# Neutron Study of Medium Transfer Bystander Effect for a Human Skin Cell Line

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*Neutron irradiations at the McMaster Nuclear Reactor have been performed to study direct and bystander effects for a human skin cell line. The neutrons are produced in the core of the swimming pool type reactor by nuclear fission; they are then partially moderated through water before exiting along a beam tube in the reactor wall. The resultant neutron spectrum has an energy distribution with an average of 1 MeV. The gamma contamination of the reactor neutron beam was designed to be less than 0.8% for a neutron dose of 2 Gy/h with the current design. Microdosimetric methods have been used to measure the neutron and gamma doses for the cell irradiations. It was found that there was no significant direct cell death occurring for a human skin cell line when the neutron dose was below 200 mGy. It was further found that neutrons did not significantly induce the medium transfer bystander effect when the pure neutron doses were kept below 200 mGy, which is much higher than the γ effective dose threshold of 3 mGy. The result is consistent with a published microdosimetric model.*

## I. INTRODUCTION

Over the past two decades, there has been considerable interest in radiation induced bystander effects, which are defined as observed biological responses in non-irradiated cells resulting from communication with irradiated cells. The phenomena have been demonstrated for numerous biological endpoints including clonogenic survival (1-3), chromosome aberration (4-6), induction of micronuclei (7), and changes in gene expression (8). A number of experimental approaches exist for the study of the bystander effect. The methods include irradiation with low fluences of α particles (9-10), a charged particle microbeam (11-16), mixing irradiated cells with nonirradiated cells (17-18), and with transferring medium from irradiated cells to non-irradiated cells (1, 3). The mechanisms, however, are still not fully understood. Two possible processes are considered to be involved in the bystander effects depending upon the degree of cell-to-cell physical contact at the time of irradiation: intracellular cell-to-cell gap junction communication or the transmission of soluble factors from the irradiated cells to the surrounding medium.

Bystander effects with physical contact between the irradiated and bystander cells have been observed with both high LET and low LET radiations. The reported medium transfer experiments, without physical contact, have all used γ rays to induce the bystander effect. The studies of the medium transfer bystander effect have been reviewed by Mothersill and Seymour (19). Only one study, however, is available regarding the induction of the medium transfer bystander effect and direct effect by low dose neutrons (20). There is no systematic study of the bystander effect induced by neutrons. It is well known that neutron radiation is significantly more efficient than γ–rays in killing cells and inducing chromosomal aberrations, gene mutations and oncogenic transformation (21-23).

However, neutron radiation is always contaminated by a contribution from γ radiation. To differentiate the bystander effects of the two radiations, Liu *et al.* established that there is an effective dose threshold for the γ ray induced bystander effect at 3 mGy (20). If there is an effective threshold, as long as the γ-ray contamination is kept below the threshold, significant observed bystander effects should be caused solely by neutrons.

No bystander effects were found to occur in human skin keratinocytes (HPV-G) at a 1 Gy neutron dose with a 0.3 Gy γ dose generated by an accelerator (20). The neutrons were produced by bombarding a thick lithium target with 2.30 MeV protons in the laboratory's Van de Graff accelerator, inducing the $^{7}$Li (p,n) $^{7}$Be reaction. This procedure produces a broad neutron spectrum extending to 600 keV as described by Aslam *et al.* (*24*) However, the neutron and γ doses were estimated from on the accelerator current settings which may not be an accurate method as this is a measure of the protons incident on the target. The neutron dose depends both on the proton current and the condition of the lithium target. As the condition of the target is not known during an irradiation, this can lead to overestimations of the neutron dose. The

neutron dose rate used in this previous study was measured as $956 \pm 96$ mGy/h. No direct and bystander effects were observed in this previous study. As bystander effects were predicted, it was therefore decided to change the neutron source for the irradiations. In the present study, we looked at the direct effect and the bystander response induced by a neutron beam from the McMaster Nuclear Reactor (MNR). The reactor generated neutron beam has a higher neutron dose rate and lower γ contamination. Clonogenic survival was used for the endpoint.

## II. METHODS

### II.A. Cell Line and Cell Culture

The HPV-G cell line has a well-characterized and stable bystander response, a reduction in cloning efficiency of ~40% over a wide range of radiation doses, and well-characterized calcium fluxes and mitochondrial effects (25-27). This makes it ideal as a reporter system (28). All cell culture procedures were performed in a class II biosafety cabinet. The cells were grown in DMEM/F12 medium containing 60 ml FBS, 5 ml penicillin-streptomycin, 5 ml L-glutamine, 15 m*M* Hepes buffer, and 1 mg/ml hydrocortisone.

### II.B. Clonogenic Assay Technique and Bystander Protocol

Cell cultures that were 85–90% confluent and that had received a medium change the previous day were selected. Cells were removed from the flasks using 0.25% w/v trypsin/1 m*M* EDTA solution (1:1). When the cells had detached, they were resuspended in medium, and an aliquot was counted using a Z2 Coulter Particle Count and Size Analyzer. Appropriate cell numbers (~300) were pipetted for plating for the receptor or bystander flasks to optimize the ratio of signal molecules to cell number. Cell survival was determined by using the clonogenic assay technique of Puck and Marcus (29). We irradiated at low cell density for the direct experiments so we could get colonies without trypsinizing and replating the cells. Flasks destined to donate medium were plated with around $5 \times 10^5$ cells per 5 ml medium to give approximately 100,000 cells per milliliter in T-25 40-ml flasks (Nunclon, Denmark). Medium was harvested 1 hour postirradiation, which took place $6.0 \pm 0.2$ hour after plating. The harvested medium was transferred to cultures containing cloning densities of cells set up at the same time as the donors. Controls for medium only and actual radiation effects were included in each experiment. Controls for transfer of unirradiated medium from densely seeded cultures to cultures seeded at cloning densities were always included. Cultures were incubated in 5 ml of culture medium in 25-$cm^2$ T-25 40-ml flasks in a humidified 37°C incubator in an atmosphere of 5% $CO_2$ in air.

### II.C. Irradiation

It was found that the characteristics of the cells could change from experiment to experiment. In order to make sure the cells were behaving as expected and showing a bystander response, a protocol was set-up to monitor the cell quality in each experiment. The protocol used two sets of cells from the same T75 flasks: one set of cells was exposed to high γ dose radiation (e.g. 3 Gy) while the other set was exposed to the neutron radiation. The γ irradiated set acted as a quality control for the cells since the γ induced bystander data were well established. A $^{60}$Co source housed in a "hotcell" in the MNR was used as the γ source. The measured response for neutrons was considered acceptable only if a bystander response was observed in the γ irradiated cells. The data were discarded when a lack of the bystander response was observed in the γ irradiated set.

Neutron irradiations were performed at beam port three of MNR. The neutrons generated in the nuclear reactor were through a fission chain reaction. The reactor neutron spectrum has an energy distribution with an average at 1 MeV. The generated neutrons are directed through six tubes, or beam ports, from the core to the user end stations. The beam ports are sealed to be water tight at the core end and direct particles from the core of the reactor to an experiment outside of the reactor shielding. By installing appropriate filters in the beam ports, the beam can be used for specific experiments. Beam port three, which was used in this study, has been designed for real time neutron radiography. The neutron beam was not filtered and the major dose contribution was from fast neutrons. Before it was extended to radiation biology research, the beam was characterized, i.e., the neutron and gamma dose rates were determined (30). The cross section of beam port three at the user end is a rectangular window 42 cm in width and 30 cm in height. There is a shutter at a depth of 61 cm from the edge of the user end station. Radiative capture of thermalized neutrons in surrounding shielding material produces a γ-ray field, so a lead shielding box was placed at the user end to attenuate the γ rays, and minimize the γ ray dose to the cells. The thickness of the shielding box on the side closest to the reactor core was 11 cm.

Experimental microdosimetric methods were used to determine both the neutron and γ-ray doses at the user end of the beam port. A 1.27-cm-diameter spherical Rossi counter (Far West Technology) was used to measure neutron and γ ray doses at a series of low reactor operating powers. This was necessary because the detector count rate is limited by pile-up effects and dead times at full reactor power. The dose rate data at full power (3 MW) could be extrapolated from the experimental values at lower powers based on the linearity between the fluence spectrum and operating

power. Analysis of the microdosimetry counter's pulse-height distribution revealed the neutron dose rate at full power (3 MW) to be $2.0 \pm 0.2$ Gy/h and the γ-ray dose to be less than 0.8% of the neutron dose. The user end station is approximately five-meters away from the nuclear reactor core while the length of the flask is 8 cm. The dose variation across the flask is therefore negligible due to the fact that the size of the flask is much smaller than the distance between the cell culture and the neutron source. Dose measurements at different days reveal that the neutron and gamma doses were constant if the operating power level was kept at 3 MW. Monte Carlo simulations and preliminary studies with a $^{3}$He detector were also performed to determine neutron spectra.

Experiments were performed in which neutron doses were in the range from 100 to 700 mGy. For each dose, the experiments were repeated three times. In each experiment, each group (donor and directly irradiated) consisted of six flasks (three for unirradiated controls and three for irradiation). All the flasks were wrapped with aluminum foil to protect cells from the exposure to light since phenol red in the culture can produce toxicity when exposed to light. This is a routine practice in our laboratory. The cultures were irradiated at room temperature. Cultures were returned to the incubator immediately after irradiation. Cells were irradiated $6.0 \pm 0.2$ hour after plating.

The two groups of cells (directly irradiated and recipients of bystander medium) were left untouched in the incubator for 10 days. Then Giemsa was used to fix the cells, and the colonies were counted.

### II. D. Medium Transfer and Incubation

This technique has been described in detail by Mothersill and Seymour (1). The previous results showed that contact times after irradiation from 30 min to 24 hour resulted in no significant differences in the toxicity of bystander medium to unirradiated cells (1). Briefly, medium was poured off from donor flasks that had been in the incubator for 1 hour after irradiation. The medium was filtered through a 0.22-μm filter to separate the cells (~10 μm in diameter) from the transferred medium. Culture medium was then removed from the flasks designated to receive irradiated medium.

## III. RESULTS

The aim of this study is to investigate the proposed existence of a neutron induced medium transfer bystander effect. The dosimetry measurements at beam port three of the MNR showed that it has advantages over the accelerator beams previously investigated: low gamma contamination (<0.8%), reasonable neutron dose rate ($2.0 \pm 0.3$) Gy/h.

The endpoint used in this study was clonogenic survival. The clonogenic survivals for the gamma and neutron sets are shown in Table I. At 100 and 200 mGy neutron dose, the corresponding gamma sets showed that significant cell death occurred in the bystander cells, which indicates the cell behaved normally in these experiments. The clonogenic survival data from neutrons show that neither the direct nor the bystander responses are significant at neutron doses of 100 and 200 mGy. The corresponding gamma set for the 400 mGy neutrons did not show the bystander effect and the neutron data were discarded. Significant cell death from the bystander effect was found to occur at 700 mGy neutron dose. However, it is difficult to assess the respective γ and neutron contributions to the bystander effect at this dose point since the γ dose estimate at this neutron dose point is only known to be less than 6 mGy. It is unknown whether the gamma dose was above or below the previously measured γ ray dose threshold of 3 mGy. More data are needed for further study. Nonetheless, neutrons did not significantly induce the medium transfer bystander effect in the HPV-G cells when the neutron doses were equal or less than 200 mGy.

## IV. DISCUSSION

### IV. A. Neutron Direct Effect

The direct effect resulting from neutron irradiation indicates that no significant cell death was observed at doses below 200 mGy while significant cell death caused by γ irradiation occurred at 100 mGy. The neutron data appears to contradict the typical values of the RBE. The RBE effect, however, is cell line dependent. In addition, this RBE estimate does not consider the influence of different dose rates. The neutron dose rate (2 Gy/h) in this study was much lower than the γ dose rate used (18 and 108 Gy/h) in previous studies. The neutron direct effect is compared with unpublished data on direct effect for the HPV-G cells (31).

Table I Survival percentages for the irradiated and bystander cells exposed to neutron irradiation at beam port three of the MNR

| | Radiation type | Dose | Survival percentages (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Direct | | | | Bystander | | | |
| | | | 1[a] | 2[a] | 3[a] | Mean | 1[a] | 2[a] | 3[a] | Mean |
| 1 | n (γ) (mGy) | 100 (<0.8) | $96 \pm 4$ | $92 \pm 16$ | $95 \pm 2$ | $95 \pm 2$ | $103 \pm 7$ | $108 \pm 5$ | $111 \pm 9$ | $107 \pm 4$ |
| | γ (Gy) | 3 | $58 \pm 5$ | $52 \pm 6$ | $59 \pm 5$ | $57 \pm 3$ | $77 \pm 5$ | $80 \pm 5$ | $89 \pm 6$ | $82 \pm 3$ |
| 2 | n (γ) (mGy) | 200 (<1.6) | $104 \pm 7$ | $95 \pm 8$ | $99 \pm 5$ | $100 \pm 4$ | $107 \pm 4$ | $96 \pm 4$ | $89 \pm 5$ | $99 \pm 2$ |
| | γ (Gy) | 3 | $66 \pm 5$ | $51 \pm 6$ | $58 \pm 7$ | $59 \pm 4$ | $83 \pm 5$ | $83 \pm 4$ | $89 \pm 3$ | $86 \pm 2$ |
| 3 | n (γ) (mGy) | 400 (3.2) | $97 \pm 7$ | N/A | N/A | $97 \pm 7$ | $77 \pm 5$ | N/A | N/A | $77 \pm 5$ |
| | γ (Gy) | 3 | $57 \pm 9$ | N/A | N/A | $57 \pm 11$ | $96 \pm 7$ | N/A | N/A | $96 \pm 9$ |
| 4 | n (γ) (mGy) | 700 (<5.6) | $62 \pm 6$ | $71 \pm 6$ | $57 \pm 5$ | $63 \pm 3$ | $86 \pm 6$ | $87 \pm 7$ | $92 \pm 8$ | $88 \pm 4$ |
| | γ (Gy) | 3 | $47 \pm 4$ | $50 \pm 4$ | $39 \pm 2$ | $42 \pm 2$ | $72 \pm 3$ | $86 \pm 4$ | $79 \pm 9$ | $77 \pm 2$ |

[a] values are means $\pm$ SEM, n=3.

Mothersill and Seymour (31) measured the neutron direct effect on the HPV-G cell line at the Medical Research Council (MRC) in England. The gamma dose contribution from the neutron beam, unfortunately, was 49% (i.e. a γ/n dose ratio, in Grays, of 0.49). The neutron dose rate from the MRC beam was 0.205 Gy/h, which was lower than the neutron beam at the MNR. The neutron direct effect on this HPV-G cell line as measured by Mothersill and Seymour at the MRC is shown in Table II. The neutron doses given to the cells were 350 and 700 mGy. The cells irradiated by the MRC beam were in microcolonies.

Table II Neutron Direct effect on the HPV-G human Keratinocytes [a]

| n Dose (mGy) | γ dose (mGy) | Plating efficiency | Survival fraction |
|---|---|---|---|
| 0 | 0 | $19.3 \pm 0.6$ | 100 |
| 350 | 172 | $17.4 \pm 0.7$ | $90 \pm 4$ |
| 700 | 345 | $7.80 \pm 0.08$ | $41 \pm 4$ |

[a] Cells irradiated as microcolonies.

The survival percentages of the neutron direct effect at the two doses (350 and 700 mGy), corrected by the controls, are shown in Fig. 1. The three points were fitted using a broken curve (shown in Fig. 1). The best fit gives survival fraction (S. F.) as

$$S.F. = 100 \exp[-(2.4 \pm 0.2) \times Dose] \quad (1)$$

If one assumes that the direct effect from a mixed field is the sum of the effects caused by each type of radiation, one can estimate the neutron direct effect by subtracting the gamma effect from the mixed effect. The gamma direct effect that was used was the mean of three data sets (20, 25, 32). The estimated direct effect solely caused by neutrons is shown as a solid curve in Fig. 1. The equation of best fit (corresponding to the solid curve in the Figure) is

$$S.F. = 100 \exp[-(0.71 \pm 0.02) \times Dose] \quad (2)$$

The estimated direct survival fractions in the neutron dose range from 100 to 700 mGy are listed in Table III. The comparison between the estimated and the measured neutron direct effect is also shown in Table III. The gamma dose contamination at 700 mGy neutron dose at the MNR beam was estimated to be less than 6 mGy. There was no significant direct cell death at 6 mGy according to previous published data (20). The comparisons at 100 and 700 mGy do show agreement between the estimated and measured survival data while the set at 200 mGy does not. The paired γ group of 400 mGy neutron dose, which worked as a quality control of the cell response, did not show a bystander response at 3 Gy. This might suggest that neutron direct survival data at 400 mGy may not reliable. Nonetheless, they show a similar trend as the neutron dose increases and are of the same order. In addition, it is hard to estimate the uncertainties involved in the estimated group. The comparison shows the agreement between the two data sets. It confirms that no significant direct cell death occurs at 100 mGy neutron dose while significant cell death happens at 700 mGy.

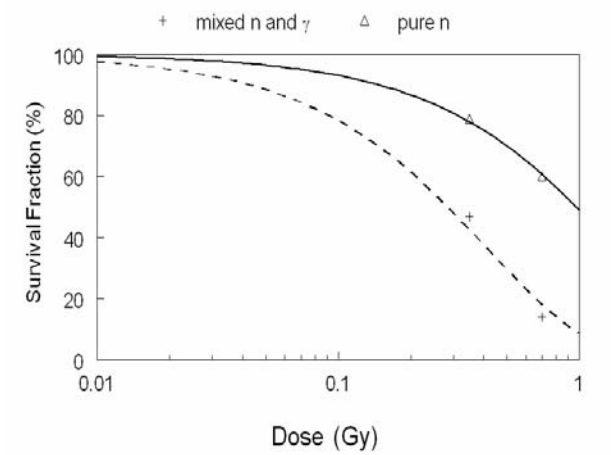


Fig. 1 Measured survival data for cells subjected to a mixed field and estimated survival data for cells subjected to a pure neutron beam. The broken line is the fitting curve for the measured data. The fitting curve for the estimated data is shown as a solid curve. The two fitting curves are shown in Eqs. (1) and (2).

Table III Survival fraction comparison of the estimated and measured neutron direct effect

| n Dose (mGy) | Survival percentages (%) Estimated | Measured |
|---|---|---|
| 100 | 93.1 | $95 \pm 2$ |
| 200 | 86.7 | $100 \pm 4$ |
| 400 [a] | 75.2 | $97 \pm 7$ |
| 600 | 65.2 | N/A |
| 700 [b] | 60 | $63 \pm 3$ |

[a] The paired γ group did not show bystander response at 3 Gy γ dose which might suggest that the data at this neutron dose point (400 mGy) may not reliable.

[b] The gamma dose contribution at this neutron dose point was estimated to be less than 6 mGy. There was no significant direct cell death at 6 mGy (20). The correction for the measured survival fraction due to γ dose contribution is negligible.

**IV. B. Neutron Bystander Effect**

Two signal emission models (exponential and bi-exponential models) have been shown (33, 34) to be plausible models for the gamma induced medium transfer bystander response observed with HPV-G cells. There is no significant difference between the two models to fit the gamma data (32). Neutron bystander data will be used to test the simpler model, exponential model. The exponential model developed in detail elsewhere (31) gives the survival fraction

$$S.F. = 100 \times \exp[-\omega(1 - e^{-D/\bar{z}_F})] \qquad (3)$$

where $D$ is dose, $\bar{z}_F$ is the frequency mean specific energy and $\omega = f(\bar{z}_F)$. Based on the exponential model, there are two possibilities to determine the parameter $\omega$ involved in the medium transfer process. If $\omega$ is proportional to the frequency mean specific energy $\bar{z}_F$, the neutron survival curve has a much lower plateau (broken curve shown in Fig. 2). If the $\omega$ involved in the neutron curve is same as that for gamma, the neutron survival curve has a higher threshold (solid curve in Fig. 2). The two possible curves are compared in Fig. 2. The $\bar{z}_F$ used in this figure was ($2.41 \pm 0.83$ Gy) which was determined from the frequency lineal energy measured using the Far West microdosimteric counter at the MNR beamport. The difference between the two curves is significant. The neutron experimental results show that no significant cell death was observed for the bystander response when the neutron doses were below 200 mGy. For the low neutron doses used, as shown in the results section, the contribution from γ rays is below the observed threshold of 2 to 3 mGy. In other words, γ induced bystander effect was negligible when the neutron doses were less than 200 mGy. These facts are inconsistent with the lower plateau case that $\omega$ is proportional to $\bar{z}_F$. The observed bystander response induced by 700 mGy reactor based neutrons easily discriminates against the first possibility so $\omega$ would have to be constant in the process of the medium transfer bystander effect.

The constant $\omega$ curve, shown in Fig. 2 as a solid curve, indicates that the neutron bystander response will not reach the plateau or saturation stage until the pure neutron dose is beyond 10 Gy. If this is true, the neutron dose rate requirement and γ dose limitation (<3mGy) greatly challenge neutron facilities. The best neutron facility we have available is the MNR which provides a 2 Gy/h dose rate with less than 0.8 % γ dose at 3 MW. The maximum neutron dose given to the cell cultures at this configuration was 700 mGy. In order to observe a significant bystander response induced by pure neutrons, the neutron dose rate has to be improved at least factor of 10, while the γ dose rate must remain the same or be lowered.

The solid curve with the frequency average specific energy $\bar{z}_F$ ($2.41 \pm 0.83$ Gy), in Fig. 2, indicates that the effective threshold for the medium transfer bystander effect induced by the reactor based neutron source is around 700 mGy. The gamma data with a much smaller $\bar{z}_F$ ($19.3 \pm 6.7$ mGy) determined a lower gamma threshold (~3 mGy). In addition, $\omega$ is a constant in both cases. One can see that $\bar{z}_F$ determines the threshold value by comparing the two curves shown in Fig. 2.

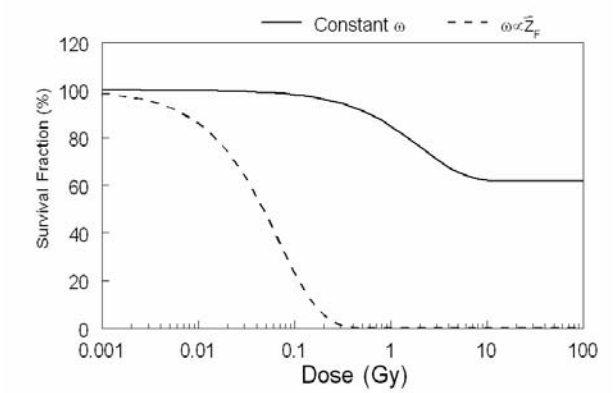


Fig. 2 Comparison of the survival curves of the bystander response induced by neutrons from two possibilities. The neutron survival curve has a much lower plateau when ω is proportional to the frequency mean specific energy $\bar{z}_F$. The neutron survival curve has a higher threshold compared with γ dose threshold if the ω is assumed to be constant in the process.

Furthermore, the exponential model could be used to predict the survival data from an accelerator based neutrons. The accelerator based neutrons have a relatively smaller $\bar{z}_F$ value ($1.65 \pm 0.57$ Gy) compared with those from the MNR. The accelerator based neutron beam has a relative lower threshold and reaches the saturation stage at a smaller dose compared to the reactor based beam. The difference of the bystander response for the two neutron beams is shown in Fig. 3. The accelerator curve indicates that significant cell death by the bystander response would occur around 5 Gy which is lower than the dose determined for the MRC. The previous study (20) indicates that both the γ contribution of the accelerator beam (7%) and the maximum neutron dose rate ($956 \pm 96$ mGy/h at 300 μA) limit further study at the Tandetron accelerator. The maximum designed current setting of the Tandetron accelerator is 1 mA which might bring the maximum neutron dose rate up to 3 Gy/h. Meanwhile more gamma shielding is required to reduce the γ contribution. It is difficult to keep a reasonable neutron dose rate (~ 3 Gy/h) with an extremely low gamma dose (~3 mGy) from the Tandetron accelerator.

High dose rate neutron facility with extremely low gamma contamination is needed to observe the neutron induced medium transfer bystander effect.

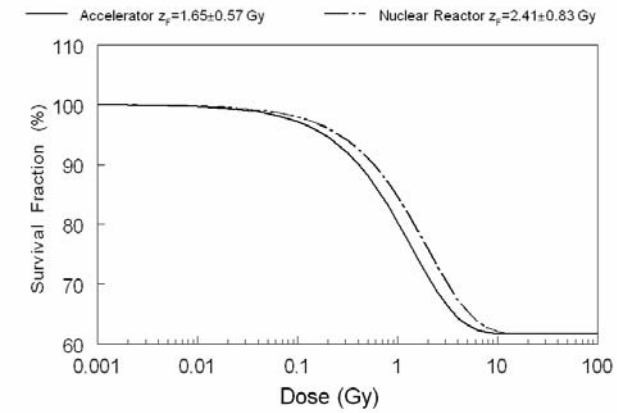


Fig. 3 Difference between the McMaster Nuclear Reactor and the Tandetron accelerator. The frequency mean specific energies for the MNR and the Tandetron accelerator were $2.41 \pm 0.83$ and $1.65 \pm 0.57$ Gy, respectively. The solid curve is for the accelerator beam while the broken line is for the reactor beam.

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