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Development of micro-CT protocols for *in vivo* follow-up of mouse bone architecture without major radiation side effects

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ABSTRACT

In vivo micro-computed tomography (micro-CT) will offer unique information on the time-related changes in bone mass and structure of living mice, provided that radiation-induced side effects are prevented. Lowering the radiation dose, however, inevitably decreases the image quality. In this study we developed and validated a protocol for *in vivo* micro-CT imaging of mouse bone architecture that retains high quality images but avoids radiation-induced side effects on bone structure and hematological parameters.

The left hindlimb of male C57Bl/6 mice was scanned *in vivo* at 3 consecutive time points, separated each time by a 2-week interval. Two protocols for *in vivo* micro-CT imaging were evaluated, with pixel sizes of 9 and 18 μm and administered radiation doses of 434 mGy and 166 mGy per scan, respectively. These radiation doses were found not to influence trabecular or cortical bone architecture in pre-pubertal or adult mice. In addition, there was no evidence for hematological side effects as peripheral blood cell counts and the colony-forming capacity of hematopoietic progenitor cells from bone marrow and spleen were not altered. Although the images obtained with these *in vivo* micro-CT protocols were more blurred than those obtained with high resolution (5 μm) *ex vivo* CT imaging, longitudinal follow-up of trabecular bone architecture in an orchidectomy model proved to be feasible using the 9 μm pixel size protocol in combination with a suitable bone segmentation technique (*i.e.* local thresholding). The image quality of the 18 μm pixel size protocol was too degraded for accurate bone segmentation and the use of this protocol is therefore restricted to monitor marked changes in bone structure such as bone metastatic lesions or fracture healing.

In conclusion, we developed two micro-CT protocols which are appropriate for detailed as well as global longitudinal studies of mouse bone architecture and lack noticeable radiation-induced side effects.

Introduction

In vivo micro-computed tomography (micro-CT) has been suggested as a valuable tool to monitor local changes in bone structure in living mice [1]. This technology can offer unique high resolution information on the temporal responses of specific bone regions to pathological or therapeutic stimuli, stipulated that the micro-CT imaging process itself has no influence on the skeletal system. Indeed, frequent or excessive exposure of the skeletal system to X-rays leads to side effects which are closely related to the radiation dose and include growth retardation, skeletal deformities, bone loss and hematological abnormalities [2,3].

Radiation can cause cell death, most likely as a consequence of irreparable DNA damage [4,5]. But also low radiation doses can result in non-lethal DNA damage, which will initiate DNA repair processes and ultimately lead to a decrease in cell proliferation [6]. It is therefore generally accepted that proliferating cells are more radiosensitive than non-proliferating cells and that less differentiated cells are more prone to radiation damage than highly differentiated cells [7]. This radiation-induced cytotoxicity has resulted in the use of radiation as a local therapy for numerous malignancies in humans.

Radiation will also harm non-malignant cells and several studies have investigated the effects of high-dose radiation on bone cells *in vitro* and *in vivo*. X-ray radiation in the range of 2.5 to 8 Gy inhibits the proliferation and activity of osteoblasts [8–11] and osteoclasts [12], while radiation doses lower than 2 Gy have a stimulatory effect on osteoclast proliferation and activity [13]. Growth plate chondrocytes and bone marrow cells are the most radiation sensitive cells because of their high proliferation rate, and radiation damage to these types of cells inhibits their proliferation and thus results in growth retardation

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and myeloid depletion, respectively [2,14]. On the other hand, the exposure of the total mouse body to a low dose radiation of 75 mGy was found to stimulate the proliferation of bone marrow hematopoietic progenitor cells as well as their mobilization into the peripheral blood [15].

In contrast to the radiation doses used in radiation therapy, the doses associated with imaging are substantially lower. To investigate the temporal changes in bone structure during a longitudinal follow-up study, multiple successive micro-CT scans are, however, required. Frequent *in vivo* micro-CT imaging, even with relatively low radiation doses per scan, can still affect bone architecture. Discrepant results have been reported concerning the effects of micro-CT-induced radiation on the bone micro-architecture and especially the hematopoietic system in rodents. In rats, 8 weekly *in vivo* scans of 939 mGy each did not alter the structural bone parameters nor the viability of the bone marrow cells [16]. In mice, however, micro-CT-induced radiation decreased trabecular bone volume of the tibiae of 8 to 10-week old mice after 4 weekly scans with 846 mGy [17], while 4 weekly scans with 1255 mGy did not exacerbate disuse-related bone loss in the femurs of 17-week old mice [18]. It is still unclear whether this inconsistency should be attributed to differences in the radiation dose, frequency and number of *in vivo* scans or a combination of these. In addition, young, growing animals may be particularly susceptible to radiation exposure [19]. Certainly in humans, this age-related radiation sensitivity has already been thoroughly described [3].

Taken together, it is critical to delineate the settings for *in vivo* micro-CT imaging of the bone architecture of mice in order to avoid side effects on bone and hematopoietic cells that could result in altered bone structure and hematological defects. However, lowering the radiation dose to avoid radiation effects may increase noise in the images and decrease the signal-to-noise ratio, or reduce image sharpness and lower the resolution [20]. Hence, image quality needs to be weighed against the radiation risks [21]. This trade-off between dose and image quality has not been investigated thoroughly in relation to *in vivo* micro-CT imaging of mouse bone architecture. In this study, we designed 2 protocols for longitudinal micro-CT studies of the skeletal system in young pre-pubertal and adult mice that are devoid of radiation-induced side effects and maintain adequate image quality.

Materials and methods

Animals and experimental design

Male C57Bl/6 mice (Janvier) were housed under standard conditions in our animal facility (Proefdierencentrum Leuven, Belgium). All procedures were approved by the Ethical Committee of the Katholieke Universiteit Leuven.

Three types of experiments were performed: a pilot experiment, an optimisation experiment and a validation experiment. In each experiment, the left hindlimb of male C57Bl/6 mice, anesthetized with isoflurane, was scanned *in vivo* with micro-CT at 3 consecutive time points, separated each time by a 2-week interval. This follow-up period of 4 weeks was chosen because it corresponds with the evaluation period used in several murine bone pathology models such as ovariectomy, castration, tail suspension and fracture repair. The right hindlimb was positioned out of the field of view of the micro-CT and served as the non-irradiated control, as the total X-ray exposure of this hindlimb was negligible (data not shown). Following the last *in vivo* scan, mice were sacrificed by cervical dislocation and, depending on the experiment, blood was collected and the spleen, tibia and femur were isolated.

In the pilot experiment, we investigated the radiation effect of the recommended micro-CT parameters on 10-week old male C57Bl/6 mice ($n=5$), an age often used as starting point in bone pathology models. The *in vivo* micro-CT parameters were 9 μm pixel size, 50 kV, 120 uA, 0.5 mm Al filter, angular rotation step 0.9°, 220 projections and an exposure time of 4.7 s with a total scan duration of 19 min. After sacrifice, dissected tibiae were imaged by *ex vivo* micro-CT after overnight fixation in 2% paraformaldehyde, and then processed for histological analysis.

In the optimization experiment, 4 and 16-week old male C57Bl/6 mice were analyzed, as age is known to change the susceptibility to radiation effects ($n=4$ per age group). For each age, an additional group of mice was included, referred to as 'reference' group, and these mice were anesthetized at each time point but did not receive any *in vivo* radiation ($n=4$ per age group). Two *in vivo* micro-CT parameters were used: (i) a pixel size of 9 μm (50 kV, 100 uA, 1 mm Al filter, angular rotation step 1°, 199 projections, exposure time 3.3 s, scan duration 12 min); (ii) a pixel size of 18 μm (50 kV, 100 uA, 1 mm Al filter, angular rotation step 0.8°, 248 projections, exposure time 1 s, scan duration 5 min). After sacrifice, the bone architecture was analyzed by *ex vivo* micro-CT and histology. Peripheral blood cell counts were determined and the *in vitro* colony forming capacity of hematopoietic progenitor cells of the bone marrow and spleen was analyzed. The *in vitro* osteogenic potential of bone marrow stromal cells and the differentiation of bone marrow hematopoietic cells into osteoclasts were assessed.

In the validation experiment, 10-week old male C57Bl/6 mice were either sham-operated or orchidectomized the day before the first *in vivo* micro-CT scan was taken ($n=5$ per group). They were anesthetized with pentobarbitone sodium (Nembutal, 50 mg/kg body weight, CEVA Santé Animale) and received buprenorfine hydrochloride as postoperative analgesic (Temgesic, 0.05 mg/kg body weight, Schering-Plough). The *in vivo* micro-CT parameters were the 9 μm pixel size parameters used in the optimization experiment, and trabecular bone architecture was analyzed and compared between orchidectomized and sham-operated mice over time.

Micro-computed tomography

The bone micro-architecture of the tibiae was assessed *ex vivo* and *in vivo* using a SkyScan 1172 and a SkyScan 1076 micro-CT system, respectively, and related software (SkyScan). *In vivo* micro-CT images were segmented using an adaptive thresholding algorithm provided by the SkyScan CTan software because the reduced image quality precluded to use global thresholding [22]. The local threshold was calculated in a circular region of radius 8 pixels around each pixel. When indicated, registration software based on mutual information was applied to align these images [23].

The scanning parameters for *ex vivo* micro-CT imaging were 5 μm pixel size, 50 kV, 200 uA, 321 projections, 0.5 mm Al filter. After reconstruction, the *ex vivo* micro-CT images were segmented using a global threshold. The global threshold was visually determined to optimally separate the bimodal histogram into bone and soft tissue. Trabecular and cortical volumes of interest were selected manually and histomorphometric parameters were calculated according to the 'Guidelines for the assessment of bone microstructure in rodents using micro-CT' [19].

Image quality

Image quality can be quantified by severable factors such as resolution and signal-to-noise ratio. Resolution describes the ability to resolve small details in the image and can be quantified as the full-width-at-half-maximum of the system point spread function [24]. The signal-to-noise ratio in micro-CT images of the different protocols is measured as the ratio of the mean intensity over the standard deviation of the intensity in a region of interest in images of a homogeneous water phantom. More details about image quality and its relationship to radiation dose can be found in Ref. [21].