ASSESSMENT OF BONE DECALCIFICATION BY QUANTITATIVE ULTRASONIC PARAMETERS IN ANIMAL MODEL

A. Fontes-Pereira*, P. Rosa*, D. Matusin*, T. Barboza**, S. Souza**, M. A. von Krüger* and W. C. A. Pereira*

* Biomedical Engineering Program/UFRJ, Rio de Janeiro, Brazil
** Laboratório de Marcação de Células e Moléculas (LMCM), Hospital Clementino Fraga Filho (HUCFF/UFRJ) and Laboratório PET/SPECT/CT Cenabio (UFRJ), Rio de Janeiro, Brazil
email: aldo.fontes@gmail.com

Abstract: Ultrasound pulse echo parameters and a simple protocol are here proposed for in vitro characterization and monitoring progressive degrees of decalcification of rat femurs. Two quantitative parameters: Integrated Reflection Coefficient (IRC) and Frequency Slope of Integrated Reflection (FSIR) were estimated from in vitro bone surface echoes in eight femur diaphysis of Wistar rats. The echo signals were acquired from three previously chosen locations along the distal lateral third of the femur diaphysis during the decalcification process by EDTA. A positive correlation with quantitative computerized tomography was found for the results. This is an indication that the proposed protocol has potential to characterize bone tissue in animal models, providing more consistent results standardizing bone characterization studies by QUS endorsing its use in humans.
Keywords: Bone, Tissue characterization, Quantitative ultrasound, Animal model.

Introduction

Bone injury may cause negative impact to an individual [1]. This is why it is relevant to develop, employ and improve tools and methods to access bone quality and monitor bone condition. Such is the case of Quantitative Ultrasound (QUS) that provides quantitative information on bone tissue integrity [1,2] and opens an additional alternative for diagnostics of bone structural diseases and also for healing process monitoring.

In literature, it is possible to found reports of several ultrasonic parameters that, with varying degrees of success characterize bone. This is an indication of lack of an adequate standardization and leads to difficulties in extrapolating results to clinical trials. There is not check list comprehending such parameters. Few studies are found using protocols in rat bones [2,3] and there are no studies in rat bones with different degree of calcification.

The aim of this work is to monitor femurs of rats in vitro during bone decalcification, based on two QUS parameters: Integrated Reflection Coefficient - IRC and Frequency Slope of Integrated Reflection - FSIR.

The choice of rat bones is because rat bone tissue is more similar to human [4], exception made for primates. Furthermore, rats are animal models for the evaluation of metabolic bone diseases [4,5] and pathophysiological conditions [4,6].

Materials and methods

Ethical Norms – The research was approved by the Ethical Committee for the Use of Laboratory Animals in Research of the Faculty of Medicine of the Federal University of Rio de Janeiro (UFRJ) (Protocol N. 18/11), following the Guidelines for Care and Use of Animals in Research [7].

Samples – The *in vitro* samples consisted of eight femurs of healthy rats (*Rattus Norvergicus Albinus*) weighting 54±0.2g. Before acquisition of signal, the femurs were maintained on average for 30 days in the presence of beetle larvae (*Dermestes Maculatus*) to totally remove the soft tissue. The marrow was completely removed too.

The demineralization was made by immersing the femurs in a 25-ml solution of ethylenediaminetetraacetic acid (EDTA) disodium salt (Sigma-Aldrich®, Missouri, USA), pH=8, at 0.376 M, for 24-h, at 25± 1.5ºC, using a new solution and new bottle in each day. After each 24-h demineralization period, the femur was assessment by quantitative ultrasound (QUS) and quantitative computed tomography (QCT) (Figure 1). This procedure was followed for 5 days, similar to Machado et al.[8]. The demineralization was made at the same environment temperature (25±1.5ºC).

Signal acquisition protocol – For characterization of femur diaphysis (22±0.2mm length), echo signals from the bone surface were acquired, with transducers and samples immersed in distilled water (20.6±0.6ºC) according to the protocol below:

- Femurs were positioned on a reflector steel plate (5.80-cm thick).
- Transducer of 5-MHz frequency (model V326, Olympus® NDT Inc., Massachusetts, EUA), diameter of 9.5 mm and 69.3 mm focal length, excited by pulse generator US-key (Lecoeur Electronique®, Loiret, FR).
- The transducer focal region placed at the distal lateral third of the femur diaphysis (Region of Interest - ROI).
- Three signals acquired in the femur, in 1.5-mm steps controlled by a stereotactic holder of 2-μ

resolution. Each parameter was calculated from the three signals for each rat femur per day, and the average of these values was considered as representative of the femur. So, each femur was characterized by two parameters per day.

• Reference signals collected from steel plate at the same distance of femurs (69 mm).

Ultrasonic parameters measurement – According to Fontes-Pereira et al. [3] to identify the bone surface echo it was necessary to determine the length of the reference echo by selecting the position of the extreme

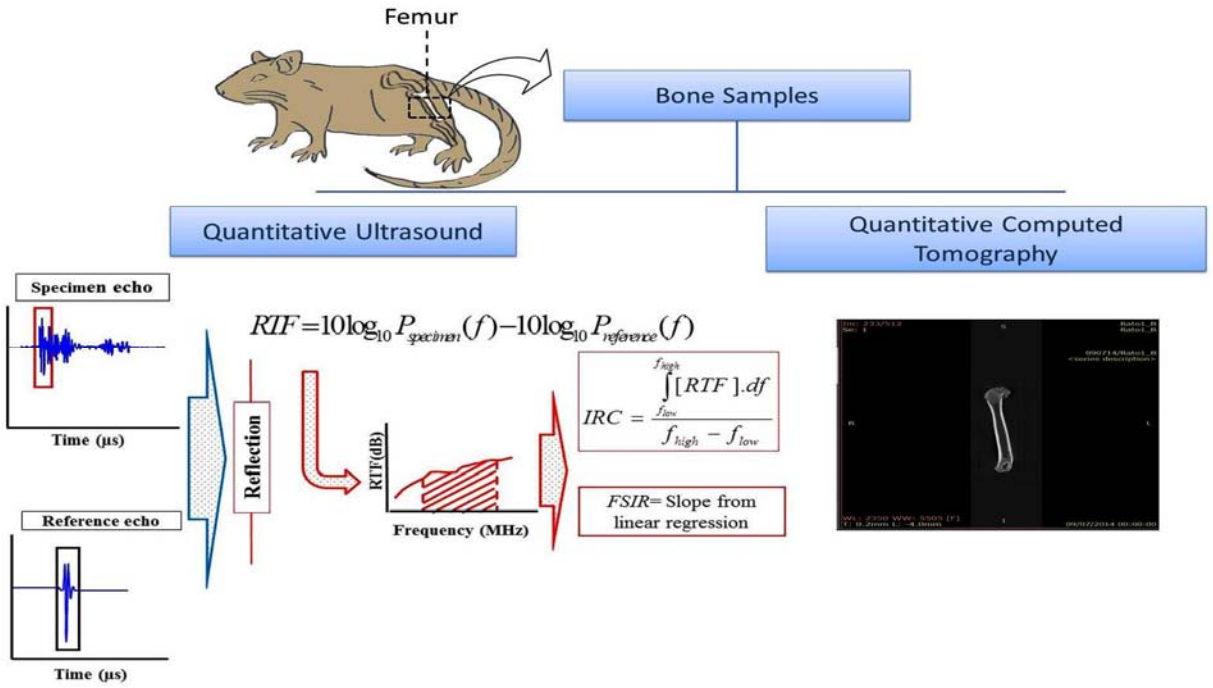

Figure 1: Setup experimental and schematic diagram of the steps to obtain each of the parameters.

limits (corresponding to 10% of its maximum amplitude). Then a rectangular window was created around the reference echo (steel plate). After that this window was used to define the limits of the bone surface echo. The algorithm was developed in Matlab® (MathWorks Inc., Massachusetts, USA) to estimate the ultrasonic parameters from femurs and reference signals. The parameters IRC and FSIR were estimated based on the Reflection Transfer Function – RTF, defined as:

$$RTF = 10\log_{10} P_{specimen}(f) - 10\log_{10} P_{reference}(f) \quad (1)$$

where $P_{specimen}$ and $P_{reference}$ are the power spectra of the signals from sample and from reference plate, respectively.

The IRC parameter expresses the average value of the reflection in a studied frequency (f) range. The integration of RTF over frequency gives the IRC, according to equation 2:

$$IRC = \frac{\int_{f_{low}}^{f_{high}} (RTF)df}{f_{high} - f_{low}} \quad (2)$$

The FSIR represents the fraction of the reflection related to each frequency and is obtained as the slope value resulting from a linear regression of the RTF versus frequency plot.

QCT acquisition – QCT was performed with a small animal PET/SPECT/CT camera (Flex Triumph, GE-Gamma Medica Ideas, Northridge, CA, USA). The protocol of acquisition was based on axial slices of 5-mm thickness, 2.3X collimation, 1 frame of 1024 slices, 75 kVp and 140 µAs, making a 4-minute total time. Eight femurs were put in the scanning plate for six days. The tomographic images were processed with the RadiAnt DICOM Viewer software (RadiAnt DICOM Viewer©, Poznań, PL) for bone density analysis (in Hounsfield units) of the femur diaphysis. These data were used as the gold standard for bone density.

Statistical Analysis – Normality was tested by the Kolmogorov-Smirnov test and equal variance test. The statistical analysis using Pearson test ($\alpha = 0.05$ level of significance and confidence interval (CI) = 95%) was applied to test the correlation between the variables. The tests were carried out using SigmaStat 3.5 (Systat Software, Inc., California, USA).

Results

The average values and standard deviations of parameters (IRC and FSIR) for each experiment are

shown in Table 1. Figure 2 shows the variation of parameters during the period of decalcification.

Pearson's correlation coefficient showed a positive correlation between surface bone density and IRC (Figure 3A) and FSIR (Figure 3B) for the five experiments.

Table 1: Average values and standard deviation of each parameter in five experiments

Rats	Parameters	Days				
		1	2	3	4	5
1	IRC	-19.85 (3.86)	-24.01 (4.41)	-17.35 (2.21)	-27.40 (3.42)	-46.33 (5.26)
	FSIR	-2.77 (0.95)	-3.07 (0.81)	-4.28 (1.63)	-6.15 (1.12)	-6.93 (1.61)
2	IRC	-15.92 (1.21)	-17.59 (3.37)	-15.51 (1.04)	-19.87(2.96)	-35.17 (2.10)
	FSIR	-5.33 (1.61)	-2.40 (1.26)	-4.59 (1.46)	-3.39 (0.88)	-5.57 (1.17)
3	IRC	-24.25 (3.06)	-20.77 (5.61)	-21.98 (2.24)	-24.29 (2.05)	-31.70 (1.59)
	FSIR	-4.11 (1.82)	-4.27 (0.36)	-3.70 (0.44)	-4.91 (1.07)	-3.00 (0.42)
4	IRC	-18.25 (5.50)	-19.35 (3.58)	-19.63 (2.43)	-26.11 (7.34)	-38.80(2.40)
	FSIR	-4.59 (2.69)	-2.08 (0.72)	-5.49 (0.63)	-3.47 (0.47)	-4.37 (2.59)
5	IRC	-13.95 (4.03)	-17.94 (2.33)	-15.04 (1.49)	-29.92 (3.91)	-30.40(1.27)
	FSIR	-3.30 (1.62)	-2.21 (1.37)	-3.89 (0.08)	-4.59 (0.32)	-5.01 (0.73)
6	IRC	-17.03 (1.22)	-29.79 (0.95)	-18.99 (0.89)	-22.75 (5.43)	-31.76(2.19)
	FSIR	-2.11 (1.67)	-3.77 (1.46)	-4.44 (1.01)	-4.81 (0.48)	-6.06 (1.23)
7	IRC	-18.22 (3.67)	-16.46 (0.68)	-22.75 (2.63)	-24.83 (3.56)	-28.68 (1.78)
	FSIR	-2.66 (2.58)	-5.61 (0.41)	-3.80 (1.06)	-5.28 (1.12)	-5.77 (0.92)
8	IRC	-22.29 (5.93)	-10.58 (2.59)	-19.36 (1.53)	-28.99 (2.73)	-31.44 (3.38)
	FSIR	-3.42 (0.66)	-2.76 (1.38)	-4.89 (0.67)	-4.51 (1.45)	-4.45 (1.22)

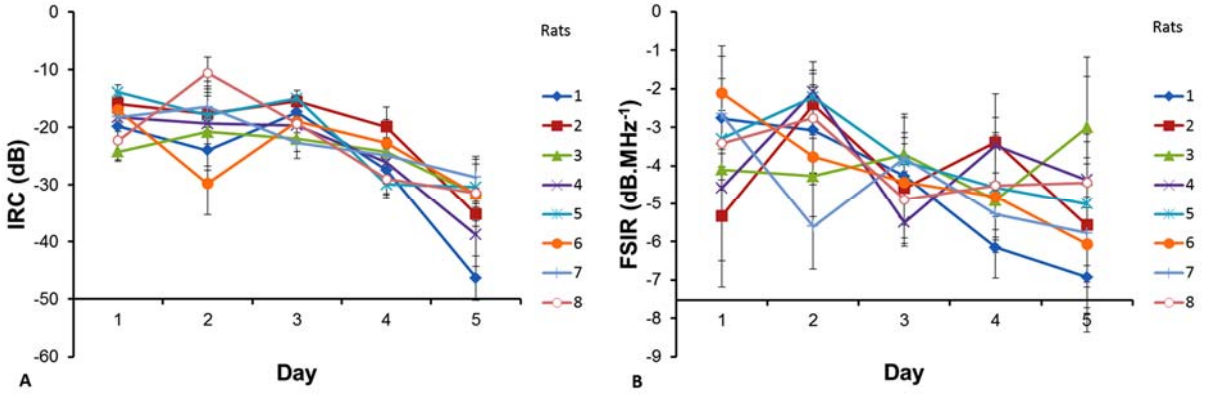

Figure 2: Variation of parameters (IRC (A) and FSIR (B)) during the period of decalcification.

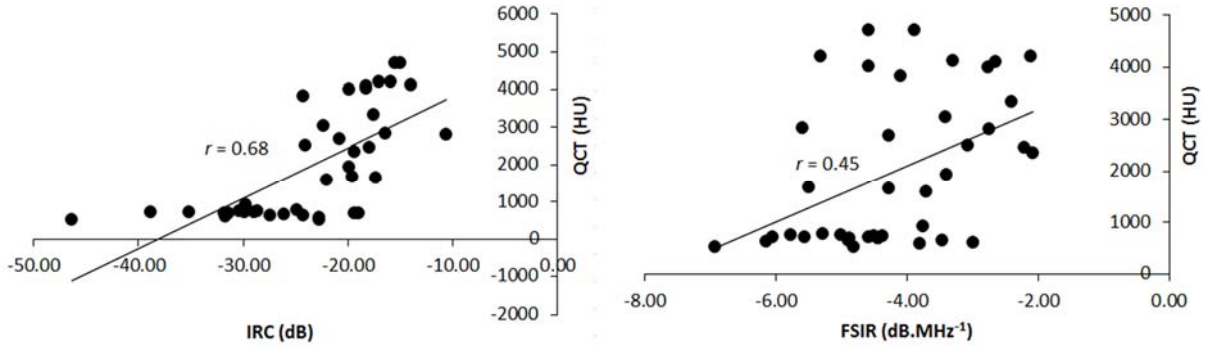

Figure 3: Pearson's correlation between surface bone density, the integrated reflection coefficient (IRC; A) and frequency slope of integrated reflection (FSIR; B). The experiment showed positive correlation between surface bone density and the parameter IRC (r=0.68; CI=0.47-0.82; P≤0.0001) and the parameter FSIR (r=0.45; CI=0.16-0.67; P=0.0036).

Discussion

The lowering of bone density in presence of EDTA shown in Figure 2 supports the feasibility of mimicking bone diseases decreasing the need of animal model.

This work represents an important contribution to the use of QUS as an adjuvant tool in diagnosis and monitoring of fracture healing and metabolic bone disease. Among the advantages of QUS in relation to QCT are the lower cost and the reduction the exposition to ionizing radiation. This is: in a series of N periodical assessments with QCT a fraction n/N can be performed with QUS. The literature on methods for the characterization of bone by QUS is extensive [1,3,8,9,10], but, to date, there is no standardization of methods.

Several analyzes have been performed, including mathematical simulations [11], in vitro studies [2] and animal models [12], however the results did not allow extrapolation to human case for many reasons, mainly lack of reproducibility. A significant contribution [8] for the advancement of bone characterization by QUS was made on bovine bone with increasing degrees of decalcification by EDTA.

In the present study, a similar experiment was performed but it was made in rat bones at different degrees of decalcification with special care for standardization and protocol consolidation with the aim of transferring information to clinical use in humans. This is the first study to do this analysis in a large sample number of animals and in bones of Wistar rats. A contribution of the present work is the employment of rat bones. This is relevant because the characteristics of rat bone tissue are more similar to human bones [4], except for primates. Furthermore, as the rats are animal models for the evaluation of metabolic bone diseases [5,4] and pathophysiological conditions [6,4]. Because the rat femur are small (3.16±0.1mm), it was necessary to use a 5-MHz transducer for acceptable resolution [3]. The precise positioning of the ultrasound beam of the three places of acquisition over the bone, chosen close enough to ensure minimal anatomical variations, was assured by a high-precision stereotactic holder.

QCT provides accurate measurements of bone density of the cortical bone which is used as a gold standard. A positive correlation between the parameters (FSIR and IRC) and bone density surface was identified. According to the correlation coefficient value, we consider this study adequate to characterize the density of the cortical bone method. The low coefficient of variation assured a good accuracy of this method.

The experiments were performed in five days, after the action of EDTA (24-h-immersion for day) to characterize and to monitor bone decalcification. Statistical tests showed a better correlation between the parameters IRC and QCT than FSIR and QCT suggesting that coincidence between QUS and QTC measuring points must be improved. However, the experiments indicate that the parameters have the potential to characterize bone. It was the first time that correlation between QUS and QCT was done. We believe it is relevant for the research of bone characterization by QUS that, applied according to the method here described, can be used in monitoring bone fracture diseases and diseases where bone loss is observed for example osteoporosis and osteopenia.

A next step would be relating the calcium concentration in the EDTA with IRC and FSIR and histomorphometry data.

Conclusion

The protocol and QUS parameters demonstrate potential to characterize and for monitoring the diaphysis of the femur rats by the method of ultrasound pulse-echo. The parameters as well as simple protocol for signal acquisition provide further predictive data of living human bone. Furthermore, this research contributes to the use of rats in future characterization studies of bone by QUS to provide results more consistent standardize the bone characterization studies by QUS and thus endorse the use of QUS in humans.

Acknowledgements

Research supported by CNPq, CAPES and FAPERJ.

References

[1] Hakulinen MA, Toyras J, Saarakkala S et al. Ability of ultrasound backscattering to predict mechanical properties of bovine trabecular bone. Ultrasound in Medicine & Biology. 2004; 30(7):919-27.

[2] Fontes-Pereira A., Rosa PT., Matusin D *et al*.. Use of Ultrasonic Parameters as Adjuvant Tool for Diagnosis and Monitoring of Bone Lesions. In: *International Work-Conference on Bioinformatics and Biomedical Engineering*; 2013 March 18-20; Granada, 2014. p. 243-50.

[3] Fontes-Pereira A, Matusin DP, RosaP et al.. Ultrasound method applied to characterize healthy femoral diaphysis of Wistar rats in vivo. Brazilian Journal of Medical and Biological Research. 2014; 47(5):403-10.

[4] Lelovas PP, Xanthos TT, Thoma SE et al.. The Laboratory Rat as an Animal Model for Osteoporosis Research. Comparative Medicine. 2008; 58(5):424-30.

[5] Blouin S, Basle MF *and* Chappard D. Rat models of bone metastases. Clinical & Experimental Metastasis. 2005; 22(8):605-14.

[6] Fontes-Pereira A J, Teixeira RC, Oliveira AJB et al.. The effect of low-intensity therapeutic ultrasound in induced fracture of rat tibiae. Acta Ortopédica Brasileira. 2013; 21:18-22.

[7] NRCoN A. Guide for the care and use of laboratory animals. 8ª ed. National Academies; 2011.

[8] Machado CB, Pereira WC, Granke M et al.. Experimental and simulation results on the effect of cortical bone mineralization in ultrasound axial transmission measurements: a model for fracture healing ultrasound monitoring. Bone. 2011; 48(5):1202-

09.
[9] Wear KA, Stuber AP, *and* Reynolds JC. Relationships of ultrasonic backscatter with ultrasonic attenuation, sound speed and bone mineral density in human calcaneus. Ultrasound in Medicine & Biology. 2000; 26(8):1311-16.
[10] Laugier P. Instrumentation for in vivo ultrasonic characterization of bone strength. IEEE Transactions on Ultrasonics Ferroelectrics and Frequency Control. 2008; 55(6):1179-96.
[11] Protopappas VC, Fotiadis DI *and* Malizos KN. Guided ultrasound wave propagation in intact and healing long bones. Ultrasound in Medicine & Biology. 2006; 32(5):693-708.
[12] Barbieri G, Mazzer N, Ribeiro EA et al.. A Comparative Analysis between Ultrasonometry and Computer-Aided Tomography to Evaluate Bone Healing. Journal of Orthopaedic Research. 2012; 30(7):1076-82.