Citation: BoneKEy Reports **6**, Article number: 855 (2017) | doi:10.1038/bonekey.2016.87

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ORIGINAL ARTICLE

Repeated irradiation from micro-computed tomography scanning at 2, 4 and 6 months of age does not induce damage to tibial bone microstructure in male and female CD-1 mice

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Long-term effects of repeated *in vivo* micro-computed tomography (μCT) scanning at key stages of growth and bone development (ages 2, 4 and 6 months) on trabecular and cortical bone structure, as well as developmental patterns, have not been studied. We determined the effect of repetitive μCT scanning at age 2, 4 and 6 months on tibia bone structure of male and female CD-1 mice and characterized developmental changes. At 2, 4 and 6 months of age, right tibias were scanned using *in vivo* μCT (Skyscan 1176) at one of three doses of radiation per scan: 222, 261 or 460 mGy. Left tibias of the same mice were scanned only at 6 months to serve as non-irradiated controls to determine whether recurrent radiation exposure alters trabecular and cortical bone structure at the proximal tibia. In males, eccentricity was lower ($P < 0.05$) in irradiated compared with non-irradiated tibias (222 mGy group). Within each sex, all other structural outcomes were similar between irradiated and non-irradiated tibias regardless of dose. Trabecular bone loss occurred in all mice due to age while cortical development continued to age 6 months. In conclusion, repetitive μCT scans at various radiation doses did not damage trabecular or cortical bone structure of proximal tibia in male and female CD-1 mice. Moreover, scanning at 2, 4 and 6 months of age highlight the different developmental time course between trabecular and cortical bone. These scanning protocols can be used to investigate longitudinal responses of bone structures to an intervention.

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Introduction

Micro-computed tomography (μCT) is an imaging technology that enables the non-destructive visualization and assessment of three-dimensional structural properties of objects at a micron scale.[1] μCT has become standard practice in rodent studies examining bone structure because of its power to visualize and quantify the three-dimensional structure of trabecular and cortical bone compartments that were previously performed using two-dimensional histological techniques. This imaging technology has also become customary because of its increasing accessibility within the bone research community, allowing for the investigation of a wide range of interventions—drugs,[2,3] diet,[4,5] exercise,[6] mechanical loading[7,8] or unloading[9]—on bone structure and morphometry.

In vivo μCT is a scanning system that permits the longitudinal assessment of bone structure and morphology in rodents such as mice and rats. Unlike *ex vivo* μCT systems that evaluate bone structure using excised samples, repeated scanning using *in vivo* μCT enables the measurement of structural changes within the same bone over a lifespan which minimizes the number of animals needed in a study and provides for a more powerful study design compared with cross-sectional analyses.

Despite the high utility for *in vivo* μCT technology in bone research, repeated exposure of the hind limb to ionizing radiation may result in tissue damage leading to a loss of bone tissue in trabecular and cortical compartments[10–12] and, ultimately, skeletal damage could exceed the effect size of the intervention.[10] Thus, while sufficient radiation exposure is

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Received 19 August 2016; accepted 10 November 2016; published online 13 January 2017

needed to achieve an acceptable image quality that enables quantitative evaluation of bone structure,[10,13] it is imperative that scanning parameters are optimized to achieve a collective dose of radiation that is low enough to ensure that tissue damage does not ensue.

In mouse studies, some have assessed the resilience of bone tissue to radiation exposure by modifying the dose and frequency of ionizing radiation emitted by *in vivo* μCT systems. For example, in male adult mice, three exposures to 776 mGy at 2-week intervals resulted in lower bone volume in the irradiated tibia compared with the non-irradiated control tibia.[10] In female adult mice, four exposures to 845.9 mGy at 1-week intervals also resulted in lower bone volume in the irradiated tibia compared with the non-irradiated control tibia.[11] These studies indicate that lower doses of ionizing radiation and/or less frequent exposures than what were applied[10,11] are required to minimize or eliminate damage to the tibia of male and female mice. In contrast to mouse studies, rat studies demonstrate that *in vivo* μCT scans performed at weekly to monthly intervals, with up to eight exposures and using doses as high as 939 mGy per scan, does not affect bone structure at the proximal tibia.[11,14,15] This apparent resilience of rat bones to ionizing radiation may be due to their larger and thicker skeletons that result in a lower absorbed dose compared with mouse bones. Nonetheless, findings obtained from rat models cannot be extrapolated to mice.

To determine the limit of radiation exposure on bone structure in mice, some studies have investigated the effects of lower doses of radiation using short-term or long-term designs. In growing and adult male mice, three exposures to 166 mGy in 2-week intervals did not result in diminished bone structure at the irradiated tibia compared with the non-irradiated control tibia.[10] In growing male and female mice, five exposures to 188 mGy per scan over a total of six weeks also resulted in no differences in trabecular bone at the lumbar vertebra compared with other male and female mice that were exposed to 188 mGy twice over six weeks.[16] Using a long-term study design, female mice exposed to 188 mGy for a total of twelve exposures between the ages of six and forty-eight weeks resulted in similar bone structure at the lumbar vertebra compared with other female mice that were scanned at half the frequency during the same age period.[17] To further test the limit of radiation exposure on bone structure, three times exposure to 434 mGy per scan in two-week intervals did not affect trabecular or cortical bone structure at the proximal tibia growing and adult male mice.[10] Collectively, these mouse studies demonstrate that the safety limit for radiation exposure for skeletal tissue in male mice range between 434 and 776 mGy when bones are irradiated three times in two-week intervals. It is uncertain whether the same safety limit for radiation exposure applies to female mice as no studies applying these doses (434–776 mGy) have been investigated.

Since scanning images obtained at a lower radiation dose (166 mGy) compared with a higher dose (434 mGy) results in images with lower resolution and significantly different values for outcomes of bone structure,[10] it is of interest to determine the effects on skeletal structure of male and female mice from repeated exposure to the same scanning parameters that provide reasonable image resolution and quality. Thus, doses higher than 166 mGy per scan are needed.[10] In addition, since most mouse studies were short (fewer than 6 weeks) in duration,[10,11,16] it is important to understand if there are consequences to bone structure over long time periods using radiation doses that provide reasonable image quality (that is, at doses >166 mGy per scan). Indeed, published μCT scanning methods[18] and guidelines on μCT scanning of rodent bones[19] acknowledge the need for longer-term studies to determine effects of repeated exposure to radiation from μCT scanning.

Thus, the objective of the present study was to determine the effect of repeated μCT scanning at 2, 4 and 6 months of age, using scanning parameters that result in acceptable image quality,[10] on trabecular and cortical bone structure in the proximal tibia of both male and female CD-1 mice. The proximal tibia was selected because it is a standard site for evaluating bone structure in rodents.[19] Because the present study aimed to scan less frequently (that is, every two months versus every two weeks[10]) to represent key stages of bone growth and development, we tested three different sets of scanning parameters that produce reasonable image contrasts. In addition, we chose to examine the skeletal responses to the same *in vivo* μCT scanning protocol and radiation dose on bone structure in both male and female mice as granting agencies such as the NIH in the US and the CIHR in Canada are increasingly interested in researchers studying sex-specific responses. A secondary objective was to characterize the developmental changes in trabecular and cortical bone from 2 through 6 months of age, which has not been previously reported for CD-1 mice.

Results

Body weight

At 2 months of age, initial body weights were similar ($P>0.05$) among all groups within males (**Figure 1a**) and females (**Figure 1b**). As expected, there was an effect of time ($P<0.001$) as the mice were growing. There were no dose ($P>0.05$) or dose $\times$ time interaction ($P>0.05$) effects on body weight throughout the study in male or female mice (**Figure 1**).

Effects of repeated irradiation on trabecular and cortical structure of proximal tibias

At 6 months of age, structural properties of trabecular and cortical bone were compared between the right tibia (that underwent irradiation at 2, 4 and 6 months of age) and left tibia (that was irradiated only at 6 months of age) within each mouse to determine whether repeated irradiation alters bone structure.

In male mice, no differences ($P>0.05$) in bone volume (BV, mm^3) (data not shown), total volume (TV, mm^3) (data not shown), bone volume fraction (BV/TV, %), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), degree of anisotropy (DA, no unit) or connectivity density (Conn.D, mm^{-3}) were observed between the tibias that received repeated irradiation (right tibias) and their contralateral controls (left tibias) (**Figure 2a**). For cortical bone structural properties, irradiated tibias resulted in lower ($P<0.05$) eccentricity (Ecc, no unit) compared with non-irradiated tibias in the 222 mGy group but not within other dose groups (**Figure 2b**). Within all radiation dose groups, no differences ($P>0.05$) in cortical bone area (Ct.Ar, mm^2; data not shown), total cross-sectional area inside the periosteal envelope (Tt.Ar, mm^2; data not shown), cortical area fraction (Ct.Ar/Tt.Ar, %), cortical thickness (Ct.Th, mm), periosteal

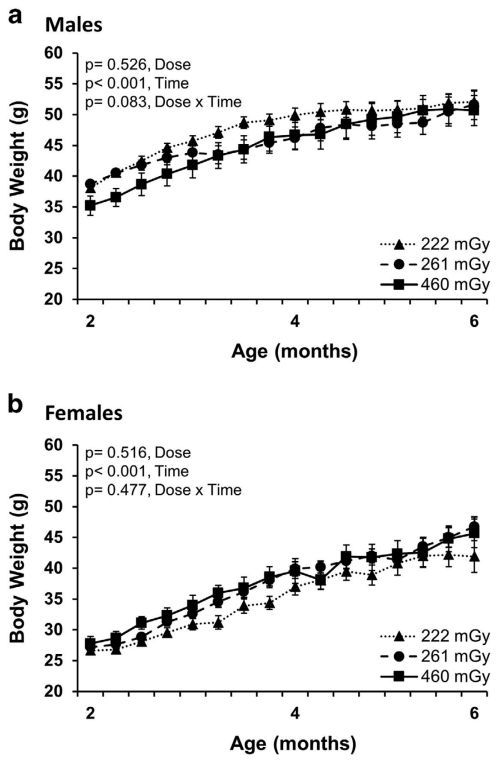

Figure 1 Body weight of male (**a**) and female (**b**) CD-1 mice throughout the study. $n = 9$–12 mice per group. Mean ± s.e.m.

perimeter (Ps.Pm, mm), endocortical perimeter (Ec.Pm, mm), and bone marrow area (Ma.Ar, mm^2) were observed between the irradiated and non-irradiated tibias (**Figure 2b**).

In females, there were no significant differences ($P > 0.05$) in trabecular structure outcomes between the irradiated (right tibias) and non-irradiated (left tibias) within each radiation dose group (**Figure 3a**). Likewise, no significant differences ($P > 0.05$) in structural or geometric properties of cortical bone (**Figure 3b**) were observed between the irradiated and non-irradiated tibias within each group.

Longitudinal observations of trabecular and cortical structure of proximal tibia

Trabecular and cortical structures of the right proximal tibias were evaluated at 2, 4 and 6 months of age. In male mice (**Table 1**, **Figure 4**), a diminution of trabecular bone structure was observed within each dose group that was more evident in the 261 and 460 mGy groups as characterized by decreases in TV ($P < 0.05$) and Conn.D ($P < 0.01$) with a concomitant increase in Tb.Sp ($P < 0.01$). In addition, main effects were observed in both the 261 and 460 mGy groups for DA ($P < 0.05$, 261 and 460 mGy groups) and Tb.N ($P < 0.01$, 460 mGy group). Variable BV and BV/TV was observed in the 222 mGy group, that increased from 2 to 4 months of age and returned closer to 2 month levels at 6 months of age (**Table 1**). Also in males, cortical bone structure increased from 2 to 6 months of age within each dose group but was more prominent in the 460 mGy group as characterized by increases in Ct.Ar ($P < 0.001$), Ct.Ar/Tt.Ar ($P < 0.001$), and Ecc ($P < 0.05$) with concomitant decreases in Tt.Ar ($P < 0.001$), Ec.Pm ($P < 0.05$), and Ma.Ar ($P < 0.001$). In the 261 mGy group, a decrease in Tt.Ar ($P < 0.05$) and increase in Ct.Th ($P < 0.05$) was observed from 2 months to 6 months of age. In the 222 mGy group, an increase in Ct.Ar ($P < 0.001$) and Ct.Th ($P < 0.05$) were observed as well as a decrease in Ecc ($P < 0.05$; **Table 1**).

In female mice, a diminution in trabecular structure was observed within each radiation dose group from 2 through 6 months of age (**Table 2**, **Figure 4**). Significant decreases in BV, BV/TV, Tb.N and Conn.D ($P < 0.05$) with a significant increase in Tb.Sp ($P < 0.05$) (**Table 2**) due to age was observed within each group. In addition, all groups experienced a significant increase in Tb.Th ($P < 0.001$) over the course of the study. The 261 and 460 mGy groups, but not the 222 mGy group, experienced a significant decrease in TV ($P < 0.01$) from 2 to 4 months that persisted to 6 months of age. DA increased ($P < 0.05$) by 6 months of age in the 460 mGy group. In female mice, cortical bone structure changed within each radiation dose group whereby Ct.Ar, Ct.Ar/Tt.Ar, and Ct.Th increased ($P < 0.05$) with increasing age (**Table 2**). In addition, the 222 mGy group experienced a decrease in Ma.Ar from 2 to 4 months of age ($P < 0.05$) while the 460 mGy group experienced decreases in Tt.Ar, Ps.Pm, Ec.Pm and Ma.Ar from 2 to 4 months of age ($P < 0.01$). No changes in Ecc were observed within any of the dose groups.

Discussion

Using 3 doses of ionizing radiation (222, 261 and 460 mGy), the present study demonstrated that trabecular and cortical bone structure in male and female CD-1 mice are resilient to repeated scanning of the tibia at 2, 4 and 6 months of age, representing key stages of growth and bone development. Even at the highest dose of radiation, no differences in trabecular or cortical bone structure were observed between the repeatedly scanned leg and its non-irradiated contralateral control. The finding that the highest of the three radiation doses studied (460 mGy) resulted in the greatest number of age-specific changes in bone microstructure agrees with previous work[10] that demonstrated that 434 mGy improved sensitivity and accuracy for imaging and evaluating bone microstructure in mice compared with a lower dose (166 mGy). Thus, future studies examining these same stages of skeletal growth and development should use scanning parameters that emit a radiation dose ranging between 434 and 460 mGy per scan.

With regards to examining the effects of repeated radiation exposure on bone structure, repeated irradiation with 222 mGy at 2, 4 and 6 months of age resulted in lower Ecc at the proximal tibia compared with the non-irradiated limb in males at 6 months of age. This may be a result due to chance since exposures to higher doses of radiation (that is, 261 and 460 mGy), which produce greater image quality (**Figure 4**),[13] did not have the same effect. In addition, the longitudinal male data of the irradiated limbs demonstrated a decrease (222 mGy dose), increase (460 mGy dose) or no change (261 mGy) in Ecc from 2 months to 6 months of age. Despite the observed difference in Ecc between the irradiated and non-irradiated tibias in the 222 mGy group, there were no other differences in trabecular or cortical bone structure regardless of dose. This finding demonstrates that the radiation doses applied in the present study did not affect trabecular or cortical bone structure in CD-1 mice.

With regards to examining the longitudinal changes in trabecular bone structure from 2 through 6 months of age,

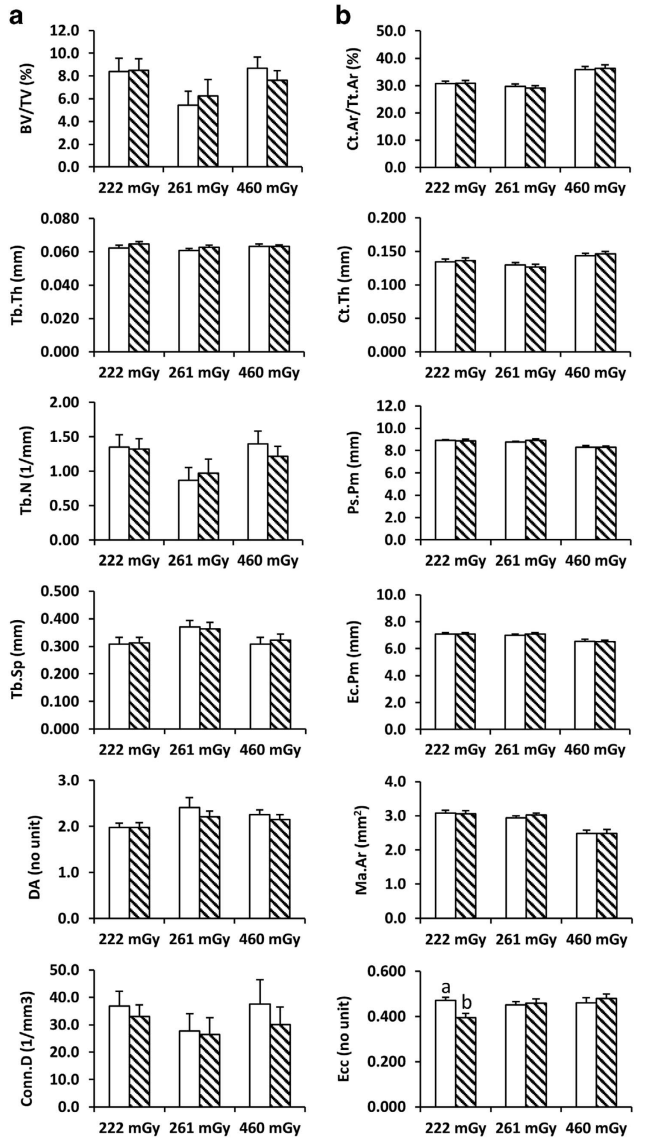

Figure 2 Trabecular (**a**) and cortical (**b**) bone structure of the irradiated right tibias (hatched columns) and non-irradiated left tibias (white columns) of 6-month-old male CD-1 mice. $n = 7$–11 mice per group. Mean ± s.e.m. Different letters denote statistical significance at $P < 0.05$ between irradiated (right) and non-irradiated (left) tibias for trabecular or cortical bone structure within each irradiation dose.

a diminution was observed in both male and female CD-1 mice. The observations that several measures of trabecular bone structure decreased from 2 to 4 months of age indicates that peak trabecular bone structure is achieved before 4 months of age. Indeed, these changes were due to the skeletal physiology of CD-1 mice rather than effects of irradiation since tibial bone structures at 6 months of age within a mouse were similar between the tibia that received repeated irradiation and the tibia that did not. In male mice, the diminution in trabecular bone microstructure was more notable with the two higher radiation doses (261 mGy, 460 mGy). In contrast, the 222 mGy radiation dose resulted in a variable change in BV/TV in male trabecular bone microstructure, resulting in BV/TV at 6 months of age that was intermediate to that at 2 and 4 months of age. This observation may be due to differences in image quality attributed to the different radiation doses (**Figure 4**).[10,13,20] In tibias of mice that were exposed to either 166 or 434 mGy of ionizing radiation by μCTscanning, image quality in the 166 mGy scans was lower than compared with that from the 434 mGy scans and resulted in trabecular bone microstructure that was significantly different between the two dose groups.[10] Similar to the males, the diminution of trabecular bone structure in females was more notable with the two higher radiation doses (261 mGy, 460 mGy). Thus, in both male and female CD-1 mice, peak trabecular bone structure is achieved before 4 months of age.

The longitudinal patterns of trabecular bone structure in female mice of other mouse strains have been evaluated using *in vivo* μCT scanning.[11,17] These studies demonstrate that trabecular bone development at the proximal tibia differs among female C3H/HEN, C57BL/6 and BALB/c inbred mice. Moreover, in comparing these data[17] with those from the present study, female C3H/HEN, C57BL/6 and BALB/c mice experienced a continued development of trabecular bone structure between two and four months of age[17] while CD-1 mice examined in the present study experienced a diminution during the same time period. Future investigation that includes various mouse strains including CD-1 is needed to determine how the longitudinal growth patterns of bone structure in CD-1 mice directly compares with other strains.

In males, a continuous development in cortical bone structure occurred throughout the study and was characterized by an increase in Ct.Th among all groups. However, mice exposed to 460 mGy exhibited the greatest changes in cortical bone structure that may be due to a higher image quality achieved by a higher radiation dose. In females, Ct.Ar/Tt.Ar and Ct.Th continued to increase through 2 to 6 months of age within all dose groups with the most notable changes occurring in the 460 mGy group. Others[11,17] have observed increases in cortical bone properties past 2 months of age in female C3H/HeJ, C57Bl/6J and BALB/cByJ mice, indicating that within several mouse strains, cortical bone development reaches its peak at a later time point compare with trabecular bone. Collectively, findings from the present study highlight the different developmental time course between trabecular and cortical bone in both male and female CD-1 mice. Thus, for both male and female mouse studies, a study duration of approximately 4 months long may be sufficient when the investigative focus surrounds the responses of trabecular bone to specific interventions. Longer studies of at least 6 months may be needed for examining effects on cortical bone.

Our 222 mGy dose was achieved by applying the same scanning parameters previously used by others to obtain a dose of 434 mGy,[10] which highlights the possibility that different scanning systems may produce distinct radiation doses using identical scanning parameters. Indeed, differences in scanning efficiencies and distances from source to object among μCT scanning systems affect the amount of ionizing radiation that reaches the scanned sample. A future study that directly compares the measured levels of radiation from different scanning systems that apply identical scanning parameters would be appropriate. Different radiation doses of distinct scanning systems may involve the type of radiation detection equipment used. The present study measured the radiation dose for each set of scanning parameters using a MOSFET portable dosimeter that was calibrated to an ion chamber while others used an ionizing chamber.[10,11] Nevertheless, radiation

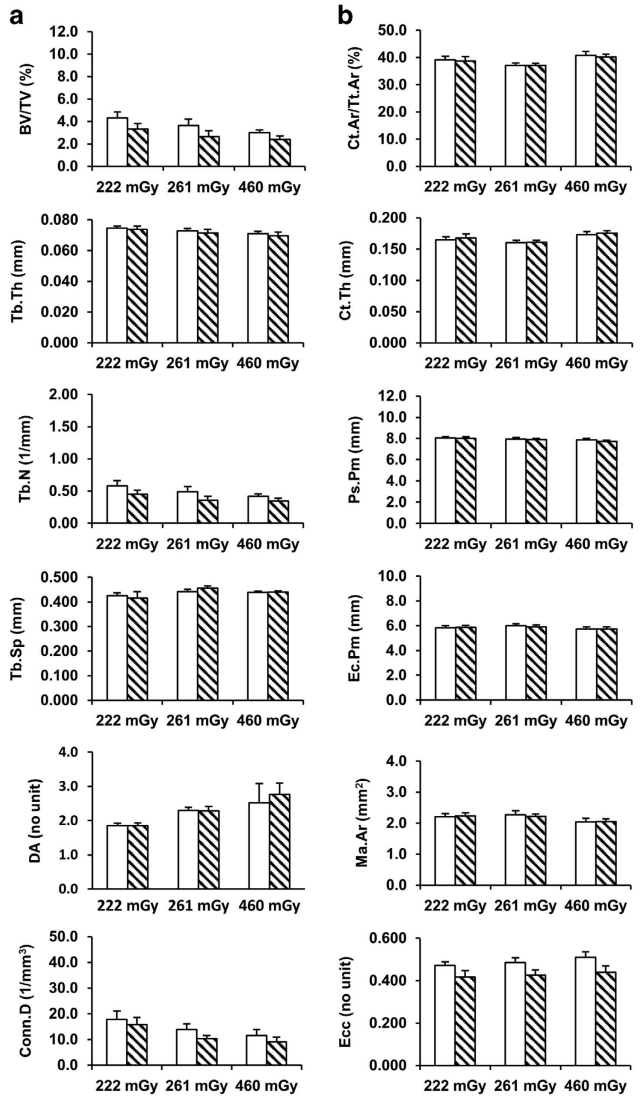

Figure 3 Trabecular (**a**) and cortical (**b**) bone structure of the irradiated right tibias (hatched columns) and non-irradiated left tibias (white columns) of 6-month-old female CD-1 mice. $n = 10$–12 mice per group. Mean ± s.e.m. There were no significant differences in any outcomes between irradiated and non-irradiated tibias for trabecular or cortical bone structure within each irradiation dose.

levels should be measured before longitudinally assessing bone structure in mice to confirm that the dose for a specific set of scanning parameters falls within a range that does not damage skeletal tissue.

The present study contains a number of strengths. First, effects on bone structure from repeated exposure to radiation by *in vivo* μCT scanning was determined using a longer-term (4-month) study design compared with previous studies, allowing longitudinal evaluation and characterization of bone structure during ages that represent key stages of growth and bone development. Second, the present study reports effects of repeated irradiation on bone structure in both male and female CD-1 mice and suggests no overall sex-specific effects using our scan protocol. Third, the effects of repeated radiation exposure were determined using three different doses of ionizing radiation.[10] Fourth, the inclusion of the non-irradiated control leg (left tibia) that was scanned only at 6 months of age serves as an internal control. This decreases variability and

Table 1 Longitudinal assessment of trabecular and cortical bone properties of the proximal tibia of male CD-1 mice

Dose (mGy)	Age (months)	*Trabecular structure*							*Cortical structure*						
		TV (mm³)	*BV (mm³)*	*BV/TV (%)*	*Tb.Th (mm)*	*Tb.N (mm⁻¹)*	*Tb.Sp (mm)*	*DA (no unit)*	*Conn.Dn (mm⁻³)*	*Tt.Ar (mm²)*	*Ct.Ar (mm²)*	*Ct.Ar/Tt.Ar (%)*	*Ct.Th (mm)*	*Ps.Pm (mm)*	*Ec.Pm (mm)*	*Ma.Ar (mm²)*	*Ecc (no unit)*
222	2	1.358 ± 0.055	0.113 ± 0.013^{b}	8.24 ± 0.79^{b}	0.058 ± 0.001^{b}	1.413 ± 0.115	0.259 ± 0.022	1.920 ± 0.070	63.98 ± 6.13^{a}	4.398 ± 0.102	1.240 ± 0.078^{b}	28.27 ± 1.78	0.105 ± 0.007^{c}	8.739 ± 0.108	6.650 ± 0.190	3.158 ± 0.114	0.461 ± 0.020^{a}
	4	1.434 ± 0.038	0.149 ± 0.015^{a}	10.30 ± 0.83^{a}	0.065 ± 0.001^{a}	1.603 ± 0.128	0.279 ± 0.018	1.882 ± 0.061	50.61 ± 6.33^{a}	4.442 ± 0.092	1.324 ± 0.042^{ab}	29.84 ± 0.80	0.127 ± 0.004^{b}	8.780 ± 0.097	7.065 ± 0.094	3.117 ± 0.078	0.436 ± 0.017^{a}
	6	1.400 ± 0.045	0.120 ± 0.018^{ab}	8.50 ± 1.02^{ab}	0.065 ± 0.002^{a}	1.319 ± 0.154	0.312 ± 0.021	1.977 ± 0.102	32.95 ± 4.37^{b}	4.411 ± 0.116	1.358 ± 0.051^{a}	30.83 ± 0.98	0.136 ± 0.004^{a}	8.890 ± 0.113	7.077 ± 0.111	3.053 ± 0.096	0.394 ± 0.020^{b}
261	2	1.474 ± 0.045^{ab}	0.157 ± 0.014^{a}	10.71 ± 0.98	0.060 ± 0.001	1.759 ± 0.137^{a}	0.249 ± 0.012^{b}	1.810 ± 0.078	66.82 ± 6.09^{a}	4.460 ± 0.080^{a}	1.280 ± 0.045	27.96 ± 1.25	0.105 ± 0.004^{b}	8.902 ± 0.100	6.973 ± 0.127	3.319 ± 0.107	0.444 ± 0.020
	4	1.451 ± 0.036^{a}	0.097 ± 0.018^{ab}	6.60 ± 1.14	0.062 ± 0.002	1.057 ± 0.172^{b}	0.348 ± 0.027^{a}	1.919 ± 0.071	34.74 ± 6.16^{b}	4.319 ± 0.144^{ab}	1.273 ± 0.065	30.05 ± 2.56	0.127 ± 0.009^{ab}	8.912 ± 0.125	6.997 ± 0.309	3.046 ± 0.178	0.451 ± 0.034
	6	1.383 ± 0.028^{b}	0.087 ± 0.020^{b}	6.26 ± 1.43	0.063 ± 0.001	0.970 ± 0.208^{b}	0.363 ± 0.024^{a}	2.211 ± 0.120	26.42 ± 6.21^{b}	4.262 ± 0.077^{b}	1.242 ± 0.042	29.11 ± 0.77	0.127 ± 0.004^{a}	8.925 ± 0.110	7.077 ± 0.116	3.021 ± 0.059	0.458 ± 0.021
460	2	1.368 ± 0.038^{a}	0.109 ± 0.011	7.98 ± 0.71	0.060 ± 0.001^{b}	1.336 ± 0.118	0.299 ± 0.014^{b}	2.723 ± 0.060	37.75 ± 3.61^{a}	4.207 ± 0.075^{a}	1.167 ± 0.035^{c}	27.75 ± 0.76^{c}	0.095 ± 0.003^{c}	8.447 ± 0.062^{a}	6.964 ± 0.087^{a}	3.040 ± 0.068^{a}	0.358 ± 0.019^{b}
	4	1.167 ± 0.055^{b}	0.091 ± 0.008	7.91 ± 0.80	0.067 ± 0.001^{a}	1.178 ± 0.126	0.324 ± 0.018^{b}	2.696 ± 0.149	25.96 ± 4.64^{a}	3.872 ± 0.100^{b}	1.280 ± 0.029^{b}	33.26 ± 1.25^{b}	0.139 ± 0.004^{b}	8.251 ± 0.096^{b}	6.533 ± 0.127^{b}	2.591 ± 0.110^{b}	0.419 ± 0.017^{a}
	6	1.137 ± 0.057^{b}	0.081 ± 0.009	7.16 ± 0.84	0.069 ± 0.001^{a}	1.026 ± 0.116	0.352 ± 0.018^{a}	2.350 ± 0.123	16.16 ± 2.93^{b}	3.913 ± 0.110^{b}	1.380 ± 0.031^{a}	35.54 ± 1.44^{a}	0.148 ± 0.003^{a}	8.367 ± 0.087^{a}	6.493 ± 0.160^{ab}	2.532 ± 0.120^{b}	0.421 ± 0.011^{ab}

Abbreviations: BV, bone volume; BV/TV, bone volume fraction; Conn.D, connectivity density; Ct.Ar, cortical bone area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree of anisotropy; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; Ma.Ar, marrow area; Ps.Pm, periosteal perimeter; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, total volume. Within each dose (that is, 222, 261 or 460 mGy), different letters within a column denote statistical significance over time. Data are expressed as mean ± s.e.m., $n = 7$–11 per group. Right tibias were irradiated at 2, 4 and 6 months of age using in vivo micro-computed tomography.

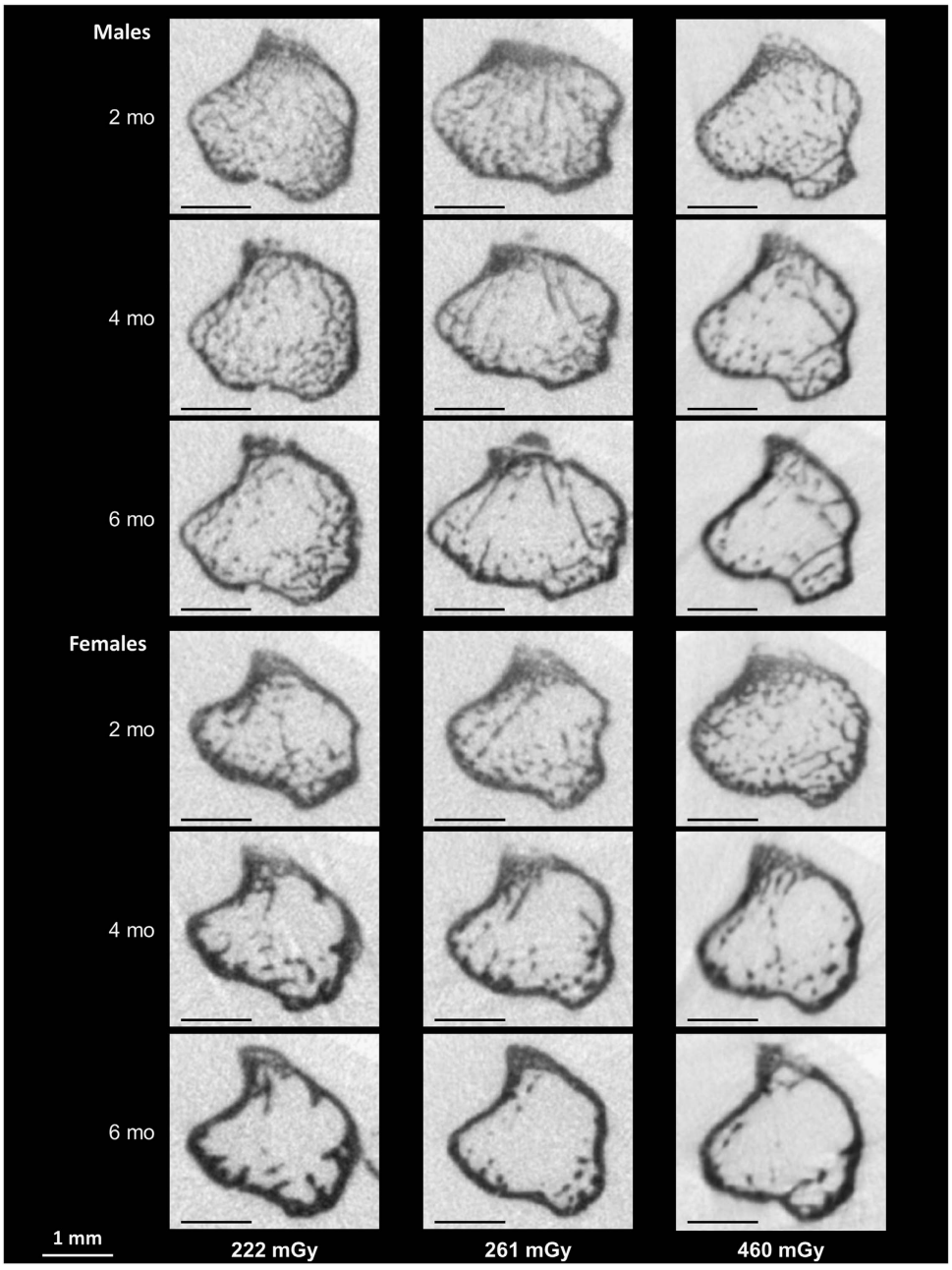

Figure 4 Representative images of proximal tibias of male and female CD-1 mice exposed longitudinally to 222, 261 or 460 mGy at 2, 4 and 6 months of age. Images within each radiation dose depict the same mouse, chosen to be representative of its respective dose group. Images were inverted to feature differences in ambient noise among radiation doses.

further strengthens the study design. Moreover, findings from the present study provide insight into the growth characteristics of trabecular and cortical bone microarchitecture in male and female CD-1 mice. A limitation to the present study include different reconstruction parameters applied to the 460 mGy group compared with the 222 and 261 mGy groups; this does not permit the direct comparison of dose effects on bone microarchitecture. However, the mice that were irradiated with 460 mGy were part of a larger study separated by ~3 months to the 222 and 261 mGy groups; similar reconstruction parameters would have resulted in unsatisfactory image contrast for the scanned images belonging to the 460 mGy group. Moreover, the purpose of the study was not to compare structural indices among groups. Rather, we sought to determine responses of tibia bone structure to a specific dose of radiation and changes with age for male and female CD-1 mice.

In conclusion, repeated irradiation of the tibia with 222, 261 or 460 mGy at 2, 4 and 6 months of age, that provide reasonable image contrast, did not affect trabecular and cortical bone structure in male and female CD-1 mice. Because scanning images obtained at a higher radiation dose improves image resolution and quality (**Figure 4**),[10] scanning parameters that emit a radiation dose of 460 mGy per scan can be applied to future studies that seek to examine skeletal growth and development in male and female mice. In addition, we showed that male and female CD-1 mice experienced trabecular

Table 2 Longitudinal assessment of trabecular and cortical bone properties of the proximal tibia of female CD-1 mice

Dose (mGy)	*Age (months)*	*Trabecular structure*							*Cortical structure*						
		TV (mm^3)	*BV (mm^3)*	*BV/TV (%)*	*Tb.Th (mm)*	*Tb.N (mm^{-1})*	*Tb.Sp (mm)*	*DA (no unit)*	*Conn.Dn (mm^{-3})*	*Tt.Ar (mm^2)*	*Ct.Ar (mm^2)*	*Ct.Ar/Tt.Ar (%)*	*Ct.Th (mm)*	*Ps.Pm (mm)*	*Ec.Pm (mm)*	*Ma.Ar (mm^2)*	*Ecc (no unit)*
222	2	1.062 ± 0.080	0.066 ± 0.005^a	6.43 ± 0.66^a	0.059 ± 0.001^c	1.071 ± 0.095^a	0.326 ± 0.009^b	2.086 ± 0.087	48.54 ± 3.10^a	3.551 ± 0.117	1.108 ± 0.060^b	31.69 ± 2.14^b	0.108 ± 0.006^c	7.816 ± 0.127	5.890 ± 0.195	2.444 ± 0.143^a	0.455 ± 0.023
	4	0.971 ± 0.043	0.056 ± 0.007^a	5.64 ± 0.54^a	0.072 ± 0.001^b	0.787 ± 0.073^a	0.382 ± 0.008^b	1.852 ± 0.061	27.89 ± 3.84^b	3.504 ± 0.097	1.338 ± 0.042^a	38.34 ± 1.32^a	0.151 ± 0.006^b	7.867 ± 0.099	5.870 ± 0.146	2.166 ± 0.090^b	0.419 ± 0.028
	6	0.965 ± 0.048	0.031 ± 0.004^b	3.32 ± 0.49^b	0.074 ± 0.002^a	0.449 ± 0.066^b	0.416 ± 0.025^a	1.854 ± 0.075	15.75 ± 2.90^c	3.620 ± 0.108	1.390 ± 0.044^a	38.70 ± 1.62^a	0.168 ± 0.006^a	8.036 ± 0.126	5.874 ± 0.146	2.230 ± 0.112^{ab}	0.418 ± 0.029
261	2	1.154 ± 0.060^a	0.070 ± 0.006^a	6.02 ± 0.45^a	0.060 ± 0.001^c	1.000 ± 0.076^a	0.342 ± 0.011^c	1.994 ± 0.049	43.56 ± 3.26^a	3.654 ± 0.118	1.030 ± 0.030^c	28.70 ± 1.56^c	0.101 ± 0.004^c	7.910 ± 0.127	5.988 ± 0.149	2.624 ± 0.139	0.507 ± 0.035
	4	1.059 ± 0.056^b	0.048 ± 0.010^b	4.33 ± 0.77^b	0.068 ± 0.002^b	0.625 ± 0.107^b	0.410 ± 0.016^b	2.031 ± 0.096	21.77 ± 3.84^b	3.571 ± 0.098	1.216 ± 0.018^b	34.34 ± 0.96^b	0.138 ± 0.138^b	7.892 ± 0.105	6.022 ± 0.147	2.355 ± 0.095	0.438 ± 0.023
	6	1.006 ± 0.042^b	0.027 ± 0.006^c	2.62 ± 0.53^c	0.072 ± 0.002^a	0.356 ± 0.062^c	0.457 ± 0.008^a	2.288 ± 0.127	10.32 ± 1.33^c	3.514 ± 0.094	1.299 ± 0.026^a	37.12 ± 0.75^a	0.160 ± 0.003^a	7.903 ± 0.103	5.913 ± 0.135	2.215 ± 0.079	0.426 ± 0.025
460	2	1.140 ± 0.075^a	0.080 ± 0.010^a	6.92 ± 0.49^a	0.060 ± 0.001^b	1.158 ± 0.083^a	0.333 ± 0.014^c	2.816 ± 0.110^{ab}	29.47 ± 2.53^a	3.778 ± 0.141^a	1.207 ± 0.027^b	32.41 ± 1.42^b	0.111 ± 0.005^c	8.007 ± 0.128^a	6.213 ± 0.162^a	2.571 ± 0.149^a	0.501 ± 0.033
	4	0.916 ± 0.049^b	0.034 ± 0.005^b	3.66 ± 0.43^b	0.071 ± 0.001^a	0.518 ± 0.061^b	0.425 ± 0.007^b	2.776 ± 0.222^b	18.04 ± 2.43^b	3.382 ± 0.105^b	1.316 ± 0.030^a	39.14 ± 1.18^a	0.162 ± 0.005^b	7.633 ± 0.103^b	5.660 ± 0.158^b	2.066 ± 0.097^b	0.436 ± 0.025
	6	0.903 ± 0.048^b	0.020 ± 0.004^c	2.12 ± 0.32^c	0.072 ± 0.003^a	0.287 ± 0.039^c	0.450 ± 0.004^a	3.532 ± 0.307^a	9.09 ± 2.57^c	3.400 ± 0.104^b	1.335 ± 0.025^a	39.51 ± 1.15^a	0.178 ± 0.004^a	7.695 ± 0.114^b	5.682 ± 0.169^b	2.066 ± 0.100^b	0.440 ± 0.029

Abbreviations: BV, bone volume; BV/TV, bone volume fraction; Conn.D, connectivity density; Ct.Ar, cortical bone area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree of anisotropy; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; Ma.Ar, marrow area; Ps.Pm, periosteal perimeter; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, total volume.
Within each dose (that is, 222, 261 or 460 mGy), different letters within a column denote statistical significance over time. Data are expressed as mean ± s.e.m., n = 10-12 per group.
Right tibias were irradiated at 2, 4 and 6 months of age using in vivo micro-computed tomography.

structural diminution between 2 and 4 months of age while cortical bone structure continued to increase throughout the study. Thus, studies that are 2 to 4 months in duration are appropriate for examining the effects of intervention strategies on trabecular bone development in male and female CD-1 mice while longer studies are needed to measure responses of cortical bone outcomes. These findings can be applied to future studies that investigate lifelong responses of bone structures from early life exposure to various intervention strategies.

Materials and Methods

Animals and diets

Seven-week-old male and female CD-1 mice ($n = 48$) were obtained from Charles River Canada (St Constant, QC, Canada) and housed four to five per cage (222 or 261 mGy group). Another two groups consisting of 7-week-old male ($n = 9$) and female CD-1 mice ($n = 10$) were obtained from breeding mothers (Charles River Canada) that were part of another larger study (460 mGy groups). All mice were housed in a temperature- and humidity-controlled room (22–24 °C, 50% humidity) with 12:12-h light:dark cycle and had free access to food (AIN-93G with vitamin-free casein, TD. 06706, Harlan Teklad, Mississauga, Canada)[4] and water. Body weight was monitored weekly. Animal care and use conformed to the Guide to the Care and Use of Experimental Animals[21] and the experimental protocols were approved by the Animal Care Committee at Brock University, St Catharines, ON, Canada.

Micro-computed tomography scanning of proximal tibias

At 2, 4 and 6 months of age, the right tibias were scanned using μCT (Skyscan 1176, Bruker-microCT, Kontich, Belgium) and host software (1176 version 1.1, Bruker-microCT, Kontich, Belgium). Mice were anaesthetized in an induction chamber using isoflurane (2–5%) dissolved in oxygen and then transitioned to a nose cone for maintenance of the anaesthesia (0.5–5% delivery rate) and placed on the scanning bed. Ophthalmic gel was applied to the eyes when the mouse was transitioned to the nose cone from the induction chamber to prevent dryness. The mouse was positioned on its back with its right leg extended (**Figure 5a**). The extended leg was held firmly in a piece of foam that is lined with dental wax to help prevent slippage (**Figure 5b**). The foam containing the extended leg was subsequently secured in a plastic holder that was connected to the scanning bed. The non-scanning hind limb and tail were folded away towards the head and secured alongside the animal using masking tape, and the nose cone was secured over the head (**Figure 5a**). For μCT scanning of the right tibias, an isotropic resolution of 9 μm was chosen which permits the evaluation of fine trabecular outcomes such as trabecular thickness that can measure from 47 to 141 μm in 3 to 6-month-old CD-1 mice.[4,22,23] To reduce beam hardening, a 1 mm aluminum filter was used, which simultaneously reduces the exposure of tibias to lower energy X-rays. One of the three sets of scanning parameters ($n = 9$–12 per parameter) was used. The first set of scanning parameters (222 mGy dose) consisted of 50 kV, 100 μA, 1.0° rotation step, 3300 ms exposure time. Total scan time was 12 min 10 s. These scanning parameters replicate those applied in previous work[10] to achieve acceptable image contrast for *in vivo* evaluation of bone microarchitecture in growing and adult mice. The second set of scanning parameters (261 mGy dose) consisted of 50 kV, 100 μA, 0.8° rotation step, 3300 ms exposure time. Total scan time was 15 min 11 s. These parameters were similar to the first set except for a 0.2° improvement in rotation step that resulted in a greater number of projection images acquired per scan (246 projection images versus 198 projection images). The third set of scanning parameters (460 mGy dose) consisted of 40 kV, 300 μA, 0.8° rotation step, 3350 ms exposure time, resulting in a total scan time of 16 min 23 s. The third set of scanning parameters was developed to produce reasonable image contrast for evaluation of trabecular and cortical bone microarchitecture while potentially emitting a higher dose of radiation. All scans were

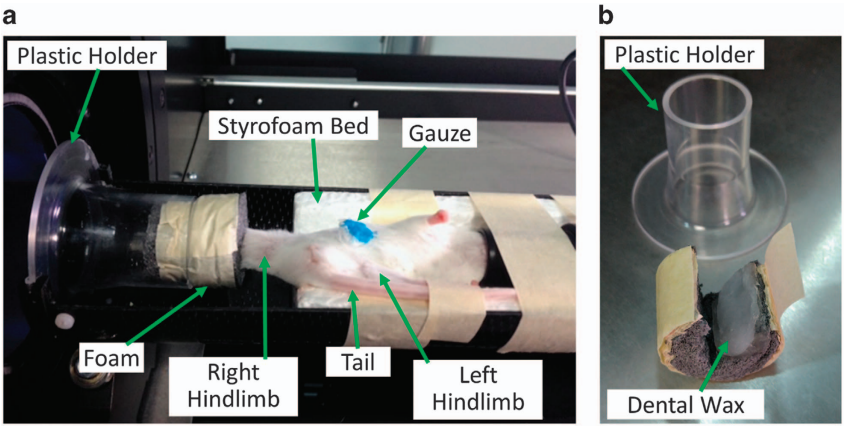

Figure 5 Positioning of an anaesthetized mouse for *in vivo* micro-computed tomography scanning (**a**). The mouse was positioned on its back on a 1 cm thick styrofoam bed secured to the scanning bed to facilitate the positioning of the right scanned leg into the iso-center of the scanning chamber. The right leg was extended and firmly held in foam and then secured in a plastic holder that was connected to the scanning bed. The non-irradiated hind limb and tail were folded away towards the head and secured alongside the animal using masking tape, and a nose cone was secured over the head to keep the mouse under anaesthesia during scanning. A piece of blue-coloured gauze was placed on the abdomen of the mouse to facilitate monitoring of real-time breathing rate during scans by a video camera secured to the distal end of the scanning bed. Closer view of the foam and plastic holder (**b**) used to secure the right leg to the scanning bed. Dental wax was added to one side of the foam holder to help secure the leg to prevent movement during scans.

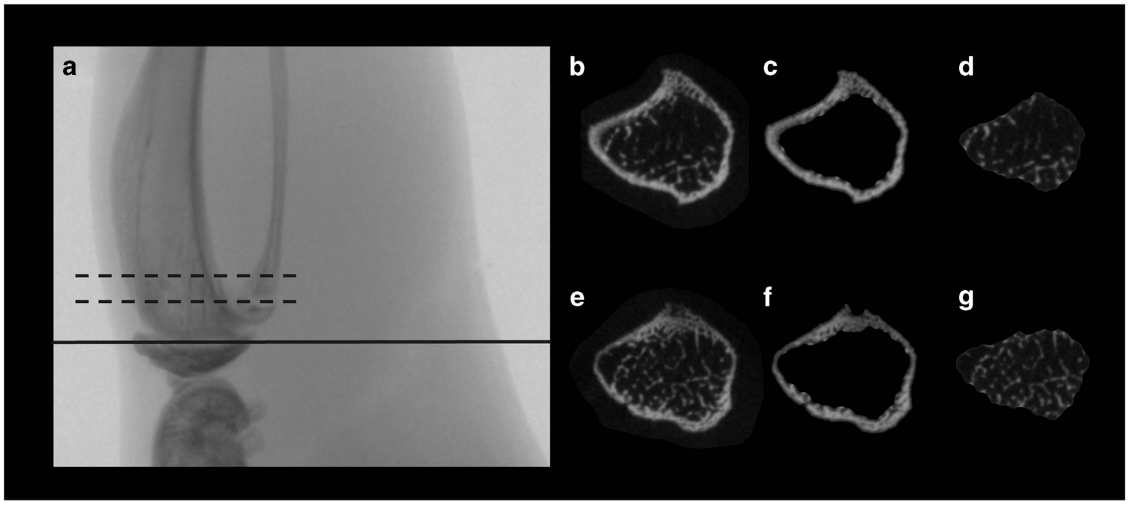

Figure 6 Representative projection image (**a**) of the right proximal tibia of a 2-month-old female CD-1 mouse irradiated with 460 mGy through micro-computed tomography scanning. Right tibias were scanned at 2, 4 and 6 months of age. The solid line represents the reference point at the growth plate from which an offset of 110 slices (0.967 mm) was made to reach the beginning of the region of interest (bottom dashed line) to ensure the growth plate was excluded from structural analyses. The region of interest for trabecular and cortical structural analyses extended 58 slices (510 um) into the diaphysis and includes the bone area shown between the two dashed lines (**a**). Transaxial sections of the beginning and end of the region of interest that comprise the proximal (**e**) and distal (**b**) slices, respectively, are depicted. Cortical (**c**, **f**) and trabecular (**d**, **g**) bone were segmented using automated processes.

performed over 180° with no frame averaging. A MOSFET portable dosimeter (TN-502RD-H, Best Medical Canada, Ottawa, ON, Canada) was calibrated to an ion chamber (10 × 5–0.6, Radcal, Monrovia, CA, USA) and then scanned in air using the high bias setting to measure the radiation dose of each set of parameters (222, 261 and 460 mGy).

At 6 months of age, the contralateral left tibias were scanned *in vivo* in the 222 and 261 mGy groups to serve as non-irradiated internal controls that we can compare directly with the repeatedly irradiated right tibias to determine whether repeated radiation exposure alters proximal tibia trabecular and cortical bone structure. For the 460 mGy group, the right leg was scanned *in vivo* at 2, 4 and 6 months of age. After the 6-month scan of the right leg, both the right and left tibias were excised, cleaned of soft tissue, and stored at − 80 °C in saline soaked gauze until *ex vivo* scanning. For *ex vivo* scanning, the right and left tibias were wrapped in parafilm and placed axially in a foam holder for μCT scanning. The *ex vivo* scanning parameters included: 9 μm isotropic pixel, 0.25 mm aluminum filter, 45 kVp, 545 uA, 0.2° rotation step applied over a 180° scan, and 850 ms exposure time. Contralateral left tibias were not assessed at 2 or 4 months of age as the primary objective of the present study was to determine the effects of repeated irradiation on bone structure using a longer study design (that is, 4 months) than what has previously been used (that is, 4–6 weeks).[10,11]

Image processing and analysis

GPU-accelerated reconstruction was used to reconstruct cross-section images from the tomography projection images using GPURe-conServer (Bruker-microCT) and NRecon Reconstruction 64-bit software (Bruker-microCT). For the 222 and 261 mGy groups, scanned images were reconstructed using variable post-alignment compensations and a dynamic image range of 0.000–0.0741 of the X-ray attenuation coefficient. Smoothing, beam-hardening and ring artifact corrections were applied to reduce signal noise. The same

reconstruction parameters and corrections were applied across all sample images within the 222 and 261 mGy groups. For the 460 mGy group, variable post-alignment compensations and a dynamic image range of 0.000–0.089 of the X-ray attenuation coefficient were applied. Smoothing, beam-hardening and ring artifact corrections were also applied to reduce signal noise and the same reconstruction parameters and corrections were used across all sample images.

Reconstructed images were reoriented (DataViewer version 1.5.0) and a region of interest (ROI) in the proximal tibia consisting of 58 transaxial slices (0.510 mm in length) was selected using CTAnalyzer software (Bruker-microCT; **Figure 6**). The ROI began 110 slices (0.967 mm in length) away from the metaphyseal side of the growth plate and extended towards the ankle. Trabecular and cortical bone compartments were automatically segmented from one another and trabecular and cortical bone structure of the saved ROI data sets were evaluated using custom processing task lists (CTAnalyzer, Bruker-microCT). For trabecular bone, adaptive thresholding with a pre-thresholding of 74 (lower) and 255 (upper) and a radius of 6 pixels was applied followed by a despeckle function to separate bone from the background. Three-dimensional μCT outcome measures of trabecular bone included BV, TV, BV/TV, Tb.Th, Tb.N, Tb.Sp, DA and Conn.D.[19] For evaluating cortical bone structure, global thresholding with a lower threshold of 102 and an upper threshold of 255 was applied to separate bone from the background. Two-dimensional μCT outcome measures of cortical bone included Tt.Ar, Ct.Ar, Ct.Ar/Tt.Ar, Ct.Th, Ps.Pm, Ec.Pm, Ma.Ar and Ecc.[19]

Statistical analyses

All data are presented as mean ± s.e.m. and all statistical analyses were performed using SPSS Statistics (version 22, IBM, Armonk, NY, USA). Comparisons within sex were performed to investigate skeletal responses to radiation and longitudinal changes with age for males and females. To determine the effects of time, radiation dose and their interaction on body weight, a repeated measures analysis of variance (ANOVA; general linear model) was performed. Differences in trabecular and cortical bone structure between irradiated and non-irradiated legs within a group were determined by a two-tailed independent samples Student's *t*-test. Longitudinal changes within each trabecular and cortical bone structural outcome were evaluated using a repeated measures ANOVA (general linear model) with a Bonferroni post-hoc test. Missing values that resulted from mouse leg movement during the scan or due to computer error were replaced with the series mean (average imputation). In male mice, this occurred a total of four times (once in the 222 mGy group at 2 months of age, twice in the 460 mGy group at 2 months of age, once in the 460 mGy at 4 months of age). In females, this occurred a total of five times (twice in the 222 mGy group at 2 months of age, twice in the 261 mGy group at 4 months of age, once in the 460 mGy group at 2 months of age). Since reconstruction parameters applied to the 460 mGy group differed to those applied to the 222 and 261 mGy groups, direct comparison of bone microarchitecture among radiation groups could not be performed.

Conflict of Interest

PS is an employee of Bruker-microCT. The remaining authors declare no conflict of interest.

Acknowledgements

WEW is a Canada Research Chair in Bone and Muscle Development. We thank the Canadian Institutes of Health Research (grant number 130544) for funding this research and the Canada Foundation for Innovation (grant number 222084) for purchase of the micro-computed tomography system. We also thank Best Medical Canada for their assistance in determining radiation dose measurements.

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