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Antitumor potential induction and free radicals production in melanoma cells by Boron Neutron Capture Therapy

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ABSTRACT

Antiproliferative and oxidative damage effects occurring in Boron Neutron Capture Therapy (BNCT) in normal fibroblasts and melanoma cell lines were analyzed.

Melanoma cells and normal fibroblasts were treated with different concentrations of Boronophenylalanine and irradiated with thermal neutron flux. The cellular viability and the oxidative stress were determined.

BNCT induced free radicals production and proliferative potential inhibition in melanoma cells. Therefore, this therapeutic technique could be considered efficient to inhibit growth of melanoma with minimal effects on normal tissues.

1. Introduction

Melanoma, a highly invasive and metastatic tumor type, has presented increasing incidence and mortality in recent years (Jemal et al., 2009). It is the most aggressive form and causes the majority of deaths from skin cancers (Mendes et al., 2008).

The disappointing results that have come from chemotherapy show 5-year survival rates for treated patients ranging from only 3% to 14% (Wolchok and Saenger, 2007). For this reason, the appropriate systemic treatment for disseminated malignant melanoma is still controversial and no defined standard exists.

BNCT is a binary treatment modality based on the use of low energy neutrons able to produce a lethal damage only to cells loaded with a sufficient number of boron-10 nuclei, while a tolerable damage occurs in cells where less boron influx occurs (Menichetti et al., 2009).This technique involves selective accumulation of ^{10}B carriers in tumor cells and subsequent irradiation with a neutron beam. High linear energy transfer alpha particles and recoiling ^{7}Li nuclei are emitted during the capture of thermal neutrons by ^{10}B nuclei and deposit their energy in a 5–9 μm range with a high relative biological effectiveness (Coderre and Morris, 1999). Thus, BNCT can be used to eliminate tumor cells whose precise location may not be fully known to prevent residual cancer cells from causing tumors to recur (Chou et al., 2009).

BNCT has been carried out in the USA, Japan, Netherlands, Argentina, Italy, Finland and Sweden among others. However, there is no specialized center for this modality in Brazil.

In this work we studied the antitumor potential and free radicals production in melanoma and normal fibroblasts cell lines induced by BNCT.

2. Materials and methods

2.1. Cell lines

Tumor cell lines of murine melanoma (B16F10), human melanoma (IPC-298, SKMEL-28 and MEWO), murine normal fibroblasts (L929) and human normal fibroblasts (FN1) were cultivated in 75 cm^2 flasks with RPMI-1640 (Cultilab) medium supplemented with 10% inactive fetal bovine serum (Cultilab), 2 mM of L-glutamine (Sigma Chemical Company) and antibiotic streptomycin 0.1 mg/ml (FontouraWyeth AS).

Adherent cell suspensions were obtained by 5 min treatment of the culture flasks with trypsin 0.2%, then inactivated in 10% fetal bovine serum (FBS) enriched medium. The non-adherent cells, centrifuged twice and resuspended in a FBS supplemented RPMI-1640 medium, at the concentration of 5×10^5 cells/ml, were seeded in 96 wells plates and allowed to grow for 24 h.

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2.2. Boronophenylalanine (BPA)

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

2.3. Cells treatment and irradiation

Tumor and normal cells were treated with different concentrations of BPA from 8.36 to 0.52 mg/ml – corresponding from 440 to 27.5 μg ^{10}B/ml. After 90 min (the best time of boron-10 uptake for these cells) they were irradiated for 120 min, using the IEA-R1 nuclear reactor operating at a power of 3.5 MW, at the Nuclear and Energetic Research Institute (IPEN, Brazil) facilities. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the position was 2.3×10^8, 4.6×10^6 and 3.5×10^7 n/cm^2 s, respectively. The gamma dose rate in air at the irradiation location was 4.2 ± 0.2 Gy h^{-1}. Before the 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 and was denominated Irradiated Control. Furthermore, one group received BPA without irradiation and was denominated BPA only. A non-irradiated and without BPA was also studied and was denominated Control Group.

Images of the control and treated cells were taken by a camera (Sony Cyber-shot 7.2 mega pixels) coupled to an optic inverted microscope (Carl Zeiss), magnified at $40\times$.

2.4. Cellular viability assay – MTT

Cellular viability of tumor and normal cell lines were determined using colorimetric methodology MTT (3-(4,5-dimethylthiazol-2-y1)2,5-diphenyl tetrazolium bromide) (Sigma), based on MTT reduction of formazan by living cells (Mosmann, 1983). The yellow tetrazolium salts (MTT) are reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color was quantified by spectrophotometric means. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. The cellular viability was determined by MTT colorimetric assay for calculation of the 50% Inhibitory Concentration ($IC_{50\%}$).

2.5. Lipid peroxidation (TBARS)

The oxidative stress over the unsaturated lipids in cell membranes was evaluated by determining the amount of malondialdehyde (MDA), which is the final product of fatty-acid peroxidation, which reacts with thiobarbituric acid (TBA) to form a colored complex. Thiobarbituric acid reactive substances (TBARSs) are quantified by spectrophotometric determination (Ohkawa et al., 1979).

2.6. Statistical analysis

The amount of produced MDA is expressed by its mean value. 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.

3. Results and discussion

3.1. BNCT induces selective tumor cell death

The results show that BNCT induces cell death mainly in tumor cell lines (see Fig. 1). The $IC_{50\%}$ values of these lines ranged from 3.31 to 6.93 mg/ml (see Table 1). This effect does not show up in normal fibroblast lines, which exhibited $IC_{50\%}$ values up to 53.06 mg/ml. All the $IC_{50\%}$ values of melanoma cell lines were smaller than those in normal fibroblast cell lines. The $IC_{50\%}$ values were calculated by straight line fittings where all R^2 values were higher than 0.95, indicating positive data correlation.

The cellular viability ($IC_{50\%}$ value) of the Irradiated Control Group does not present difference in comparison to Control Group.

These data suggest that, owing to their selective BPA uptake capability, a smaller amount of ^{10}B concentration treatment, if compared to that required by normal cells, is necessary to induce tumor cell death. In this context, the binary characteristic of BNCT could be an attractive tool to improve response better than standard radiotherapy treatment, by delivering high doses to tumor while reducing normal tissue effects, due to the different boron uptake in normal and tumor cells (Blaumann et al., 2004).

Other *in vitro* studies report intracellular accumulation of BPA in melanoma cells with concentrations around 110 μg ^{10}B/ml (Capala et al., 1996).

3.2. Free radicals production in tumor cells by BNCT

Lipidic peroxidation can be defined as a resultant cascade of biochemistries events of the action of free radicals on cellular membrane unsaturated lipids, leading to destruction of its structure and cell death (Benzie, 1996). This method constitutes one of the main mechanisms of cellular damage leading to cytotoxicity.

BNCT induces production of free radicals in melanoma cell lines. In B16F10, IPC-298 and SKMEL-28 melanomas this effect was more relevant than in MEWO cells.

In B16F10 melanoma, free radicals production was 28 times higher than that in the control group, which points to intense cytotoxicity in skin tumor cells (see Table 2).

FN1 and L929 normal cell lines did not present elevated production of free radical amounts, thus resulting in toxicity decrease in cells adjacent to the tumor.

The cells irradiated without BPA (Irradiated Control) did not produce significant amount of free radicals in all the cell lines.

3.3. Morphologic aspects of tumor and normal cells after BNCT

After incubation with BPA and thermal neutrons irradiation, the cells were photographed for morphologic analysis. It is possible to observe debris and evident cell death in the highest concentrations of BPA in B16F10 cell line. In MEWO cells there are a lack of regular morphologic characteristics, and presence of cellular fragments in supernatant. Nuclear disorder was evident in IPC-298 cells treated by BNCT and leading to cell death. SKMEL-28 cells treated by BNCT presented apoptotic bodies and cytoskeleton disarray with debris formation. (see Fig. 2)

In the L929 cell line these effects were not present, because BNCT did not induce alterations both in the cell population, in the amount of these cells and in morphologic aspects, as well. The same effects were observed in the FN1 cell line, showing, however, only a few structural modifications as decreasing confluence only at the highest BPA concentration.

The FN1 cell line showed a slight increase in cell confluence at lower BPA concentrations in relation to the control group cells. This effect may be due to stimulation by repair mechanism

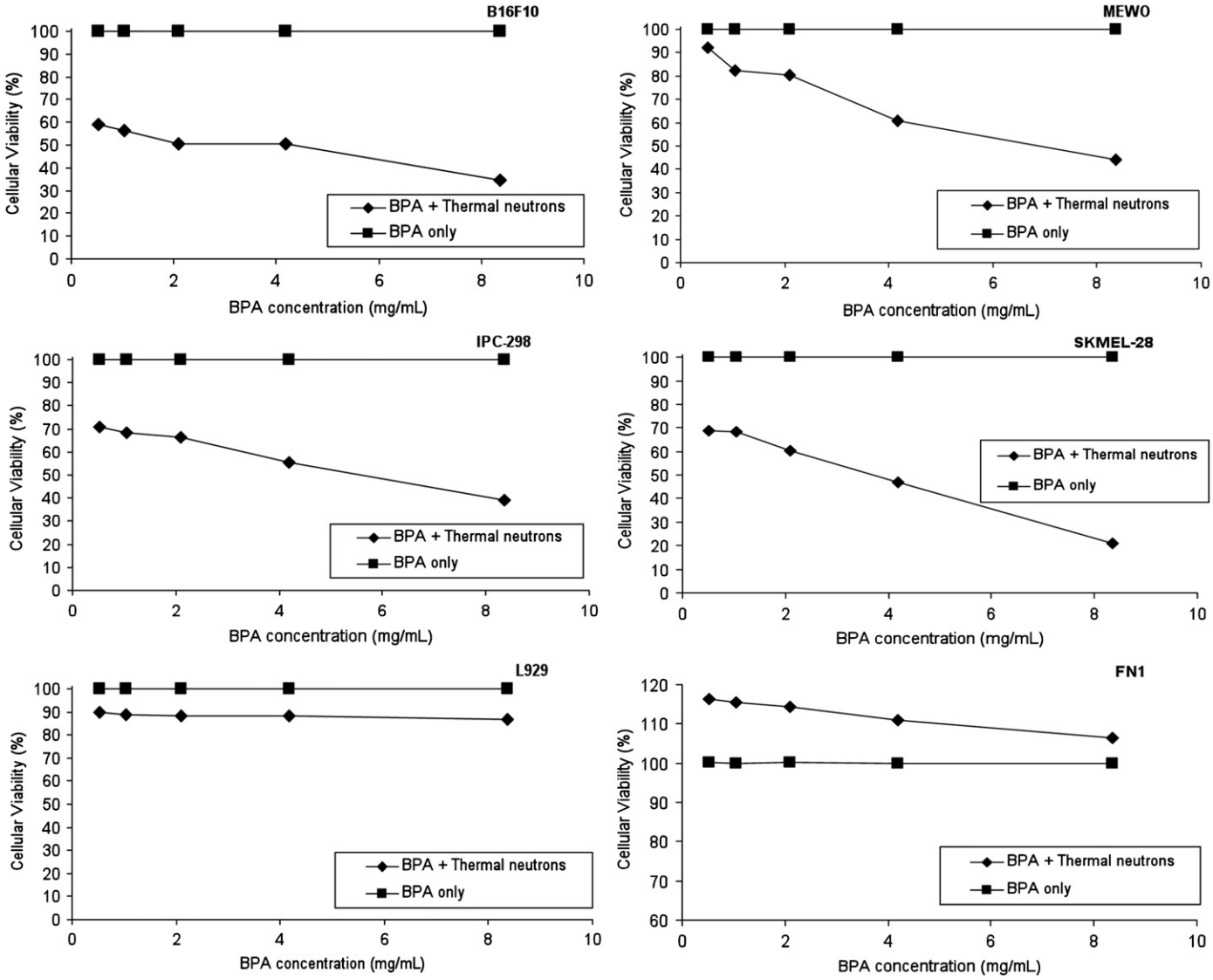

Fig. 1. BNCT cytotoxic activity on normal and neoplastic cells. The percentage of viable cells is plotted against the different BPA concentrations checked. The straight line equation and linear regression curve were calculated at Graph Pad Prism Instat 3.

Table 1
Comparison among the $IC_{50\%}$ values in tumor and normal cell lines treated by BNCT.

Cell lines	IC50%		
	BPA (mg/ml)	BPA (mM)	^{10}B (μg/ml)
B16F10	3.31	15.83	172
MEWO	6.93	33.15	360
IPC-298	5.68	27.17	295
SKMEL-28	3.71	17.75	192
L929	103.60	495.67	5387
FN1	53.06	253.86	2759

Table 2
Free radicals production following BNCT at the different BPA checked concentrations and after the only neutron irradiation.

Cell lines	Irradiated control	BPA (mg/ml)				
		8.36	4.18	2.09	1.045	0.52
B16F10	1.03	28.65	25.08	20.28	14.87	2.79
MEWO	2.50	11.04	10.50	10.11	7.11	5.79
IPC-298	1.02	14.19	14.07	11.28	10.34	9.31
SKMEL-28	1.02	20.08	15.03	13.84	13.59	11.90
L929	1.13	2.32	2.00	1.71	1.68	1.39
FN1	1.13	2.90	2.69	2.36	1.73	1.16

activation of cells that received low BPA concentrations, inducing the factor growth production and cell survival.

Data show that melanoma cells are much more damaged than normal cells by the BNCT treatment. This is most likely due to the higher ^{10}B levels accumulated by the neoplastic cells with respect to the normal fibroblasts.

The cells of the Irradiated Control presented few alterations and small cell damage, explaining the presence of these findings in normal fibroblasts. Furthermore, there were no significant changes in the parameters studied in normal and tumor cells that received only BPA without irradiation with thermal neutrons with respect to the Control group.

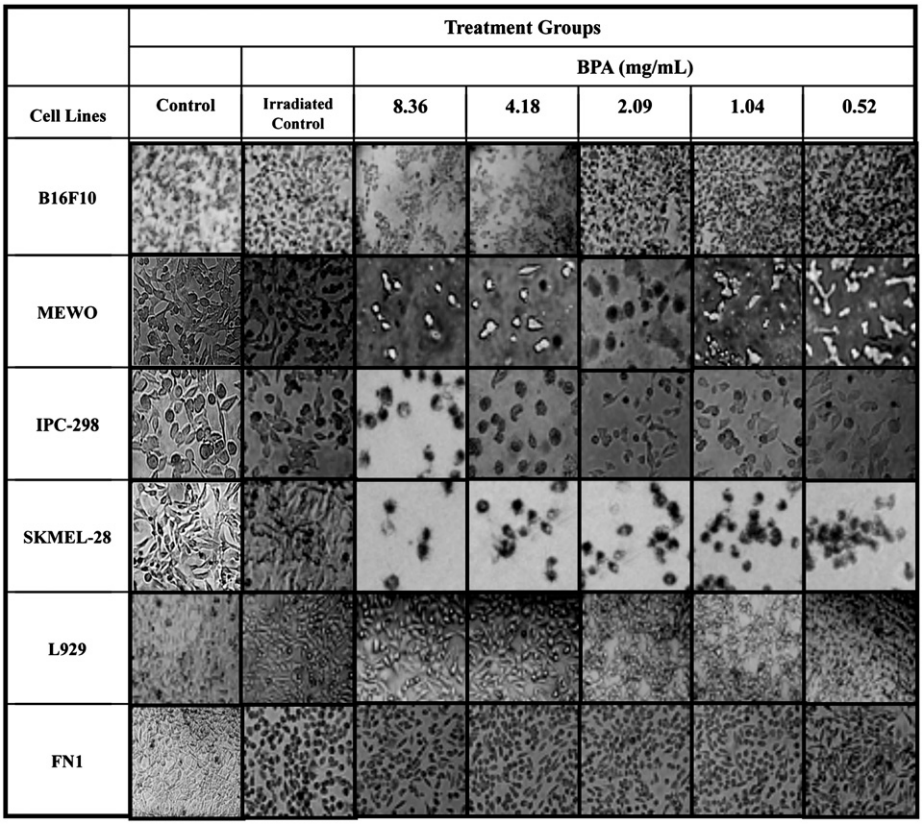

Fig. 2. Morphological aspects of cell lines treated with BNCT at all concentrations. Apoptotic bodies and confluence decrease are evident only in the BNCT treated group. Irradiated control is similar to Control group.

4. Conclusions

BNCT induces cell death mainly in tumor cells, while preserving normal tissues. In melanoma cells it produces high amounts of free radicals leading to tumor membrane degeneration and destruction. This therapy damages tumor cells selectively, with minimum effects on normal adjacent tissue.

Acknowledgments

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