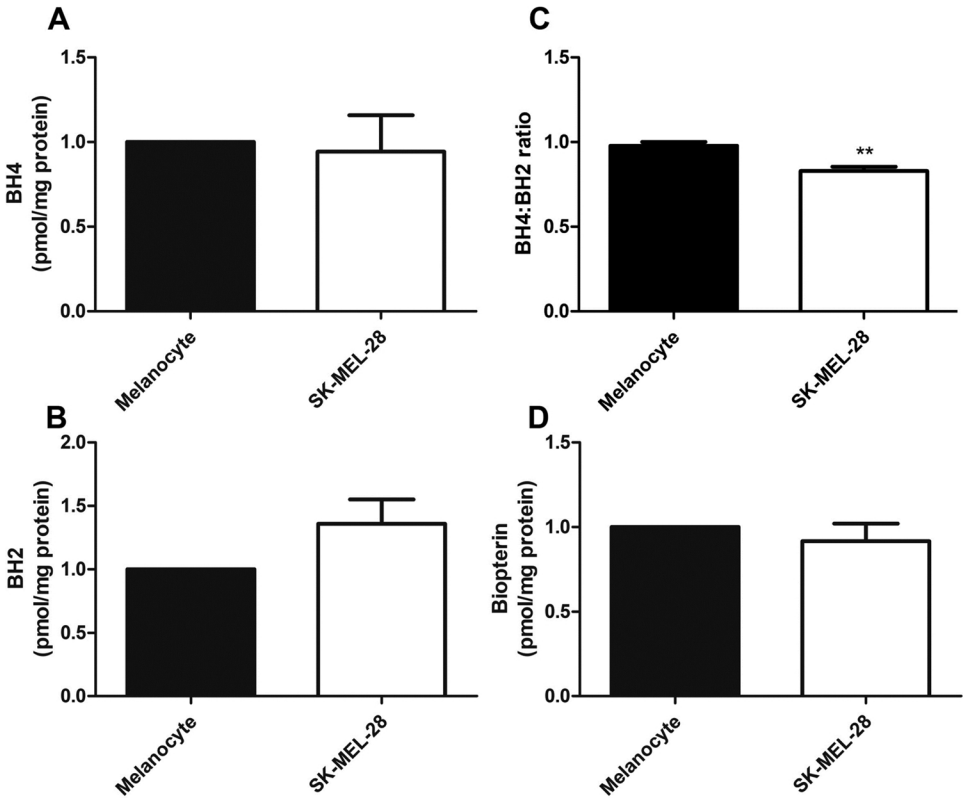

Fig. 1. BH4:BH2 ratio is lower in SK-MEL-28 metastatic melanoma cell line compared to melanocytes. The amount of BH4 (A); BH2 (B), BH4:BH2 ratio (C) and total biopterin (D) of melanocytes and SK-MEL-28 metastatic melanoma cells were determined by HPLC. Values are reported in the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and *p* values were based on Students' *t-test* **p < 0.01.

although it is not statistically significant in SK-MEL-28 (Fig. 1B) and decreased in Mel14 and Mel25 (Fig. S1B). Nevertheless, BH4:BH2 ratio (Fig. 1C) is lower in SK-MEL-28 melanoma cell compared to melanocytes, which suggests the uncoupling of nitric oxide synthase in these cells, nevertheless in Mel14 and Mel25 BH4:BH2 ratio is not altered (Fig. S1C).

3.2. Metastatic melanoma cells exhibit increased expression of nitric oxide synthases

It has been shown only in endothelial cells that increased eNOS protein expression in is also associated with enzyme uncoupling by a stoichiometric disagreement between eNOS enzyme and BH4 concentration (Ozaki et al., 2002; Bendall et al., 2005; Crabtree et al., 2009). Moreover, although increased expression of all NOSs has been described in different melanoma cells lines, this superexpression was not correlated with superoxide anion production or uncoupling. We observed increased expression of eNOS (Figs. 2A and 2SA) and iNOS (Figs. 2B and 2SB) in SK-MEL-28, Mel14 and Mel25 melanoma cells when compared to melanocytes, suggesting that uncoupling of NOS in tumor cells may be related to NOS-BH4 stoichiometry disturbance as described in endothelial cells.

3.3. Metastatic melanoma cells show increased superoxide anion levels and decreased nitric oxide concentration

To compare $O_2^{\cdot -}$ levels between melanocytes and metastatic melanoma cell lines, we used the fluorescence indicator DHE and performed flow cytometry, fluorimetric assay or HPLC analysis. By all of these approaches, we found increased superoxide anion levels in Mel14, Mel25 and SK-MEL-28 metastatic melanoma cell lines when compared to melanocytes (Fig. 3A, B and C). We assessed intracellular nitric oxide levels by flow cytometry using the fluorescence indicator DAF or by Griess reaction and the results demonstrated a decrease in nitric oxide levels in Mel14, Mel25 and SK-MEL-28 metastatic melanoma lines comparing to melanocytes (Fig. 3D and E). We also evaluated the extracellular nitric oxide concentration by NO analyzer and observed that Mel14 and Mel25 tumor cells have decreased amounts of NO in comparison to melanocytes (Fig. 3F).

3.4. Tetrahydrobiopterin impairs superoxide anion and increases nitric oxide production in metastatic melanoma cells

An increase in superoxide anion, a decrease in nitric oxide levels and lower BH4:BH2 ratio in metastatic melanoma cells when compared to melanocytes implicates NOS dysfunction in these tumor cells. Several data have reported that uncoupling NOS in endothelial cells is associated with increased superoxide anion production and that BH4 treatment restored NOS function (Schmidt and Alp, 2007). It is important to note that treatment with BH4 promotes the recoupling of the enzyme without considerable off-target antioxidant effects (Vásquez-Vivar, 2009). Here, we treat tumor cells with exogenous BH4 and evaluate a possible NOS recoupling. Initially, the amount of BH4 in melanoma cells following treatment with BH4 was analyzed by HPLC and exhibited an approximately fifteen-fold increase after 2 h or thirty-fold increase after 96 h (Fig. 4A) thus demonstrating the efficiency of BH4 supplementation. Although BH2 levels also augmented (Fig. 4B), the BH4:BH2 ratio (Fig. 4C) was greater in treated compared to untreated cells. As expected, the concentration of total biopterins also increased after BH4 treatment (Fig. 4D). Metastatic melanoma cells Mel14 (Fig. 5A) and SK-MEL-28 (Fig. 5B) showed impaired superoxide production in the presence of BH4 as indicated by flow cytometry using DHE. We observed these same results when SK-MEL-28 cells were treated with L-sepiapterin, a precursor of BH4 (Fig. S3A). In addition, BH4 treatment increased NO levels in Mel14 cells as measured by flow cytometry using DAF staining (Fig. 5C) and in SK-MEL-28 as evaluated in the Griess reaction (Fig. 5D). NO levels remained unchanged in SK-MEL-28 treated with L-sepiapterin (Fig. S3B).

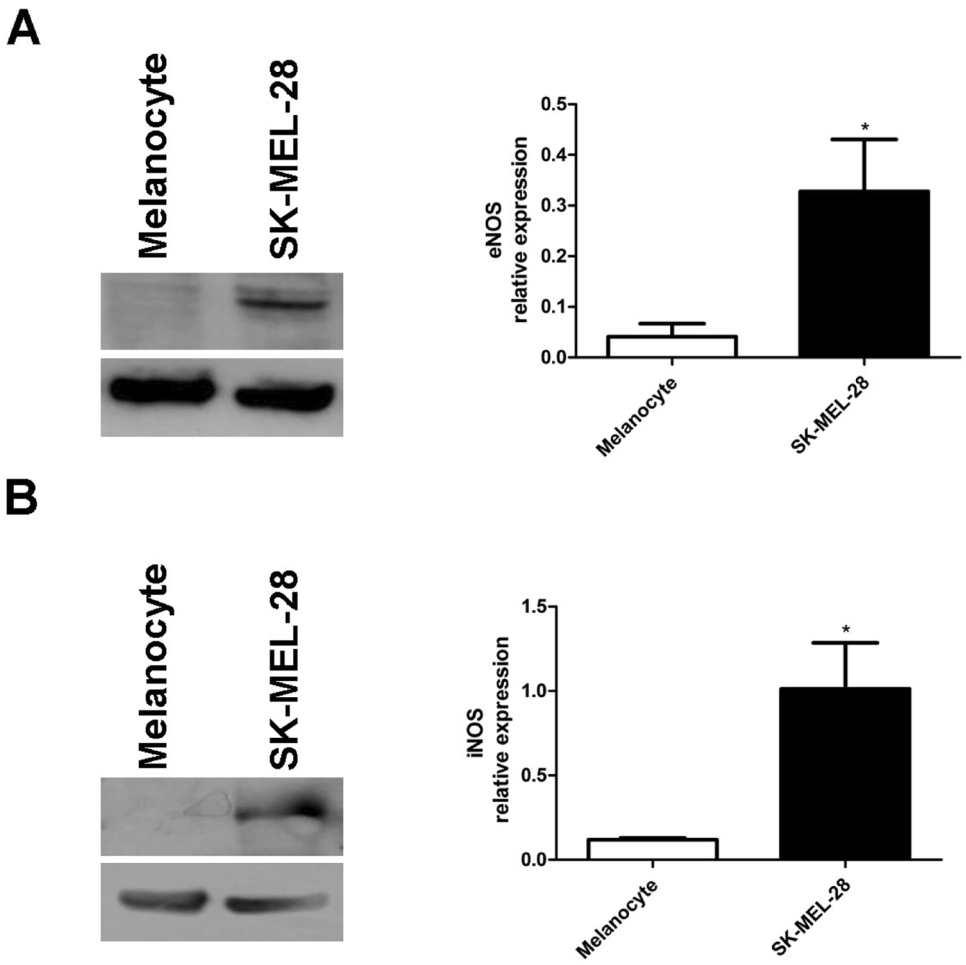

Fig. 2. Metastatic melanoma cell exhibits increased expression of nitric oxide synthases. Expression of eNOS (A) and iNOS (B) in SK-MEL-28 metastatic melanoma cells and melanocytes were determined by western blot using specific antibodies. The images show representative results of three independent experiments. The experiments were performed in triplicate and *p* values were based on Students' *t-test* *p $<$ 0.05.

3.5. Tetrahydrobiopterin decreases cell viability of metastatic melanoma cells

To evaluate the effects of superoxide anion reduction and NO increase on BH4-treated tumor cells, we initially analyzed cell viability of the SK-MEL-28 cell line treated with BH4 by using a direct method (Trypan Blue). This result allowed us to determine the exponential growth curve for the control and BH4 group. We also calculated the doubling time of each group according to the exponential growth curve parameters and observed that BH4 treatment increased doubling time from 23.33 to 60.18. The results also showed a decrease in the viability rate of the BH4-treated group when compared to the control group, at 72 h and 96 h (Fig. 6A). In the cell viability assay using an indirect method (MTT assay), the viability rate in the BH4-treated group also decreased when compared to that in the control group, at 48 h, 72 h and 96 h (Fig. 6B). L-sepiapterin treatment also induced a decrease in cell viability of SK-MEL-28 melanoma cells as showed by tripan blue exclusion assay (Fig. S4A) and by MTT (Fig. S4B). Additionally, we evaluated the viability of Mel14 (Fig. 6C) and Mel25 (Fig. 6D) metastatic melanoma cell lines using MTT and also observed a decrease in the number of viable cells in the presence of BH4 after 48 h, 72 h and 96 h. Important to note, that BH4 treatment did not modify melanocyte viability (Fig. S5).

In the colony formation assay, by measuring the absorbance of the solubilized dye, we found that SK-MEL-28 (Fig. 7A and B), Mel14 (Fig. S6A) and Mel25 (Fig. S6B) melanoma cells treated with BH4 showed a decrease in clone formation capability as compared to the control group within a 9-day interval. Similarly, when measuring sphera area in the spheroid-forming assay, we found that BH4-treated SK-MEL-28 cells have a decreased size in comparison to the control group within 14 days (Fig. 7D and E).

3.6. Tetrahydrobiopterin induces apoptosis through caspase-3/7 activation

The next step was to investigate if the decrease in SK-MEL-28 cell viability by BH4 treatment was a result of cell cycle progress impairment or cell death induction. As shown in Fig. 7, BH4 treatment for 96 h triggered apoptosis in SK-MEL-28 cells in adherent circumstances (Fig. 7C), the same conditions of viability and colony formation assay or when submitted to adhesion impediment (Fig. 7F), a status that resembles tumorsphere assay, as indicated by CellEvent™ Caspase 3/7 green detection reagent. These results indicate that the impairment competence to form colonies or tumorspheres observed in BH4 treated SK-MEL-28 melanoma cells are caused by apoptosis.

4. Discussion

The contribution of $O_2^{\cdot -}$ produced by NADPH oxidase and dysfunctional mitochondria in melanoma development has been extensively demonstrated (Govindarajan et al., 2007; Yamaura et al., 2009; Barbi de Moura et al., 2012; Rodrigues et al., 2016). More recently, we have suggested that uncoupled NOS is involved in murine melanocyte transformation and in the acquisition of murine melanoma aggressive characteristics (Campos et al., 2007; Melo et al., 2011). BH4

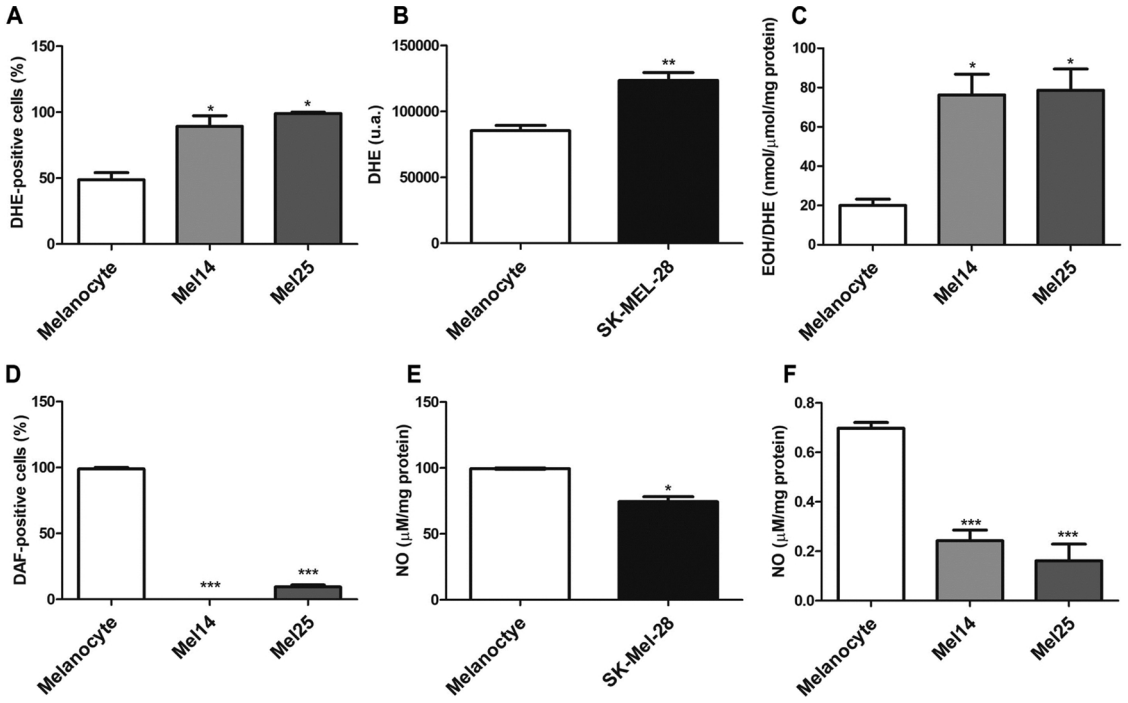

Fig. 3. Metastatic melanoma cells show increased superoxide anion levels and decreased nitric oxide concentration. Superoxide anion levels in melanocytes and Mel14, Mel25 and SK-MEL-28 metastatic melanoma cells were analyzed using DHE by flow cytometry (A), fluorimetric assay (B) and HPLC (C) and intracellular NO levels were evaluated by flow cytometry using DAF (D) or by Griess reaction (E). The extracellular NO concentration was determined by NO analyzer (F). The experiments were performed in triplicate and *p* values were based on Students' *t-test* or by One-Way ANOVA test followed by Dunnett's post-test *p < 0.05, **p < 0.01, ***p < 0.001.

is an indispensable cofactor for the synthesis of NO, promoting NOS dimerization and stability. When in its uncoupled state, NOS is accompanied by an increase in $O_2^{\cdot -}$ production and a decrease in NO generation (Alderton et al., 2001). Although these mechanisms are well defined in endothelial cells, there are few studies in the literature demonstrating the role of BH4 in tumor cell biology. Accordingly, we evaluated whether uncoupling of NOS caused by BH4 deficiency contributes to characteristics acquired during melanoma progression.

Oxidation of BH4 is complex, since it is first oxidized to BH3 (trihydropterin) and subsequently to BH2. This process, in addition to directly reducing BH4 bioavailability, indirectly affects NOS activity, since the products resulting from this oxidation, such as BH2, although have affinity for the enzyme, has no cofactor activity and can compete and displace BH4. This leads to an increase in $O_2^{\cdot -}$ production and a decrease in NO generation (Schmidt and Alp, 2007; Bendall et al., 2014). Some authors showed that the stoichiometric relationship between the amount of NOS and BH4 content and the intracellular ratio between BH4 and BH2, rather than the absolute concentration of BH4, are the determinants of normal NOS function, even in the absence of pathologies in endothelial cells (Bendall et al., 2005; Crabtree et al., 2009). However, decreased BH4 is sufficient to trigger superoxide anion production by NOS and impair NO synthesis leading to vascular abnormalities since BH4 is essential to stabilize NOS (Meininger et al., 2004; Du et al., 2008). Besides that, NOS stabilization and nitric oxide production are dependents of caveolin-1, a protein found on caveolas that is often lost in melanoma cells (Karuppiah et al., 2011). We found that BH4 levels in SK-MEL-28 metastatic melanoma cell line do not differ from those in melanocytes (Fig. 1A). The same holds true relative to BH2 (Fig. 1B) and total biopterin (Fig. 1D) concentration. Nevertheless, BH4:BH2 ratio (Fig. 1C) is lower in metastatic melanoma cell line SK-MEL-28. By other hand, BH4:BH2 ratio do not change in Mel14 and Mel25 melanoma cells when compared to melanocytes (Fig. S1C), but BH4, BH2 and biopterin concentration decrease (Fig. SA, SB and SD). Although BH4:BH2 ratio is not decreased in Mel14 and Mel25 melanoma cells when compared to melanocytes, decreased BH4 amount in melanoma cells is associated with impaired NO synthesis and superoxide anion production by NOS, since BH4 treatment reversed them. Moreover, increased expression of eNOS (Fig. 2A and 2SA) and iNOS (Fig. 2B and 2SB) was observed in SK-MEL-28, Mel14 and Mel25 melanoma cells compared to melanocytes. Therefore, uncoupling of nitric oxide synthase in SK-MEL-28 metastatic melanoma cells seems to be a result of decreased BH4:BH2 ratio, while in Mel14 and Mel25 metastatic melanoma cells decreased BH4 concentration, and enhanced NOS expression leading to a stoichiometry disbalance between BH4 concentration and NOS protein levels.

We found that Mel14, Mel25 and SK-MEL-28 metastatic melanoma cell lines had increased $O_2^{\cdot -}$ levels (Fig. 3A, B and C) and decreased NO levels (Fig. 3D, E and F) when compared to melanocytes, reinforcing the uncoupled state of NOS in metastatic melanoma cells. In addition to these results, we found that BH4 treatment decreased $O_2^{\cdot -}$ levels and increased NO levels in Mel14 (Fig. 5A and C) and SK-MEL-28 (Fig. 5B and D) tumor cell lines, suggesting that BH4 might recouple NOS in these cells. Treatment with L-sepiapterin impaired only superoxide anion production, but did not restore NO levels (Fig. S3), suggesting a partial coupling and indicating that BH4 supplementation is more effective than L-sepiapterin in these melanoma cell lines. Different models of studies have shown the efficacy of BH4 and L-sepiapterin supplementation in NO and O_2^{-} changes, through recoupled eNOS and thereby reestablishing endothelial function and improving the treatment of cardiovascular diseases (Bendall et al., 2014). These studies support our results that NOS uncoupling can be reverted by BH4 treatment in tumor cells.

Our next step was to evaluate how NOS uncoupling contributes to characteristics acquired during tumor development treating melanoma cells with BH4 and restoring NOS function. We found that at 48 h, 72 and 96 h there was a significant reduction in cell viability among BH4-treated SK-MEL-28 (Fig. 6A and B), Mel14 (Fig. 6C) and Mel25 cells (Fig. 6D). We also observed a reduction in cell viability of SK-MEL-28

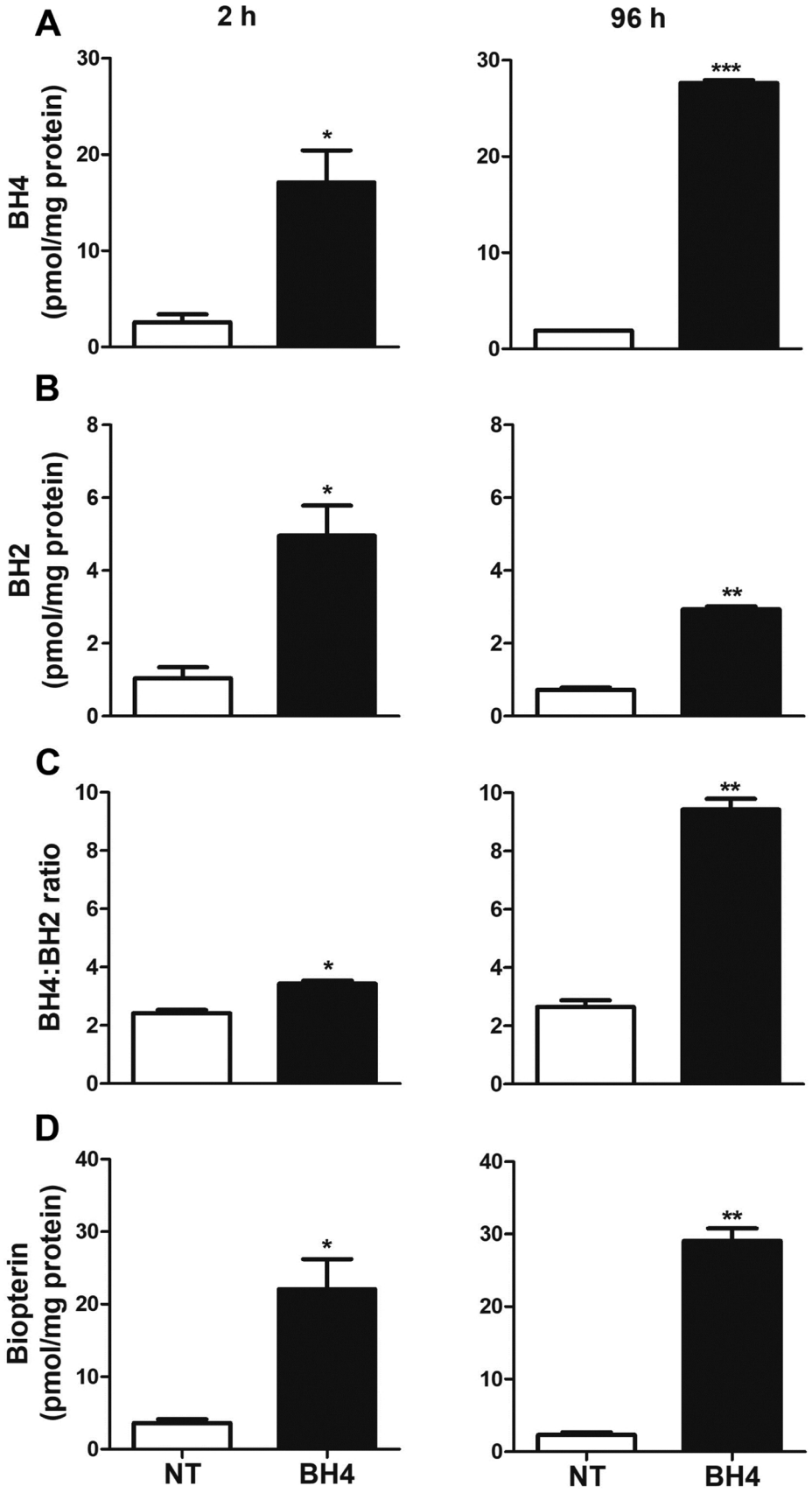

Fig. 4. Tetrahydrobiopterin treatment was effective to restore intracellular BH4 levels in metastatic melanoma cells. SK-MEL-28 cells were treated or not for 2 h and 96 h with 40 μM BH4 and the amount of BH4 (A), BH2 (B), BH4:BH2 ratio (C) and total biopterin (D) were determined by HPLC. The experiments were performed in duplicate and *p* values were based on Students' *t-test* *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

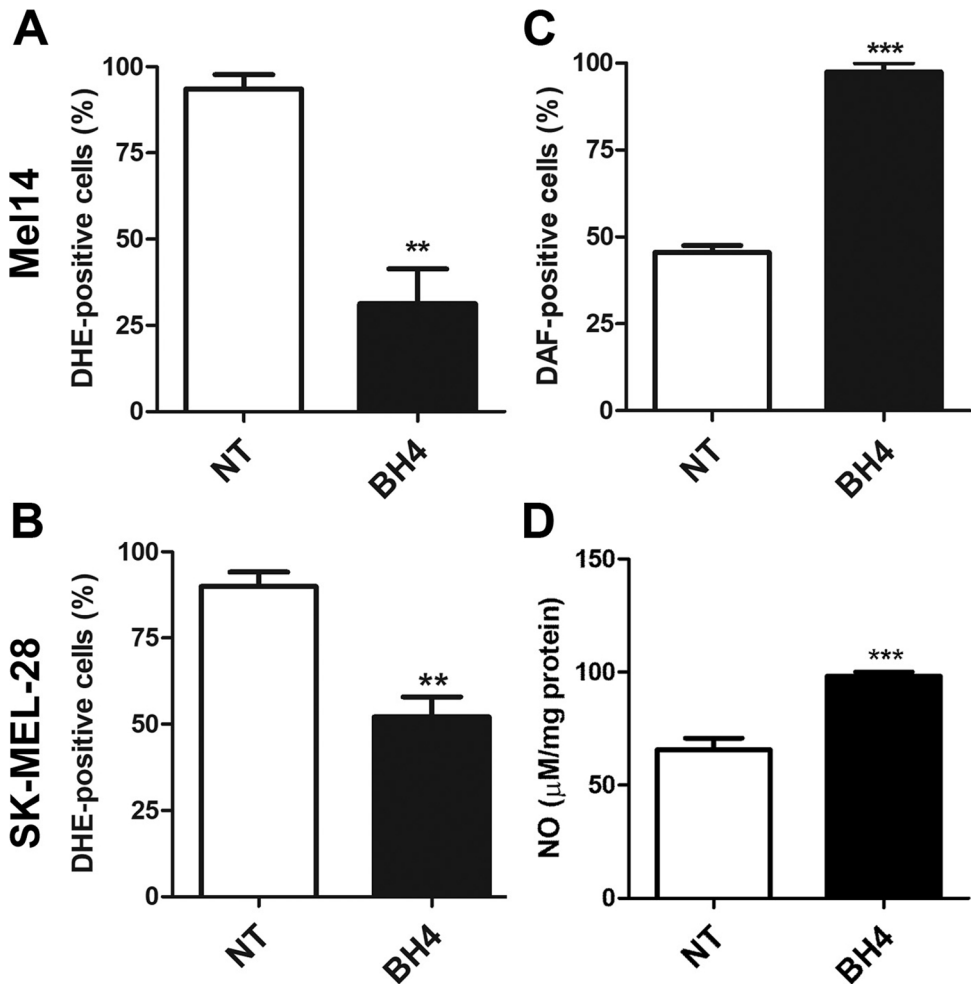

Fig. 5. Tetrahydrobiopterin abrogates superoxide anion and increases nitric oxide production by metastatic melanoma cells. Mel14 and SK-MEL-28 metastatic melanoma cells were treated or not for 2 h with 40 μM BH4 and intracellular superoxide anion levels were analyzed by flow cytometry using DHE (A and B) and NO levels by flow cytometry using DAF (C) or by Griess reaction (D). Values are reported in the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and *p* values were based on Students' *t-test* *p < 0.05, **p < 0.01, ***p < 0.001.

treated with L-SEPIAPTERIN (FIG. S4). In melanocytes, BH4 supplementation did not change cell viability (Fig. S5). This result is in accordance with literature data, since studies have shown that BH4 treatment has different functions in normal and tumor cells. Interestingly, an increase in the viability of normal endothelial cells treated with L-sepiapterin (Marinos et al., 2001; Shimizu et al., 1999) and with concentration of up to 50 μM of BH4 (Du YH et al., 2008). We also evaluated the ability of cells to form colonies and observed that, after 9 days, treatment with BH4 significantly decreased the number of colonies of the SK-MEL-28 cell line when compared to the untreated group (Fig. 7A). The same holds true for Mel14 and Mel25 cell lines after BH4 treatment (Fig. S6). Finally, we evaluated the ability of SK-MEL-28 melanoma cells to form tumors through spheroid cultures assay and saw a decrease in tumor diameter when they were treated with BH4 (Fig. 7C). It has been reported that in this assay is possible to analyze the capability of tumor cells to grow *in vivo*, since 3D models as tumor spheroids cultures are more representative of tumor microenvironment, therefore, it is feasible to propose that BH4 treatment has the potential to impair melanoma development *in vivo*. Corroborating with this hypothesis, BH4 treatment increased caspase 3/7 activation, leading to apoptosis in adherent and non-adherent conditions (Fig. 7B and D). Moreover, HUVEC cells submitted to conditions leading to eNOS uncoupling, also showed decrease in $O_2^{\cdot -}$ and increase in NO levels, but decreased caspase 3 activation when treated with BH4, indicating that in normal cellsBH4 supplementation may be antiapoptotic, contrary to what we have seen in melanoma cells (Fig. 7C and F) (Quagliaro et al., 2007).

Although the correlation between BH4 levels and tumor progression is not yet well understood, it is known to depend on tumor histology and staging. In breast carcinoma, increased BH4 and GTPCHI expression is associated with tumor development, since high BH4 levels caused by GTPCHI overexpression in tumor-associated fibroblasts leads to proliferation, angiogenesis and tumor growth in mice (Chen et al., 2010, 2016). It was demonstrated that GTPCHI expression is elevated in breast tumors, both in the stroma and epithelium, and is related to poorer prognosis. In a study of ovarian cancer cells, treatment with L-sepiapterin, a BH4 salvage pathway precursor, and the consequent increase in BH4 and NO concentration have been shown to increase proliferation and migration rates of these cells. However, when these same ovarian cancer cells were stimulated by VEGF-A (vascular endothelial growth factor) and subsequently treated with L-sepiapterin, tumor proliferation and migration were inhibited, showing that activation of different signaling pathways interfere with BH4 participation in tumor progression and can in part explain its dual role (Cho et al., 2011). Corroborating with our data that BH4 has an anti-tumorigenic role, Cardnell et al. observed that mice treated with L-sepiapterin showed a reduction in the number but not in the size of colorectal tumors induced in an azoxymethane/SDS colorectal cancer mouse model. Accordingly, the authors suggested that L-sepiapterin acts to limit tumor initiation, but not to restrict its progression (Cardnell et al., 2013). Rabender et al., recently related the association between NOS

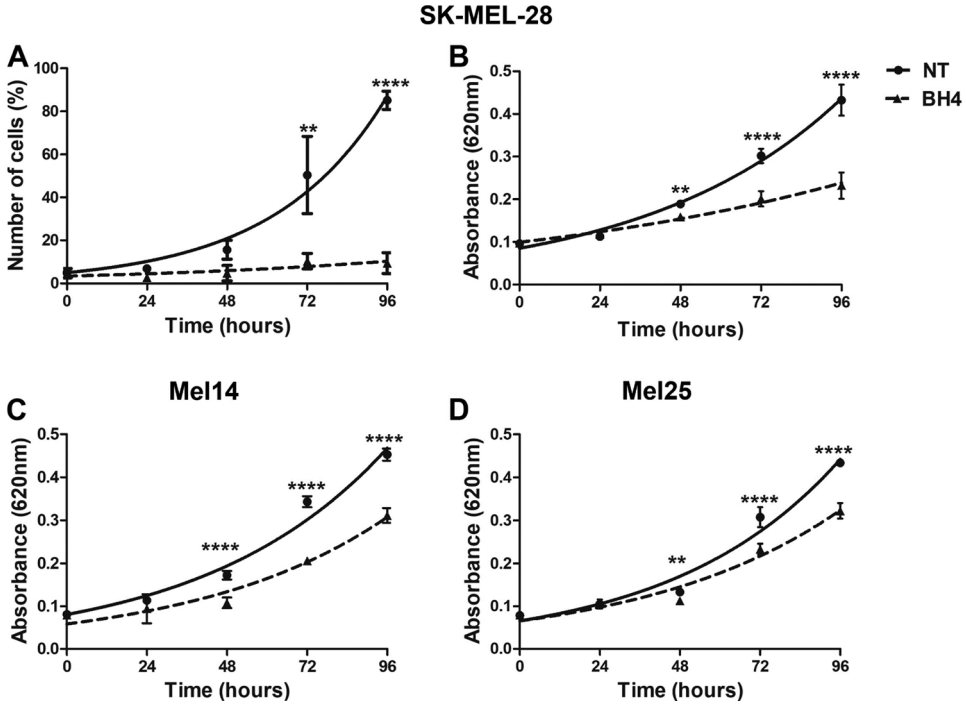

Fig. 6. Tetrahydrobiopterin decreases cell viability of metastatic melanoma cells. SK-MEL-28 metastatic melanoma cells were treated (BH4) or not (NT) for 24, 48, 72 and 96 h with 40 μM BH4 and viable cells were evaluated by trypan blue dye exclusion assay (A). The same treatment was performed in SK-MEL-28 (B), Mel 14 (C) and Mel 25 (D) metastatic melanoma cells and analyzed by MTT. The experiments were performed in triplicate and the results are representative of three experiments. *p* values were based on Two-Way ANOVA test followed by Sidak's multiple comparisons test $^{**}p < 0.01$, $^{***}p < 0.0001$.

uncoupling and tumor progression. They found decreased BH4:BH2 ratio in different tumor cell lines and xenographic tumors *in vivo* compared to normal tissues. Additionally, they observed that treatment of breast tumor cells with sepiapterin increased cGMP levels and decreased $O_2^{\cdot -}$ levels, suggesting that NOS is thus uncoupled in neoplasms (Rabender et al., 2015). Taken together these data denote that BH4 can act as a pro or anti-tumorigenic molecule, depending of tumor microenvironmental and activated signaling pathways. The participation of $O_2^{\cdot -}$ in malignant transformation is related to its ability to regulate signaling pathways involved in promoting proliferation and apoptosis resistance (Gorrini et al., 2013; Liu et al., 2011; Kitagishi and Matsuda, 2013). $O_2^{\cdot -}$ can also act on RAS and BCL-2 signaling, thus

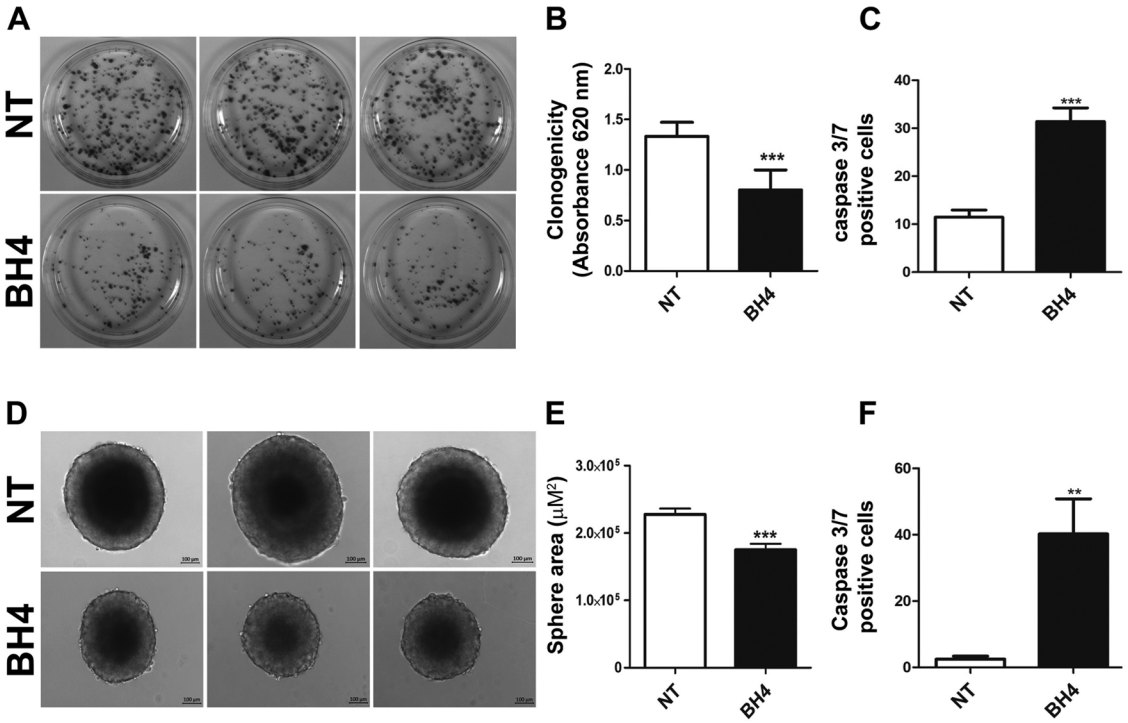

Fig. 7. Tetrahydrobiopterin decreases cell viability of metastatic melanoma cells by inducing apoptosis. SK-MEL-28 metastatic melanoma cells were treated or not with 40 μM BH4 for 9 days and the formation of clones was visualized by clonogenicity assay (A and B). SK-MEL-28 metastatic melanoma cells were also treated or not with 40 μM BH4 for 14 days and the formation of tumorspheres were evaluated by bead diameter measure (D and E). To evaluate apoptosis, SK-MEL-28 metastatic melanoma cells were treated or not with 40 μM BH4 for 96 h in adherent circumstances (C) or submitted to anchorage impediment (D) and was analyzed by CellEvent™ Caspase 3/7 green detection reagent. Values are reported in the bar graphs and expressed as the means ± S.D. The experiments were performed in triplicate and *p* values were based on Students' *t-test* $^{**}p < 0.01$, $^{***}p < 0.001$.

contributing to inhibiting apoptosis in tumor cells (Pervaiz and Clement, 2007). Superoxide anion abrogation was shown to inhibit the activation of Ras/Rac/Erk signaling pathway in murine melanocytes and impair melanocyte malignant transformation associated with sustained stress conditions (Molognoni et al., 2013). NO is also an important second messenger and depending on its concentration, NO may play opposite roles in apoptosis and consequently in tumor progression. High NO levels have been correlated with apoptosis in tumors, on the other hand, low levels have been correlated with promoting tumor growth (Ekmekcioglu et al., 2005; Reynolds et al., 2013). This dual role of NO in apoptosis pathways has been reported on melanoma. Human melanoma A375-S2 cells were treated with evodiamine, which in turn induces NO production, an increase in p53 activation and, consequently, apoptosis (Yang et al., 2008). By using a specific inhibitor of iNOS, aminoguanidine, in metastatic melanoma cell lines isolated from patients, there was observed a change in mitochondrial membrane potential and reduction in BCL-2 expression levels, in addition to greater release of caspase 1 and greater activity of caspase 3, thereby triggering an increase in the apoptotic rate of these cell lines (Salvucci et al., 2001). Furthermore, studies have shown that the apoptotic capacity of NO can be inhibited by interaction with $O_2^{\cdot -}$. Studies with mesangial cells and murine macrophages revealed that apoptosis caused by NO is inhibited when there is simultaneous production of $O_2^{\cdot -}$, indicating that the balance between NO and reactive oxygen species can regulate this process (Brüne et al., 1997; Brüne, 2005).

It had been demonstrated that treatment by intravenous administration with BH4 500 μg/min, a dose sufficient to reach BH4 plasma concentration that have been show to achieve maximal NO production *in vitro* which was 50 μM (Chen et al., 1995), improve endothelial-dependent vasodilation to acetylcholine in patients with hypercholesterolaemia (Stroes et al., 1997), diabetes (Heitzer et al., 2000a) and chronic smokers (Heitzer et al., 2000b). In this study, we also observed increased NO concentration in melanoma cells after treatment of 40 μm BH4, so we believe that the transposition of this concentration into *in vivo* cause the same effect. However, pharmacokinetic studies are necessary to evaluate the effects observed *in vitro*.

A synthetic preparation of 6R-BH4 is available as sapropterin dihydrochloride (Kuvan®, BioMarin Pharmaceutical Inc., Novato, CA, USA). Sapropterin dihydrochloride was approved by the Food and Drug Administration (FDA) in 2007 and by the European Medicines Agency (EMA) in 2008 for the treatment of phenylketonuria (PKU) in patients with BH4-responsive PKU (BH4 is also the cofactor of phenylalanine hydrolase) or BH4 deficiencies. In patients with BH4-responsive PKU, clinical trials have demonstrated significant decrease in blood phenylalanine concentration using doses of 20 mg/kg without adverse effects. The full prescribing information of Kuvan® recommended that the starting dose of Kuvan is 10 mg/kg taken once daily for patients with 1 month to 6 years and 10–20 mg/kg taken once daily for patients with 7 years and older. It had been demonstrated that 20 mg/kg given orally reached after 1–4 h 400 nmol h/L approximately, equivalent to 130 μg h/L and after 24 h approximately 3 μmol h/L, equivalent to 957 μg h/L (Fiege et al., 2004) when the concentration of phenylalanine decreases, showing the effect of the drug (Muntau et al., 2019). Based on the information above, the administration of 40 μM BH4 would be sufficient to achieve promising results being administered orally or intravenously, however, studies *in vivo* are necessary to evaluate this afirmation.

Accordingly, based on our results, we postulate that NOS uncoupling in human metastatic melanoma cells caused by decrease in BH4:BH2 ratio or BH4 concentration and by stoichiometric imbalance between BH4 amount and NOS protein levels contributes to melanoma progression by altering the balance between NO and $O_2^{\cdot -}$ levels and consequently redox homeostasis. BH4 treatment leads to NOS recoupling, promoting an increase in intracellular NO levels and a reduction in $O_2^{\cdot -}$ levels, which could trigger apoptotic pathways. As already discussed, the role of BH4 in tumor progression is not yet well defined, with the divergences reported across different studies indicating that it may function in an anti- or pro-tumoral fashion what would be explained, in part, by the dual role that both NO and $O_2^{\cdot -}$ play in apoptotic and proliferative pathways and also the change or not in the balance between them.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105592.

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