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FULL PAPER

Evaluation of local, regional and abscopal effects of Boron Neutron Capture Therapy (BNCT) combined with immunotherapy in an ectopic colon cancer model

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We previously demonstrated, for the first time, the abscopal effect of Boron Neutron Capture Therapy (BNCT) in an ectopic model of syngeneic colon cancer in BDIX rats.

Objective: The aim of the present study was to evaluate the local and regional therapeutic efficacy and abscopal effect of BNCT mediated by boronophenyl-alanine, combined with Bacillus Calmette-Guerin (BCG) as an immunotherapy agent in this model.

Methods: The local effect of treatment was evaluated in terms of tumor response in the irradiated tumor-bearing right hind flank. Metastatic spread to tumor-draining lymph nodes was analyzed as an indicator of regional effect. The abscopal effect of treatment was assessed as tumor growth inhibition in the contralateral (non-irradiated) left hind flank inoculated with tumor cells 2 weeks post-irradiation. The experimental groups BNCT, BNCT + BCG, BCG, Beam only (BO), BO +BCG, SHAM (tumor-bearing, no treatment, same manipulation) were studied.

Results: BNCT and BNCT + BCG induced a highly significant local anti-tumor response, whereas BCG alone induced a weak local effect. BCG and BNCT + BCG induced a significant abscopal effect in the contralateral non-irradiated leg. The BNCT + BCG group showed significantly less metastatic spread to tumor-draining lymph nodes *vs* SHAM and *vs* BO.

Conclusion: This study suggests that BNCT + BCG-immunotherapy would induce local, regional and abscopal effects in tumor-bearing animals. BNCT would be the main effector of the local anti -tumor effect whereas BCG would be the main effector of the abscopal effect.

Advances in knowledge: Although the local effect of BNCT has been widely evidenced, this is the first study to show the local, regional and abscopal effects of BNCT combined with immunotherapy, contributing to comprehensive cancer treatment with combined therapies.

INTRODUCTION

Boron Neutron Capture Therapy (BNCT) combines selective tumor uptake of ^{10}B compounds and neutron irradiation. The capture reaction between ^{10}B and a thermal neutron gives rise to the formation of short-range (5–9 μm), high Linear Energy Transfer (LET) α and lithium-7 particles. BNCT protocols are designed to maximize the tumor-specific boron dose component, proportional to the concentration of boron and produced in the above-mentioned reaction, and minimize background dose that affects tumor and normal tissue alike.[1] Background dose in the facility used to perform the irradiations results from the protons produced in the interaction of thermal neutrons with the ^{14}N present in the tissue and from the γ field, both structural and generated in the interaction of thermal neutrons with the hydrogen also present in the tissue.

Clinical trials of BNCT for different tumor types and localizations such as glioblastoma multiforme, melanoma and head and neck tumors have shown therapeutic efficacy with room for improvement.[2–6] The optimization of BNCT for different pathologies continues to be a field of much needed research. In this sense, our group has performed translational and pre-clinical research in different animal models to improve the safety and efficacy of BNCT protocols for different pathologies.[7–15]

We described, for the first time, the abscopal effect of BNCT mediated by borono-phenylalanine (BPA) in an ectopic model of colon cancer in BDIX rats.[16] The abscopal effect was originally described by Mole (1953)[17] as the "out-of-field" inhibitory effect of standard radiotherapy on tumor growth.[18,19] Demaria et al[20] suggested that the abscopal effect would be mediated by radiation-induced immune responses. Tumors create an immunosuppressive environment that would allow them to escape destruction by the immune system. Radiotherapy would have the capacity to induce immunogenic cell death, leading to cross-priming of tumor-specific T cells that would induce an *in-situ* tumor vaccine effect.[21] More recently, Khan et al[22] described immunomodulatory effects of BNCT that contribute to tumor growth inhibition.

The immune system can be stimulated by the administration of attenuated or genetically modified microorganisms, in turn causing tumor regression.[23] Within this context, Bacillus Calmette-Guerin (BCG) would act as an immune stimulator and has been shown to effectively treat certain malignancies.[24,25] Treatment with BCG does not entail major complications or compromise survival, being the gold-standard treatment for non-muscle invasive bladder cancer.[26] In addition, BCG alone is not an effective therapy in more aggressive pathologies like invasive bladder tumors. However, studies in preclinical models demonstrated that it improves tumor response to γ irradiation through the enhancement of local and systemic immune anti tumor effects.[25]

Having provided proof of principle of the abscopal effect of BNCT alone employing an ectopic model of colon cancer in BDIX rats,[16] the aim of the present study was to evaluate, for the first time, the local and regional therapeutic efficacy and the abscopal effect of BNCT combined with BCG as an immunotherapy agent in the same model. The knowledge of these effects would conceivably contribute to the comprehensive treatment of cancer with combined therapies.

METHODS AND MATERIALS

Experimental model

A total of 100 male or female adult BDIX rats (Charles River Lab., MA, USA), 170–250 g body mass (bm) were used throughout. The animals were housed as described previously.[11] All rats were injected subcutaneously (sc) in the right hind flank, under ketamine (36.5 mg/kg bm)-xylazine (5.4 mg/kg bm) anesthesia, with 1×10^6 DHD/K12/TRb syngeneic colon cancer cells (ECACC, UK) in 100 µl of F-10-DMEM culture medium (GIBCO) using a syringe with a 27-gauge needle. Based on previous studies,[16] all experiments were performed 3–4 weeks post-inoculation. At this time, the animals have developed vascularized, measurable subcutaneous tumor nodules. This time point was considered T0.

Animal experiments were carried out in accordance with the guidelines of the National Institute of Health in the USA regarding the care and use of animals for experimental procedures and with protocols approved by the National Atomic Energy Commission Animal Care and Use Committee (CICUAL-CNEA #10/2018).

Experimental groups

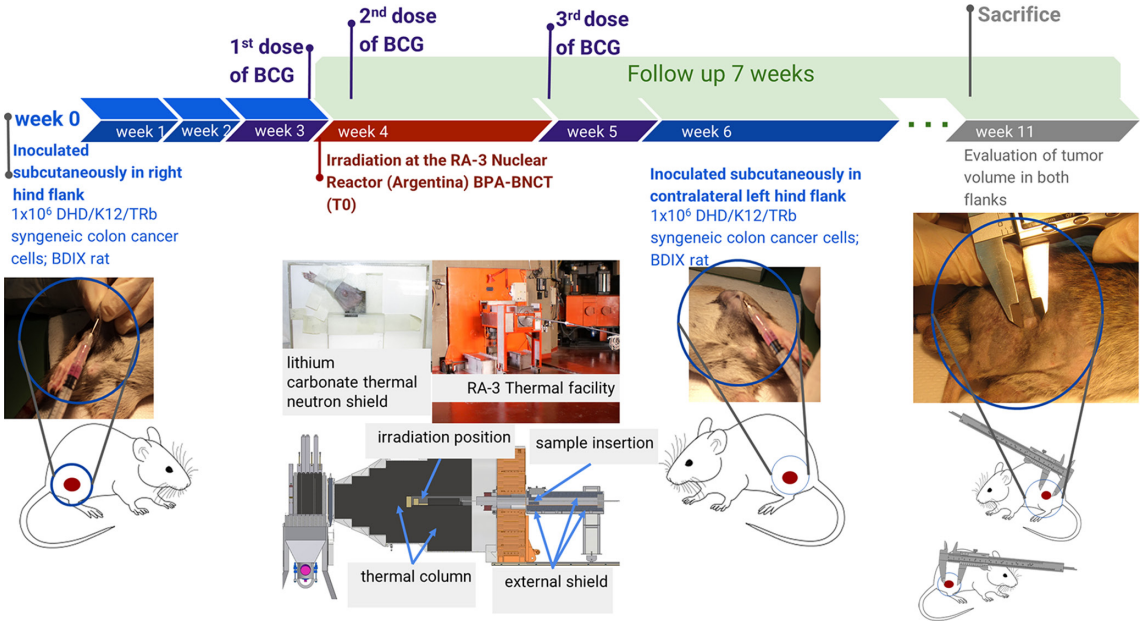
3–4 weeks post-inoculation of DHD/K12/TRb colon carcinoma cells, a total of 100 tumor-bearing rats were assigned at random to each of the following experimental groups:

- BNCT-group: tumor-bearing rats injected with BPA (46.5 mg ^{10}B/kg bm) intravenously (i.v.) and irradiated at the RA-3 Nuclear Reactor (Argentina) as described below.
- BNCT + BCG-group: tumor-bearing rats treated similarly to the BNCT-group plus three intratumoral applications (3 days pre-BNCT, 1 and 7 days post-BNCT) of BCG (strain Pasteur 1173P2; 0.2 mg/0.1 ml per injection, viability 6×10^5 colony forming units [CFU]).
- BCG-group: tumor-bearing rats treated with BCG only as described above.
- Beam only-group (BO-group): tumor-bearing rats exposed to the same neutron fluence as the BNCT-group, without boron compound administration.
- (BO + BCG)-group: combines the two previous protocols.
- SHAM-group: untreated tumor-bearing animals exposed to the same manipulation.

In-vivo BNCT

3–4 weeks (depending on Reactor logistics) post-inoculation of DHD/K12/TRb colon carcinoma cells, the rats in the corresponding groups (BNCT, BNCT + BCG, BO, BO + BCG) were irradiated at the thermal neutron facility constructed by the National Atomic Energy Commission at the RA-3 research and production reactor in Buenos Aires.[11,27] Irradiations were performed under ketamine (36.5 mg/kg bm)-xylazine (5.4 mg/kg bm) anesthesia. The animals were inserted into a near-isotropic neutron field while the reactor was in normal operation. A self-powered neutron detector (SPND),[28] was used to perform neutron flux measurements at a monitor position during each irradiation and calculate exposure time to reach the prescribed dose. Dosimetric calculations were performed employing dosimetry data for the RA-3 facility reported by Farías.[29] In the case of the groups treated with BNCT, BPA was administered iv at a dose of 46.5 mg ^{10}B/kg bm in the jugular vein under ketamine (36.5 mg/kg bm)-xylazine (5.4 mg/kg bm) anesthesia as previously described.[30] The right leg bearing the tumor nodule was locally irradiated 3 h post-administration of BPA. A lithium carbonate thermal neutron shield (enriched to 95% in lithium-6), developed and fabricated by our group, was used to protect the body of the animals from thermal neutrons while exposing the tumor-bearing leg through a collimated aperture. The schematic representation of the experimental protocol is shown in Figure 1. An absorbed dose of 7.6 Gy was administered to exposed skin as the

Figure 1. Schematic representation of the experimental protocol.



"organ-at-risk" based on skin radiotolerance data.[31] In Table 1, we show the prescribed absorbed doses from the different radiation components, the total absorbed BNCT dose and the gross boron concentration data employed for dose calculations.[16] Irradiation time to reach the prescribed dose was approximately 13–16 min. The thermal neutron fluence at the irradiation position was 4.2 $\times 10^{12}$ n cm^{-2}, while the thermal neutron fluence at all locations within the shield container was at least a factor of 20 lower than the fluence on the exposed leg.

In the case of the groups that include the BCG protocol, three intratumoral applications of BCG (strain Pasteur 1173P2; 0.2 mg/0.1 ml per injection, viability 6 $\times 10^5$ CFU) were given 3 days pre-BNCT, 1 and 7 days post- BNCT, or at matched time points in the BCG only group.

Two weeks post-irradiation (or at the matched time point in the non-irradiated SHAM and BCG only groups), 1 $\times 10^6$ DHD/K12/TRb cells were injected sc in the contralateral left hind flank in all BDIX rats. The choice of this time interval was based on experimental studies of abscopal effect of standard radiotherapy by Zenkoh et al[32] which suggest that the immune responses that are responsible for the abscopal effect in certain cases set in 2 weeks after treatment. A two-stage tumor cell inoculation protocol was used to avoid a potential influence of γ irradiation (not shielded by the lithium carbonate device) on tumor growth inhibition.[33] The response of the tumor in the right leg (irradiated in the corresponding groups) was used to evaluate the local effect of treatment whereas the inhibitory effect on tumor growth in the left leg (not exposed to irradiation in any of the groups) was used to evaluate the abscopal effect of treatment. The metastatic spread to tumor-draining lymph nodes was used as an endpoint to monitor the regional effect of treatment.

Follow-up

Tumor volume was determined by external caliper measurement of the three largest orthogonal diameters (d) and calculated as d1 x d2 x d3 as previously described[16] in the right leg pre-irradiation and once a week post-irradiation until sacrifice at 7 weeks (or at matched time points in the non-irradiated groups). Throughout follow-up and at the end of the treatment, the post/pre-treatment tumor volume ratio was calculated to evaluate the local response. Similarly, tumor volume was measured weekly in the contralateral left flank to assess a potential influence of treatment in the right leg on tumor growth in the left leg. The potential inhibitory

Table 1. Boron concentration in tissue[16] used for boron dose calculation and absorbed dose (Gy) expressed as mean [min;max] as indicated

Tissue	ppm ^{10}B mean ± SD	Induced protons	Total γ ray dose	Boron dose	Total BNCT dose
Leg (Skin)	18.2 ± 2.4	1.0 [0.9;1.0]	1.1 [0.9;1.3]	5.5 [5.0;5.7]	7.6 [6.8;8.1]
Tumor	20.1 ± 4.5	1.0 [0.9;1.0]	1.1 [0.9;1.3]	6.0 [5.6;6.3]	8.1 [7.3;8.7]

BNCT, Boron Neutron Capture Therapy; SD, standard deviation.
Induced protons result from the interaction of thermal neutrons with the nitrogen present in the tissue, total γ ray dose includes both structural γ rays and those generated in the interaction of thermal neutrons with the hydrogen present in the tissue, and boron dose includes the α and Li-7 particles generated in the capture reaction between ^{10}B and a thermal neutron.

effect on tumor development in the left leg induced by treatment in the right leg was taken as an indicator of abscopal effect. The end point to evaluate tumor growth in the left leg for each group was incidence of animals with a tumor volume ≤50 mm^3.

The percentage of animals with metastatic spread to the tumor-draining lymph nodes in the right irradiated leg was analyzed at the time of sacrifice as an indicator of regional effect. Macroscopic evaluation and confirmatory histological analysis were performed in each case. This study was carried out in the last rounds of treatment once the detection and dissection technique of the draining lymph node were optimized.

Clinical signs and local toxicity were monitored throughout. Local toxicity was assessed employing a radiation dermatitis scale adapted from the National Cancer Institute Common Terminology Criteria for Adverse Events and the Radiation Therapy Oncology Group.[34]

Statistical analysis

All statistical analyses were performed using a 5% level of significance. For Normality tests we used Shapiro–Wilk and Kolmogorov–Smirnov tests, whereas for homoscedasticity (to compare treatment group variances), we used Hartley (Maximum variance) test.

We used Kruskal–Wallis non-parametric test with *post hoc* Dunn´s test to compare right or left tumor volume for each treatment group (baseline and over time for each week evaluated), because this variable does not fit a Normal distribution. For relative measurements such as post-/pre-tumor volume (compared to baseline at time point T0), that fit a Normal distribution, we used ANOVA and *post hoc* Tukey´s test.

For categorized variables such as left leg tumor volume (≤or>than 50 mm^3) and lymph node macroscopic and histological features (positive or negative in terms of metastatic spread), we compared treatment group proportions with Chi-Squared or z tests for proportion differences.

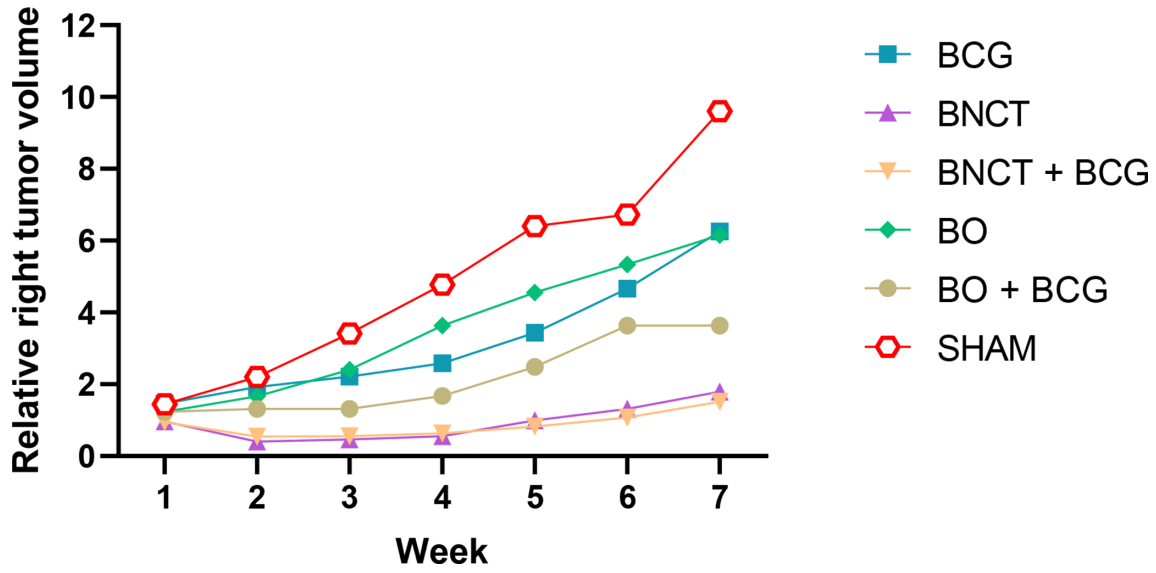
RESULTS

The initial (pre-treatment) conditions (tumor volume and body mass) of all the experimental groups were compared using a Kruskal–Wallis test (since they did not fit a normal distribution $p > 0.05$) and no statistically significant differences were found ($p = 0.3$), rendering them comparable.

No clinical systemic signs of toxicity were identified in any of the experimental groups. Local toxicity was observed only in the exposed skin of the BNCT and BNCT + BCG groups. It involved reversible Grade 3 dermatitis in the thigh (moist desquamation and bleeding induced by minor trauma) and reversible Grade 4 dermatitis in the more radiosensitive soles of the feet (moist desquamation, ulceration and spontaneous bleeding). The peak of dermatitis was observed approximately 10 days post-BNCT and lasted 5–7 days, followed by healing. When necessary, the animals were medicated with analgesics and antibiotics to mitigate the symptoms of dermatitis.

The relative right leg tumor volume (relative to pre-treatment tumor volume) was compared between the experimental groups at each time point, as shown in Figure 2. At each time point, the ratios were calculated individually for each tumor and then used to determine the mean ratio for each group. Fixed effects (Type III) two-way ANOVA showed a significant interaction between group and week ($p < 0.0001$) and a significant effect for group ($p = 0.008$) and for week ($p < 0.0001$). Simple effects for treatment group were evaluated within each time point (weeks 1–7) through Tukey´s multiple comparison test, where significant differences between at least some of the groups became apparent 3 weeks after treatment. In particular, significant differences were observed between the BNCT groups (BNCT and BNCT + BCG)

Figure 2. Temporal evolution of post-/pre-treatment tumor volume ratio in the right leg from 0 to 7 weeks. Error bars indicating the SD were omitted for image clarity.



and the remaining groups (SHAM, BO, BO +BCG and BCG). The greatest difference between BNCT and BNCT + BCG vs SHAM and BO ($p < 0.05$ in all cases) was observed at weeks 6 and 7.

The curves (Figure 2) show the evolution in time of relative tumor volume in the right leg for each group, revealing the trend in each case and the differences between some of the experimental groups at each time point (week) evaluated. The values of relative tumor volume over time showed that both the BNCT and BNCT + BCG values were very small and significantly different from SHAM at weeks 6 and 7, revealing a robust local response to treatment for both groups. While BCG alone did exert a certain degree of local effect on tumor (albeit not statistically significant) *vs* SHAM, a statistically significant inhibitory effect on tumor growth was achieved when BNCT and BCG were combined. However, BCG did not seem to contribute to local tumor control beyond the local effect achieved by BNCT alone (local tumor control was almost identical for BNCT and BNCT + BCG). Conversely, BNCT did contribute to local tumor control when combined with BCG compared to BCG alone. However, this difference between BNCT + BCG and BCG did not reach statistical significance. Within this context, BNCT would provide the main contribution to local tumor control when combined with BCG. BO and BCG exhibited a slight and similar inhibitory effect on tumor growth *vs* SHAM that did not reach statistical significance. The combination of BO and BCG seemed to favor tumor control compared to each treatment alone, albeit not significantly. The Sham mean tumor volume ratio progressed from over 1 at 1 week to almost 10 at 7 weeks. This implies that, left untreated, the tumor volume increased almost 10 times on average in 7 weeks. The volume ratio for BNCT and BNCT + BCG was always close to 1, *i.e.* the tumors remained stable over time. At 7 weeks, the tumor volume ratio of the SHAM group was six times greater than that of the groups treated with BNCT (BNCT and BNCT + BCG). Likewise, between the second and fourth weeks the post-/pre-treatment ratios for the BNCT and BNCT + BCG groups were below 1, showing a reduction in tumor volume from its initial value. At weeks 5, 6 and 7, these values recovered their pre-treatment value or increased a little. This finding suggests that a second treatment around the fourth week could prevent or delay tumor recurrence or even achieve complete tumor remission.

Table 2 shows absolute tumor volume values pre-treatment and at 7 weeks post-treatment. These values are presented only as a reference because in contrast with Figure 2, they fail to represent the behavior of individual tumors with respect to their pre-treatment volume. If we evaluate absolute tumor volume at 7 weeks, as an indicator of local effect of the treatment, we see significant differences between both BNCT and BNCT + BCG groups *vs* both the SHAM control group and the BO group ($p < 0.05$ in all cases). Furthermore, the BNCT + BCG group also exhibited differences with the BCG alone group that reached statistical significance in this analysis ($p < 0.05$), providing additional evidence of the main role of BNCT in local tumor control. Follow-up ended at 7 weeks because the SHAM group exceeded admissible tumor growth after that.

Table 3 shows the percentage of animals with a tumor volume ≤50 mm^3 in the left leg that was assessed for each of the experimental groups at the end of follow-up. This time-point corresponds to 11 weeks post-inoculation and 7 weeks post-treatment for the right leg, and 5 weeks post-inoculation for the left leg (Figure 1 above).

The abscopal effect was identified as an inhibitory effect on tumor growth in the left contralateral leg (untreated). The percentage of animals with a tumor volume ≤50 mm^3 was evaluated with a Chi-Squared analysis and significant differences were observed between BCG and BNCT + BCG *vs* SHAM ($p = 0.0005$ and $p = 0.02$ respectively). Likewise, BCG showed significant differences with BO ($p = 0.02$). BO, BO + BCG and BNCT exhibited some degree of abscopal effect *vs* SHAM. However, this effect did not reach statistical significance. BNCT + BCG did not differ significantly from BCG alone, revealing that BCG would be mainly

Table 2. Absolute tumor volume values in the right leg for each of the experimental groups pre-treatment and at 7 weeks

Groups (N)	Tumor volume (mm3) Pre-treatment Mean ± SD Median[Min;Max]	Tumor volume (mm3) 7 weeks post-treatment Mean ± SD Median[Min;Max]
SHAM (20)	178 ± 123 137 [19;441]	1178 ± 754 945 [307;3591]
BO (10)	258 ± 128 233 [123;586]	1545 ± 1258 1248 [551;4708]
BO + BCG (8)	219 ± 113 248 [54;372]	938 ± 864 890 [0;2697]
BCG (21)	157 ± 125 128 [14;381]	1018 ± 1317 598 [0;5321]
BNCT (20)	207 ± 174 154 [31;722]	394 ± 468 368 [0;2017]
BNCT + BCG (21)	156 ± 99 119 [39;405]	229 ± 439 12 [0;1932]

BCG, Bacillus Calmette-Guerin; BNCT, Boron Neutron Capture Therapy; BO, beam only.
Tumor volume in the right leg expressed as the mean ± SD and Median [Min;Max]. *N* = number of animals.

Table 3. Number of animals with tumor volume $\leq 50\,mm^3$ for the left (non-irradiated) leg and percentage for each group

Groups (*N*)	*# of animals with tumor volume* $\leq 50\,mm^3$ *(%)*
SHAM (*20*)	1 (5%)
BO (*10*)	1 (10%)
BO + BCG (*8*)	3 (37%)
BCG (*21*)	12 (57%)
BNCT (*20*)	3 (15%)
BNCT + BCG (*21*)	9 (43%)

BCG, Bacillus Calmette-Guerin; BNCT, Boron Neutron Capture Therapy; BO, beam only.
N = number of animals.

responsible for inducing an abscopal effect in the BNCT + BCG protocol. Although BNCT alone would generate some degree of abscopal response, it would be less powerful than that induced by either BCG alone or BNCT + BCG. These data reveal that BCG and BNCT + BCG induced a statistically significant abscopal effect in the contralateral non-irradiated leg and that BCG would be the principal effector of the abscopal response.

Table 4 shows the regional effect of treatment expressed as the incidence of animals without metastatic tumor-draining lymph nodes in terms of macroscopic and histological evaluation. This regional effect would combine the abscopal effect that results from local tumor treatment and the local effect of radiation in the exposed area. Metastatic nodes were enlarged and indurated and were considered macroscopically positive when visual inspection revealed pale, high density, nodular areas. All the lymph nodes that were considered macroscopically positive also proved to be positive on histological analysis. Conversely, some of the lymph nodes that were considered negative on macroscopic assessment exhibited a varying degree of metastatic invasion on histological analysis. Most of the lymph nodes that were positive on histological analysis, exhibited only small areas of metastasis in the BNCT and BNCT + BCG groups whereas in the case of the BO and SHAM groups, the lymph nodes were completely replaced by tumor tissue. In the latter case, only the border of the lymph node was identifiable as shown in Figure 3. Metastatic spread to tumor-draining lymph nodes was significantly inhibited by BNCT + BCG *vs* the SHAM group as revealed by macroscopic ($p < 0.05$) and histological ($p = 0.01$) analysis. Similarly, metastatic spread was significantly inhibited by BNCT + BCG *vs* BO both macroscopically and histologically ($p = 0.02$ and $p = 0.001$ respectively). BNCT, BCG and BO + BCG exhibited some degree of regional effect *vs* SHAM and BO. However, this effect did not reach statistical significance. Although we did not detect statistically significant differences between BNCT + BCG and BCG, a trend is evident and suggests that BNCT + BCG would exert a more potent regional effect than BCG alone. In fact, the combination of BNCT and BCG would be more effective than either of the treatments alone. Both BNCT and BCG seemed to contribute importantly to the statistically significant regional effect of BNCT + BCG. These data are reported as preliminary but contributory and indicative that future studies in this sense are warranted.

DISCUSSION

It is known that when ionizing radiation is applied to a primary tumor, it can induce immunogenic cell death which may in turn trigger a cytotoxic immune response against the primary tumor and its metastasis.[25,35,36] This phenomenon, known as abscopal effect, has been described in association with localized standard radiotherapy.[37] Proof of principle of the abscopal effect of BNCT was provided for the first time by our group.[16] More recently, Kahn et al[22] also demonstrated an immunomodulatory effect of BNCT, as it induced an anti -tumor phenotype in peripheral blood mononuclear cells.

BCG has successfully been used as an immunotherapy agent or as an immunological adjuvant against human neoplasms.[38,39] It is an immune stimulator that was shown to improve the anti tumor immune response in combination with ionizing radiation, inducing a systemic immune response to both the primary tumor and metastases.[25]

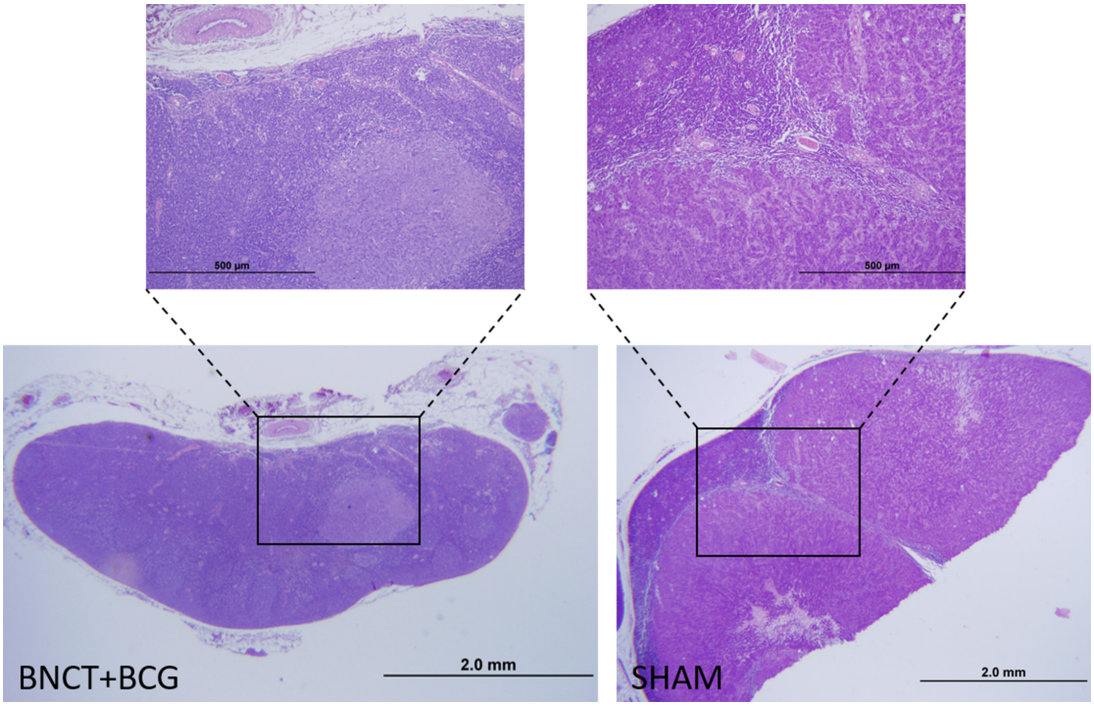
In the present study we examined, for the first time, the local, regional and abscopal effects of BNCT combined with BCG as an anti -tumor immune stimulator. The use of an experimental model that employs the inoculation of syngeneic colon cancer cells in immunocompetent rats was essential to examine abscopal effect that it is dependent on a functional immune system.[20,33,40] The fact that BPA-BNCT is approved for use in patients[4] and that

Table 4. Number of animals with negative lymph nodes (-) in terms of macroscopic and histological evaluation, total number of animals (N) and % of animals with negative lymph nodes for each of the experimental groups

Groups	*Macroscopic analysis of lymph nodes -/N (%)*	*Histological analysis of lymph nodes -/N (%)*
SHAM	4/9 (44%)	1/9 (11%)
BO	3/9 (33%)	0/9 (0%)
BO + BCG	3/6 (50%)	2/6 (33%)
BCG	5/9 (56%)	4/8 (50%)
BNCT	6/9 (67%)	3/9 (33%)
BNCT + BCG	10/11 (91%)	8/11 (73%)

BCG, Bacillus Calmette-Guerin; BNCT, Boron Neutron Capture Therapy; BO, beam only.

Figure 3. Microphotograph of a lymph node corresponding to an animal in the BNCT + BCG group. A small area of metastasis can be observed in the follicular area of the lymph node. Microphotograph of a lymph node corresponding to an animal in the SHAM group. The lymphatic tissue has almost completely been replaced by tumor tissue with the typical features of colon adenocarcinoma: atypical glandular structures with scarce stroma. There is only a small portion of remaining lymphatic tissue in the subcapsular area. H&E, scale bar is shown in the images. BCG, Bacillus Calmette-Guerin; BNCT, Boron Neutron Capture Therapy



BCG is approved as an immunological adjuvant in the development of anti -tumor vaccines,[38] contributes to bridge the gap between translational research and a clinical scenario.

We showed that BNCT alone and BNCT + BCG induced a highly statistically significant local tumor response. BNCT has been widely shown to induce local tumor control,[11] mainly as a result of cell death induced by the direct action of the high LET α and Li particles on DNA, that in turn leads to complex, clustered DNA damage that is difficult to repair. While BCG alone exhibited weak local tumor control, it failed to significantly enhance the local effect of BNCT when BNCT and BCG were combined, rendering BNCT mainly responsible for local tumor control. The combination of BCG and BNCT might act as an "anti -tumor vaccine" where BNCT would trigger the process of tumor antigen generation and BCG would promote antigen presentation in an inflammatory microenvironment. Within this context, Antonelli et al. (2020)[41] demonstrated that BCG therapy enhanced an anti-tumor effect induced by tumor-specific T cells in the bladder tumor microenvironment. Robust regional and abscopal effects of BNCT + BCG were observed. BCG would be the main effector of the abscopal effect since BCG alone exhibited a powerful abscopal effect that was not enhanced by the combination of BNCT + BCG. In the case of the regional effect, both BNCT and BCG would contribute importantly, given that the regional effect of BNCT + BCG was more pronounced than for either treatment alone.

BCG alone has been used as a local sensitizer to overcome hypoxia-associated radioresistance, inducing the local production of the free radical Nitric Oxide[42,43] and improving the local and abscopal response to ionizing radiation in the context of low LET radiation.[25] Furthermore, BCG has been used for local immunomodulation. The combined treatment with immunomodulation and radiofrequency ablation (RFA), resulted in a complete cure of local and distant colorectal carcinoma in an experimental model. The lack of an effective distant immune response in patients treated with RFA alone supports this new combined treatment strategy.[44] In the case of BNCT, the main radiation dose component is high LET and as such, induces direct damage to DNA rather than indirect damage associated to the production of free radicals.[1] In a high-LET radiation scenario, the local effect of BCG (albeit weak) would be mainly related to its immune-stimulator effect. In addition, pre-clinical studies showed that double strand DNA damage activates innate immune signaling pathways,[45] suggesting that BNCT would contribute to an abscopal effect.

In the experimental conditions employed herein, the immune-stimulator effect of BCG alone would not, on average, be enough to induce a statistically significant local tumor response. This weak local effect of BCG alone might be related to the high tumor burden at the time of treatment as previously described. It is known that close contact between the *Mycobacterium* and cancer cells is required for successful therapy.[25,46] It has been

described that with a high tumor burden, immune cells alone are not effective in controlling disease and complementary therapies are necessary.[47]

Regarding the similar and statistically significant abscopal effect of BCG and BNCT + BCG groups *vs* SHAM the reduction of tumor bulk in the right, treated leg could contribute to reduce the suppressive immune response developed by tumor cells, in turn favoring abscopal response.[16] It is known that cell death leads to the release of endogenous damage-associated molecular patterns (DAMPs) that contribute to the priming of the immune system by triggering dendritic cells, in turn improving antigen presentation to T cells that are present in the lymph nodes. This presentation stimulates an adaptive immune response against tumor cells, that impacts locally and systemically affecting the same tumor in unirradiated sites.[48–50] Other authors reported that immunogenic cell death induced by ionizing radiation alone or combined with immunotherapy would elicit local and abscopal effects.[47] The systemic immune response induced by BCG alone would suffice to induce a robust abscopal effect, inhibiting or reducing the development of experimental "out-of-field" tumors. BCG primary tumor inoculation was the most powerful strategy to induce an abscopal effect, alone or in combination with BNCT. These observations are supported by clinical studies from other authors that conclude that the use of BCG as tumor vaccine in combination with ionizing radiation therapy help to control the distant disease in locally advanced recurrent hepatocellular carcinoma and in advanced breast cancer.[51]

To analyze the regional effect (a combination of abscopal and local effects) induced by our treatment protocols, we studied the metastatic spread to tumor-draining lymph nodes. The regional therapeutic efficacy in terms of a statistically significant reduction in metastatic spread was achieved only with the combined BNCT + BCG treatment, suggesting that both BNCT and BCG play an important role in triggering a regional effect.

As reported by Hatanaka et al,[52] BNCT would be ideally suited to integrate a multimodal tumor treatment. It is capable of inhibiting tumor growth, has low local and systemic toxicity compared to conventional chemo- and radiotherapy and it is not immunosuppressive. Extensive studies are necessary to elucidate the best combination of radiotherapy and immune therapies to induce effective and long-lasting tumor response and abscopal effect.[47] Within this context, a reduction in local toxicity would allow for dose-escalation to tumor and an increase in therapeutic advantage without exceeding radiotolerance.

BNCT mediated by BPA clinical trials are currently mainly devoted to treating glioblastoma and head and neck cancer.[6,53,54] BPA-BNCT in dog patients with spontaneous head and neck cancer with no other therapeutic option showed the potential value of BNCT in veterinary medicine.[55] Clinical studies also demonstrated that BNCT would be useful to treat melanoma.[56,57] Experimental studies have shown that BNCT could be expanded to treat prostate and breast cancer.[58,59] None of these targets would require complex *ex-vivo* irradiations as described for liver metastases and have been proved clinically feasible.[60] Experimental and clinical studies in veterinary and human patients showed that BCG would also be useful to treat the above mentioned illnesses.[61–64] In this sense, and based on the results of this study, the combination of BNCT + BCG for the treatment of these tumors in a clinical scenario could be a promising approach.

While the efficacy of reactor based BNCT has been confirmed for certain malignancies, the difficulty to install a nuclear reactor in a hospital environment encouraged the BNCT community worldwide to work on the development of accelerator-based neutron sources for BNCT. Several accelerators destined for hospital placement have been introduced and have already afforded encouraging results, conceivably favoring widespread BNCT clinical trials.[65]

Ongoing studies by our group are addressing the issue of minimizing dermatitis and/or enhancing local therapeutic efficacy of BPA-BNCT using the seaweed extract Oligo-Fucoidan (Hi-Q Marine Biotech International Ltd). Given that multimodal tumor treatments would be the best suited to optimize local and systemic response, translational studies in this area are pivotal to optimize outcome. Tailored studies are necessary to determine the radiation doses and sequence that will maximize immune stimulation and to establish the best combination of immunostimulatory molecules and radiation that will neutralize radioinduced immunosuppression.[49]

CONCLUSIONS

The present results show that the best option for local control of disease is BNCT (alone or combined with BCG). On the other hand, BCG is the best strategy to control the distant disease by immunological activation and the induction of a powerful abscopal response. Regarding the regional spread of the disease to the lymph nodes, it is the combined treatment of BNCT + BCG that provides the most promising results. The findings on regional spread, albeit preliminary, are contributory and warrant further studies. Multimodal therapies would be the best option for the comprehensive treatment of this type of disease.

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