OPERATIVE MODALITIES AND EFFECTS OF BNCT ON LIVER METASTASES OF COLON ADENOCARCINOMA

A Microscopical and Ultrastructural Study in the Rat*

T. Pinelli¹, S. Altieri¹, F. Fossati¹, A. Zonta¹, C. Ferrani²,
U. Prati², L. Roveda², S. Ngiateju Tata², S. Barni², P. Chierì¹,
R. Nano², and D. M. Ferguson¹

¹INFN, Pavia, Italy
Dept. of Nuclear and Theoretical Physics, University of Pavia, Italy
²Dept. of Surgery, Div. of General Surgery, University of Pavia, Italy
³Dept. of Animal Biology, University of Pavia, Italy. Centre of Study for
Histochemistry, C.N.R., Pavia, Italy
⁴Mayo Clinic, Rochester, Minnesota

1. INTRODUCTION

During the biennial ISNCTC Symposia held in Bremen (1988)¹ and Kobe (1994)² we introduced the idea of a novel BNCT application. Its basic concept is the thermal neutron treatment of an explanted organ previously isolated and maintained in an extracorporeal condition before reimplantation in the same donor patient (organ autotransplant). In those years some of us were refining the liver autotransplant technique later applied to several clinical cases.² Therefore our efforts were directed to the liver metastases therapy as the first application of the method;³⁴¹⁷ in the future such a therapy could be extended to every organ suitable for autotransplant surgery.

The colon adenocarcinoma is one of the most frequent tumours afflicting the human population. There is an estimated number of nearly 600,000 cases per year in the world and 152,000 of those cases are ascertained in USA.³ In one third of such cases the diagnosis is late and the survival rate 5 years after diagnosis is not higher

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than 40–50%. Liver metastases are responsible of this poor clinical outcome in two thirds of the cases. They are often multiple and diffuse, as a consequence surgically unresectable. Particularly disappointing is the consideration that in most cases the primary tumour in the colon can be excised easily while the liver is the only site of metastatic diffusion.

The irradiation of the entire isolated liver offers the following major positive aspects:

- An accurate knowledge of the tumour distribution is not necessary. The method guarantees the treatment of the whole organ with the same efficacy, even in the case of a microscopic infiltration. The probability of recurrent tumours is reduced to the minimum.
- The entire body of the patient (except liver) is preserved from any radiation damage.
- The liver, being washed before the irradiation, does not suffer any damage deriving from neutron interaction with the boron lasting in the blood.
- The radioactive dose absorbed during the treatment can be made substantially uniform in the entire liver volume. In fact, due to the isotropic nature of the neutron field inside the thermal column, it's not difficult to realize a satisfactory flat distribution of the neutron flux by an opportune arrangement of scattering and absorbing materials around the irradiation position (section 2).

Nevertheless these important advantages are associated to the crucial problem of preserving healthy tissues. After the therapy, the liver should support all its functions.

The feasibility of our project, beyond the surgery problems, has been defined by the following assumptions:

1) The total radioactive dose released in tumour must be larger than in normal tissues by a factor 2.5 at least.
2) The total dose given to the healthy tissues must be lower than the tolerance level (15 Gy-Eq).
3) The lowest local dose absorbed by the tumour must exceed the level of 40 Gy-Eq.
4) The treatment length must be largely compatible with the survival time of the patient in anhepatic phase.

The tolerance level of the hepatic parenchyma treated by BNCT has not been investigated until now. Values from 25 to 30 Gy are reported\(^6\) for the case of photon single dose given to the whole liver. During the current clinical trials of BNCT for brain tumours, the upper limit of radiation dose to normal brain varied from 8.9 to 14.8 Gy-Eq.\(^7\) We assumed as tolerance dose 15 Gy-Eq. This value represents a conservative choice being the RBE of 14.3 MeV neutron for liver tissues\(^8\) more than 30% larger in comparison with that of thermal neutron beam for brain tissue.\(^9\) The lower dose to be given to the tumour represents a median value when compared with those used to treat brain tumours in the current clinical trials.\(^10\)

Our preliminary calculations and measurements\(^11\) evidenced, as necessary conditions to proceed according to these assumptions, the following protocol prescriptions:
A) The thermal neutron flux in the whole volume of the liver undergoing the neutron irradiation must be uniform with a minimum value of $10^8 \text{ cm}^{-2} \text{sec}^{-1}$.

B) The boron distribution in the liver must realize the condition $T = \frac{C_l}{C_u} \geq 4$. $C_l$ and $C_u$ represent $^{10}B$ concentration in tumour and normal tissues respectively.

$T$, the tumour over healthy tissue $^{10}B$ concentration ratio, is a time function whose behaviour is of the maximum importance in order to satisfy this second prescription in the most convenient manner.

C) The $^{10}B$ concentration in tumour must be larger than 20 ppm. This lower threshold allows the tumour tissues to absorb more than 40 Gy-Eq in a short time fitting an adequate and safe treatment.

2. METHODS AND RESULTS

The original structure of our thermal column was modified in order to realize a channel with a well-moderated neutron field. A strong reduction of gamma background has been obtained by bismuth screens in front of this channel (upper left side of Figure 1). We simulated the irradiation of a typical size liver shielded by a heavy water wall containing 35 ppm of $^6Li$ as represented in the upper right side of the same figure.

Figure 1 describes the main features of the neutron field in the volume of the liver model used both for experimental measures and simulation by the MCNP-4B code. The model results have been preliminary standardized and validated by absolute flux measurements inside a pig liver situated at the irradiation position.

The neutron space distribution along the longitudinal axis (x) of the reactor thermal column is shown in the figure for 4 vertical levels ($Z = 0, 1.5, 2.5, 4.5 \text{cm}$). Corresponding cross distributions are not reported being substantially constant inside the entire volume of our liver model (a hepatic-equivalent spherical bowl having a 6 cm height and 30 cm base diameter).

The obtained volume distribution evidences a satisfactory uniformity. The peak and lowest values are $8.2$ and $4.5 \times 10^8 \text{ cm}^{-2} \text{sec}^{-1}$. Obviously the dose distribution has the same behaviour. In addition at half-time of the one-shot treatment we have the possibility to rotate the organ by a 180° angle around z axis, so producing an improved uniformity; the difference between the peak and lowest values is reduced to 20% peak dose.

Results reported in Figure 1 evidence that our irradiation facility can largely satisfy the protocol prescription A. In particular, the neutron flux value allows to conclude the irradiation in about 12 min. Such a time is negligible when compared to the survival of the patient in the hepatic state (longer than 8 hours).²

Due to the crucial prescription B, we dedicated noticeable efforts to the study of boron uptake by hepatic tissues. Using a rat model we performed a large statistical analysis on a population of 100 rats (of these 83 resulted useful for our analysis). Details of methods concerning tumour induction, boron administration, boron concentration measurements in tumour and healthy hepatic tissues were described elsewhere²,¹,¹,¹ as well as the other operative instruments and procedures we utilized during this particular work. We recall the following protocol² adopted to study the liver and tumour boron uptake:
Schematic view of the Triga Mark II reactor of the University of Pavia

Figure 1. Neutron flux distribution in the longitudinal symmetry plane of a liver model (upper right). Data as obtained by a MCNP-4B code have been checked by experimental measurements. Open circles show the neutron flux behaviour when the D₂O-²Li solution is replaced by air. On the upper left side of the figure the modified thermal column is shown inside a sketch of the reactor structure.

a) Tumour induction in the rat by inoculating 10⁶ cells of colon carcinoma (section 3) by an intrasplenic injection.
b) 10 days later a dose of 300 mg ^9B - BPA/Kg body weight (section 3) is intravenously injected.
c) After a time t from the injection the animal is sacrificed and its liver is extracted, then washed.
d) Samples of both tumour and normal tissues are cut in thin samples and submitted to histologic analysis and computerized imaging to evaluate the tumour percentage in each sample.

e) The boron concentration is evaluated with a 0.5 ppm resolution by a spectroscopic analysis of alpha particles released by the $^{10}B(n,\alpha)^7Li$ inside the reactor thermal column.

Figure 2a,b,c detail the definitive data we obtained on boron uptake by liver tissues.

**Figure 2.**

a) Time distribution of $\bar{T}$, the mean value of boron concentration ratio of tumour over healthy tissues ($T = \frac{C_T}{C_H}$). The variable $t$ is the time elapsed between the BPA infusion and the sacrifice of experimental animals. b) Time distribution of the probability to get values $T > 4$ at the time $t$ after BPA infusion (300 mg/Kg dose). c) Time distributions of boron concentration in tumour (red) and healthy tissues (blue). $T$, $t$ and the BPA dose are the same as in Figures 2a and b.
Figure 2a shows the time distribution of $\bar{T}(t)$, the mean value of boron concentration ratio in tumour over healthy tissues. We see that in the time interval from 1 to 6 hours the prescription B is largely fulfilled.

In Figure 2b we can consider in probabilistic terms the possibility to get satisfactory values of $T$ ($T \geq 4$) at a certain value of $t$. When the variable $t$ is included in the interval 2–4 hours we meet high probabilities, going from 80 to 90%, to achieve values of $T$ larger than 4. More precisely, inside this interval, the mean value $\bar{T}$ ranges from 4 to 6 (Figure 2a).

In Figure 2c we observe the diagrams describing the time behaviour of $^{10}$B concentration in both cases of tumour and healthy tissues ($C_T, C_H$). The remarkable values assumed by the neoplastic tissues in the interval $1 \leq t \leq 4$ hours make the results fully compatible with the prescription C of the treatment protocol.

By assuming a neutron fluence of $5 \cdot 10^{12} cm^{-2}$ we can calculate the tumour and healthy tissue doses using the following equations:

$$D_H = 7.3 + 0.59 \cdot C_H$$

$$D_T = 7.3 + 1.71 \cdot C_T$$

where 7.3 Gy-Eq is the background dose, 1.71 Gy-Eq/ppm is the dose produced by the $^{10}\beta(n,\alpha)^7Li$ process when the boron concentration in tumour is 1 ppm, 0.59 Gy-Eq/ppm is referred to the concentration of 1 ppm in the normal tissue. The biological effectiveness of the undue radiations and the products of $^{10}\beta(n,\alpha)^7Li$ reaction are given in ref. 13 for brain normal and tumour tissues.

RBE values of particles released in Boron Neutron Capture process are not available for liver parenchyma, nevertheless a significant check of the project feasibility can be realized by using reasonable approximations. We find in literature$^{10,14}$ the RBE of 14 MeV neutron for rat hepatic parenchyma and intestine; in both cases the value is about 2 while for rat spinal cord RBE of the thermal beam (single dose) is 1.4. In addition the RBE value of neutron beam and the CBE value of BNC irradiation for brain white matter are roughly equal.$^{15}$ We may conclude that our dose calculations seem to be useful to verify the project feasibility.

With the sake of evaluating the RBE correct value for the normal liver tissues, we are experimentally studying the dose-effect relation by the usual ex vivo-in vitro method.

From Figures 2b and 2c we realize that the treatment is made in conditions of feasibility with 75–85% probability when $t$ value is inside the interval from 2 to 4 hours.

The conclusive data elaboration of the complete experiment is in good agreement with the preliminary results we gave progressively in various occasions.$^{2,16,17}$

The situation determined by our study on the treatment feasibility is graphically resumed in Figure 3. Here five straight lines are drawn. Three of these lines represent the assumptions 1, 2 and 3 of section 1.

The remaining two are the limits of that portion of the plane where $T$ assumes the favorable values from 4 to 6. Then the dashed area represents the polygon whose internal points are associated to a practicable application of our method. Let's now consider the realistic example relative to the irradiation starting at $t = 2$ hours. The comparison between Figures 2a and 2b indicates a probability around 80% to get a $T$ value larger than 5. The same probability can be attributed to the typical values of boron concentration $C_H = 5.8$, $C_T = 30.1$ ppm (Figures 2a and 2c). In the plane of Figure 3 the assumed example is represented by the point $P$, conveniently positioned inside the dashed polygon.
This is a synthetic indication that we are in condition to satisfy the feasibility protocol with a probability larger than 80%. Though satisfactory, this situation could be improved eventually by a convenient increase of the BPA dose up to 400–450 mg per Kg.

3. EXPERIMENTAL BNCT

After the positive results obtained in determining the feasibility parameters of the treatment we passed to analyse, by "in vitro" and "in vivo" methods, the effects induced on liver tissues by the BNCT procedure.

We considered as essential this third phase of our project for the following reasons:

i) To get a first realistic check on the correspondence between the damages induced by the treatment and those expected according to our project protocol. Of particular importance is the comparison between the radiation damage produced by BNCT in tumour and normal hepatic tissues.

ii) To get, by direct biological observation, a first answer for the problem regarding the boron uptake into the deepest tissue of those metastases having a relevant size. This problem could be originated by the poor blood circulation through such tissues.

iii) To realize a first approaching of our project to the clinical application.

- In vitro method consisted in the analysis of the viability of boron enriched colon carcinoma DHDK cell line, after neutron irradiation.
The viability was determined using a technique that is a measure of the membrane permeability associated with cell death. The viability probe used is the anti-cytokeratin (CK) MoAb.16

Flasks containing $10^5$ cells of colon carcinoma DHDK 12 TRb cell line in 20 ml of medium were treated for two hours with $^{10}BPA$-fructose complex containing 60 mg D.L-p-boronphenylalaninehydrochloride (100 mg BPA combined with 20 ml fructose in 0.3 M solution. The pH is adjusted to 7.4-7.5 with NaOH 2N solution). The BPA was supplied by BBI, Raleigh, NC, USA with a $^{10}B$ enrichment more than 95%.

Harvested cells were washed three times in medium and then split in two aliquots. One was irradiated with the neutron fluence of $7 \cdot 10^{12} cm^{-2}$, while the other one was used as control. The equivalent dose corresponding to the given fluence is 58.1 Gy-Eq.

Soon after the irradiation, $10^6$ cells were treated for 15 min with 200 µl of 1:10 diluted MoAb anti-CK MNF116 (DAKO). After washing, the cells were incubated with the secondary antibody IgG FITC (SIGMA) conjugated for 15 min, then fixed in 10 ml of 70% ethanol. One hour before analysis the cells, washed twice in PBS, were counterstained with 1 ml of 5 µg/ml Propidium Iodide (PI). A two-parameter analysis of the DNA content (PI-red fluorescence) versus CK positive dead cells (FITC-green fluorescence) was performed on a FACStar (Becton Dickinson) equipped with a 5 W argon ion laser. The same procedure was used for the control cells. Moreover, aliquots of $2 \cdot 10^6$ cells were cultured again and analyzed after 24 and 48 hours with the previous described method.

The two-parameter analysis shows that the percentage of CK+ dead cells evaluated on the irradiated and non-irradiated cell lines did not evidence any difference. Such a result proves that the neutron irradiation cannot be considered responsible of observable membrane damages. More interesting information (Figure 4) is obtained from the DNA content histograms deduced from the same analysis. Immediately after irradiation (t = 0) no difference can be noticed between the DNA histograms concerning irradiated and control cells respectively. After t = 24 hours we observe in both cases cells with DNA stainability lower than that of G0G1 cells; such findings could represent debris and/or apoptotic cells. This situation in the non-irradiated cells might be caused by cell suffering due to the procedure they have been submitted. The third histograms in figure (t = 48 hours) show a different result. A permanent signal of suffering is still evident in the irradiated population while control cells have almost completely restored the initial condition (t = 0).

The radioactive dose we administered results adequate to induce severe and probably irreversible damages in neoplastic cells during their thermal neutron treatment.

The above results are to be intended as preliminary ones. More experiments are planned for extending our observations to longer time after treatment and using more sensitive reagents for apoptotic cells.

- **In vivo method** to analyze the effects of neutron irradiation in tumour and normal tissue consisted in the application of our entire procedure in a rat model. The objectives of the experiment were:
  - Developing a technique to optimize the induction of hepatic metastases.
  - Setting up a microsurgical technique of liver transplantation.
  - Completing a whole procedure of boron compound infusion.
  - Irradiated liver grafting into a syngeneic/allogeneic rat after the heptectomy.
  - Evaluating the cellular damage induced by the administered radioactive dose.
A colon carcinoma was induced in the experimental BD-IX strain rat by inoculation in the spleen of $2 \cdot 10^7$ cells from the DHDK cell line according to the method described by Caignard and coll.\textsuperscript{19} During the splenic inoculation the left branch of the portal vein was clamped. In this way each animal gave samples of both tumour and healthy tissues.

Multiple and sometimes confluent liver metastases were induced in the right lobe of syngeneic rats during the following days. Ten days after the inoculation a D,L p-\textsuperscript{10}BPA dose of 300mg/Kg was infused through the dorsal penis vein over a time of about 5 minutes. The used BPA-fructose complex was the same as in "in vitro" trials.

After 2 hours from boron infusion, the animal was sacrificed and its liver extracted, perfused with Beltzer solution, then irradiated inside the thermal neutron facility of our reactor. As in the case of the "in vitro" analysis the liver received a neutron fluence of $7 \cdot 10^{12} \text{cm}^{-2}$. Assuming boron concentration $C_T = 30.1$ and $C_U = 5.8 \text{ppm}$ in tumour and normal tissues (see section 3), the corresponding doses $D_T$ and $D_U$ are 82.5 and 15.0 Gy-Eq respectively.
Table 1. Experimental rats as stratified according to the “in vivo” treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group A (healthy rats)</th>
<th>Group B (rats with liver metastases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>Liver transplant only (control)</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>BPA + liver transplant (control)</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>BPA + neutron irradiation + liver transplant</td>
<td>2</td>
<td>19</td>
</tr>
</tbody>
</table>

Hepatectomy was previously performed on a syngeneic or allogeneic recipient rat maintained in general anesthesia. After having been irradiated, the isolated liver was then transplanted orthotopically in this last animal using a modification of the Kamada method. After a preset time the animal was sacrificed and liver rapidly removed. Samples of both tumour and healthy tissues were cut according to a common geometry and submitted to either histological or ultrastructural analysis.

The “in vivo” analysis schedule included 39 experiments variously designed in order to control each important step of the whole procedure.

All donor animals were BD-IX strain rats. The experimental animals were included into two groups. Group A consisted of 12 healthy rats, while group B contained 27 rats with liver metastases. Each group was then stratified according to the treatment as reported in Table 1.

Morphologic observations were performed both in light and electron microscopy. They were all made within 6 days from the liver transplant so to avoid the consequences of immunological intolerance in allogeneic grafts. Such observations were extended to a statistically significant number of both healthy and tumoral samples, before and after the neutron irradiation.

The results of the entire analysis, after comparison with the control population, can be resumed with the following overall conclusions:

- Cancerous samples from non irradiated animals evidence with a noticeable continuity good intrusive properties of the inoculated tumour. Such an indication is suggested by a significant amount of colon carcinoma cells observed both in free diffusive state and in aggregate nodules (Figure 5a and b). Moreover, the neoplastic cells have been seen either as a proliferating undifferentiated phenotype or as a pseudo-differentiated phenotype (Figure 5c and d).

Figure 6 gives a typical view of samples obtained from the liver submitted to the complete procedure (Metastases induction, BPA infusion, hepatectomy on the donor, neutron irradiation, transplant in the recipient animal). In particular this figure refers to a recipient animal sacrificed 4 days after the liver transplant.

Electron microscopy shows, with a significant frequency, severe damages both in neoplastic sinusoidal cells and nodules while the parenchyma cells (hepatocytes) are only occasionally found in a suffering state whose seriousness is difficult to establish.

4. DISCUSSION

The modified structure of the neutron facility together with the definitive results of our boron uptake study have determined an irradiation protocol largely practicable for our BNCT novel application:
Figure 5. Light (a,b) and electron microscopy (c,d) of liver samples in pre-irradiation condition (after tumour inoculation and boron administration). Tumour cells lasting within the sinusoidal space after the liver washing are indicated by arrows in a,c and d micrographs. Still evidenced by arrows we can observe various tumour nodules in micrograph b.

- **BPA dose:** D,L p-10BPA dose of 300 mg/Kg body weight
- **Neutron irradiation starting time from BPA infusion:** 2 hours
- **Neutron fluence over the liver surface:** $5 \cdot 10^{15} \, cm^{-2}$
- **Neutron irradiation length:** 12 min

This protocol allows, with a probability near 80%, to give doses of 10.7 and 59.0 GY-Eq to healthy and tumour tissues respectively. The value of tumor over healthy tissue ratio $\frac{D_T}{D_H}$ results to be 5.5, more than the double we require for a safe treatment (2.5 at least).
The "in vitro" analysis of radiation effects on colon carcinoma tumour cells, points out a different result between irradiated cells and the control population. After 48 hours from irradiation, while control cells show a complete recovery of the initial conditions, a permanent state of suffering is still evident in the irradiated population. This could be indication of debris and/or apoptotic cells due to severe damages induced by the neutron irradiation.

Interesting findings of our preclinical "in vivo" trials derive from microscopic and ultrastructural analyses. In particular 4–6 days from the irradiation the neoplastic cells, either singly dispersed or structured in nodules, appeared, with a discrete frequency,
strongly damaged and in many cases in apoptotic state. The normal hepatocytes, on the other side, were never observed in condition clearly indicative of irreversible damages.

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