

## Trabecular bone changes induced by fast neutrons versus gamma rays in mice

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### ABSTRACT

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**Background:** The trabecular bone changes in the tibia of C3H/HeN mice were measured 12 weeks after whole body irradiation with various doses of fast neutrons (0-2.4 Gy) or <sup>137</sup>Cs-generated gamma-rays (0-6 Gy). **Materials and Methods:** Serum calcium, phosphorus, estradiol concentration and alkaline phosphatase activity were measured. Tibiae were analyzed using microcomputed tomography. Biomechanical property and osteoclast surface level were measured. **Results:** There was a significant relationship between the loss of bone architecture and the radiation dose, and the best-fitting dose-response curves were linear-quadratic. Mean relative biological effectiveness (RBE) values (Ref. gamma) of 2.05 and 2.33 were estimated for fast neutron irradiation in trabecular bone volume fraction and bone mineral density, respectively. There was a substantial reduction in osteoclast surface level in tartrate-resistant acid phosphatase-stained histological sections of tibial metaphyses in irradiated mice with high dose of neutrons. **Conclusion:** There was a significant relationship between the loss of bone architecture and the radiation dose. The difference of osteoclastic bone resorption may represent a contributor to the low RBE in high dose of irradiation level relative to that of low dose level.

**Keywords:** Neutrons, trabecular bone, osteoclast, relative biological effectiveness.

### INTRODUCTION

In adults, high dose local radiation therapy has been associated with bone loss, with the changes usually being detected radiographically (1). The primary effect of radiation on bone is atrophy, which in the case of this rigid organ involves a reduction in the number of functioning structural components to the tissue without a reduction in size. There are several primary factors that need to be considered in the pathogenesis of radiation-induced changes in bone, vascular changes, bone matrix, and cellular changes (2). Such changes are at the forefront of the development of spontaneous fractures after

irradiation (3).

The biological effects of fast neutrons in the normal tissues and tumors are of interest for clinical radiotherapy, for radiation protection purposes, and as an aid to the basic understanding of the radiation-induced inactivation of cells, whether by low or high linear energy transfer (LET) radiations. In general, the biological effects of high-LET radiations are greater than those of low-LET radiations. The variation in the relative biological effectiveness (RBE) with dose, oxygenation, cell cycle parameters, and from one tissue to another have been well documented (4). Early studies observed the effects on bone of low

-LET radiations, such as gamma rays, and several studies have shown that high-LET radiations are more effective biologically than low-LET irradiations<sup>(5-7)</sup>. However, there have been few studies of bone responses to radiation exposure at higher ionizing density, such as neutrons, and the RBE of neutrons on bone changes remains unclear.

In this study, cyclotron-derived fast neutrons with peak energy of 35 MeV were used, and we evaluated the RBE for fast neutron-induced changes in the bone using C3H/HeN mice compared with the results of parallel experiments using  $\gamma$ -rays.

## MATERIALS AND METHODS

### *Experimental animals*

Eight-week-old female C3H/HeN mice were obtained from a specific pathogen-free colony at Orient Bio Inc. (Seoul, Korea) and allowed 1 week for quarantine and acclimatization. The Institutional Animal Care and Use Committee at Chonnam National University approved the protocols used in this study, and the animals were cared for in accordance with the Guidelines for Animal Experiments. The animals were housed in a room that was maintained at  $22 \pm 2$  °C and relative humidity of  $50 \pm 5\%$ , and with artificial lighting from 08:00 to 20:00 h and with 13–18 air changes per hour. The animals were housed in groups of three per polycarbonate cage, and were given tap water and commercial rodent chow (Samyang Feed, Seoul, Korea) *ad libitum*.

### *Irradiation*

The neutrons were generated from the Korea Cancer Center Hospital (Seoul, Korea) cyclotron, using the proton-beryllium reaction. The estimated forward neutron spectra established peak energy of 35 MeV. The mean dose rate was 94 mGy/min for neutrons. The contamination of  $\gamma$ -rays was estimated as 14.2% of the neutron dose. For measuring the absorbed doses and the dose distribution of fast

neutron beam, we used two kinds of ion chambers according to the guideline proposed by International Commission on Radiation Units and Measurements<sup>(8, 9)</sup>. Exposure to  $^{137}\text{Cs}$ -generated  $\gamma$ -rays was conducted with Gammacell (Nordion, Ottawa, Canada). The mean dose rate of  $\gamma$ -rays was 2 Gy/min. Sixty mice were irradiated to the whole body with 0, 0.4, 0.8, 1.6, or 2.4 Gy of neutrons or 0, 1.0, 2.0, 4.0, or 6.0 Gy of  $\gamma$ -rays (6 mice for each dose). They were sacrificed 12 weeks after irradiation.

### *Grip strength assessment*

Grip strength was assessed using a grip strength meter (GSM) designed by IWOO-Systems (Seoul, Korea). For testing, mice were gently held so their back legs were supported with one forelimb lightly restrained. The paw being tested was brought to the bar, the mouse was allowed about 1 s to establish a grip, and the mouse was then gently pulled back in one smooth motion until grip was released. The time between trials averaged 2 s. Positive grips were scored when the mouse grasped the bar immediately with all fingers and, after release, the paw was relaxed and not clenched. Gripping force was defined as the maximum force recorded on the GSM before the mouse released the bar. Mice were given four trials per session.

### *Blood and tissue examination*

The blood samples from all the groups were withdrawn by the abdominal vein method to assess biochemical parameters. The animals were then sacrificed using ether anesthesia, and the left tibia was collected, cleaned of all non-osseous tissue, measured for length and weight, fixed in 10% neutral formalin for 48 h and stored in 70% ethanol. Tibia length was considered as the maximal distance between the proximal condyles and malleolus. The freshly isolated right tibiae were assessed for their biomechanical strength. The freshly isolated bones (tibia) were assessed for their biomechanical strength using the tensile strength testing apparatus. Three-point bending tests were performed using an Instron 3344

(Instron, Norwood, MA, USA). Lateral surface of the tibia at the tibio-fibular junction was placed upon the first point and proximal tibia upon the other. A rounded press head compressed the middle of the tibial shaft until fracture occurred. Serum calcium (Ca) and inorganic phosphorus (P) concentrations and serum alkaline phosphatase (ALP) activity were measured on an automatic analyzer (Fuji Dri-chem, Fujifilm, Tokyo, Japan) using a diagnostic slide. The levels of estradiol (E<sub>2</sub>) were determined using a specific and sensitive double-antibody radioimmunoassay kit (Estradiol Coat-A-count, Diagnostic Products Corp., Los Angeles, CA, USA) on a gamma-ray counter (EG & G, Wallace, Finland). Morphological measurements, including bone volume fraction (bone volume/tissue volume, BV/TV), trabecular thickness/separation/number (Tb.Th, Tb.Sp, Tb.N), structure model index (SMI), cortical BV and mean polar moment of inertia (pMOI) were calculated from the resulting microcomputed tomography ( $\mu$ -CT) data for each mouse using a Skyscan 1172 apparatus (Skyscan, Kontich, Belgium). The regions of interest for analysis were the proximal tibia metaphysis. User-defined contours were outlined on every fifth slice of a 150 slice region extending 2.5 mm distally from the growth plate, starting at the point where the growth plate tissue was no longer visible in the grayscale computed tomography (CT) slice. The proximal 90 slice region was used when analyzing the trabecular bone, and the most distal 60 slices were used when analyzing the cortical bone. For quantification of the trabecular volumetric mineral density (BMD), the  $\mu$ -CT was calibrated using two standard phantoms with a density of 0.25 and 0.75 g/cm<sup>3</sup>. The image slices were reconstructed and analyzed using CTan analyzer software (Skyscan). Following tomographic analysis, the tibiae were decalcified using a formic acid solution and embedded and cut into sagittal sections with a thickness of 3  $\mu$ m. Each slide was stained with tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA) to identify the osteoclast surface and counterstained with methyl green. Histomorphometric evaluation

was performed from captured micrographs (400X) throughout the metaphysis, starting approximately 0.25 mm distal from the growth plate (to exclude the primary spongiosa) and extending a further 0.5 mm. Osteoclast surface measurements were quantified relative to total trabecular bone surface (Oc.S/BS).

### Statistical analysis

The statistical significance of differences between the results in irradiated and control groups was determined by the two-tailed Student's *t*-test using a Graph PAD In Stat (GPIS) computer program (GraphPad Software, San Diego, CA, USA). A value of  $p < 0.05$  was considered statistically significant. The data are reported as the mean  $\pm$  standard error (SE).

## RESULTS AND DISCUSSION

Grip strength and body weight did not differ among the five groups. No dose-dependent differences were apparent among the five groups with regard to mechanical property, lengths and weights of tibia (data not shown).

The effects of neutron irradiation on serum biochemical markers are summarized in figure 1. As compared with unirradiated group, there was no dose-dependent difference in all of the markers.

$\mu$ -CT revealed that proximal tibial metaphysis from irradiation group had less trabecular bone compared to the sham group. Figure 2 shows the percent of control of bone change for each dose. Bone loss was markedly enhanced by irradiation. The dose-response curves were analyzed using the best-fitting curve model. The dose-response curves were linear-quadratic and a significant relationship was found between the bone change and dose. Taking the controls into accounts, the lines of best fit are as follows:

### $\gamma$ -rays:

$$BV/TV \quad y = 0.429D^2 - 13.91D + 100 \quad (r^2 = 0.971)$$

$$BMD \quad y = -0.265D^2 - 8.046D + 100 \quad (r^2 = 0.960)$$

### Neutrons:

$$BV/TV \quad y = 2.157D^2 - 27.12D + 100 \quad (r^2 = 0.997)$$

$$BMD \quad y = 2.851D^2 - 22.475D + 100 \quad (r^2 = 0.986)$$

Where  $y$  = % of control and  $D$  = the irradiation dose in Gy.

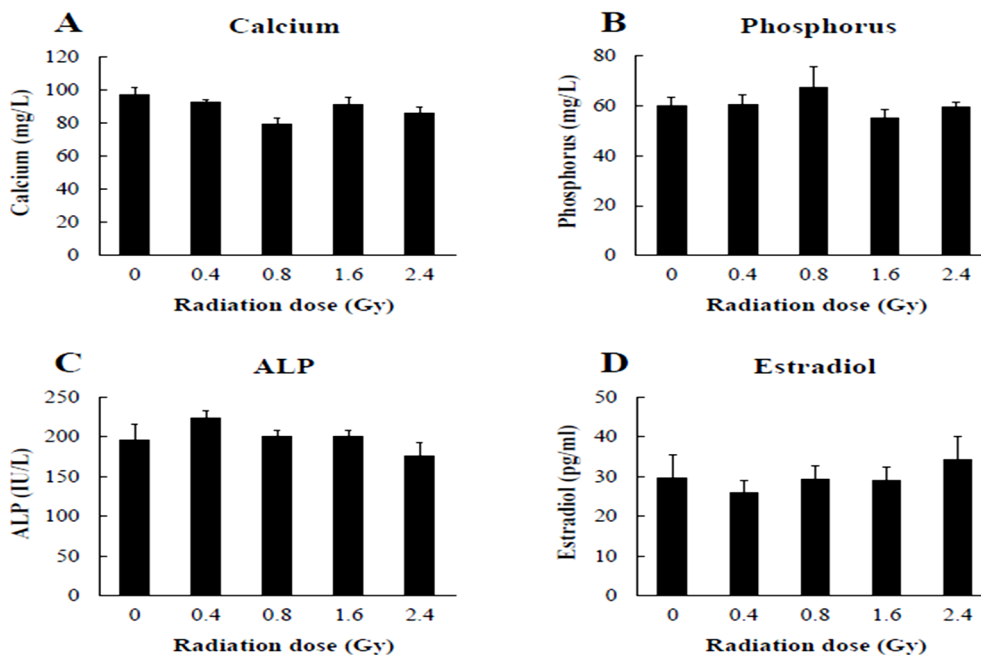


Figure 1. Dose-related changes in serum biochemical markers at 12 weeks after neutron-irradiation. (A) calcium, (B) phosphorus, (C) ALP, (D) estradiol. The data are reported as the mean  $\pm$  SE (n=6).

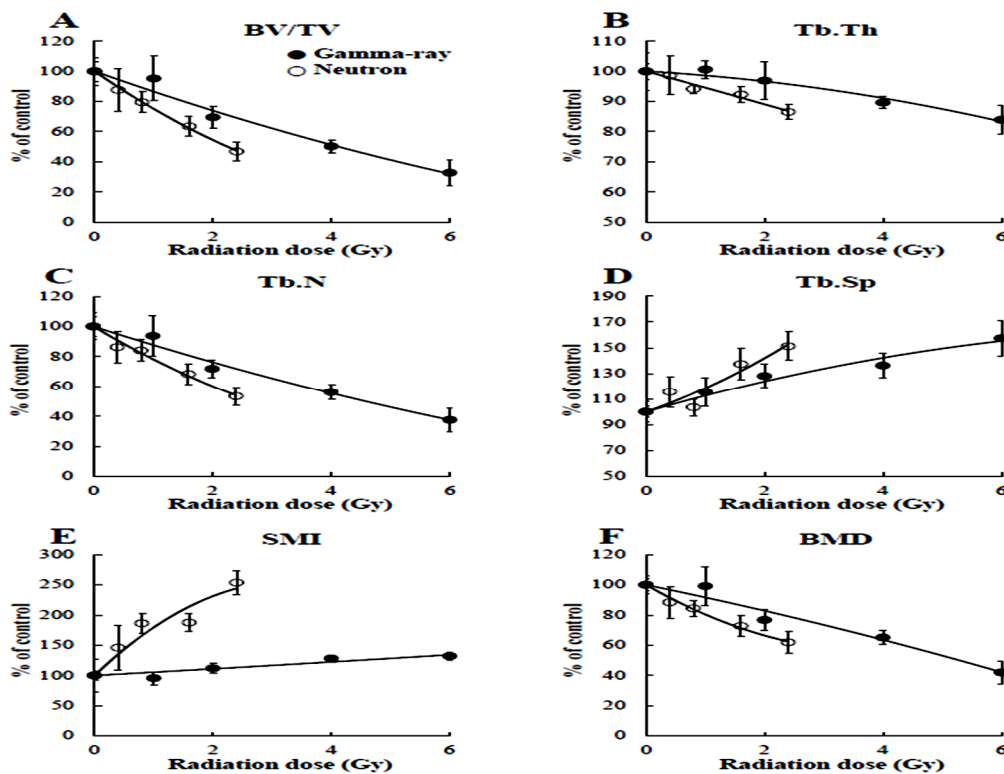


Figure 2. Dose-response curves for changes in trabecular bone properties at 12 weeks after exposure to neutrons (o) or  $\gamma$ -rays (●) in mice tibia. Data (% of control) are reported as mean  $\pm$  SE (n=6). (A) BV/TV: bone volume fraction; (B) Tb.Th: trabecular thickness; (C) Tb.N: trabecular number; (D) Tb.Sp: trabecular separation; (E) SMI: structure model index; (F) BMD: trabecular volumetric mineral density.

Since the neutrons are mixed neutron- $\gamma$  radiation, the induced rate by neutrons ( $V_{n+\gamma}$ ) can be approximated by  $V_{n+\gamma} = pV_n + (1-p)V_\gamma$ , where  $p$  is the fraction of the neutron dose contributing to the total dose of fast neutrons,  $V_n$  is the value induced by neutrons, and  $V_\gamma$  is the value induced by  $\gamma$ -rays.  $V_{n+\gamma} = pV_n + (1-p)V_\gamma$  can be rewritten as  $V_n = V_\gamma + (V_{n+\gamma} - V_\gamma) \div p$ . When analyzed by the linear-quadratic model, the lines of best fit of the theoretical dose-response to neutrons are as follows:

$$BV/TV \quad y = 2.443 D^2 - 29.306 D + 100 \quad (r^2 = 0.996)$$

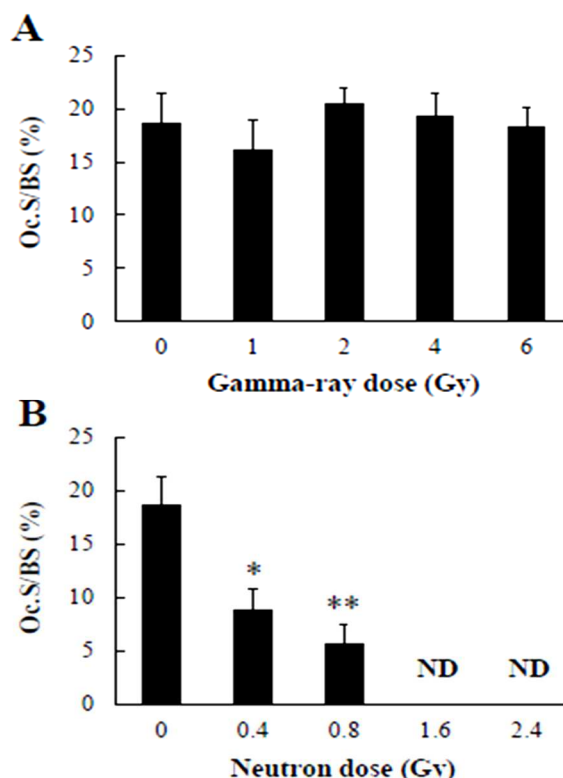
$$BMD \quad y = 3.366 D^2 - 24.863 D + 100 \quad (r^2 = 0.984)$$

In order to determine the RBE of neutrons compared with  $\gamma$ -rays, the equation,  $y = aD^2 + bD + c$  was transformed as  $D = [-b \pm \sqrt{b^2 - 4a(c-y)}] \div 2a$ . The RBE of the neutrons were obtained from this equation. Mean neutron RBE values (Ref. gamma) of 2.05 and 2.33 was estimated for irradiation of trabecular bone volume fraction and of bone mineral density, respectively (table 1).

TRAP-stained sections of bone were used to quantify osteoclast surface measurements within tibia metaphyses. The Oc.S/BS was markedly decreased by neutron irradiation, whereas the change of that was mild in the irradiation group with  $\gamma$ -rays (figure 3).

No significant differences were apparent between the control and irradiation groups with regard to the cortical bone microarchitecture (data not shown).

The present results demonstrate that bone loss is significantly increased from adult C3H/HeN mice at 12 weeks after a single whole-body dose of fast neutron or  $\gamma$ -ray irradiation. In addition, it is dose-dependent.



**Figure 3.** Dose-related changes in osteoclast surface as a percentage of total bone surface (Oc.S/BS). (A) gamma-ray, (B) neutron. Data are expressed as mean  $\pm$  SE (n=6). ND: not detected. \*P<0.01, \*\*P<0.05, vs unirradiated control group.

**Table 1.** Empirical and theoretical values for the decline frequency of bone volume fraction and BMD in trabecular bone of tibia by  $\gamma$ -rays ( $V_\gamma$ ), neutron- $\gamma$  mixed radiation ( $V_{n+\gamma}$ ) and neutrons ( $V_n$ ).

Parameter and Frequency (% of sham control)	Required dose (Gy) of $V_\gamma$ ( $DV_\gamma$ )*	Required dose (Gy) of $V_{n+\gamma}$ ( $DV_{n+\gamma}$ )*	Required dose (Gy) of $V_n$ ( $DV_n$ )*	RBE	
				( $DV_\gamma/DV_{n+\gamma}$ )	( $DV_\gamma/DV_n$ )
<b>BV/TV</b>					
90	0.74	0.38	0.35	1.95	2.11
70	2.32	1.23	1.13	1.89	2.05
50	4.12	2.24	2.06	1.84	2.00
Mean value				1.89	2.05
<b>BMD</b>					
90	1.20	0.47	0.43	2.55	2.79
75	2.84	1.34	1.20	2.12	2.37
60	4.35	2.71	2.37	1.61	1.84
Mean value				2.09	2.33

\*Calculated from best fitting linear-quadratic model. BV/TV: bone volume fraction; BMD: trabecular volumetric mineral density.

Osteopenia and osteoporosis is perhaps the most serious clinical complication of radiotherapy for the treatment of cancers. Since 50% of all cancer patients receive radiation therapy <sup>(10)</sup>, it is of clinical importance to understand the mechanisms underlying these iatrogenic complications.

The biological effectiveness of high-LET radiation is generally much greater than that of low-LET radiation. This is referred to as the RBE. The same dose of high-LET particles (e.g. fast neutrons, carbon ions) will usually produce a greater biological result in living systems than low-LET particles (e.g. photons or protons) <sup>(11,12)</sup>. A wide range of RBE values have been reported for fast neutrons <sup>(13)</sup>, and RBE values have been estimated for multiple tissues and organs, including skin (small follicle: 2.09, large follicle: 2.15), intestine (base crypt: 4.03, total crypt: 3.87), and testis (semiferous tubules: 5.18) exposed to 35 MeV neutrons <sup>(14)</sup>. In this study, neutron RBE values of 2.05 (BV/TV) and 2.34 (BMD) were obtained in the relative frequency of bone change, which means that the bone changes in mouse tibia is sensitive to a difference in radiation quality. On the other hand, Hamilton et al. <sup>[15]</sup> reported no differences between low-LET (<sup>60</sup>Co  $\gamma$ -rays, LET = 0.23 keV/ $\mu$ m; <sup>1</sup>H<sup>1+</sup>, 250 MeV/n, LET = 0.4 keV/ $\mu$ m) and high-LET radiations (<sup>12</sup>C<sup>6+</sup>, 290 MeV/n, LET = 13 keV/ $\mu$ m; <sup>56</sup>Fe<sup>26+</sup>, 1 GeV/n, LET = 148 keV/ $\mu$ m), and no differences for bone loss were observed at the dose of 2 Gy. Ten-week-old, female C57BL/6 mice were used and the bones harvested at 110 days after irradiation. The mice we used in this study were 9 weeks of age, so it would seem quite possible, that the dose dependent differences in parameters may be due as much to an inhibition of normal bone formation as to loss of bone. Therefore, it appears that the RBE for bone change depends on strain and age of mice, duration of experiment, radiation type, intensity and/or dose.

In this study, the neutron group showed significantly reduced osteoclast surface compared with the gamma ray group. Neutron irradiations had a more marked effect on

osteoclast activity than gamma irradiations. These observations suggest that neutron irradiations induce differential modulation of osteoclast response. The present study is the first report of a dose-response relationship of neutron-induced bone loss in the tibia of mice. The yield of the bone changes was linear-quadratically related to the dose. Generally, the dose-effect relationship in the biological response induced by neutrons is best fit to a linear model, while low-LET radiation-induced response fits a linear-quadratic model. However, most of the data has been derived from *in-vitro* studies with acute high irradiation dose. Several *in-vivo* experiments that demonstrated the dose-response curves of some neutron-induced tissue injuries fit to the linear-quadratic model, such as the normal tissues reported by Lee *et al.* <sup>(14)</sup>, Broerse and Barendsen <sup>(16)</sup> and International Agency for Research on Cancer (IARC) <sup>(17)</sup>.

Although some studies have shown significant correlation between grip strength, biomechanical property and BMD <sup>(18,19)</sup>, there was no significant correlation among these markers in the current study. The absence of an effect of radiation on cortical bone parameters in the present study is in agreement with previous findings <sup>(20)</sup>. Other studies investigating cortical bone after irradiation have found changes in cortical bone strength and porosity after very high doses <sup>(21, 22)</sup>. We investigated parameters of BV and pMOI in cortical bone; the results confirmed that bone loss after neutron and  $\gamma$ -irradiations is specific to trabecular bone in the dose range of this study.

## CONCLUSION

In this study, cyclotron-derived fast neutrons with peak energy of 35 MeV were used, and we evaluated the RBE for fast neutron-induced changes in the bone using C3H/HeN mice compared with the results of parallel experiments using  $\gamma$ -rays. This study established a linear quadratic dose-effect relationship of bone change in the tibia of

C3H/HeN mice for fast neutrons and  $\gamma$ -rays. Based on the dose-response data, the RBE values of fast neutrons (Ref. gamma) were estimated to be 2.05 for BV/TV and 2.34 for BMD. Further mechanistic studies on the neutron-induced changes of bone and osteoclastic activity will be needed to extrapolate the experimental data for radiation protection in humans.

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**Conflicts of interest:** none to declare.

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