Synchrotron radiation X-ray microtomography and histomorphometry for evaluation of chemotherapy effects in trabecular bone structure

R Alessio\textsuperscript{1}, L P Nogueira\textsuperscript{2}, A P Almeida\textsuperscript{1}, M V Colaço\textsuperscript{2}, D Braz\textsuperscript{1}, C B V Andrade\textsuperscript{3}, C Salata\textsuperscript{3}, S C Ferreira-Machado\textsuperscript{4}, C E de Almeida\textsuperscript{1}, G Tromba\textsuperscript{5} and R C Barroso\textsuperscript{2}

\textsuperscript{1}COPPE - Universidade Federal do Rio de Janeiro, P.O. Box 68509, 21945-970, Rio de Janeiro, Brasil.
\textsuperscript{2}Laboratório de Física Médica – Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier, 524 PJLF, sl 3007F, Rio de Janeiro, Brasil.
\textsuperscript{3}Laboratório de Ciências Radiológicas – Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier, 524 PHLC, sl 136, Rio de Janeiro, Brasil.
\textsuperscript{4}Departamento de Biologia Geral, Universidade Federal Fluminense, Outeiro de São João Batista, s/n, Niterói, Brasil
\textsuperscript{5}Sincrotrone Trieste ScpA, Strada Statale S.S. 14 km 163.5, 34012 Basovizza, Trieste, Italy

E-mail: liebertrj@gmail.com

Abstract. Three-dimensional microtomography has the potential to examine complete bones of small laboratory animals with very high resolution in a non-invasive way. One of the side effects caused by some chemotherapy drugs is the induction of amenorrhea, temporary or not, in premenopausal women, with a consequent decrease in estrogen production, which can lead to bone changes. In the present work, the femur heads of rats treated with chemotherapy drugs were evaluated by 3D histomorphometry using synchrotron radiation microcomputed tomography. Control animals were also evaluated for comparison. The 3D tomographic images were obtained at the SYRMEP (SYnchrotron Radiation for MEdical Physics) beamline at the Elettra Synchrotron Laboratory in Trieste, Italy. Results showed significant differences in morphometric parameters measured from the 3D images of femur heads of rats in both analyzed groups.

1. Introduction

Chemotherapy often causes significant bone loss, marrow adiposity and haematopoietic defects. One of the side effects caused by some chemotherapy drugs is the induction of amenorrhea, temporary or not, in premenopausal women, with a consequent decrease in estrogen production. It leads to bone changes, similar to those presented in osteoporosis [1].

The osteoporosis is defined as a systemic skeletal disease characterized by low bone mass, microarchitectural deterioration and changes of bone tissue, resulting in increased bone fragility and susceptibility to fracture risk. It is generally accepted that trabecular bone strength depends not only on
bone volume but also on its structures, which consist of connected bony plates. The investigation of a rat model for osteoporosis from X-ray microtomography has already been described previously [2, 3, 4].

Imaging techniques based on microtomography (µCT) enable three-dimensional (3D) non-destructive analysis of bone microarchitecture. µCT technique can be used to image and to quantify trabecular bone and this quantification has the capability to address the role of trabecular architecture on the mechanical properties of bone. The use of rat requires even higher spatial resolution, just because it has thinner bone structures. Trabecular diameter in rats is less than 100 µm while in humans, greater than 120 µm. To this goal, the combination of synchrotron radiation (SR) with µCT technique yields notable advantages, including high spatial resolution and coherence what can be achieved due to the high intensity of synchrotron facility and the natural collimation of the beam, and the wide spectral bandwidth allows continuous tuning of energy from a few keV to several tens of keV [5, 6].

For quantification, a volume of interest (VOI) has to be chosen in the way that it is comprised only of trabecular bones. For this VOI, the following parameters can be computed: bone volume-total tissue volume ratio (BV/TV), trabecular thickness (Tb.Th (mm)), trabecular separation (Tb.Sp (mm)), trabecular number (Tb.N (mm⁻¹)) and structure model index (SMI). Different parameterization methods may be applied to extract quantitative architecture parameters from the sample such as in conventional histology. But this technique is somehow tedious and time-consuming, as it includes sample sectioning (the sample is destroyed) and the parameters are visually assessed in two dimensions, so the third dimension has to be added using the basis of stereology. In 3D computed microtomography, the histomorphometric parameters are assessed from 3D images of the bone structures, non-destructively, fastly, and precisely [7, 8, 9]. This technique was pioneered by Feldkamp and co-workers [10].

This work aims to evaluate changes in trabecular bone architecture of rats treated with a combination of chemotherapy drugs, using 3D computed microtomography and histomorphometry.

2. Materials and methods

2.1. Specimens

In this study, the experimental animals were ten adult female Wistar rats at 90 days of age, divided randomly into two groups, with n = 5 each. The treated group received doses of docetaxel and cyclophosphamide drugs (G1) while control group (G0) was sham treated. At the beginning of the study, the animals weighed an average of 200 g. The rats were acclimatized with standard conditions of temperature (25°C) and light-controlled environment (12 h light-dark cycles). Food and water were provided ad libitum. The treatment lasted for 1 month in cycles of 4 and 7 day of intervals between them. At the same time, untreated animals of the same age were sham treated as control. The rats were sacrificed by direct heart KCl injection at 150 days post-treatment, at 240 days of age, and femurs were excised, cleaned and let to air dry for at least 72 hours. Ethics permission to utilize the animals for the research described in this paper was obtained from Ethics Committee on Animal Research of the State University of Rio de Janeiro (Process CEA/010/2012). Figure 1 illustrates the location from where images were acquired.
2.2. Synchrotron radiation computed microtomography (SR-µCT)

All the specimens were imaged using the new high resolution microCT setup which has been recently available at the Synchrotron Radiation for Medical Physics (SYRMEP) beamline of the Elettra Synchrotron Light Laboratory (Trieste, Italy). The SYRMEP light source is one of the bending magnets of ELETTRA. The horizontal acceptance covered by the front-end light-port is 7 mrad. The useful energy range is 8.5-35 keV. Typical flux measured at the sample position at 17 keV is about 1.6×10⁸ ph/mm²s, with a stored electron beam of 300 mA when ELETTRA is operated at 2 GeV, while it is 5.9×10⁸ ph/mm²s, with 140 mA at 2.4 GeV. A custom-built ionization chamber, placed upstream to the sample, is used to determine the exposure on the sample, and hence to calculate the delivered dose [11]. In order to optimize the performances of the µCT setup for high resolution experiments in the SYRMEP beamline, 25-µm thick single crystal of cerium-doped lutetium–aluminum garnet (Lu₃Al₅O₁₂) scintillator screen (Crytur, Czech Republic) was coupled to an air-cooled 16 bit CCD camera (Photonic Science, KAI 4022M CCD, 2048 x 2048 full frame) via a visible light microscope optics (LEICA), effective pixel size of 1.03 µm. This system designed to achieve up to 2 µm spatial resolution was used in white/pink X-ray beam mode that provides a nearly parallel, laminar-section X-ray beam with a maximum area of 100 mm (horizontal) x 6 mm (vertical) at a distance of about 15 m from the source.

For each bone sample, 1800 radiographic images were acquired over an angular range of 360° with angular step of 0.2°. Sample-detector distance was set to 9 cm and scanning time was approximately 1h for each sample. The sample was positioned so that the region of femur head lied on the field of view of the detector, not been necessary to cut the sample. Palladium (0.047 mm) and Silicon (1.5 mm) filters were used to cut the low energy X-ray components therefore, the average energy was around 24 keV. The 2D radiographies are normalized by using flat (images without the samples) and dark (background) images. This procedure allows one to take into account incident beam non-uniformities and to correct fixed noise due to the efficiency of the detector elements. After radiographic data acquisition, the SYRMEP Tomo Project software was used to reconstruct the slices [12]. The reconstruction was performed using filtered back projection with Shepp Logan filter. The quantitative analysis was performed in a VOI with 200 x 158 x 500 pixels, starting 0.5 mm below the top of femur head. The ImageJ® software [13] was used to render the 3D volumes and BoneJ software (ImageJ plugin) [14] was employed for quantifying the samples.

![Figure 1. Femur head - location from where the microtomographic images were obtained.](image-url)
2.3. Morphologic parameters

Prior to quantification of morphologic parameters, the images had to be binarized so that voxels corresponding to bone could be distinguished from those of background. To distinguish bone tissue from marrow and background, an optimal threshold for each specimen was determined. The method used is implemented in ImageJ, called IsoData, also known as Iterative Intermeans [15].

The morphologic parameter BV/TV was calculated by the number of foreground (bone) voxels divided by the total number of voxels in the image. The 3D model-independent parameter Tb.Th was computed based on the calculation of the local thickness volume [16]. The local thickness $\tau(n)$ at any point $n \in \Omega \subseteq \mathbb{R}^3$ is defined as the diameter of the largest sphere containing the point $n$ and which is completely inside the structure $\Omega$, i.e:

$$\tau(n) = 2 \max \{ r / n \in \text{sph}(x, r) \subseteq \Omega, x \in \Omega \}$$

This definition was given in the context of the continuous space utilizing the Euclidean distance. The trabecular separation Tb.Sp was computed using the same process but applied to the complement of the binary trabecular bone structure. The number of trabeculae, Tb.N, was determined using:

$$Tb.N = \frac{BS}{2TV}$$

BS, the surface of trabecular bone, was calculated using a triangular surface mesh by marching cubes and bone surface area can be assumed to be the sum of the areas of the triangles making up the mesh [17].

An estimation of the plate-rod characteristic of the structure is achieved using the Structure Model Index, SMI [18]. This parameter is calculated by a differential analysis of a triangulated surface of a structure and is defined as

$$SMI = 6 \frac{BV}{BS} \frac{dBS}{dr}$$

where $dBS/dr$ is the surface area derivative with respect to a linear measure $r$, relative to the half-thickness. For an ideal plate and rod structure the SMI value is 0 and 3, respectively. For structure with both plates and rods of equal thickness the value is between 0 and 3, depending on the volume ratio between rods and plates.

3. Results

The femoral head samples were scanned to reveal the two-dimensional (2D) and three-dimensional (3D) trabecular microstructure. Figure 2 presents the 2D and 3D microstructure of a typical specimen from G0 and G1 groups. The 3D rendered images, together with 2D sections of the G1 group (Figure 2) reveal the possible changes in the microarchitectural cancellous femoral head. It can be seen that the microstructure of femoral head in the chemotherapy group showed a greater spacing between the trabeculae and loss of interconnections, compared with control group.
Figure 2. Two-dimensional trabecular microstructure from SR-µCT images of rat femoral head (a) and 3D ROI from where histomorphometry was computed: (1) Control group and (2) Chemotherapy group.

On the original 3D image, morphometric indices were determined directly from the binarized volume of interest (VOI). Statistical differences among groups were evaluated with Student's t-test. P-values less than 0.05 were considered to be significant. Table 1 indicates the mean values and respective standard deviations of the different trabecular parameters for both groups. The statistical significance between the two groups is reported in the last line through the p-value.

The results reveal a significant decrease in BV/TV as well as an increase in spacing (Tb.Sp) but not significant for rats treated with chemotherapeutic drugs. This fact can be clearly seen in microtomographic images of the samples of this group (Figure 2). The trabecular thickness (Tb.Th) increased 7% in the G1 group, compared to the control one, but not significantly. The number of trabeculae per millimeter (Tb.N) decreased of 13% in G1 group compared with control one (P = 0.095). Bone volume to total volume ratio (BV/TV) decreased 31% (P = 0.006) with a consequent increase of 36% (P = 0.063) in trabecular spacing (Tb.Sp). The Structure Model Index (SMI) increased significantly 154% (P = 0.02) in G1 group comparing to G0, showing values varying between 0.244 and 2.240, indicating that trabeculae appear to have mixed plate-rod structures. The lowest value obtained (0.244) is closer to the plate-like, while the highest value (2.240) resembles rod-like structures. The deterioration of cancellous bone structure due to aging and diseases such as osteoporosis is characterized by a conversion from plate elements to rod elements. Consequently the terms "rod-like" and "plate-like" are frequently used for a subjective classification of cancellous bone [19]. Group treated with chemotherapy presented SMI of 1.840, indicating that structures are closer to rod-like, while control group presented SMI of 0.724, meaning more plate-like structures which reflects higher mechanical strength.
Table 1. Histomorphometric indices for femur head.

| Groups     | BV/TV    | Tb.Th (mm) | Tb.Sp (mm) | Tb.N (mm⁻¹) | SMI       |
|------------|----------|------------|------------|-------------|-----------|
| G0 (n = 5) | 0.494 ± 0.041 | 0.028 ± 0.002 | 0.076 ± 0.019 | 8.454 ± 0.880 | 0.724 ± 0.480 |
| G1 (n = 5) | 0.339 ± 0.040 | 0.030 ± 0.005 | 0.103 ± 0.021 | 7.379 ± 0.921 | 1.840 ± 0.598 |

P value  
- P = 0.006ᵃ  
- P = 0.460  
- P = 0.063  
- P = 0.095  
- P = 0.02ᵃ

ᵃ Significantly different (P < 0.05)

4. Conclusions

The use of synchrotron sources with considerably higher flux opens up new possibilities in the analysis of 3D images from bone samples, in particular to access quantitative analysis of trabecular microarchitecture. Three-dimensional SR-µCT was used to investigate bone architecture in femur head site. After image acquisition, 3D image processing technique was used to fully exploit 3D data. The advance in three-dimensional imaging techniques allows studying the organization of trabeculae in great detail. Concerning the obtained results for control and treated bones from same skeletal site, a certain declining bone volume fraction was achieved. The relative bone loss (decreased BV/TV) between chemotherapy group and control group in the femur head accompanied by lower Tb.N and higher Tb.Sp seems to indicate that chemotherapy induced bone loss and it is manifested through decreased connectivity and loss of thin trabecular elements. SMI seems to indicate that structures are tending to turn from plate to rod-like as chemotherapy is applied, suggesting a lower mechanical strength in the bones of chemotherapy treated animals.

In conclusion, the results obtained could be used in forming the basis for comparison of the bone microarchitecture and the resulting 3D high resolution images along with histomorphometric quantification can be a valuable tool for predicting bone fragility.

Acknowledgments

The authors thank the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support. This work was supported by ICTP-ELETTRA Users Programme (Projects SYRMEP 2007834 and 20090192). These images were obtained during a visit (R.C.B.) within the Associate Scheme of the Abdus Salam International Center for Theoretical Physics (ICTP). We acknowledge the SYRMEP group for help during image acquisition and reconstruction.

References

[1] Marcus R, Feldman D, Dorothy N and Clifford J 2008 *Osteoporosis* **2** 1337
[2] Barbier A and Martel C 1999 *J. Bone Miner Metab* **17** 37
[3] Laib A, Barou O, Vico L, Lafage-Proust M, Alexandre C and Ruegsegger P 2000 *Med Biol Engng Comput* **38** 326
[4] Martín-Badosa E, Elmoutaquakkil A, Nuzzo S, Amblard D, Vico L, Peyrin F 2003 *Medical Imaging and Graphics* **27** 447
[5] Muller R 2009 *Nat. Rev. Rheumatol.* **5** 373
[6] Margaritondo G and Meuli R 2003 *Eur. Radiol.* **13** 2633
[7] Nogueira L, Almeida A P, Braz D, Andrade C B, Salata C, Tromba G, Almeida C E and Barroso R C 2012 *Appl. Radiat. Isot.* **70** 1296
[8] Nogueira L, Almeida A P, Braz D, Andrade C B, Tromba G, Almeida C E and Barroso R C 2011 *AIP Conf. Proc.* **39** 1437
[9] Nogueira L, Braz D, Oliveira L F, Pinheiro C J G, Dreossi D, Tromba G and Barroso R C 2010 *Micron* **41** 990
[10] Feldkamp L A, Goldstein S A, Parfitt M A, Jesion G and Kleerekoper M 1989 *J. Bone Miner. Res.* **4** 3
[11] Abrami A, Arfelli F, Barroso R C, Bergamaschi A, Billè F, Bregant P, Brizzi F, Casarin K, Castelli E, Chenda V, Dalla Palma L, Dreossi D, Fava C, Longo R, Mancini L, Menk R H, Montanari F, Olivo A, Pani S, Pillow A, Quai E, Ren Kaiser S, Rigon L, Rokvic T, Zanetti A and Zanini F 2005 *Nuclear Instruments & Methods in Physics Research A* **548** 221.
[12] Montanari F 2003 http://www.ts.infn.it/physics/experiments/syrma/SYRMEP/Tutorial tomo program.ppt
[13] Schneider A, Rasband W S and Eliceiri K W 2012 *Nat. Meth.* **9** 671
[14] Doube M, Kłosowski M M, Arganda-Carreras I, Cordelieres F, Dougherty R P and Jackson. J 2010 *Bone.* **47** 1076
[15] Riddler W and Calvard S 1978 *IEEE Tran. Syst. Man. Cybern* **8** 630
[16] Hildebrand T and Ruegsegger P 1997 *J Microsc.* **185** 67
[17] Lorensen W E and Cline H E *Comput Graph (SIGGRAPH 87 Proceedings)* 1987 **21** 163
[18] Hildebrand T and Ruegsegger P 1997 *Comput Meth Biomed Engng* **1** 15
[19] Ding M and Hvid I 2000 *Bone* **26** 291