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Cell cycle arrest, extracellular matrix changes and intrinsic apoptosis in human melanoma cells are induced by Boron Neutron Capture Therapy

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ABSTRACT

Boron Neutron Capture Therapy (BNCT) involves the selective accumulation of boron carriers in tumor tissue followed by irradiation with a thermal or epithermal neutron beam. This therapy is therefore a cellular irradiation suited to treat tumors that have infiltrated into healthy tissues. BNCT has been used clinically to treat patients with cutaneous melanomas which have a high mortality. Human normal melanocytes and melanoma cells were treated with BNCT at different boronophenylalanine concentrations for signaling pathways analysis. BNCT induced few morphological alterations in normal melanocytes, with a negligible increase in free radical production. Melanoma cells treated with BNCT showed significant extracellular matrix (ECM) changes and a significant cyclin D1 decrease, suggesting cell death by necrosis and apoptosis and cell cycle arrest, respectively. BNCT also induced a significant increase in cleaved caspase-3 and a decrease in the mitochondrial electrical potential with selectivity for melanoma cells. Normal melanocytes had no significant differences due to BNCT treatment, confirming the data from the literature regarding the selectivity of BNCT. The results from this study suggest that some signaling pathways are involved in human melanoma treatment by BNCT, such as cell cycle arrest, ECM changes and intrinsic apoptosis.

Significance

Metastatic melanoma remains a highly lethal disease, with an incidence that continues to increase faster than any other cancer and almost adjuvant treatments fail to control this malignancy. Boron Neutron Capture Therapy was used is this work with selective treatment for melanoma cells with minimum effects in normal cells. This therapy induces cell death by apoptosis and cell cycle arrest only in melanoma cells.

1. Introduction

Boron Neutron Capture Therapy (BNCT) is a binary treatment modality that involves the selective accumulation of boron carriers in a tumor, followed by irradiation with a thermal or epithermal neutron beam (Monti Hughes et al., 2011). The neutron capture reaction with a boron-10 (^{10}B) nucleus yields high linear energy transfer (LET) particles, alpha and ^{7}Li, with a range of 5–9 μm, thus BNCT is a putative cell-selective radiation therapy with the potential to control local recurrences of malignant tumors (Nakamura et al., 2011). These particles can only travel very short distances and, as such, release their damaging energy directly to the tissue that contains the boron compound. Cell death is triggered by the release of these charged particles, which create ionisation tracks along their trajectories, thereby resulting in cellular damage (Toppino et al., 2013).

BNCT has two advantages. Firstly, the dose of radiation given in the neutron beam can be quite low; secondly, the local decay and action allow the surrounding healthy tissue to be spared damage due to radiation (Barth et al., 2005).

BNCT has been used clinically to treat patients with cutaneous melanomas (Mishima, 1996). These patients were either not candidates for, or had declined, conventional therapy (Barth et al., 2004). Melanoma is the most aggressive skin cancer and frequently involves distant and locoregional spread, usually with no efficient

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treatment (Menéndez et al., 2009). Metastatic melanoma remains a highly lethal disease, with an incidence that continues to increase faster than any other cancer (González et al., 2004). Almost all adjuvant treatments fail to control this malignancy (Pawlik and Sondak, 2003).

BNCT has a strong local radiotherapy effect. The efficacy of the method in cancer therapy requires sufficient accumulation of boron into the tumor and an irradiation in tumor location (Joensuu et al., 2011). Only cells that have 10-boron are damaged by thermal neutrons. So, this therapy is a cellular radiation suited to treat local tumors or those infiltrate near healthy tissues (Esposito et al., 2008).

BNCT could be an attractive tool to improve response over the standard radiotherapy treatment delivering high dose to tumor while reducing normal tissue effect, due to the different boron uptake in normal and tumor cells (Menéndez et al., 2009).

There are no published results about the BNCT effect on normal melanocytes compared to melanoma cells, and these data are extremely important to know the effectiveness of BNCT versus the side effects incidence in healthy tissues. There is also no data about signaling pathways involved in the melanoma treatment. The aim of this study was to evaluate the selectivity and signaling pathways involved in melanocytes and melanoma treatment with BNCT.

2. Materials and methods

2.1. Cell lines

A human melanoma tumor cell line (SK-MEL-28) was cultivated in 75 cm^2 flasks with RPMI-1640 (Cultilab) medium supplemented with 10% inactivated fetal bovine serum (Cultilab), 2 mM L-glutamine (Sigma Chemical Company) and 0.1 g/mL streptomycin (FontouraWyeth AS). A human primary culture of melanocytes isolated from foreskin was cultivated with 254CF medium (Life Sciences®), supplemented with 10% HMGS growth factors (Life Sciences) and 0.1 mg/mL streptomycin (FontouraWyeth AS) as previously described (Fernandez et al., 2005).

Adherent cell suspensions were propagated by treatment of the culture flasks with 0.2% trypsin and then inactivated in 10% fetal bovine serum (FBS)-enriched medium. The nonadherent cells were centrifuged twice, resuspended in medium and then seeded in plates and allowed to grow for 24 h.

2.2. Boronophenylalanine (BPA)

^{10}B-enriched (>99%) BPA was purchased from KatChem and converted to a fructose 1:1 complex to increase its solubility (Coderre et al., 1994).

2.3. Cells treatment and BNCT irradiation for MTT and lipid peroxidation tests

Melanocytes were seeded in 96-well plates at concentration of 10^5 cells/mL and allowed to grow for 24 h. They were then treated with different concentrations of BPA, from 40 to 0.52 mg/mL, which corresponds to 2100–27.5 μg ^{10}B/mL for MTT assay and from 8.32 to 0.52 mg/mL, which corresponds to 440–27.5 μg ^{10}B/mL for lipid peroxidation test. After incubation with BPA for 90 min, the cells were irradiated at the BNCT research facility at the Nuclear and Energetic Research Institute (IPEN, Brazil) Coelho et al., 2002 for 120 min using the IEA-R1 nuclear reactor operating at a power of 3.5 MW. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the irradiation position were $(2.31 \pm 0.03) \times 10^8$, $(4.60 \pm 0.10) \times 10^6$ and $(3.50 \pm 0.10) \times 10^7$ n/cm^2 s, respectively. The gamma dose rate in air at the irradiation site was 3.50 ± 0.80 Gyh^{-1}. Before irradiation, the BPA-enriched incubation medium was removed and the cells were washed in 0.9% saline solution. Another cell group was irradiated without BPA (beam only) and was designated as the "irradiated control". A non-irradiated and BPA-free group was also studied and was designated as the "control". Images of the control and treated cells were recorded by a camera (Sony Cyber-shot 7.2 mega pixels) coupled to an optic inverted microscope (Carl Zeiss), magnified by 40×.

2.4. Cells treatment and BNCT irradiation for soluble collagen quantification and flow cytometry tests

Melanocytes and SK-MEL-28 melanoma cells were seeded in 24-well plates at a concentration of 10^5 cells/mL and allowed to grow for 24 h. SK-MEL-28 melanoma cells were treated with 3.7 mg/mL BPA in all flow cytometry tests (this value is equivalent to 192.0 μg ^{10}B/mL), which corresponds to the inhibitory concentration of 50% (IC_{50}) for this compound in this cell line (Faião-Flores et al., 2011a). Melanocytes were treated with 34.4 mg/mL BPA in all flow cytometry tests (this value is equivalent to 1.8 mg ^{10}B/mL), which corresponds to the IC_{50} for this compound in this cell line. After 90 min of incubation with BPA, the cells were irradiated at the BNCT research facility at the Nuclear and Energetic Research Institute (IPEN, Brazil) Coelho et al., 2002 for 30 min, using the IEA-R1 nuclear reactor operating at a power of 3.5 MW. The analysis was performed 6 h after BNCT treatment. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the irradiation position were $(2.31 \pm 0.03) \times 10^8$, $(4.60 \pm 0.10) \times 10^6$ and $(3.50 \pm 0.10) \times 10^7$ n/cm^2 s, respectively. The gamma dose rate in air at the irradiation site was 3.50 ± 0.80 Gy h^{-1}. Before irradiation, the BPA-enriched incubation medium was removed and the cells were washed in 0.9% saline solution. Another cell group was irradiated without BPA (beam only) and was designated as the "irradiated control". A non-irradiated and without BPA group was also studied and was designated as the "control".

2.5. Cellular viability assay – MTT

The cellular viability of the melanocytes was determined using a colorimetric methodology known as MTT (3-(4,5-dimethylthiazol-2-y1)2,5-diphenyl tetrazolium bromide) (Sigma) Mosmann, 1983. MTT is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color is then quantified by spectrophotometry. After irradiation, the culture medium was removed for lipid peroxidation (LPO) analysis and 10 μL MTT reagent (5 mg/mL) (Sigma–Aldrich Corp.) was added to each well. The plates were incubated at 37 °C for 3 h, protected from light. Blue formazan crystals thus formed were pelleted to the bottom of the well by centrifugation, separated from the supernatant and dissolved in 150 μl of dimethylsulfoxymide. The optical density at 540 nm was determined by absorbance spectrometry using a microplate reader. A linear relationship between cell number and absorbance was established, enabling an accurate, straightforward quantification of changes in proliferation. The mean values of several experiments were presented in a linear regression model, and the IC_{50} was calculated.

2.6. Lipid peroxidation (LPO)

The oxidative stress on unsaturated lipids in cell membranes was evaluated by determining the amount of malondialdehyde (MDA), which is the final product of fatty-acid peroxidation that reacts with thiobarbituric acid (TBA) to form a colored complex. Thiobarbituric acid reactive substances (TBARS) were quantified

by spectrophotometric determination (LPO method) Ohkawa et al., 1979. The supernatant of the samples obtained after irradiation and before carrying out the MTT method was used for LPO. This experiment was performed 6 h after thermal neutron irradiation.

2.7. Soluble collagen quantification by Sirus Red assay

The Sirus Red cytochemical staining test evaluates the quantity of collagen in a sample (Koren et al., 2001). The dyes used for the Sirus Red test react specifically with basic groups in the collagen molecule (Junqueira et al., 1979; Pickring and Boughner, 1991).

After irradiation, plates with the supernatant (metabolized culture medium) of melanoma cells and melanocytes were placed in an incubator at 37 °C overnight without lids to dry the contents. Then, saturated Bouin solution (Koren et al., 2001) was added in each well, and the samples were incubated for 1 h at the room temperature. The dye was removed and 300 μL distilled water was added. The plate was dried at room temperature for approximately 2 h. After this period, 200 μL 0.1% picrosirius dye was added for 1 h, protected from light. The dye was removed and 250 μL 0.01 M HCl was added. After that, the HCl solution was removed, and the samples were incubated with 150 μL 0.1 M NaOH for 30 min. The optical density of the samples was read at 550 nm in a spectrophotometer. This experiment was performed 6 h after thermal neutron irradiation.

2.8. Assessment of mitochondrial membrane potential by flow cytometry

Rhodamine 123 is a cationic lipophilic fluorescent chemical that accumulates specifically in mitochondria of living cells (Kennady et al., 2004). Thus, this agent binds only in metabolically active mitochondria, resulting in a fluorescent emission. After irradiation, the culture medium was removed and adherent cells were trypsinized. Melanoma cells and melanocytes were pelleted by centrifugation at 1800 rpm for 10 min and resuspended in 5 μL Rhodamine 123 (5 mg/mL) for 30 min at 37 °C. The cells were then washed with phosphate-buffered saline (PBS) and resuspended in FACS flow buffer (Becton Dickinson). The samples were analyzed for fluorescence (FL-1H detector) on a Becton Dickinson FACScan flow cytometer using Cell Quest software. This experiment was performed 6 h after thermal neutron irradiation.

2.9. Cyclin D1 quantification by flow cytometry

The type D cyclins (with their partner CDKs) form a regulatory unit of the G1/S transition that is frequently impaired in neoplásicas (Li et al., 2006). After irradiation, the culture medium was removed and adherent cells were trypsinized. Melanoma cells and melanocytes were pelleted by centrifugation at 1800 rpm for 10 min and incubated with 1 μg specific Anti-cyclin D1 antibody (Santa Cruz, USA) and 10 μL Triton X-100 (0.1%) for 1 h at 4 °C. The cells were then resuspended in FACS Flow buffer. The samples were analyzed for fluorescence (FL-1H detector) on a Becton Dickinson FACScan flow cytometer using Cell Quest software. This experiment was performed 6 h after thermal neutron irradiation.

2.10. Annexin-V assay

Annexin V is a small Ca^{2+}-dependent protein with high affinity for phosphatidylserine (PS) Vermes et al., 1995. In normal living cells, PS is located in the inner layer of the cell membrane only, but in apoptotic cells this phospholipid is translocated to the outer leaflet. PS exposure on the surface of cells functions as tags for specific recognition for phagocytosis by macrophages or neighboring cells (Fadok et al., 1992). Annexin V was used to detect apoptosis at an early stage in the cells together with propidium iodide, which binds to DNA in cells that have lost membrane integrity (necrotic or late apoptotic cells).

After treatment, the cells in the supernatant and the adherent cells were washed with PBS and binding buffer (10 mM HEPES pH7.5 containing 140 mM NaCl and 2.5 mM CaCl2) and stained with 1 μg annexin V-FITC (Santa Cruz Biotechnology, USA) and 18 μg/mL of propidium iodide (PI) (Sigma–Aldrich Corp.). Each sample was analyzed by flow cytometry using the FL-1 and FL-2 channels to distinguish the apoptotic, necrotic, and viable cell populations. The analysis was performed on a FACSCalibur flow cytometer using the Cell Quest software (FACSCalibur; Becton Dickinson). The caspase-3 inhibitor zDEVD-fmk (Becton Dickinson, USA) was prepared as stock solution in 100% DMSO (100 mM). Final concentration in serum-free medium was 1 mM for zDEVD-fmk. Cells were incubated with caspase inhibitor 1 h before addition of BPA. Control wells were incubated with corresponding DMSO concentration.

2.11. Cleaved caspase-3 activity by flow cytometry

Caspases represent a family of cysteine proteases that are common downstream effectors of apoptosis (Chen et al., 2001). After irradiation, the culture medium was removed and adherent cells were trypsinized. Melanoma cells and melanocytes were pelleted by centrifugation at 1800 rpm for 10 min and incubated with 1 μg of specific Anti-caspase 3 PE antibody (Santa Cruz, USA) and 10 μL of Triton X-100 (0.1%) for 1 h at 4 °C. The cells were then resuspended in FACS Flow buffer. The samples were analyzed for fluorescence (FL-1H detector) on a Becton Dickinson FACScan flow cytometer using Cell Quest software. This experiment was performed 6 h after thermal neutron irradiation.

The caspase-3 inhibitor zDEVD-fmk (Becton Dickinson, USA) was prepared as stock solution in 100% DMSO (100 mM). Final concentration in serum-free medium was 1 mM for zDEVD-fmk. Cells were incubated with caspase inhibitor 1 h before addition of BPA. Control wells were incubated with corresponding DMSO concentration.

2.12. Statistical analysis

The values are expressed as the mean ± standard deviation (s.d.). The data were analyzed using one-way analysis of variance (ANOVA), and significant mean differences were determined using multiple comparisons by the Tukey–Kramer test at the $p < 0.05$ level. Significant differences between the control and treated groups are indicated by $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

3. Results

3.1. BNCT induced both low levels of cell death and free radical production in normal melanocytes

Melanocytes treated with BNCT showed low levels of cell death. The IC_{50} value was 34.4 mg/mL, which corresponds to 1.8 mg/mL ^{10}B (Fig. 1). The cellular viability (IC_{50} value) of the irradiated control did not show any significant difference compared to the control group.

After BNCT treatment, the melanocytes exhibited an increase in free radical production, and this increase was greater only when higher BPA concentrations were used (Fig. 2). However, the increase in free radical production in the highest BPA concentration used was approximately only 1.5 times higher than that of the control group. The lower BPA concentrations did not show significant differences. The irradiated control also did not exhibit any differences compared to the control group.

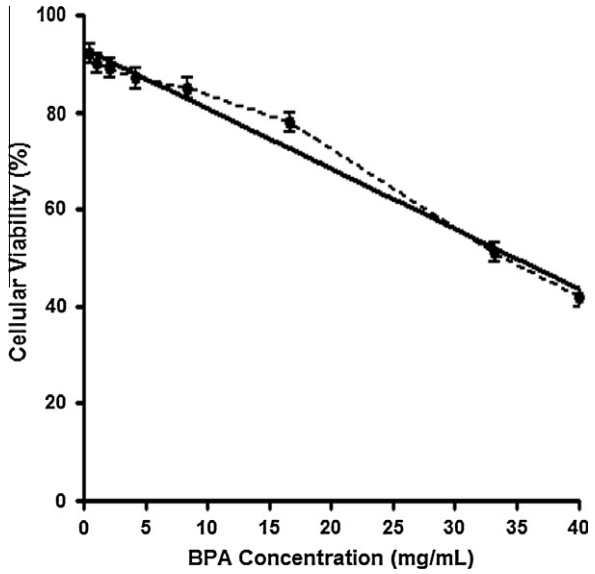

Fig. 1. Cellular viability of normal melanocytes after BNCT. The percentage of viable cells was plotted against the different BPA concentrations followed by thermal neutron irradiation. The linear regression curve and IC_{50} were calculated using Graph Pad Prism Instat 3 software. The IC_{50} value estimated was 34.4 mg/mL (approximately 1.8 mg ^{10}B). Data were obtained from three independent experiments and results were represented as mean ± s.d.

The normal melanocytes were photographed for morphological analysis after BNCT treatment. None of the BPA concentrations induced morphological changes. The presence of apoptotic bodies, debris formation and cytoskeleton disarray was also not detected (Fig. 3). Only the highest BPA concentration showed a slight decrease in confluence, which is consistent with the free radical production observed when using this concentration. The cells of the irradiated control presented insignificant alterations and little cell damage.

3.2. Extracellular matrix changes in melanoma cells after BNCT

After BNCT, the extracellular matrix of normal melanocytes and melanoma cells was analyzed by Sirus Red staining. The extracellular matrix of melanoma cells treated with BNCT showed dramatic changes, as evidenced by a decrease in soluble collagen synthesis (Fig. 4). The decrease of soluble collagen synthesis in melanoma cells treated with BNCT was approximately 2.7 times higher than in the control group. Melanocytes did not present any differences in soluble collagen synthesis after BNCT treatment. Additionally, the irradiated group did not show significant differences in comparison with the control group in these normal and tumor cell lines.

3.3. The mitochondrial electric potential decreases after BNCT in melanoma cells

BNCT induces a decrease of the mitochondrial electric potential, thereby causing cell death in SK-MEL-28 melanoma cells. After BNCT, the melanoma cells had their mitochondrial electric potential reduced by approximately 12.3 times compared to the control group (Fig. 5). Melanocytes treated by BNCT did not show significant differences in this electric potential. These data confirm the cellular viability assay, which provided a high IC_{50} value for normal melanocytes. The irradiated group also did not present differences compared to the control group for either cell line.

3.4. BNCT induces cell death by necrosis and apoptosis in melanoma cells

After BNCT treatment, melanocytes and melanoma cells were observed as to the ability in necrosis and apoptosis induction (Fig. 6A). SKMEL-28 melanoma cells treated by BNCT showed approximately 50% of cell population in necrosis and in late apoptosis (Fig. 6B). After zDEVD-fmk inhibitor addition, the necrosis population was increased, whereas apoptosis population was decreased. Cells treated with this inhibitor showed reduced capacity in apoptosis induction. This is due to the ability of this caspase-3 inhibitor to provoke high influence in the both apoptotic pathways.

Melanocytes did not present significant differences in necrosis or apoptosis in comparison to the control and irradiated control groups (Fig. 6C).

3.5. Cell cycle progression decreases in melanoma cells after BNCT

The cyclin D1 marker was used to quantify cell cycle progression in the G1-S phases. BNCT was able to induce a decrease in cy-

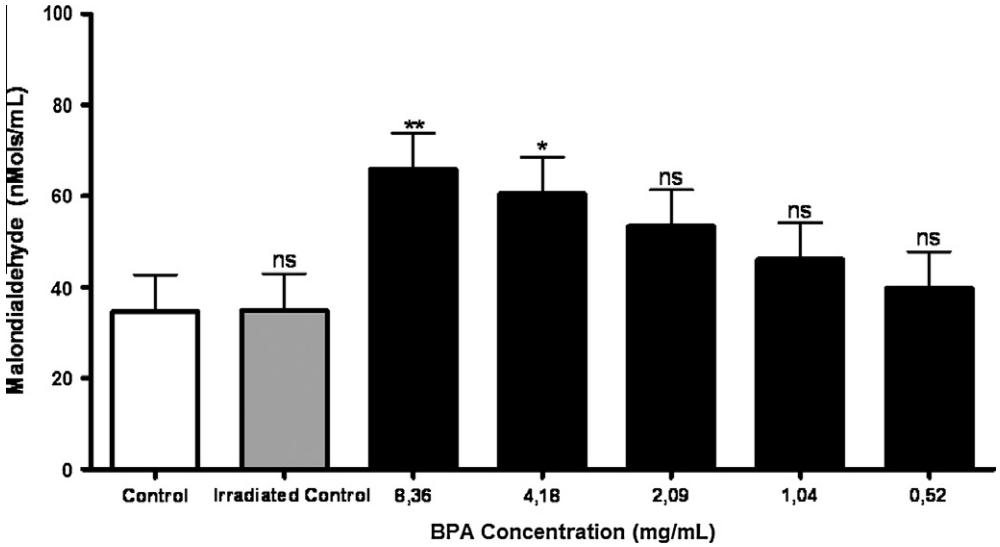

Fig. 2. Malondialdehyde production in normal melanocytes after BNCT at different BPA concentrations and after neutron irradiation alone (irradiated control) compared to cells without treatment (control). Data were obtained from three independent experiments and results were represented as mean ± s.d. ns: not significant compared to control. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control.

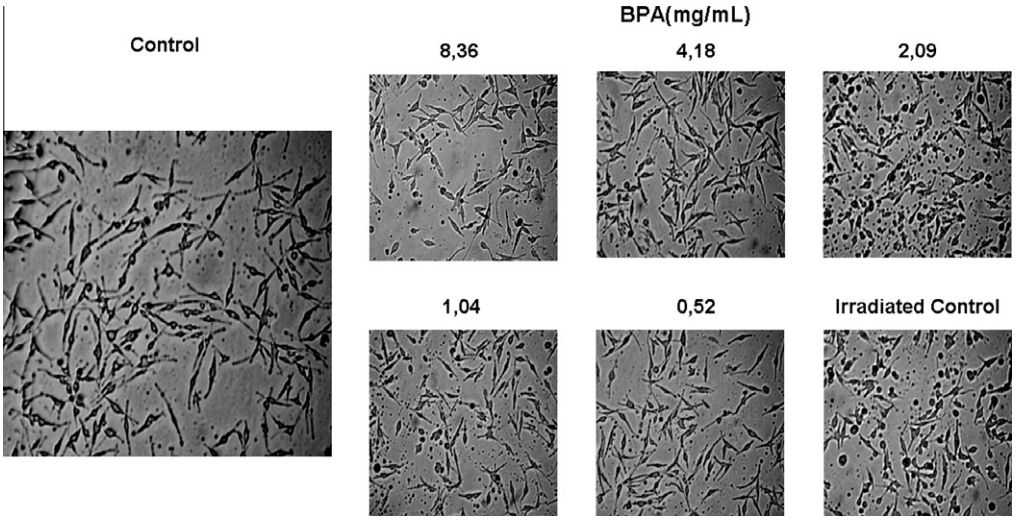

Fig. 3. Morphological aspects of normal melanocytes after BNCT at all BPA concentrations compared to the control group and irradiated control. Magnification: 40×.

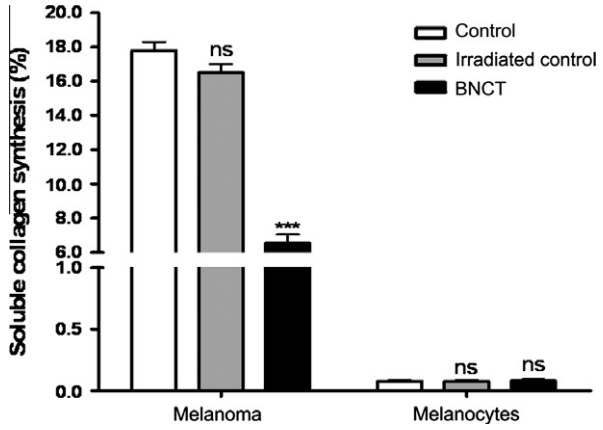

Fig. 4. Soluble collagen synthesis in melanoma cells and melanocytes after BNCT and neutron irradiation alone (irradiated control) compared to cells without any treatment (control). Data were obtained from three independent experiments and results were represented as mean ± s.d. ns: not significant compared to control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.

clin D1 expression only in melanoma cells. In normal melanocytes this progression decrease was not significant (Fig. 7A). There were no significant changes in cyclin D1 expression in melanocytes. The irradiated control did not present significant alterations in this marker in either cell line.

3.6. Apoptosis induction by cleaved caspase-3 in melanoma cells after BNCT

Cleaved caspase-3 was used to verify the presence of cell death by the apoptosis pathway. In melanoma cells, BNCT was able to induce significant caspase-3 cleavage, indicating apoptosis activation (Fig. 7B). There was a small decrease of cleaved caspase-3 in melanocytes after BNCT treatment. The irradiated control group did not exhibit any significant differences compared to the control group for either cell line, thus confirming all previous results shown in this work.

To confirm whether or not caspase-3 activation is involved in the apoptosis of cells triggered by BNCT, it was used the caspase inhibitor zDEVD-fmk before BNCT treatment. The results indicated that BNCT induces caspase-3 activity increase and apoptosis without the caspase inhibitor. After treatment with BNCT and the zDEVD-fmk, the inhibition of BNCT-mediated caspase-3 activation was accompanied by the moderate necrosis expression increase.

4. Discussion

Advancements in cancer therapy depend on the development of new drugs, drug delivery systems and target therapies. A major challenge in drug delivery is to increase the efficiency by which a compound can deliver the maximum amount of a therapeutic agent to the tumor while minimizing any adverse effects to normal cells (Chakrabarti1 et al., 2012). To fully develop its treatment potential, BNCT requires the combination of a suitable thermal neutron flux and a selective uptake of ^{10}B in the target tissue. The latter condition is more critical because none of the boron carriers used for experimental or clinical purposes so far have shown optimal selectivity for cancer cells compared to normal cells (Menichetti et al., 2009). The BNCT treatment induced moderate malondialdehyde production only at the highest concentration of BPA in melanocytes. The other concentrations, along with the irradiated control, did not manifest an appreciable increase of malondialdehyde, demonstrating that this therapy did not influence free radical production in normal cells. In SKMEL-28, B16F10, IPC-298 and MEWO melanoma cells there were high malondialdehyde production (at least 10–30-fold increase) after BNCT treatment in the same conditions (Faião-Flores et al., 2011a).

It should be emphasized that the main criterion for the development of successful boron-containing compounds in cells is a high selectivity of these compounds for cancer cells over normal cells (Gnewuch and Sosnovskym, 2002).

There is a decrease in normal melanocytes viability only in the highest BPA concentrations followed by neutron irradiation. The IC_{50} found here was significantly high compared to SKMEL-28 melanoma cells: IC_{50} = 34.4 mg/mL and 3.7 mg/mL in normal melanocytes and melanoma cells SKMEL-28, respectively. These results confirm the most selectivity of BPA for tumor cells *in vitro* without inducing high cell death in normal cells, which has been reported elsewhere (Faião-Flores et al., 2011a, 2012; Menichetti et al., 2009). The increased BPA selectivity *in vivo* was studied in mice bearing melanoma tumors consisting of B16F10 cells, and the study found that the liver, heart and lungs do not take up boron from BPA, whereas other organs, such as the spleen and brain, captured minimal quantities of this compound (Faião-Flores et al., 2011).

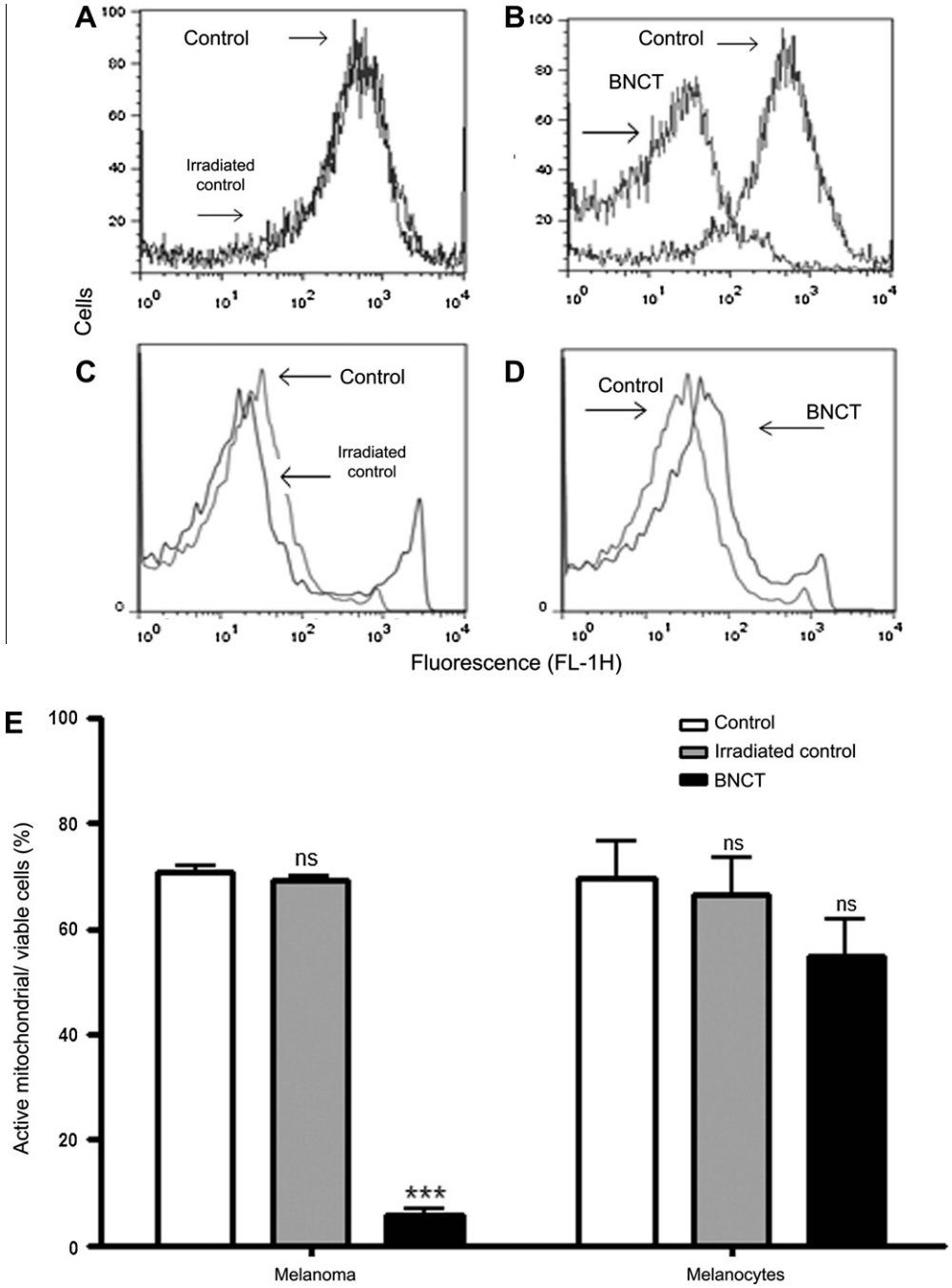

Fig. 5. Representative overlaps of fluorescence intensity and percentage values from melanoma cells and normal melanocytes marked with Rhodamine 123 analyzed by flow cytometry. The overlaps represent (A) control melanoma cells compared to irradiated group, (B) control melanoma cells compared to BNCT, (C) control melanocytes compared to irradiated group and (D) control melanocytes compared to BNCT. (E) Percentage values of melanoma cells and normal melanocytes with active mitochondria and/or viable cells are shown as the median ± s.d. Data were obtained from three independent experiments ns: not significant compared to control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.

Melanoma cells are strongly resistant to many chemotherapeutic drugs, as demonstrated by their ability to block apoptosis and stimulate tumor progression (Soengas and Lowe, 2003). The survival of adherent cells depends on an uninterrupted connection with the components of the extracellular matrix (ECM), such as laminin and fibronectin (Makino et al., 2000). The interactions between cells and ECM are crucial for cell behavior, growth and death (Wunrau et al., 2009). The detachment of adherent cells from the ECM can induce apoptosis almost immediately, a process known as *Anoikis* (Grossman et al., 2001). Despite melanoma being able to produce low amounts of collagen, the tumor development and invasion into adjacent tissue is often accompanied by increased architectural disorder of the extracellular matrix (ECM) and cellular components especially at the invasive front of the neoplastic mass (van Kempen et al., 2008). BNCT induced a decrease in collagen synthesis in nearly 60% of melanoma cells without affecting normal cells, involved with cell detachment of ECM, which followed by apoptosis, could suggest cell death by *Anoikis*.

The observation of mitochondrial bioenergetics, among other parameters, is important to establish the mechanisms by which therapy may cause cell death (Wallace and Starkov, 2000). The electronic gradient between the mitochondrial membranes during

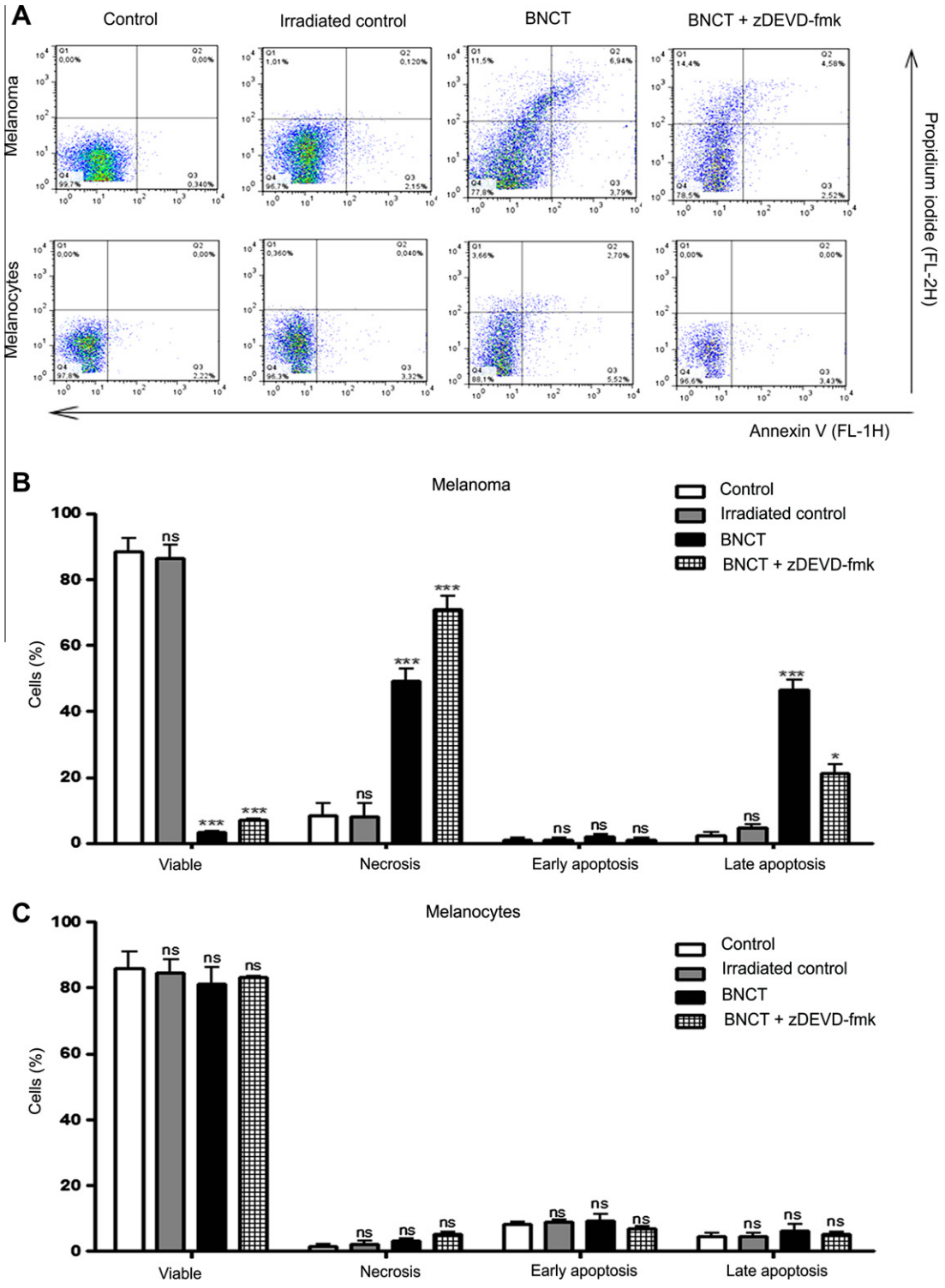

Fig. 6. (A) Representative histograms of SKMEL-28 melanoma cells and melanocytes of control, irradiated control, BNCT and BNCT + zDEVD-fmk stained with annexin V (FL-1H axis *X*), and propidium iodide (FL-2H axis *Y*) for cell quantification. The distribution (mean ± s.d) is the number of viable, necrotic and apoptotic (B) melanoma cells and (C) normal melanocytes. Data were obtained from three independent experiments ns: not significant compared to control. $^{*}p < 0.05$; $^{***}p < 0.001$ compared to control.

metabolism is known as mitochondrial electric potential ($\Delta\psi$) Chen et al., 2009. The $\Delta\psi$ is reduced when mitochondrial energy metabolism is disrupted, notably during apoptosis (Fuller and Arriaga, 2003). We note that BNCT induced a decrease of mitochondrial electric potential in melanoma cells by approximately 7 times compared to the control group. This same result was not observed in normal melanocytes. The irradiated control did not present any differences in either cell line.

The BNCT cytotoxic effect is mediated through many mechanisms, which include interaction and damage of DNA followed by activation of DNA damage-induced signaling pathways. These pathways culminate in cell cycle arrest and/or apoptosis, necrosis,

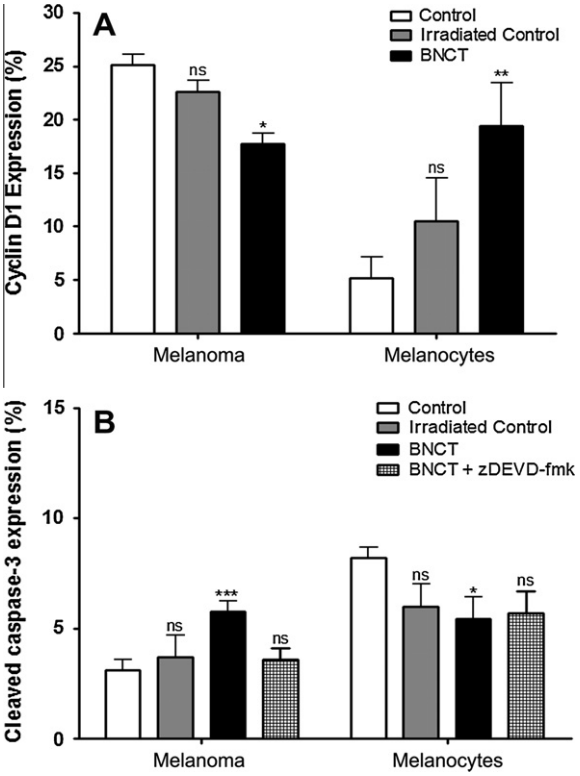

Fig. 7. Expression of cell markers in melanoma cells and normal melanocytes (median ± s.d.) by flow cytometry. (A) Cyclin D1 expression after BNCT treatment and neutron irradiation alone (irradiated control) compared to cells without any treatment (control). (B) Cleaved caspase-3 expression after BNCT treatment and neutron irradiation alone (irradiated control) compared to cells without any treatment (control). The zDEVD-fmk inhibitor was used as caspase-3 inhibitor in BNCT treated cells. Data were obtained from three independent experiments and results were represented as mean ± s.d. ns: not significant compared to control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.

autophagy or mitotic catastrophe (Debatin and Krammer, 2004; Okada and Mak, 2004). For this reason, some melanoma cells after BNCT treatment presented substantial necrosis expression increase, possibly by cellular communication between neighboring cells and due to the limited BNCT efficacy, which is almost exclusively for cells carrying ^{10}B irradiated by thermal neutrons. This way, the apoptotic cascade signaling was interrupted.

The molecular mechanism of cyclin D1 induction during the cell cycle is of central importance in understanding cell proliferation control. Cyclin D1 is expressed at high levels in the middle and at the end of the G1 phase of the cell cycle. High levels of cyclin D1 in G1 promote entry into S phase and downregulation of this marker indicates cell cycle progression arrest and in some cases may result in cell death by apoptosis (Faião-Flores et al., 2011b; Baker et al., 2005). BNCT caused a decrease in cyclin D1 expression only in the melanoma cells and did not interfere with the G1 phase of normal melanocytes. It known that BNCT can induce cell cycle arrest at the G1 and G2 checkpoints in another cell lines as human oral squamous cell carcinoma (Kamida et al., 2008). BNCT can induce cell cycle arrest and apoptosis in both p53 wild-type or p53 mutant cells. However, p53 wild-type cells are more susceptive to cell death than p53 mutant cells (Fujita et al., 2009). These data can explain the cell death in SKMEL-28 melanoma cells that possess p53 wild-type. Thus, cell cycle arrest and associated cell death would contribute to the decrease in cell viability caused by BNCT.

Moreover, BNCT was able to induce an increase in cleaved caspase-3, another marker of cell death by apoptosis, in this tumor cell line. This confirms further results where BNCT also induced apoptosis in a caspase 3-dependent manner, with PARP cleavage in tumor cells (Kamida et al., 2008). These results have also been reported in murine melanoma cells (Sauter et al., 2002), and now, in this study, they have also been confirmed in human melanoma cells, showing that BNCT is effective against tumor cells.

5. Conclusions

BNCT can potentially target tumor tissue selectively, sparing normal cells damage due to radiation. This therapy did not induce significant changes in free radical production or in the morphological characteristics of normal melanocytes. Furthermore, this therapy decreased collagen synthesis, suggesting that ECM changes took place in melanoma cells. Cyclin D1 and the mitochondrial electric potential were significantly reduced, whereas cleaved caspase-3 levels increased only in the melanoma cells. These results suggest that both the intrinsic apoptosis pathway and cell cycle arrest are involved in this antitumor therapy. Thus, BNCT could be used to treat many tumors, inducing cell death specifically in tumor tissues while protecting healthy tissues.

Conflicts of interest

None.

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