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LUCAS HENRIQUE DE SOUZA TEIXEIRA

EFEITO DOS PARÂMETROS DE AQUISIÇÃO DO MICRO-CT NA AVALIAÇÃO DO REPARO ÓSSEO

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EFEITO DOS PARÂMETROS DE AQUISIÇÃO DO MICRO-CT NA AVALIAÇÃO DO REPARO ÓSSEO

Trabalho de conclusão de curso apresentado a Faculdade de Odontologia da UFU, como requisito parcial para obtenção do título de Graduado em Odontologia

Orientadora: Profª. Drª. Priscilla Barbosa Ferreira Soares

Co-orientadora: Drª. Milena Suemi Irie

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RESUMO

O objetivo foi avaliar o efeito da resolução e tipo de filtro utilizado no escaneamento por microtomografia computadorizada (micro-CT) e da experiência do examinador na análise do reparo ósseo em ratos. Defeitos foram criados nas tíbias de 5 ratos e escaneados seguindo os seguintes parâmetros: filtro de alumínio 0,5 ou 1,0 mm de espessura; e resolução de 6 ou 12 μm. Examinadores experientes (n=5) e não experientes (n=5) avaliaram a fração de volume ósseo (BV/TV) e a espessura das trabéculas (Tb.Th) dos 20 escaneamentos obtidos. Os dados foram analisados por ANOVA de 3 fatores e teste de Tukey (□=0.05). Diferença entre os examinadores experientes e não experientes foi observada na análise de BV/TV (P<0,001). No grupo de examinadores experientes, não houve diferença signficativa para BV/TV entre as análises em 6 e 12 μm (P=0,900). No grupo não experiente os resultados foram significativamente diferentes (P<0,001). Para Tb.Th, houve diferença entre os resultados dos escaneamentos de 6 e 12 μm (P<0,001). Não foi observada diferença (P=0,9) entre o grupo experiente e não experiente nas análises em 6 μm, diferentemente das análises em 12 μm, em que houve diferença (P<0.001). O tipo de filtro não apresentou efeito nas análises realizadas. Com base nos resultados, fica evidente que a resolução e a experiência dos examinadores podem afetar os resultados morfométricos (BV/TV e Tb.Th) de análises por micro-CT de reparo ósseo em ratos. Os dados sugerem que a experiência do examinador foi fator mais crítico para BV/TV, enquanto que a resolução apresentou maior efeito para Tb.Th.

Palavras-chaves: Microtomografia de Raios-X; Osso e Ossos; Cicatrização de Feridas; Modelos animais; Osso Esponjoso.

SUMÁRIO

Authors: Milena Suemi, Iriea; Rubens, Spin-Netob; Lucas Henrique, de Souza Teixeirac; Gustavo, Davi Rabelod; Nayara, Teixeira de Araújo Reise; Priscilla, Barbosa Ferreira Soaresf

a Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Avenida Pará s/n°, Campus Umuarama, Bloco 4L, Bairro Umuarama, 38400-902, Uberlândia, Minas Gerais, Brazil. E-mail: milena.irie@gmail.com ORCID: https://orcid.org/0000-0002-2919-2910

b Department of Dentistry and Oral Health, Section of Oral Radiology, Health, Aarhus University, Aarhus, Denmark. E-mail: rsn@dent.au.dk ORCID: https://orcid.org/0000-0002-9562-0610

c Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Avenida Pará s/n°, Campus Umuarama, Bloco 4L, Bairro Umuarama, 38400-902, Uberlândia, Minas Gerais, Brazil. E-mail: lucashenriquest@hotmail.com ORCID: https://orcid.org/0000-0002-5228-4653

d Dentistry Department, Federal University of Santa Catarina, Rua Delfino Conti SN, Trindade, Florianópolis, SC, 88040-370, Brazil. E-mail: drgustavorabelo@yahoo.com.br ORCID: https://orcid.org/0000-0001-9511-5078

e Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Avenida Pará s/n°, Campus Umuarama, Bloco 4L, Bairro Umuarama, 38400-902, Uberlândia, Minas Gerais, Brazil. E-mail: atnayara@gmail.com ORCID: https://orcid.org/0000-0002-5201-8320

f Department of Periodontology and Implantology, School of Dentistry, Federal University of Uberlândia, Avenida Pará s/n°, Campus Umuarama, Bloco 4L, Bairro Umuarama, 38400-902, Uberlândia, Minas Gerais, Brazil. E-mail: pbfsoares@yahoo.com.br ORCID: https://orcid.org/0000-0002-4492-8957

Corresponding author: Priscilla Barbosa Ferreira Soares. +55 3432258119; e-mail: pbfsoares@yahoo.com.br

INTRODUCTION

Microcomputed tomography (micro-CT) analysis is a nondestructive method that provides three-dimensional reconstructions of interior structures and other bone properties.[1,2] A strong correlation between micro-CT and histomorphometric analysis has been reported in several studies.[3,4] Micro-CT has been widely used in research fields of bone metabolism, repair, and regeneration.[5] Acquisition and analysis of bone volumes using micro-CT consists of the following steps: a) scout view and preprocessing on 2D section visualization; b) sample scanning; c) segmentation and 3D reconstruction; and d) microstructure quantification and analysis. In each stage of this process, some variables, such as resolution and the use of filters may affect the morphological outcomes.[6] A guideline based on the need for standardized terminology and consistent reporting of parameters analyzed was published,[1] and described that besides following the manufacturer-specific instructions for regular quality control, images should be inspected visually to identify possible scanning artifacts. In this way, the influence of scanning and image processing during analysis and its influence on the results still needs to be accessed.

Image resolution is determined by voxel size. Morphological assessment of thinner structures, such as rat bone trabeculae (20-70 μm), can be affected by resolution.[3,7] Scanning small structures with low resolution can underestimate bone mineral density and overestimate its thickness.[8] Most micro-CT systems provide a resolution on the order of 6-73 μm.[9] Ideally, the smallest voxel size (highest resolution) should be used in animal experiments; however, using a small voxel size increases the scanning duration and data generation, being sometimes too much time-consuming. Moreover, the amount of radiation must be considered when *in vivo* micro-CT scanning is applied.[6]

Other acquisition parameter that influences on the quality of the results is the use of filters. They may minimize artifacts present in the images. Beam hardening is an artifact produced by a polychromatic X-ray beam with different energy spectra. When the X-ray beam propagates through the sample, the low-energy portion stops in the surface area, while the high-energy portion goes inside the sample. This phenomenon manifests as a higher-density image of the surface area of the sample. It is possible to minimize this artifact at the reconstruction stage. However, by placing a metal filter between the X-ray and the sample

during image acquisition, the lower energy portion of the beam is filtered. The ideal filter and filter thickness to use will also depend on the sample size and density.[1]

The region-of-interest (ROI, e.g., the specific site where bone healing will be assessed in this study) should be delimited and separated from the other structures across the acquired field of view.[5,1011,12] This process could be done manually or in an automatic fashion. After ROI delimitation, determining a grayscale threshold (0-255) distinguishes bone from nonbone, a process called image segmentation (or binarization). This process can be performed by means of local or global values. Most commonly, global thresholding is performed, in which a chosen value (Hounsfield units or g/cm^3) distinguishes bone (above the threshold) from nonbone (below the threshold). The threshold is selected either visually, by analyzing the density of the histogram, or by setting a threshold value that will result in a volume dataset equal to the volume of the original bone sample.[13] Local thresholds are based on the neighboring values of each voxel[14] or based on the local minima and local maxima values of the selected ROI. Diverse methods applying local threshold definition have been reported[15,16] to overcome the limitations related to low resolution and nonhomogeneous samples that affects global thresholding. However, both processes (ROI delimitation and threshold setting) can be influenced by the examiner's experience.

The aim of this study was to i*nvestigate whether* the acquisition voxel size, filter thickness, and operator experience affect the morphometric outcome of bone repair evaluation assessed by means of micro-CT. The null hypothesis was that the acquisition voxel size, filter thickness, and examiner experience have no effect on the outcome.

2 MATERIALS AND METHODS

This study was approved by the Research Ethics Committee (approval number 093/17) of the institution and was carried out in strict compliance with the ethical principles for the care and use of laboratory animals, also according to the ARRIVE guidelines. Cortical bone defects of 1.6 mm in diameter were created using a cylindrical burr (Neodent®, Curitiba, Brazil) at a standardized location on the tibias of 5 Wistar rats. The animals were euthanized 7 days after surgery, and the right tibias were covered with moist gauze containing phosphate-buffered solution and stored in plastic tubes at -20 °C until scanning. The tibiae were positioned in the

sample holder and left at room temperature before the scans. Micro-CT scan of the 5 tibiae (Fig. 1) were acquired with a desktop SkyScan 1272 high-resolution 3D X-ray microscope based on micro computed tomography (micro-CT) technology (Bruker, Kontich, Belgium).

Each sample was repeatedly scanned using the following acquisition parameters: voxel sizes of 6 μm and 12 μm, and aluminum filter thicknesses of 0.5 mm and 1.0 mm (n = 5). Image reconstruction was performed with NRecon software (version 1.6.6.0, Bruker, Kontich, Belgium). A single set of parameters was chosen visually based on minimum artifacts, irrespective of the tested group. Ring artifact correction was set at 9, smoothing at 1, and beam hardening correction at 0%. The reconstructions included the entire lesion, as follows: new bone formation inside the tibia canal and at the lesion site was manually delimitated in 2D slices (Fig. 2), from the bottom to the top of the lesion borders, delineated by a single examiner (LHST).

Morphometric data (bone volume ratio, BV/TV, and trabecular thickness in μm, Tb.Th) was evaluated by 5 experienced and 5 non-experienced examiners and assessed for each acquisition parameter. The non-experienced examiner's group included operators with experience in micro-CT analysis, but none has ever performed bone analysis before the study. The experienced examiner's group included researchers who had performed micro-CT analysis on rat bone tissue in previous experiments and other bone analysis. BV/TV and Tb.Th analyses were performed using the CTAn software (version 1.18.4.0, Bruker). Segmentation of the trabecular bone within the lesion area was manually performed by each examiner. The entire lesion was defined by interpolation of the ROIs delineated by each examiner. Thresholds for segmentation of bone and nonbone (maximum and minimum gray levels) were visually and individually defined for each set of acquisition parameters. Then, BV/TV and Tb.Th measurements were calculated.

Statistical analysis was performed using SigmaPlot® (SigmaPlot v13.1; Systat Software Inc.) considering a significance level of α=0.05. The influence of operator experience (experienced and non-experienced examiners), filter thickness (aluminum 0.5 and 1 mm) and scanning voxel size (6 and 12 μm) on BV/TV and Tb.Th were assessed using three-way analysis of variance (ANOVA) with Tukey's post hoc test. Intraclass correlation coefficients (ICCs) for absolute agreement among total tissue volume (TV) measurements

were calculated[17] (MATLAB MathWorks, Natick, MA) to evaluate interexaminer reliability16 for cortical and trabecular bone segmentation in both the experienced and non-experienced groups

Results

The ICC between the experienced examiners was 0.84, indicating good reliability. Thus, ICC between the non-experienced examiners indicated poor reliability (0.06). The mean and standard deviation values for BV/TV and Tb.Th are shown in Tables 1 and 2, respectively. The factor interactions (operator experience, voxel size and filter) for BV/TV (Table 3) and Tb.Th are described in Table 4.

BV/TV

The operator experience ($p < .001$), the voxel size ($p < .001$) and the interaction between these factors ($p = .009$) had significant effects on the BV/TV values (Table 3). The analysis performed by experienced examiners resulted in significantly lower BV/TV values (17.8 ± 4.3) than that performed by non-experienced examiners (31.2 ± 2.1, $p < .001$). The effect of voxel size depended on the examiner experience. No significant difference was observed for the experienced examiners after scanning at voxel sizes of 6 (15.8 ± 4.5) and 12 µm (15.6 ± 5.2) ($p = 0.900$). However, for the non-experienced examiners, the analysis performed on the 12 µm volumes (32.7 ± 15.5) resulted in higher BV/TV than did that performed on the 6 µm group (25.1 ± 14.2, $p < .001$). The effects of the filter and its interactions with tested factors were not significant. No significant interaction was found among the 3 factors ($p = 0.500$).

Tb.Th

The examiner ($p < .001$), the voxel size ($p < .001$) and the interaction between factors ($p = .040$) had significant effects on the Tb.Th values (Table 4). The analysis performed by the experienced examiners resulted in lower Tb.Th values (0.04 ± 0.01) than that performed by the non-experienced examiners (0.05 ± 0.02, $p < .001$). The effect of the examiner depended on the voxel size of the volumes analyzed. There was no difference between experienced and non-experienced operators in the 6 µm group ($p = .900$); however, the analysis performed on 12 µm volumes resulted in higher ($p < .001$) Tb.Th values for the experienced examiners

(0.06±0.02) than for the non-experienced examiners (0.05 ± 0.01). The filter and interactions between 2 or 3 factors had no significant influence on Tb.Th values (p = .900).

DISCUSSION

The present study investigated whether the acquisition voxel size, filter thickness, and operator's experience influence on the results of the morphometric evaluation of bone volume and trabecular thickness, using an experimental model of bone repair in the rat tibiae. Both morphometric parameters of BV/TV and Tb.Th demonstrated some dependency upon examiner experience and acquisition voxel size. Regarding the filter thickness, this parameter had no effect on BV/TV and Tb.Th measurements. Thus, the null hypothesis that the acquisition voxel size, filter thickness, and experience of examiner parameters have no effect on the outcome of the morphometric evaluation was partially rejected. In this way, the present study pointed out two critical factors that should be considered for micro-CT analysis of a site with new bone formation: the acquisition voxel size and the experience of the examiners.

Several segmentation methods have been described to separate trabecular from cortical bone in micro-CT volumes.[10,17,18] Automated segmentation to distinguish cortical from trabecular bone is not possible for bone repair sites in some specific models (mostly on the diaphysis of long bones), once the cortical contour is not intact and a detailed delimitation of the lesion edges cannot be achieved. If a ROI (e.g., standardized circle) is defined in the cancellous region, the analysis can be underestimated when only a fraction of the ROI (i.e., the trabecular bone undergoing healing) is included.[10] An automated method to identify and separate the callus and/or newly formed bone, original cortical bone, and the marrow portion without requiring the delimitation of specific ROIs was proposed previously.[19] The authors applied global thresholding for each structure visually determined by two independent examiners and by the associated histogram. This method was not time consuming; however, it did not provide volume-dependent micro-CT assessments of parameters such as BV/TV. In this case, the most accurate method for nonintact cortical analysis is manual drawing of contour lines on the outer edge of the lesion. Therefore, creating a VOI by interpolating several ROIs is feasible considering the rupture in the cortical bone, the dimensions of which diverge in each section.

The ICC for total volume (TV) indicated good reliability between the VOIs of the experienced examiners. However, such good reliability was not observed between non-experienced examiners. This finding supports the difference in BV/TV outcomes between those groups. Bone volume fraction is one of the main morphological parameters for bone repair evaluation, and it depends on the total volume.[1] The results from the present study demonstrated that detailed volume of interest (VOI) delimitation by an experienced and calibrated examiner is critical for bone volume fraction analysis of a healing area. The relevance of examiner experience is also emphasized over the effect of resolution. BV/TV was influenced by the acquisition voxel size only for the non-experienced examiners. The analysis performed by an experienced examiner showed no difference between voxel sizes of 6 μm or 12 μm.

The influence of operator's experience was also observed for Tb.Th measurements. However, this difference was solely observed in the 12 μm group, indicating that Tb.Th analysis of bone repair in rats with the 6 μm voxel size volumes imply less bias than the examiner. When acquiring volumes at a larger voxel size, the bone surface is blurred, especially for trabecular structures within a healing area (which have higher resorption rates and even a thinner trabecula).[20] This approach makes the binarization process more prone to bias, which was evident when the examiners had no previous experience in bone morphometric analysis. Longo *et al.* (2017) compared 9 μm and 18 μm voxel sizes in both *in vivo* and *ex vivo* micro-CT.[8] The authors demonstrated that analysis with smaller voxel size volumes led to lower Tb.Th measurements in rat trabeculae. Similar results were found in the present study, in which a significant difference was observed between the 6- and 12-μm groups for Tb.Th. The findings of the present study supported the hypothesis that thinner trabecular structures are blurry when scanning at a larger voxel size, culminating in an increase in the mean measured thickness.

The effect of the acquisition voxel size on trabecular structures has also been demonstrated in human cadavers' investigations.[21] While the effect of acquisition voxel size has been observed at larger dimensions, such as 41 μm in micro-CT compared to high-resolution peripheral quantitative computed tomography (HQ-pQCT) with acquisition voxel sizes of 41, 82 and 123 μm, the results of the present study revealed the same effect

even for a narrow difference in the voxel size during image acquisition. A smaller voxel size may allow more accurate segmentation of the trabecular structure, which results in more accurate quantification of the trabecular microstructure parameters. The acquisition resolution should always be chosen based on the size of the structure being analyzed as well as the size of the expected microarchitecture changes that the experimenter aims to quantify.[22] However, the resolution might be critical if it involves remodeling areas. In this way, the findings from the present study have demonstrated that scanning at a 6 μm or 12 μm voxel size was not a limiting factor for BV/TV with calibrated examiners; rather, scanning at a 6 μm voxel size is a determinant for the Tb.Th measurements of a healing bone area.

In the present study, a global threshold for each acquisition parameter was used. The cortical bone was kept out of the analyzed area, and only trabecular bone within the lesion was analyzed, providing a homogenous structure analysis. A local threshold method was proposed[23] which was validated by histological analysis. The authors concluded that the performance of global threshold methods is equal to that of local thresholds when analyzing high-resolution scans of homogenous structures. However, when nonhomogeneous samples are analyzed (e.g., both thick cortices and thin trabeculae) or when the scan resolution is relatively low, the efficiency of the local threshold method overcomes that of the global methods. When analyzing high-resolution volumes of a homogeneous bone sample, as done in the present study, subjective thresholding performs similarly to objective thresholding. Nonetheless, definition of a reliable threshold should be performed considering 2D slicewise comparisons to the original, regardless of the segmentation method. Visual inspection of the segmentations to ensure that trabecular connectivity is maintained while excluding noise is crucial for micro-CT analysis.[21] It was also reported that segmentation limitations could be mitigated by high-resolution scanning,[22] corrections for beam hardening, and the implementation of a density-based thresholding method.

The effect of beam hardening can be reduced by placing a metal filter during scanning and applying corrective algorithms during reconstruction.[24] In the present study, the beam hardening correction was set at 0% to verify the effect of the filter without algorithm correction. However, no difference was observed in either parameter (BV/TV or Tb.Th) between 0.5- and 1 mm-thick aluminum filters. It has been demonstrated that beam hardening

leads to fewer artifacts for morphology than densitometric measurements.[25] However, one of the limitations of the present study is that bone mineral density was not assessed, to serve as a reference standard (i.e., the truth); thus, we can only assume that aluminum filter thickness did not influence the morphometric outcomes. Despite this limitation, the importance of a single experienced examiner for all the samples to avoid bias in the morphometric outcomes was clear.

CONCLUSION

Considering the limitations of the study design, it was possible to conclude:

- Acquisition voxel size (6 and 12 μm) and operators' experience influenced the outcome of the results obtained for trabecular thickness and bone volume ratio in a site of bone repair in an experimental model.
- Individual experience of the operator in micro-CT analysis is more critical for BV/TV, whereas for Tb.Th, voxel size has a major effect.
- It is recommended that high-resolution acquisitions should be used whenever possible, aiming to provide the most accurate measurements of bone microstructure parameters in an area on an active repair process.

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TABLES

Table 1. Mean BV/TV values (SDs) and results of Tukey HSD test

Examiner	6 µm voxel size		12 µm voxel size	
	0.5mm filter	1.0mm filter	0.5mm filter	1.0mm filter
Experienced	15.5 (4.0)^{Aa}	16.0 (5.0)^{Aa}	16.1 (5.6)^{Aa}	15.1 (4.9)^{Aa}
Non-experienced	25.4 (14.1)^{Ba}	24.8 (14.5)^{Ba}	32.4 (15.1)^{Bb}	32.9 (16.1)^{Bb}

Different uppercase letters in vertical columns indicate significant differences; different lowercase letters in horizontal rows indicate significant differences; Tukey HSD test (P<.05).

Table 2. Mean Tb.Th values (SDs) and results of Tukey HSD test

Examiner	6 µm voxel size		12 µm voxel size	
	0.5mm filter	1.0mm filter	0.5mm filter	1.0mm filter
Experienced	0.04 (0.01)^{Aa}	0.03 (0.01)^{Aa}	0.06 (0.02)^{Ab}	0.06 (0.02)^{Ab}
Non-experienced	0.03 (0.00)^{Aa}	0.03 (0.00)^{Aa}	0.05 (0.01)^{Bb}	0.05 (0.00)^{Bb}

Different uppercase letters in vertical columns indicate significant differences; different lowercase letters in horizontal rows indicate significant differences; Tukey HSD test (P<.05).

Table 3. Three-way ANOVA interactions for BV/TV measurements

Source of variation	*P* values
Operators x Filter	0.800
Operators x Voxel size	0.009*
Filter x Voxel size	0.800
Operators x Filter x Voxel size	0.500

*The mean difference is significant at the 0.05 level.

Table 4. Three-way ANOVA interactions for Tb.Th measurements

Source of variation	*P* values
Operators x Filter	0.400
Operators x Voxel size	0.040*
Filter x Voxel size	0.400
Operators x Filter x Voxel size	0.900

*The mean difference is significant at the 0.05 level.

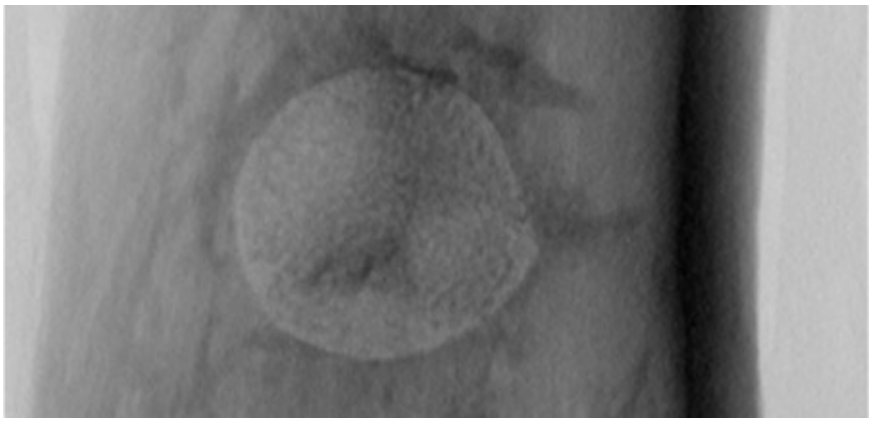

Fig. 1 Micro-CT scout view of the tibia with the cortical defect (6 μm voxel size at 70 kV, 142 μA, a 0.2 rotation step, and a 1 mm aluminum filter).

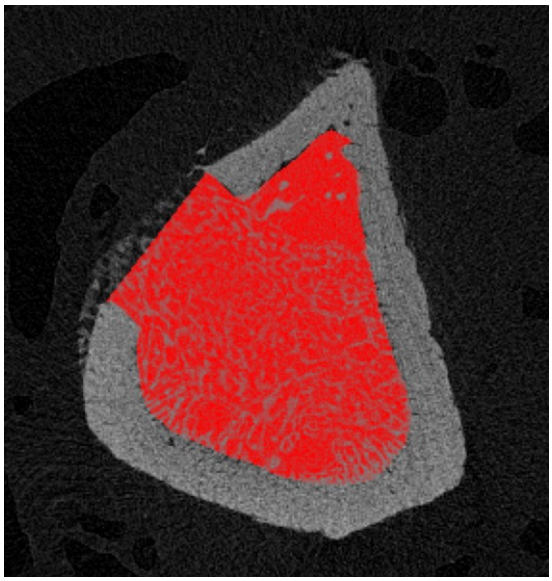

Fig. 2 Demonstration of the region of interest (ROI) delimitation of the defect area.