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Dual Energy X-Ray Absorptiometry in Small Rats with Low Bone Mineral Density

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Abstract. The feasibility of dual energy X-ray absorptiometry (DXA) using the Norland XR-26 Mark II bone densitometer for measurements of bone mineral content (BMC) and bone mineral density (BMD) in small rats was evaluated. Thirty-two young, isogenic, Lewis rats (weights from 119 g to 227 g) were used; normal rats (n = 7) and rats with low BMD obtained from three different vitamin D-depleted models (n = 25). DXA measurements were performed using the special software for small animals. Duplicate scans of excised femurs performed at 2 mm/second (pixel size of 0.5 mm × 0.5 mm) were very precise measurements with a coefficient of variation (CV) below 1.6% in animals with normal BMD; in rats with low BMD, the CV was significantly higher (P = 0.02–0.04), 7.8% and 4.4% for BMC and BMD, respectively. Regression analysis demonstrated that these measurements were related to the ash weight (R^2 > 98.6%). The CV for measurements of the lumbar spine at 10 mm/second (pixel size 0.5 mm × 0.5 mm) was 2.6% and 2.2% for BMC and BMD, respectively in rats with normal BMD, and again higher (P = 0.03–0.14) in rats with low BMD, 7.3% and 4.7%, respectively, for BMC and BMD. Even though low CVs were obtained for total body duplicate scans (scan speed of 20 mm/second and a pixel size of 1.5 mm × 1.5 mm), the measurements were problematic for accuracy because of an overestimation of both BMC and the area of bone. Using these scan parameters the measurements of total body bone mineral could not be recommended in small rats with low BMD.

Key words: Small rats — Bone mineral density — Bone mineral content — Dual energy X-ray absorptiometry — Methodological evaluation

An increasing number of investigators use dual energy X-ray absorptiometry (DXA) for measurements of bone mineral density (BMD) and bone mineral content (BMC) in various experimental studies using rats [1–4]. In several studies, the reliability of DXA measurements of tiny rat bones have been evaluated in studies using adult rats with normal bone density [5–13]. However, methodological studies evaluating the feasibility of DXA in small, young animals with low BMD have previously only been performed in few studies [14–16]. Therefore, we investigated the precision and accuracy of DXA measurements at different skeletal sites and evaluated the effect of scan speed and resolution in rats with extremely low BMD, using a standard DXA scanner supplied with software for small animal studies.

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Material and Methods

Experimental Animals

Isogenic Lewis rats (Møllegården, Roskilde, Denmark) were used for all DXA measurements. With the aim of creating rats with a large range of different degrees of low BMD, animals from four different study groups were used:

1. Weanling rats (n = 12) placed on a Vitamin D-depleted low calcium (0.15%) and high phosphate (1.5%) diet for 3 weeks. All animals with a mean body weight of 121 g (range: 119–124 g) reached the endpoint.
2. Weanling rats (n = 12) were parathyroidectomized (PTX) during pentobarbital anesthesia and then placed on the same vitamin D-depleted diet as group 1 for 3 weeks. PTX was considered successful when ionized calcium (Ca) was below 1.00 mmol/liter and plasma parathyroid hormone (PTH) was below 20 pg/ml. Only six animals with a mean body weight of 122 g (range: 121–124 g) reached the endpoint.
3. Weanling rats (n = 12) placed on the same vitamin D-depleted diet as group 1 for 9 weeks. Seven animals with a mean body weight of 121 g (range: 120–124 g) reached the endpoint.
4. A control group of animals (n = 8) placed on a standard diet [vitamin D (1 IU/g), Ca (0.9%), and phosphate (0.7%)] for 9 weeks. Seven animals with a mean body weight of 211 g (range: 191–227 g) reached the endpoint.

At the end of each study period the animals were sacrificed by pentobarbital anesthesia and ex sanguinated. Whole blood samples were immediately analyzed for ionized Ca, and plasma samples were frozen at −20°C for subsequent measurements of rat PTH. The animals were all frozen in the same position with limbs in an outward direction and stored at −20°C for later DXA scanning. After scanning, the rats were thawed at room temperature and the right femur was exarticulated. The excised femurs were trimmed free of all soft tissue and DXA scannings were performed. Then the isolated femurs were ashed in a furnace for 26 hours. During the first 2 hours the temperature was 200°C and during the remaining 24 hours the temperature was kept at 600°C. The ashed femurs were weighed using a high precision scale (Sartorius, Guttingen, Germany).

Bone Densitometer

BMC (g) and BMD (g/cm^2) were measured by DXA with a standard bone densitometer (Norland XR-26 Mark II, Norland Corp.,

Table 1. Mean (95%-CL) CV for duplicate DXA measurements performed in excised rat femurs of the low density animals and healthy controls

	Low density	Normal density	*P*-value
Scan speed: 2 mm/s			
N[a]	13	6	
BMD	0.031 (0.023–0.042)	0.068 (0.066–0.069)	
CV_{BMC}	7.8% (4.4%;11.2%)	1.6% (−0.1%;3.4%)	0.02
CV_{BMD}	4.4% (2.5%;6.4%)	1.2% (−0.4%;2.9%)	0.04
CV_{Area}	4.3% (2.8%;5.8%)	1.3% (0.9%;1.8%)	0.01
Scan speed: 10 mm/s			
N[b]	6	7	
BMD	0.034 (0.030–0.036)	0.079 (0.076–0.084)	
CV_{BMC}	8.2% (0.4%;16.0%)	1.5% (0.5%;2.4%)	0.04
CV_{BMD}	5.5% (1.1%;9.9%)	1.1% (0.5%;1.8%)	0.02
CV_{Area}	5.1% (0.3%;9.9%)	1.4% (0.7%;2.1%)	0.06
Scan speed: 2 and 10 mm/s			
N[b]	6	7	
BMD (2mm/s)	0.025 (0.023–0.027)	0.069 (0.066–0.075)	
CV_{BMC}	3.8% (−2.1%;9.8%)	5.2% (3.3%;7.2%)	0.56
CV_{BMD}	21.4% (15.3%;27.5%)	9.8% (8.7%;10.9%)	<0.0005
CV_{Area}	23.1% (18.7%;27.5%)	4.9% (3.6%;6.2%)	<0.0005

Scans (n = 32) using a scan speed of 2 mm/s and a pixel size of 0.5 × 0.5 mm were performed. At a constant pixel size, scanning was repeated with the same scan speed (n = 19) and at a scan speed of 10 mm/s (n = 13). The *P*-values (*t*-test for unpaired data) for comparison of mean CV between low and normal density groups are shown. The mean-(range) BMD (g/cm^2) for each group of animals is shown

[a] Vitamin D depleted 9 weeks (n = 7), Vitamin D depleted + PTX (n = 6), and healthy controls 9 weeks (n = 6)

[b] Vitamin D depleted 9 weeks (n = 6), and healthy controls 9 weeks (n = 7).

WI, USA) with collimator diameters of 1.9 mm and 2.0 mm, respectively, at the X-ray source and the detector. All scans were performed using the flexible small animal scan option (software version 2.5.0), and all duplicate scans were performed with full repositioning between each scanning. All scans were performed in a standardized way using the same area (20 × 30 cm) of the scanner table. According to manufacturer's recommendations the densitometer was calibrated daily by a dual material standard and a lumbar spine phantom. The coefficient of variation (CV) during a 3-month period based on 66 scans performed on the spine phantom was 0.8% for BMD and 0.6% for BMC.

Bone Mineral Measurements

Duplicate scans of the total body using a scan speed of 20 mm/second and a pixel size of 1.5 × 1.5 mm (17 minutes) were performed in frozen animals (n = 19). An additional scanning of the total body (n = 12) was performed at a scan speed of 10 mm/second using a pixel size of 1.0 × 1.0 mm (50 minutes). Duplicate scans of the lumbar spine (n = 19) were performed in the frozen animals at a scan speed of l0 mm/second using a pixel size of 0.5 × 0.5 mm (4 minutes). Bone mineral was measured in the distal part of the lumbar spine using a region size of 1.0 × 2.0 cm in all animals. The excised femurs (n = 32) were scanned on the same area of the scanner table in an identical position using a scan speed of 2 mm/second and a pixel size of 0.5 × 0.5 mm (5 minutes). At a constant pixel size, repeat scans were done at both 2 mm/second (n = 19) and 10 mm/second (1 minute) (n = 13).

Statistics

The precision of the method was evaluated from duplicate measurements with calculation of the CV (CV = SD/mean × 100%). The results are given as the mean CV together with the 95% confidence limits for the mean (95% CL). *T*-test for unpaired data was used for comparison of mean CV between groups with normal and low BMD, respectively. The accuracy of the method was evaluated using linear regression analysis. Results are presented as a scatter plot of the data together with the regression line with 95% prediction limits. Furthermore, calculations of the coefficient of determination (R^2), the standard error of the estimate (SEE), and the *P*-value for the *t*-test were performed.

Results

Precision

DXA measurements of the excised femurs performed at 2 mm/second were very precise with CV values below 1.6% in the group of animals with normal BMD. In the rats with low BMD, the CV values for these measurements were significantly higher, i.e., 7.8% and 4.4%, respectively, for BMC and BMD (Table 1). When comparing the measurements performed at the two different scan speeds (2 and 10 mm/second), it was obvious that the determination of the area of bone at 10 mm/second was not accurate (especially in the low BMD group, CV = 23.1%) and thus strongly influenced the calculation of BMD (CV = 2l.4%) (Table 1). Measurement of BMC at both 2 and 10 mm/second in both groups turned out to be accurate and with CV values of 1.5–8.3% (Table 1).The precision obtained from duplicate scans of the lumbar spine at 10 mm/second in rats with normal BMD was 2.6% and 2.2%, respectively, for BMC and BMD. Again the CV was higher in the group of rats with low BMD (Table 2).In normal rats, a CV of 2.8%, 0.8%, and 3.4% was obtained for total body duplicate measurements of BMC, BMD, and area of bone, respectively, using a scan speed of 20 mm/second and a pixel size of 1.5

Table 2. Mean (95%-CL) CV for duplicate DXA measurements performed in rat total body and lumbar spine

	Low density	Normal density	*P*-value
Total body			
Scan speed: 20 mm/s			
N[a]	12	7	
BMD	0.090 (0.080–0.110)	0.100 (0.098–0.107)	
CV_{BMC}	5.8% (3.4%;8.2%)	2.8% (0.5%;5.0%)	0.08
CV_{BMD}	3.4% (1.6%;5.3%)	0.8% (0.2%;1.3%)	0.03
CV_{Area}	6.9% (3.0%;10.8%)	3.4% (1.0%;5.8%)	0.17
Scan speed: 10 and 20 mm/s			
N[b]	12		
BMD (10 mm/s)	0.090 (0.071–0.095)		
CV_{BMC}	19.3% (14.6%;23.9%)		
CV_{BMD}	5.5% (2.9%;8.1%)		
CV_{Area}	17.3% (11.5%;23.1%)		
Lumbar spine			
Scan speed: 10 mm/s			
N[a]	12	7	
BMD	0.050 (0.037–0.064)	0.120 (0.105–0.133)	
CV_{BMC}	7.3% (2.5%;12.2%)	2.6% (0.3%;4.9%)	0.14
CV_{BMD}	4.7% (3.1%;6.4%)	2.2% (0.7%;3.6%)	0.03
CV_{Area}	5.9% (2.7%;9.1%)	3.9% (0.5%;7.3%)	0.37

Repeated scans of the total body were performed at a scan speed of either 20 (pixel size 1.5 × 1.5 mm) or 20 and 10 mm/s (pixel size 1.0 × 1.0 mm) respectively, and repeated scans of the lumbar spine were performed at a scan speed of 10 mm/s (pixel size 0.5 × 0.5 mm). The *P*-values (*t*-test for unpaired data) for comparison of mean CV between low and normal density groups are shown. The mean (range) BMD (g/cm^2) for each group of animals is shown

[a] Vitamin D depleted 9 weeks (n = 6), Vitamin D depleted + PTX (n = 6), and healthy controls 9 weeks (n = 7)

[b] Vitamin D depleted 9 weeks (n = 6), and Vitamin D depleted + PTX (n = 6).

× 1.5 mm. In rats with low BMD, however, the CV for all three measured bone parameters was significantly higher than in normal rats (Table 2). When the measurements in rats with low BMD, performed at 20 mm/second, were compared with measurements performed at 10 mm/second (pixel size 1.0 × 1.0 mm) in the same animals, the CV for BMC and area of bone was 19.3% and 17.3%, respectively, thus indicating inaccurate determination of both BMC and area at 20 mm/second (Table 2).

Accuracy

BMC and BMD of the excised femurs measured at a scan speed of 2 mm/second were closely related ($R^2 > 98\%$) to ash weight of the bones, and the 95% prediction limits for the regression line were narrow, thus indicating that these DXA measurements could be considered accurate (Fig. 1). BMC ($R^2 = 93.0\%$) and BMD ($R^2 = 94.1\%$) of the lumbar spine obtained from DXA measurements and performed at a scan speed of 10 mm/second (pixel size 0.5 × 0.5 mm) showed a significant relation to the ash weight of the excised femurs, and the prediction limits indicated that the DXA measurements can be used as an accurate measure of bone mineral in the skeleton of small rats (Fig. 2).The relation of total body BMD and ash weight of the excised femurs ($R^2 = 46.3\%$) showed that the DXA measurements were not suitable for accurate measurements of bone mineral although a significant relation was found (Fig. 2). The

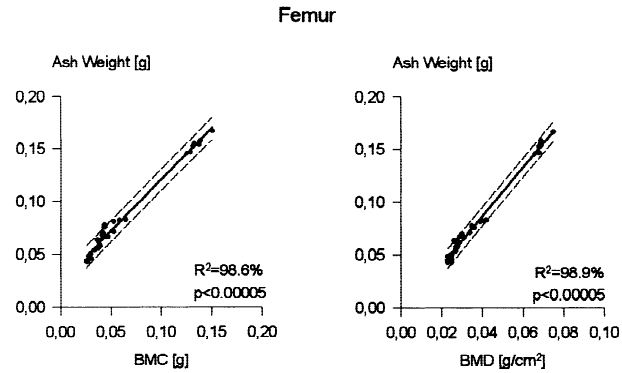

Fig. 1. Results of regression analysis evaluating the relation between ash weight of the excised femurs and BMC and BMD measurements, respectively, of the excised femurs (n = 32, scan speed 2 mm/s, pixel size 0.5 × 0.5 mm). SEE is 5.9% and 5.3%, respectively, for prediction of ash weight from measurements of BMC and BMD.

relation between total body BMC and ash weight of the excised femurs ($R^2 = 93.4\%$) showed that even though the R^2-value was high and the prediction limits relatively narrow, the scatter plot indicated that the regression equation describing the relation between BMC and ash weight in the osteopenic rats (ash weight = 5.6×10^{-2} BMC + 0.02) was different from the equation describing the variation in the group of healthy, normal rats (ash weight = 6.0×10^{-4} BMC + 0.15) (Fig. 2).

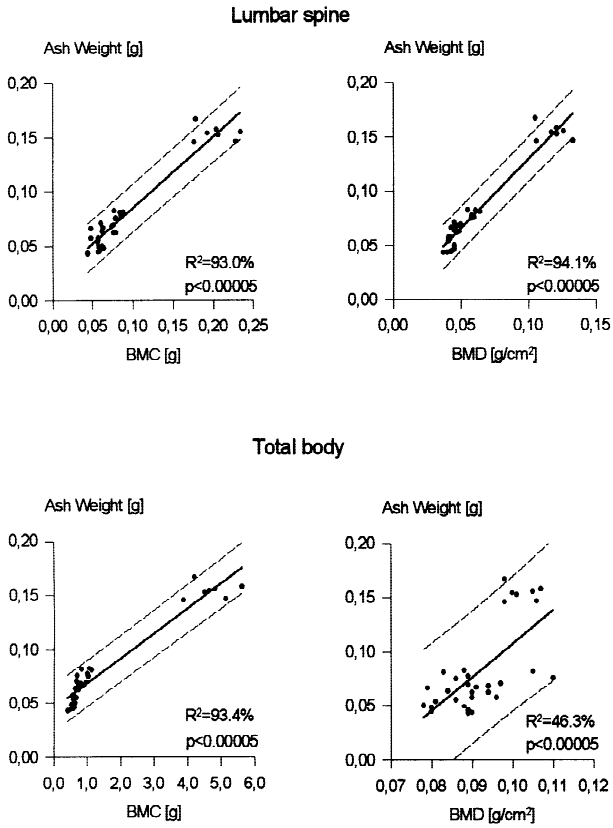

Fig. 2. Results of the regression analysis evaluating the relation between ash weight of the excised femurs and DXA measurements of the lumbar spine (n = 32, scan speed 10 mm/s, pixel size 0.5 × 0.5 mm) and total body (n = 32, scan speed 20 mm/s, pixel size 1.5 mm × 1.5 mm respectively). SEE is 13.1% and 12.2% for prediction of ash weight from measurements of lumbar spine BMC and BMD, respectively, and 12.8% and 36.5% for measurements of total body BMC and BMD, respectively.

Discussion

The accuracy for the application of DXA in rats has previously been evaluated by several other investigators for measurements in, respectively, the lumbar spine [5, 11, 15, 17], total body [11, 12, 14], and femur [7, 8, 12, 16–18]. In all studies it was concluded that the accuracy was excellent and R^2-values between 58% and 99% were found. However, with few exceptions [14–16] none of them calculated a measure of the individual variability around the regression line (SEE or prediction limits for the regression line). It is the measure of individual variability that is the best estimate of the accuracy error [19]. With the exception of the study by Casez et al. [14], all the above-mentioned studies evaluating the accuracy of DXA in rats used animals with bone density above the levels seen in our animals with extremely low BMD. Rozenberg et al. [16] evaluated the accuracy for DXA measurements of excised tibia and the femur bones using a Hologic-QDR-1000 bone densitometer with special software and collimator for small animals. The relation between BMC and bones with ash weight ranging from 0.085 to 0.492 g resulted in r-values higher than 0.99 and very low SEE. In the present study, DXA of the excised femurs (ash weights 0.43–0.167 g) were also shown to have an excellent accuracy for both BMD and BMC with R^2 above 98%, however, the scatter of data points around the regression line, especially in the rats with low bone density, was larger in our study, thus our 95% prediction limits were larger. We found that in young, small but normal rats, measurements of both BMC and BMD of the excised femurs at a scan speed of 2 mm/second were very precise, with CVs of 1.6% and 1.2%, respectively. Also, rats with very low BMD could be measured using these scan parameters, although the CVs were increased by 4–5 times. If the measurements were performed at a scan speed of 10 mm/second the CV was still on the same level, however, the altered scan parameters introduced severe problems with the accuracy for measurements of BMD because of problems with calculation of the bone area. The CV for DXA in normal rats of the present study was at the same level as in previous studies [7, 8, 12, 13, 16, 17]. No previous studies have evaluated the precision for DXA in rats with a bone density as low as in the present study. Keenan et al. [18], who evaluated the accuracy for DXA measurements of excised femurs of 5–6 month-old rats fed on a low vitamin D diet, found BMD values far above the level in our normal density rats, indicating that the relatively large precision errors in our low density animals may be the result of a combination of both low BMD and smaller size bones. The precision of our measurements of total body bone mineral at a scan speed of 20 mm/second (pixel size 1.5 × 1.5 mm) turned out to be approximately at the same level as in excised femurs. However, evaluation of the accuracy showed that these measurements were unreliable not only for rats with low BMD but also in our normal density group. The accuracy problems for measurements in the low density group were confirmed when the total body scans were related to ash weight of femurs and when scans were repeated at 10 mm/second (pixel size 1.0 × 1.0 mm). Previous studies [7, 12] have shown that precise and accurate measurements of rat total body bone mineral are possible with DXA, and using a densitometer (Hologic QDR 1000) equipped with a standard collimator (2.3 mm diameter) Casez et al. [14] documented that precise and accurate DXA measurements can be performed also in small rats with low density if a sufficient scan resolution is used. In the present study the precision for DXA of the lumbar spine again showed to be better in normal density rats compared with low density rats. In normal density rats the precision was at the same level as in previous studies [6, 15, 16]. Furthermore, the evaluation of the relation to ash weight of the excised femurs indicated that the selected scan parameters were appropriate for accurate measurements. When DXA is performed in small rats with low BMD using the Norland XR-26 Mark II bone densitometer we recommend that the measurements be performed on defleshed femurs using a pixel size of 0.5 × 0.5 mm and a scan speed of 2 mm/second. If DXA is performed on frozen rat cadavers, measurements of the lumbar spine (scan speed 10 mm/second, pixel size of 1.0 × 1.0 mm) is preferred. The precision and accuracy for measurements of rats with low BMD of both the excised femur and lumbar spine were on an acceptable level when used for evaluation of bone mineral changes in groups of animals. The total body measurements (scan speed 20 mm/second, pixel size of 1.5 × 1.5 mm), although they appeared to be relatively precise, suffered from problems of inaccuracy because of an overestimation of both BMC and the area of bone. Total body bone mineral measurements using the Norland scanner, as performed in the present study, cannot be recommended when measuring rats with low BMD. All researchers working with DXA in rats should first perform thorough

methodological evaluations. The precision and accuracy should not only be estimated in a small number of normal rats, but should also include rats with the lowest density of the study, and the selection of scan parameters should be taken into consideration.

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