Folia biologica (Kraków), vol. **52** (2004), No 1-2

The Limitation of DEXA Analysis for Bone Mass Determination in Mice

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Accepted January 27, 2004

DICKSON G. R., ŁUCZAK M., WŁODARSKI K. H. 2004. The limitation of DEXA analysis for bone mass determination in mice. Folia biol. (Kraków) **52**: 125-129.

An increase in femoral and tibio/fibular bone mass following periosteal membrane stimulation by Moloney sarcoma virus inoculation into thigh muscles of mice was measured *in situ* on formalin fixed excised hind limbs using a Hologic 4500A Fan Beam X-ray bone densitometer adapted for small bone samples. These results were verified by measurements of constant dry bone mass of the same bones liberated from soft limb tissues by NaOH hydrolysis. There was no consistent data correlation found between the DEXA scan and dry bone mass evaluations. It is concluded that the sensitivity of the DEXA measurement is unsuitable when assessing very small bone samples, weighing merely 20-30 mg.

Key words: DEXA bone mass evaluation, dry bone mass evaluation in mice, mu-MSV osteogenesis.

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There is a need, by investigators, to measure bone mineral in small animal bones, when these are used as models for the study of local effects of agents, e.g. hormones, growth factors, inflammatory-mediated cytokines or tumour development (BAUSS *et al*. 2001; LE ROY *et al*. 2002; WŁODARSKI 1990, 1991; WŁODARSKI & GALUS 1992; WŁODARSKI *et al*. 1991, 1993). The development of tumours following injection of oncogenic Moloney sarcoma virus (Mu-MSV) into murine and rat limb muscles leads to the rapid proliferation of osteoprogenitor cells, resident in the periosteal membranes of bones- adjacent to the tumour and resulting in periosteal osteogenesis (WŁODARSKI & REDDI 1986; WŁODARSKI & THYBERG 1984; WŁODARSKI *et al*. 1979, 1981). Within 5-6 days post Mu-MSV inoculation into mouse thigh muscles, the periosteum becomes thickened and composed of several layers of osteoblasts and fibroblasts, whereas in the contralateral, non-infected bones the periosteum is composed only of 1-2 layers of fibroblast-like cells. Deposition of osteoid begins within this hyperplastic periosteum and in some cases chondrogenesis is observed inside this activated periosteum (WŁODARSKI 1989).

The phenomenon of periosteal membrane, stimulated by Mu-MSV has previously answered the question whether heterotopically induced bone ossicles develop a true periosteal membrane or merely an innert connective tissue capsule (WŁODARSKI & REDDI 1989). After 2-3 weeks, newly-formed cancellous bone covers the old original cortex in varying proportions to its periphery. In some cases it becomes 3-5 times thicker than the original. This orthotopically induced bone formation sometimes affects the whole femoral and tibial/ulnar bone edge but more often is asymmetric. It always affects the site adjacent to the Moloney tumour (WŁODARSKI 1989; WŁODARSKI *et al*. 1979, 1981, WŁODARSKI & REDDI 1986, WŁODARSKI & THYBERG 1984).

The immunologically mediated, spontaneous regression of the Moloney sarcoma is accompanied by the end of periosteal growth and the start of cancellous bone remodelling. The regression starts on days 12-14 post-virus inoculation and is completed by the disappearance of tumours around the 3rd to 4th week post-virus inoculation. This regression refers to soft tissues only and not to the sarcoma-induced cancellous bone formation, which once induced persists for at least 6 months

post-virus inoculation, being accompanied by cancellous bone remodelling.

The yield of the Mu-MSV induced bone could be measured by histomorphometry or by dry bone mass estimation following soft tissue hydrolysis in 0.1 N KOH (WŁODARSKI *et al*. 1981) and the results expressed as a gain versus the contralateral control.

DEXA is a valuable non-invasive clinical tool which was applied by PHILLIPS *et al*. (2000), for assessment of the effectiveness of therapeutic treatment in severe osteogenesis imperfecta, using the oim mouse as a model.The therapeutic effect of administration of human insulin-like growth factor has been investigated in murine calvaria by BAUSS *et al*. (2001) and they reported an increase in bone mass after evaluation by DEXA analysis.

The primary goal of the present communication was to test the usefulness of bone scanning DEXA (equipped with a software package optimized for the analysis of small animal bones and operated in high resolution mode) for assessing bone mass changes in murine limb bones after exposure to the Mu-MSV sarcoma. Later the same samples were chemically isolated and dried bones were weighted on an analytical scale in order to enable comparison with the DEXA results.

Materials and Methods

Twelve male outbred MIZ strain 4 month-old mice were used for these studies, in accordance with the Medical Academy Ethics Committee guidelines for the care and use of laboratory animals. We compared the sensitivity of DEXA scan bone measurements with the dry bone mass weight of the same samples.

The animals were injected into their right thigh muscles with 0.2 mL of a standard solution of murine Moloney sarcoma virus (Mu-MSV) in RPMI 1640 medium. The left thigh muscles of the contralateral control limbs were injected with virus-free medium. The animals were observed daily and tumour development was recorded. Eleven animals were sacrificed on day 26, and one on day 48 post-inoculation. Both hind limbs were excised, fixed in 10% neutral formalin, and the DEXA evaluation of femur and tibia/fibular bones was performed.

Determination of bone mass and density by dual X-ray absorptiometry in the whole shank region was a modification of the technique described earlier (WAHNER & FOGELMAN 1994). The analysis was performed using a Hologic-4500A Fan Beam X-ray Bone Densitometer. Calibration was performed using a Small Animal Step Phantom, so that the scanner collected several lines of air before encountering the thin step of the Phantom. On completion of the calibration scan, the system automatically analyses the step phantom and updates the calibration record. The values for Bone Mineral Density (BMD, gm/cm^2) and Bone Mineral Content (BMC, grams) of paired bones were quantified under uniform conditions. DEXA examination was carried out once and the specimens were not repositioned.

Following densitometry, the formalin fixed limbs were hydrolyzed in 0.1 N NaOH at 64^{o}C, the isolated bones were washed in distilled water, dried thoroughly for 24 hrs at 64^{o}C and weighed on an analytical balance to an accuracy of 0.1 mg. This procedure provided constant weights thus there was no need to do multiple measurements of dry bone mass on specimens and for simplicity the isolated and dried bones were not incinerated.

The scans were assessed in two regions (R1 and R2), which corresponded to the femur and tibia/fibula. From the data obtained for the right, Mu-MSV treated limbs, the corresponding values for the contralateral control limbs were subtracted and the differences in bone weight were expressed as a percentage of the contralateral control values; a (+) denoting an increase in Mu-MSV induced limb bones, whilst (-) indicated a loss in virus exposed bones against a contralateral control.

Results

Following an intramuscular inoculation of Mu-MSV into the limb, a tumour began to appear on day 7-8 post inoculation. On day 17th ten out of twelve animals developed a tumour. Later this regressed spontaneously, so that on day 26 the soft tissue tumours vanished completely and the right limbs were macroscopically indistinguishable from the contralateral, medium-only injected controls. Thus, ten out of twelve mice developed tumours and in two animals the induction failed. The dry mass of bones roughly corresponded to the size of the tumours, as estimated by the arbitrary scale. A comparison of bone yield by the DEXA readings and by dry bone mass measurements in shank and femoral bones are presented in the Tables 1 and 2, respectively. No statistical analysis was performed in this study, as the aim of this report was to find out whether the DEXA method was equally as sensitive for measuring subtle changes in bone mass estimation compared to the dry bone mass evaluation, rather than the analysis of tumour/bone dynamics.

Discussion

The development of a tumour following the Mu-MSV injection was noted in 10 out of twelve

Table 1

Changes in the shank bones (R-2 area) exposed to the MSV-tumour as measured *in situ* by DEXA and by isolated bone weight

Nr of sample	Gain (+) or loss (-) in:			
	Bone area (%)	BMC (%)	BMD (%)	Weight in mg (%)
1.	-23.8	-32.0	-11.2	+2.0
2.	-34.0	-31.0	+4.8	+0.8
3.	+46.0	+79.0	+21.0	+11.3
4.	+25.5	+31.0	+4.6	+2.1
5.	+20.3	+5.0	-9.8	0.0
6.	+16.6	+8.8	-6.6	+2.7
7.	-13.9	-6.1	+9.4	+4.9
8.	+29.4	+32.6	+2.4	+61.8
9.	+91.0	+107.0	+10.0	+80.3
10.	+86.0	+91.0	+2.3	+4.1
11.	+15.8	+8.4	-6.3	+2.3
12. (47 days)	+31.0	+43.0	+48.0	+8.9

Table 2

Changes in femoral bones (R-1 area) exposed to MSV tumour as evaluated by DEXA and by isolated bone weights

Nr of sample	Gain (+) or loss (-) in:			
	Bone area (%)	BMC (%)	BMD (%)	Weight in mg (%)
1.	+1.0	+5.5	+3.9	+1.7
2.	+0.7	+4.3	+3.2	+1.6
3.	+37.0	+27.3	-7.3	+4.1
4.	0.0	+8.5	+8.6	0.0
5.	-10.5	-6.8	+4.1	+2.1
6.	-7.4	+6.0	+14.7	+2.4
7.	-1.0	+14.0	+16.0	+4.1
8.	+59.0	+68.0	+8.0	+3.0
9.	-15.9	-12.0	+7.5	0.0
10.	+22.2	+8.7	-11.0	+3.6
11.	+43.6	+31.0	-9.0	+1.2

animals, thus the tumourgenicity of this virus stock was satisfactory, although in our other experiments a 100% positive response was observed (WŁODARSKI *et al*. 1979, 1981, 1984). In this context it is noteworthy that the tumours formed were of moderate size and their regression was slightly faster than expected. If the Mu-MSV injection failed to develop a tumour, no changes in the local bones were observed (WŁODARSKI *et al*. 1981), so by observing the kinetics of tumour development in the present experiment in two cases no bone changes were found due to lack of tumour development as predicted. In two cases reported here, no bone mass gain (0.0% and 0.8%) was confirmed by the dry bone mass weight. With a big variation between animals we covered a wide range of incremental bone mass changes from non-responding through moderate to highly responding mice and have compared the sensitivity of DEXA against dry bone mass measurements.

The scan analyses of bone area, bone content and bone mineral density gave very inconsistent results, often indicating loss instead of gain. Moreover the scan results were not consistent with the results of dry bone mass measurements, which showed either no dry bone mass increase (0.0-0.8% of bone gain) or low (2.1-2.7%), moderate (4.1-11.3%) and very pronounced (61.8%-80.3%) dry bone mass gain. For the highest bone mass weight increase estimated as 61.8% and 80.3% the scan BMC estimation showed 32.6% and 107% increase. However similar increases in scan values (91% and 79%) were estimated for samples showing only 4.1% and 11.3% dry bone mass weight gain, against contralateral controls. A similar lack of bone scan consistency and of dry bone weight applied to femoral bone readings. From the above analyses we conclude that the DEXA method of measuring bone area, bone mineral content and bone mineral density is unreliable when formol fixed murine limbs are examined.

In our paper on murine bones exposed to Freund's adjuvant-induced inflammatory reaction (WŁODARSKI & DICKSON 2002) we applied the DEXA method as a supplement to histology and bone ash weight measurements for *in situ* bone mass evaluation. The use of DEXA signalled the possibility that this technique could be applied for murine systems, since the DEXA data was in line with otherwise documented postulations. DEXA analysis was of minor importance to the paper, as in the whole set of experiments it was applied to only a few animals. However, encouraged by the preliminary report of a positive correlation between DEXA and dry bone mass measurement, in a mouse model, we sought to apply the DEXA technique to other experimental systems in mice. To our surprise the current findings indicate that such correlation was incidental only, since in many cases no correlation at all has been found between DEXA and dry bone mass data for the same bones.

The adaptation of DEXA for studying the excised limb bones of small animals and for estimating bone mass in human iliac crest biopsy specimens was reported by WAHNER and FOGELMAN (1994). It was established, that the use of a Hologic machine for measuring micro-bone mineral density of cancellous bone gave good linear correlation ranging in weight from 188 to 599 mg over the measurement range (r= 0.997). The meas-

ured mass by scan (BMC (g)) overestimated (5-15%) the true ash weight.

The dry bone mass of femoral, tibial and ulnar bones in adult mice is much lower than the bone samples used by WAHNER and FOGELMAN (1994), approximately 20-30 mg. Our bone samples were 7-20 times smaller than their iliac crest biopsy measurements. Moreover, they scanned their bone samples in 100% etahanol, whilst our limb bones examined by DEXA, were encased in the formol-fixed flesh and skin. Investigating excised rat humeri by DEXA KASTL *et al*. (2002) reported the method as overestimating ash weight by 5%-9%. Their findings showed a high comparability between DEXA and ash weight for the measurement of BMC and general agreement between their *in vivo* and *in vitro* observations. They dealt, however, with relative homogeneity of bone in sibling male rats with BMC varying approximately 25%. This led KASTL *et al*. (2002) to conclude that larger variations may cause larger error as demonstrated by MAKAN *et al*. (1997).

There are now available specialized portable, small animal DEXA scanners. FINK *et al*. (2000) reported that this unit provides an accurate measurement of BMC in guinea-pigs. Unfortunately the mouse, however, is at least 12-20 times lighter than the guinea-pig and the bones are accordingly smaller.

These two factors: i.e. much smaller bone quantity and the presence of soft tissue around the bones might significantly contribute to the failure of DEXA to accurately measure the changes in murine limb bones in our study, although bone size is likely to be the most important factor. This study has significant implications for longitudinal investigations on mouse bones *in situ* for which we conclude the DEXA method is unsuitable. The literature supports, as a satisfactory approach, DEXA analysis of bones *in vivo* and of excised bones, *in vitro*, for assessement of BMD in longitudinal and end-point studies (KASTL *et al*. 2002) respectively for animals the size of rats and larger. Although we did not investigate *in vitro* DEXA analysis of mouse bones freed of all soft tissues, the close agreement between *in vivo* and *in vitro* readings for rats tends to argue against the DEXA approach for mouse bones, based on the inconsistency of our current findings. The presence of formol-fixed tissues, surrounding limb bones seems to be of minor importance when we consider it is present equally in both limbs. At the time of sample harvest, the Mu-MSV induced tumours had regressed completely and macroscopically no differences were visible in the diameter or amount of soft tissues in paired limbs.

It might seem paradoxical, but at present one of the simplest and most reliable methods for measuring subtle changes in bone mass in mice is the measurement of dry bone mass weight using an analytical balance. PHILLIPS *et al*. (2000) determined BMD for the entire mouse and not for individual bones, thus they have measured changes in the whole skeleton and not for its part. For mice DEXA analysis seems to offer a reliable approach only when the whole skeleton or spine is assessed (BENES *et al*. 2000; PHILLIPS *et al*. 2000), although caution should be exercised since sensitivity and application of the method is problematic. PHILLIPS *et al*. (2000) reported use of a customized mouse ultra-high resolution whole body software package (Hologic) in their studies. While Hologic produce ultra-high resolution whole-body and regional protocols for small animal bones, their product is stated as being optimized for the rat, and the maufacturer does not produce a mouse protocol and cannot support studies on mice performed by modification of their equipment (personal communication Hologic, November 2002). Also Hologic supply a small animal step phantom for calibration of the DEXA machine for their high resolution small animal bone protocols and do not recommend use of a human phantom for this purpose.

Our results focus only on the limitations of DEXA for the analysis of mice bones and do not pertain to other species. For rodents bigger than mice, eg. rats, hamsters and guinea-pigs which have a much higher bone mass, DEXA analysis appears to provide use of an acceptable and reliable procedure which offers validated output data (WAHNER & FOGELMAN 1994; FINK *et al*. 2000).

Acknowledgement

Mrs Ewa WIŚNIEWSKA is acknowledged for laboratory work, Mr. Hugh TAGGART for access to the DEXA scanner and The Royal Society London for grant support to GRD enabling purchase of the high resolution DEXA package. Supported by grant 1M15/NK 2002 to KHW.

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