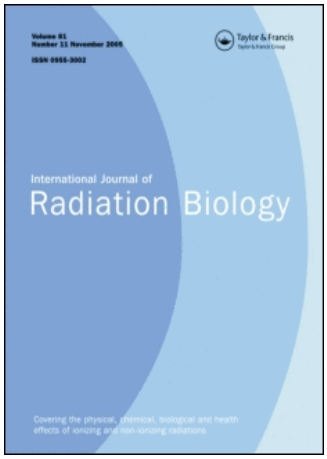

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Effect of Boron Neutron Capture Therapy (BNCT) on normal liver regeneration: Towards a novel therapy for liver metastases

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Abstract

Purpose: The effect of Boron Neutron Capture Therapy (BNCT) on normal liver regeneration was examined in the Wistar rat. The model used is clinically relevant to a novel technique proposed for the treatment of multifocal non-resectable liver metastases in man. The success of the technique also requires that BNCT should not significantly impair regeneration of normal hepatocytes.

Materials and methods: The effect of therapeutic doses of boronophenylalanine (BPA), GB-10 ($Na_2{}^{10}B_{10}H_{10}$) and (GB-10 + BPA) and of BNCT mediated by these boron delivery agents on normal liver regeneration and liver function in the Wistar rat was examined using partial hepatectomy as the regenerative stimulus. The end-points evaluated were body weight, liver weight/body weight ratio, DNA synthesis in terms of 5-bromo-2′-deoxyuridine incorporation, hemogram, kidney function in terms of blood urea nitrogen and creatinine levels, liver function in terms of serum albumin, total and direct bilirubin and liver enzymes (alanine transaminase and aspartate transaminase) and liver histology/architecture.

Results: BNCT mediated by BPA, GB-10 or (GB-10 + BPA) did not cause alterations in the outcome of normal liver regeneration, regenerated liver function/proliferation or histology/architecture.

Conclusion: The BNCT protocols, at the physical doses selected, did not impair the capacity of normal liver hepatocytes to regenerate.

Keywords: *BNCT, multifocal liver metastases, liver regeneration*

Introduction

Boron Neutron Capture Therapy (BNCT) is classically described as a binary treatment modality that involves the selective accumulation of ^{10}B carriers in tumors followed by irradiation with a thermal or epithermal neutron beam. The high linear energy transfer α particles and recoiling ^{7}Li nuclei resulting from the $^{10}B(n, \alpha)^{7}Li$ capture reaction have a range of 5–9 μm in tissue and a high relative biological effectiveness (RBE). In this way, BNCT would potentially target tumor tissue selectively, sparing normal tissue (Coderre & Morris 1999). Clinical trials of BNCT for the treatment of glioblastoma multiforme or melanoma using boronophenylalanine (BPA) or sodium mercaptoundecahydrododecaborane (BSH) as the boron compounds that have been performed and/or are under way in the U.S.,

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Europe, Argentina and Japan (e.g., Barth et al. 2005) have shown a potential, albeit inconclusive, therapeutic advantage for this technique.

More recently, BNCT was also used for the treatment of liver metastases from colorectal cancer. In cases of multifocal, non-resectable, bilobar liver metastases that do not respond to chemotherapy, the only option is palliative treatment. This therapeutic failure is particularly disappointing considering that in most cases the primary tumor in the colon can be successfully excised and liver is often the only site of metastatic spread (Nano et al. 2004). Thus, the development and evaluation of novel therapeutic strategies are needed. The 'Project for Advanced Treatment of Organs by Means of Neutron Irradiation and Autotransplant (TAOrMINA project)' developed a new method for the treatment of multifocal, non-resectable liver metastases based on whole liver *ex-situ* BNCT mediated by BPA, followed by whole liver autograft. This technique reportedly controlled metastatic liver nodules in 2 treated cases (Zonta et al. 2006). However, it poses undesirable surgical risk to the patient due to the prolonged anhepatic phase it involves.

This risk would be arguably inadmissible when it is associated to a therapeutic option that is undoubtedly promising but has not been unequivocally proven to date. The Roffo Institute liver surgeons (JEC) herein propose a novel BNCT technique based on partial liver autograft that would not involve an anhepatic phase. In this way it would pose a drastically lower surgical risk to the patient and would allow the prosecution of studies to evaluate the therapeutic advantage of BNCT for the treatment of multifocal, non-resectable, bilobar liver metastases. Conceivably, BNCT could be used to treat the undetectable micrometastases that would cause up to 40% of the patients to recur solely in the liver after surgical resection (Kavolius et al. 1996). Assuming metastatic spread to all the liver, the proposed technique is based on the administration of the boron compound/s, removal of left lateral section (segments II and III) for irradiation with thermal/epithermal neutrons at the pre-established time post-administration of the boron compound/s, followed by reimplantation of the BNCT-treated portion of the liver. Once a therapeutic effect on the metastatic nodules (expected to occur at approximately 21 days [Kreimann et al. 2001b, Trivillin et al. 2004, 2006]) and adequate liver function are verified in the BNCT-treated segments, right portal blood flow would be occluded by portal vein embolization to induce partial atrophy of the untreated right liver. The working hypothesis is that the atrophy of the right, untreated, diseased liver would stimulate regeneration of the left, BNCT-treated, 'cured' liver to regenerate a healthy liver mass, allowing for the eventual resection of the remaining portion of the diseased right liver.

This technique does not involve an anhepatic phase. Furthermore, postoperatory liver function is guaranteed by the untreated liver mass and does not depend on the BNCT-treated segments. The partial liver autograft strategy proposed employs a combination of classical surgical techniques for allotransplantation (Bismuth & Houssin 1985, Lodge et al. 2000). However, the technique requires *sine qua non* that normal hepatocytes exposed to BNCT at dose levels that would be typically encountered in normal tissue during therapy should be able to regenerate healthy liver following a regenerative stimulus. It is known that radiation inhibits the regenerative process induced by subtotal hepatectomy as described by Dave et al. (1991) and Geraci and Mariano (1994) for 10 and 15 Gy of photon irradiation respectively. These authors studied the effect on liver regeneration of radiation in previously subtotally hepatectomized animals rather than the effect of radiation followed by partial hepatectomy 21 days later, as in the present study. This difference and the difficulties in calculating dose equivalences preclude a direct comparison of dose ranges between these studies and the present study but does alert to a potential detrimental effect of BNCT on liver regeneration. Thus, the potential inability of normal hepatocytes exposed to BNCT to regenerate and restore liver mass and function could jeopardize the working hypothesis of the technique proposed.

The aim of the present study was to evaluate the effects of BNCT mediated by BPA and GB-10 ($Na_2{}^{10}B_{10}H_{10}$), either alone or in combination, on the short-term regenerative capacity of normal rat liver after partial hepatectomy. Partial hepatectomy, 21 days post-BNCT, was the technique used to provide the regenerative stimulus. The TAOrMINA project (Zonta et al. 2006) employed BNCT mediated by BPA. Previous studies have revealed remarkable tumor control with no normal tissue radiotoxicity in an experimental model of oral cancer and in spontaneous nasal cancer in feline patients treated with BNCT mediated by BPA, GB-10 and (GB-10 + BPA) (Kreimann et al. 2001a, 2001b, Trivillin et al. 2004, 2006, Heber et al. 2004, 2006, Rao et al. 2004). These findings suggested the need to explore, in addition to the BPA-BNCT protocol, the effect of these novel BNCT protocols on the regenerative capacity of normal liver.

Materials and methods

All work with experimental animals was performed in strict compliance with the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals'

of the National Academy of Sciences (NIH publication 86-23 revised 1985).

Method validation

The generally expected, virtually constant, ratio between liver and body weight is 3.0–3.5% in normal Wistar rats. In order to validate this for animals used in the present study, a group of 10 normal Wistar rats, of the same age, body weight and strain were used for this purpose. In addition, in order to check that after partial hepatectomy (approximately 70%) in keeping with Higgins and Anderson (1931), this liver to body weight ratio was maintained, a further group of 18 normal Wistar rats were used. The liver/body weight ratio was evaluated 9 days after surgery (Higgins & Anderson 1931).

Biodistribution studies

Biodistribution studies were conducted to perform the corresponding dosimetric calculations. BPA (Ryscor Science, North Carolina, USA) was administered intraperitoneally (ip) as a bolus injection at a dose of 15.5 or 31.0 mg B/kg bw. GB-10 (provided by Neutron Therapies, LLC, San Diego, CA, USA) was administered as a bolus injection at a dose of 50 mg/kg bw in the surgically exposed jugular vein under ketamine (57 mg/kg bw) – xylazine (9 mg/kg bw) anesthesia. In both cases blood and tissue samples were removed 3 hs post-administration based on previous biodistribution studies by our laboratory (Kreimann et al. 2001a, Heber et al. 2004). For the combined administration protocol, GB-10 was administered intravenously (i.v.) at a dose of 34.5 mg B/kg bw and BPA was administered at a total dose of 31 mg B/kg bw as fractionated ip injections over a 3 hr period to simulate an infusion. It was not necessary to anesthetize the animals to administer BPA intraperitoneally. However, GB-10 was administered under ketamine-xylazine anesthesia as described above. Thus, the BPA injections delivered after i.v. administration of GB-10 in the combined administration protocol were given under anesthesia. Fractionated i.p. injections of BPA rather than an in bolus BPA injection were given in combination with i.v. GB-10 because they were previously shown to improve boron biodistribution (Heber et al. 2004). Blood and tissue samples were taken 3 h post-administration of GB-10 or BPA in the single compound administration protocols and 3 h post-administration of GB-10 and 1.5 h after the last i.p. injection of BPA in the combined compound administration protocol (Heber et al. 2004). Some 4–7 animals were assessed per group. The samples were processed and boron content was evaluated by inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin Elmer, Gainesville, Florida, USA) as previously described (Heber et al. 2004).

Effect of boron compounds on normal liver regeneration

The boron compounds were administered as described above to each of the experimental groups: BPA (15.5 mg B/kg), BPA (31.0 mg B/kg), GB-10 and (GB-10 + BPA). A group injected with anesthesia alone, ketamine (57 mg/kg bw) – xylazine (9 mg/kg bw), was evaluated in addition to an absolute control, non-injected group run simultaneously. Some 3–7 animals were assessed per group. Twenty-one days post-administration of the boron compounds, partial hepatectomy was performed as previously described (Higgins & Anderson 1931). Nine days later (Higgins & Anderson 1931), the animals were sacrificed and the liver was resected and weighed to evaluate regeneration. Thirty minutes prior to sacrifice 5-bromo-2′-deoxyuridine (BrdU) was administered, a pyrimidine analogue of thymidine used to evaluate DNA synthesis (Dolbeare et al. 1983). Liver samples were processed for histological analysis and for immunohistochemical demonstration of BrdU employing the peroxidase-antiperoxidase technique (Sternberg et al. 1970). BrdU-positive cells were counted in approximately 60–100 fields per section at ×400 magnification. Blood samples were taken to evaluate hemogram (hemoglobin concentration, red blood cell, white blood cell and platelet counts), kidney function in terms of blood urea nitrogen and creatinine levels and liver function in terms of serum albumin, total and direct bilirubin and liver enzymes (alanine transaminase and aspartate transaminase). Body weight was monitored daily throughout.

Effect of BNCT on normal liver regeneration

The animals were taken by plane to Bariloche, a city 1600 km south-west of Buenos Aires, to be irradiated with the thermalized epithermal beam at the RA-6 Nuclear Reactor. The irradiation conditions are presented in Table I. Based on boron content (reported in the Results section), irradiation times were calculated to administer the maximum irradiation dose to the liver without exceeding the radiotolerance, established by earlier work, of the different organs in the treatment volume. Of particular concern in the rat model is the dose to the kidney due to its proximity to the liver and its high boron content. However, this would not be a concern in a clinical scenario where BNCT would be performed ex-situ. Since each boron compound administration protocol resulted in different boron content values, the maximum exposure time for the dose-limiting

tissues varied from protocol to protocol. Thus, the maximum dose (resulting from the maximum exposure time tolerated by the dose-limiting tissues) that could be delivered to the liver varied for each protocol.

The boron compounds were administered as described above. At the times post-administration of the boron compounds described above, the abdomen of each animal was placed in the center of the beam port, which is 15 cm in diameter. The head and posterior portion of the body remained radially outside of the beam, partially shielded by the lead and borated polyethylene beam aperture delimiter (Figure 1).

Thermal neutron flux at the position of the liver was 7×10^8 n/cm^2-sec. The control group was sham-irradiated. Each group comprised 6–7 animals. The animals were brought back to Buenos Aires and 21 days after irradiation, as described in point 2, they were submitted to partial hepatectomy (Higgins & Anderson 1931). Nine days after surgery the animals were killed to evaluate liver regeneration, DNA synthesis, liver histology and function and kidney function as described in point 3.

Statistical analysis of the data was performed by Student's *t*-test. Statistical significance was set at $p = 0.05$.

Results

Method validation

In the 10 control rats, weighing 225.8 ± 33.7 g body weight, the liver to body weight ratio was 3.6 ± 0.4%. Nine days after partial hepatectomy in 18 rats, the liver to body weight ratio was

Table I. Irradiation conditions for normal liver.

Protocol	Physical absorbed doses (Gy)				Total physical absorbed dose (Gy)	Irradiation time (min)
	Fast neutrons	Gamma photons	Induced protons	Boron component		
BPA-(31.0 mg B/kg)-BNCT	0.8	3.0	0.5	3.1	7.4	55
GB-10-BNCT	1.1	4.1	0.7	2.0	7.9	75
(GB-10 + BPA)- BNCT	0.5	1.9	0.3	2.7	5.4	35
Beam only	1.1	4.1	0.7	–	5.9	75

Figure 1. Rats positioned at the RA-6 beam port for local irradiation. The abdomen is placed at the center of the beam port (15 cm in diameter, indicated by the white circle) (A). The head and posterior end of the body of the rat remain radially outside of the beam, shielded by the lead and borated polyethylene beam aperture delimiter schematically shown in (B). The arrows in B indicate the position of the beam port.

$3.4 \pm 0.4\%$, indicative of full liver regeneration (Higgins & Anderson 1931).

Biodistribution studies

For the physical dose calculations, boron biodistribution data for each protocol were obtained for blood, liver, skin, tongue, spleen, lung, intestine, kidney and spinal cord (Table II).

Effect of boron compounds on normal liver regeneration

Normal liver regeneration reached control values in terms of liver weight/body weight ratio for all the boron compound administration protocols ($p > 0.05$). A group injected with anesthesia alone, ketamine (57 mg/kg bw) – xylazine (9 mg/kg bw), was evaluated in addition to an absolute control, non-injected group run simultaneously (Table III).

No significant differences in body weight, BrdU-positive nuclei counts, hemogram values, kidney function, liver function or liver histology/architecture were observed at the time of complete liver regeneration between the animals treated with the boron compounds and controls (data not shown).

Effect of BNCT on normal liver regeneration

Normal liver regeneration reached control values in terms of liver weight/body weight ratio for all the BNCT/irradiation protocols ($p > 0.05$) (Table IV).

The exposed animals exhibited some mild, reversible, toxic effects related to irradiation such as weight loss and diarrhoea: four animals in the BPA (31.0 mg B/kg)-BNCT protocol died and one was killed due to its general decline in health 4 days post-BNCT. The biochemical assays on the blood samples of the animal that was sacrificed prematurely suggested radioinduced nephropathy as a possible cause of death, conceivably as a result of the high boron content (69.7 ± 12.4 ppm) in the exposed kidneys in this group. The 2 remaining animals that were followed in the BPA (31.0 mg B/kg)-BNCT protocol had regained control weight and clinical status at the time of partial hepatectomy and exhibited complete regeneration of the liver mass at 9 days post-hepatectomy (Table IV). No significant differences in body weight, BrdU-positive nuclei counts, hemogram values, kidney function or liver histology/architecture were observed at the time of complete liver regeneration between the animals treated with the BNCT/irradiation protocols and controls (data not shown). Figure 2 shows a representative example of the absence of significant histological alterations in the regenerated liver of all BNCT-treated animals compared to the regenerated liver of untreated animals.

Concomitantly, a mild 1.8- to 2.7-fold increase in liver enzymes (alanine transaminase and aspartate transaminase) was observed in all the irradiated groups 9 days post-hepatectomy vs. the control group submitted to partial hepatectomy alone.

Discussion

The initial set of experiments validated the methods used in the present study relative to previously

Table II. Boron content in ppm (Mean $\pm$ SD).

Tissue/protocol	BPA-*a* (15.5 mg B/kg)	BPA-*b* (31 mg B/kg)	GB-10 (50 mg B/kg)	BPA (31 mg B/kg) + GB-10 (34.5 mg B/kg)
Blood	5.7 ± 1.3	11.9 ± 1.8	13.0 ± 5.5	21.9 ± 3.4
Lip mucosa	13.0 ± 4.0	38.8 ± 12.2	13.3 ± 9.9	*
Tongue	9.4 ± 1.2	33.3 ± 13.8	9.9 ± 5.7	27.9 ± 1.9
Skin	7.4 ± 2.7	19.1 ± 6.9	13.4 ± 10.8	28.0 ± 2.4
Liver	8.1 ± 2.1	17.9 ± 0.6	8.5 ± 6.2	24.9 ± 1.9
Spleen	11.4 ± 3.6	22.6 ± 2.7	12.6 ± 5.5	32.3 ± 2.6
Kidney	34.1 ± 8.7	69.7 ± 12.4	23.2 ± 10.7	122.0 ± 11.5
Intestine	11.2 ± 3.1	19.2 ± 3.4	10.6 ± 9.2	23.6 ± 3.5
Lung	10.1 ± 3.6	21.7 ± 6.2	14.0 ± 6.3	27.8 ± 3.4
Bone marrow	4.8 ± 2.0	8.9 ± 3.6	4.9 ± 5.3	7.4 ± 2.4

*not available.

Table III. Liver weight/body weight ratio, for each of the protocols of administration of boron compounds as indicated (Mean $\pm$ SD).

BPA-*a* (15.5 mg B/kg)	BPA-*b* (31 mg B/kg)	GB-10	GB-10 + BPA	Anesthesia	Control
2.8 ± 0.2	2.7 ± 0.1	2.8 ± 0.2	2.9 ± 0.1	2.9 ± 0.2	3.0 ± 0.4

published work (Higgins & Anderson 1931). The present study has demonstrated that normal hepatocytes exposed to boron compounds or BNCT can retain their capacity to respond to a regenerative stimulus and restore liver mass and liver function in the short-term. Future studies are needed to explore long-term effects and thus confirm the stability of recovery.

BNCT to the liver was at the maximum physical doses that could be tolerated by adjacent sensitive organs such as the kidney and gut. The BNCT physical doses used (5.4–7.9 Gy as shown in Table I) closely resemble or exceed the physical dose of 6.1 Gy to normal liver (personal communication) associated to the therapeutically effective tumor dose employed in the TAOrMINA project (Pinelli et al. 2002, Zonta et al. 2006).

This study was not designed to explore potential alterations in the kinetics of the regenerative process. The present data reveal that normal liver treated with boron compounds or BNCT at the physical dose levels tested can exhibit complete regeneration of liver mass at the same time after partial hepatectomy as untreated liver. The mild increase in liver enzymes observed in irradiated animals at the end of the regeneration process might be indicative of earlier, reversible effects. Admittedly, liver enzyme kinetics was not examined. However, the lack of histological alterations concomitant with this mild increase in liver enzymes and the absence of associated alterations would suggest recovery of liver function rather than impending liver failure. Future studies are warranted to explore potential reversible effects that might occur shortly after treatment but do not affect the outcome of the regeneration process, and also to evaluate the long-term effect of BNCT on the structure and function of regenerated liver.

The model used for this study is widely accepted, highly reproducible and allows for the study of different experimental variables (Higgins & Anderson 1931). However, the need to irradiate a large fraction of the animal's body to assess the effect of BNCT on liver regeneration is a constraint in this experimental model. This will obviously not be a concern in a clinical scenario where treatment will be performed *ex-situ*. The potential constraint lay in being able to deliver a dose to normal liver compatible with the dose it would receive in clinical BNCT without exceeding the tolerance of the animals. The aim in choosing the doses of BNCT for the different protocols was to deliver the maximum physical dose to liver that it was estimated would be compatible with animal tolerance, based on boron content in the different organs for the different protocols.

Within this context, the present data provide evidence that at the total physical doses delivered, BNCT mediated by BPA, GB-10 or (GB-10 + BPA) does not impair the capacity of normal hepatocytes to regenerate healthy liver, as seen at 9 days post-hepatectomy, the time of full liver regeneration in non-BNCT treated animals.

BNCT would be a potentially attractive technique to treat liver metastases in that it offers a mechanism

Table IV. Liver weight/body weight ratio, for the different BNCT/irradiation protocols and control group as indicated, 9 days post hepatectomy (Mean ± SD).

BPA-*b*-BNCT (31 mg B/kg)	GB-10-BNCT	(GB-10 + BPA)-BNCT	Beam only	Control
3.5 ± 0.1	3.3 ± 0.3	3.1 ± 0.3	3.4 ± 0.3	3.5 ± 0.4

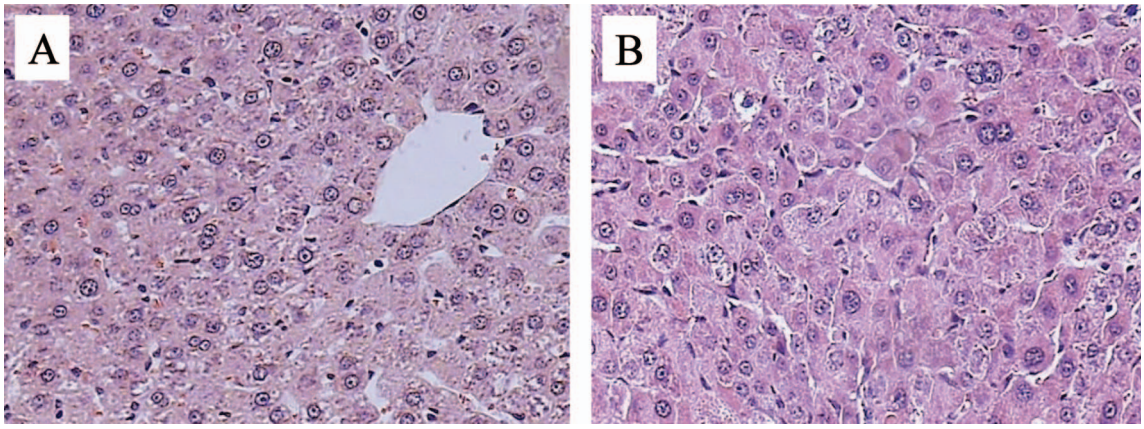

Figure 2. Light microscopy image of liver tissue 9 days post-hepatectomy in a control rat (A) and in a rat treated with BNCT mediated by GB-10 (B). No significant changes were observed in liver histology between control and BNCT treated animals for any of the BNCT protocols evaluated. Hematoxylin-eosin stain, original magnification ×400.

for targeting the undetectable micrometastases, a major challenge in oncological therapy. This notion has encouraged international efforts to devise a way to apply the technique safely and effectively (e.g., Allen et al. 1997, Suzuki et al. 2004, Nievart et al. 2006, Zonta et al. 2006). The assessment of the potential applicability of the technique proposed to the treatment of primary liver cancer would require additional studies to evaluate the response of an overall diseased liver as opposed to healthy liver surrounding metastatic nodules. The present study would support further development of the proposed novel BNCT technique for the treatment of multifocal liver metastases based on autograft and regeneration in that it provides evidence that normal, BNCT-treated hepatocytes retain their capacity to regenerate liver mass and function.

Conclusion

BNCT, at the total physical doses delivered, does not impair the capacity of normal hepatocytes to regenerate healthy liver, as seen at 9 days post-hepatectomy. This holds true for all three BNCT protocols, i.e., BNCT mediated by BPA, GB-10 and (GB-10 + BPA). We previously reported the therapeutic efficacy of these BNCT protocols in oral carcinoma, a tumor of epithelial origin like liver metastases of colorectal cancer. Thus, this finding may be of potential use in the development of novel techniques for the treatment of non-resectable liver metastases of colorectal cancer.

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