Lacunarity: a complementary research tool on bone biology

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Histological analysis of bone samples have been used to evaluate the effect of different therapies on bone diseases, as well as the influence of dental implant design or orthodontic forces on bone remodeling processes. Several analytical approaches have been applied as research tools. However, few studies leading into account the relationship between architectural parameters such as lacunarity and the functionality of several biological systems. Lacunarity is a mathematical quantitative technique used to evaluate the canal system organization and robustness. This parameter associated to traditional morphometric analysis such as the percentage of bone matrix is useful to evaluate the alterations on cortical bone after different experimental treatment models. The aim of the present study was to describe lacunarity algorithm as complementary tool to image analysis on cortical bone decalcified and histologically processed. Rabbit tibia subject to radiotherapy was used as experimental model. The animals were divided in control (nonirradiated) and test group (irradiated with 15 Gy cobalt-60). Seventy five days after irradiation the animals were euthanized and tibia segments were routinely processed to histological analysis. Pictures of bone sections were taken and had their bone channels segmented and called regions of interest (ROI). Images were analyzed through developed algorithms using the SCILAB mathematical environment, getting percentage of bone matrix, ROI areas, ROI perimeters, their respective standard deviations and lacunarity. Statistical analysis revealed significant differences (p<0.05) in bone matrix percentage, area and perimeters of the channels, their respective standard deviations and lacunarity between the groups. The results demonstrated that the radiotherapy causes reduction of bone matrix and modifies the morphology of bone channels network, which confirm the relevance of the methodology to appreciate the mechanisms associated to bone turnover. The association of these histomorphometric parameters shows great promise for improving the comprehension of bone metabolism in further studies.

Keywords: lacunarity analysis, cortical bone, bone matrix

Introduction

Histological analysis of bone samples have been used to evaluate the effect of different therapies on bone diseases [1,2], the influence of bone substitutes and technical procedures for bone repair and augmentation [3-5], as well as the influence of dental implant design [6] or orthodontic forces [7,8] on bone remodeling processes. Several analytical approaches have been applied for histomorphometric evaluation on decalcified or undecalcified bone sections [1, 7, 9-10]. Stain techniques for undecalcified sections, such as Von Kossa’s or Goldner’s are used to visualize the osteoid matrix [11]. Another frequently used technique is tetracycline pulse labeling for epifluorescence, which reflects the rate of mineralization [7,10]. A large number of histological and immunohistochemical stains can be employed on decalcified sections in order to evaluate cortical and trabecular bone [11,12], specific matrix proteins related to biomineralization [11], and cell viability [2]. The current literature shows a variety of parameters able to be assessed by histomorphometry [9], including total bone volume [1,2,8,9], osteoid and mineralizing surface [1,2], percentage of mineralized matrix [4,5], resorptive surface [11], osteoclast and osteocyte count [2,3], etc. However, these simple surface-based histomorphometry methods don’t lead into account the intricate network formed by bone cells and bone cylinders named osteons or Haversian systems, in which the blood vessels are entrapped, nourishing the cells [13]. The organization of bone canals systems is crucial to development and maintenance of the cellular framework within whole bones [13, 14], reflecting on their structure and biomechanical properties [15]. The concepts related to bone vascularization and its spatial organization, when transformed in mathematical models, might be valuable to medical treatment and surgical procedures [13-15]. Fractals and lacunarity are architectural parameters to describe the intricate organization on cortical and trabecular bone [13-14,16-17]. However, little attention has been directed to the relationship between architectural parameters such as lacunarity and the functionality of several biological systems [18]. Lacunarity is potentially useful to cortical bone measures due its potentiality to describe spatial distribution of real data sets which demonstrate heterogeneity of structure, as demonstrated by Viana et al (2009)[13] and Rabelo et al. (2010)[15]. This parameter associated to traditional morphometric analysis such as the percentage of bone matrix could help to explain the earliest pathophysiological changes on cortical bone after different experimental treatment models, such as gamma (γ) radiation to head and neck malignances treatment [15].

Irradiated bone shows moderate bone resorption with osteoclastic activity, characterized by the presence of lacunae of cortical bone reabsorption near the medullary portion [19] and an increase in porosity [20]. These structural changes promoted by radiotherapy on bone and its lower regeneration capacity [21] might be harmful to bone micro-structure,
which turn it an interesting model to histomorphometric analysis using lacunarity algorithm. Therefore, the aim of the present study was to analyze the characteristics of the irradiated bone channels network using lacunarity algorithm as complementary tool to image analysis on cortical bone decalcified and histologically processed.

Material and Methods

This study is committed to showing the feasibility of texture measures, specifically lacunarity algorithm, to evaluate the effects of some therapies under bone architecture and biology, also demonstrating the complementarity of this method with traditional histomorphometric parameters. For testing purposes, 14 young rabbits (*Oryctolagus cuniculus*), with average weight of 3.5 Kg were used. The project was conducted after Institutional Animal Care and Use Committee at Federal University of Uberlândia approval. The animals were separated in two groups: Irradiated and non-irradiated (control) and the irradiated group received, in the tibia region, bilaterally, a single acute exposure of 15 Gy (Co<sup>60</sup>, AECL MEDICAL, Phoenix Model, Kanata, Ontario, Canada) at 80 cm. The euthanasia was performed 75 days after irradiation by anesthesia overdose.

Bone biopsies with 1 cm of diameter were removed from the tibias, fixed with a 10% phosphate buffered (0.1M) solution, pH 7.2, decalcified in 4% EDTA and embedded in paraffin. For each sample, three nonconsecutive sections (5µm thick, separated by 100 µm) were cut perpendicular to the long axis of the bone core and stained with hematoxilin and eosin to histomorphometric analysis. The images were obtained using a microscope (Olympus BX 40) in conjunction with a digital camera (OLY6200 Olympus) connected to a PC microcomputer through a 3153 data Translation card. One image with objective lens of 10X and four images with objective lens of 40X were obtained in each section. The images captured with an objective lens 40X were assessed to osteocyte and empty osteocyte lacunae counting, which was performed by using the ImageJ software (ImageJ 1.40g, Wayne Rasband, National Institutes of Health, USA). These parameters were measured twice for each field in a total of 672 captured images. Numeric values were recorded in tables and a percentage was made from the total of elements.

The images captured with objective lens of 10X were used to implement lacunarity algorithm. The images were processed to eliminate artifacts (Fig A) and a binary image of the bony component was obtained (Fig B). A total of 168 images were analyzed using a methodology proposed by Oliveira et al. (2006), which consists in a histological analysis performed by algorithms developed in the SCILAB mathematical environment, considering several morphological features of bone channels and image texture characterization. Each image was segmented and analyzed separately. The segmented bone channels were called regions of interest (ROI). The bone matrix and ROI features were analyzed according to the following variables: percentage of bone matrix by area (the black pixels amount in the image from which is possible to estimate the density of bone matrix in the material), ROI area (the average of the pixels number inside the ROI); standard deviation of the ROI area; ROI perimeter (the average of the sum of the distance between pixels along each ROI border, considering two types of adjacencies: linear and diagonal); standard deviation of the ROI perimeter and lacunarity.

![Fig. A Digitalized histological image.](image-url)
Data were initially evaluated using Kolmogorov-Smirnov. The results through bone matrix and ROI features were analyzed by means of the non-parametric Mann Whitney test (error probability, 5%) and the percentage of osteocytes empty lacunae were analyzed by means of the parametric unpaired, two-tailed Student’s t test.

**Results**

Bone sections in irradiated and control groups maintained their histological references, as harvesian channels, osteocytes and empty lacunae, showing basophilic tidemarks probably related to bone remodeling process in both.

The data showed significant difference in bone matrix area between the control (96.71%) and irradiated (94.25%) groups, between area and perimeters of the channels and their respective standard deviations (p<0.05). The lacunarity showed significant difference among the control (16.48) and irradiated (13.80) groups. There was no significant difference in percentage of empty osteocyte lacunae (Table 1).

**Table 1** – Bone matrix area, ROI area and perimeter, standard deviation (SD), Lacunarity and Empty osteocytes lacunae.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bone Matrix area (%)</th>
<th>ROI area</th>
<th>ROI perimeter</th>
<th>ROI perimeter SD</th>
<th>Lacunarity</th>
<th>Empty Osteocyte Lacunae (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiated</td>
<td>94.25</td>
<td>535.8</td>
<td>1245</td>
<td>97.74</td>
<td>69.34</td>
<td>13.80</td>
</tr>
<tr>
<td>Control</td>
<td>96.71</td>
<td>310.8</td>
<td>311.4</td>
<td>88.08</td>
<td>43.20</td>
<td>16.48</td>
</tr>
<tr>
<td>p</td>
<td>0.0003*</td>
<td>0.0173*</td>
<td>0.0072*</td>
<td>0.0437*</td>
<td>0.0039*</td>
<td>0.014*</td>
</tr>
</tbody>
</table>

**Discussion**

In the last years, researches enrolling biological aspects of bone behavior have progressively strengthened the assumption that the bone tissue constituents are organized in intricate fashion [13-17, 22,23], increasing the useful of computational mathematic methods on histomorphometric analysis [13,14,23,24]. This study used lacunarity as mathematician tool to evaluate challenges promoted by γ radiation in bone structure.

Lacunarity and fractal are analytical methods proposed to investigate complex systems in a cellular biological context [18]. Both methods are quantitative techniques and have no observer dependency [16,17]. In current study, just lacunarity was applied to image measures, as previously demonstrated by Dougherty and Hennebry (2002)[23]. Though the lacunarity have been originally developed to describe a property of fractals, they not show self-similarity, which allows to apply lacunarity in images that show a limited fractalness [17,18,23]. In a general sense, lacunarity characterize the spatial organization of an image that may be manifest by texture [18], as observed to the intricate network formed by Haversian and Volkman’s canals [13-15].

**Fig. B** Binary image obtained through algorithms developed in the SCILAB mathematical environment.
Previous studies have been successfully used lacunarity analysis to evaluate the trabecular network in cancellous bone [23,24] and to determine the canals system organization on cortical bone [13-17]. These studies have tried to estimate the bone texture and spatial organization using lacunarity measures on 2-D images histological sections and recently, on 2-D images obtained by direct digital radiographs [16,17] and computed tomography [24]. The present research evaluates lacunarity on decalcified cortical bone sections stained with hematoxilin and eosin, but the same methodology might be performed in undecalcified thick sections.

The basic principles used here to lacunarity assessment have applicability to cortical sections prepared with fluorescent dyes as tetracycline or stained for immunohistochemical. The same approach for different histological methods is possible because the images need to be pre-processed to derive binary images. Additionally, artifacts created during histotechnological steps might be eliminated by semi-automatic computer procedures to reduce the presence of noise and blurring within the image system [24]. If used in adequate fashion, these correction procedures don’t change the lacunarity sensitivity, which allow its application in parallel to conventional histomorphometric techniques to appreciate other parameters as bone volume, osteoid and mineralizing surface, mineral apposition values, cell count.

In present study the same sections were used to obtain images in 10X and 40X, which were used to evaluate the percentage of bone matrix and osteocyte/lacunae count, respectively. Images obtained in 10X were used to implement lacunarity algorithm and to measure some traditional histomorphometric parameters. The former supply information regarding bone formation rate which indirectly demonstrate the cell activity, while the latter is related to bone channel system distribution and organization. Structural changes in bone and its healing process after radiation has been characterized as changes in composition and replacement of its components in many studies [20,26,27], but this research described a new way to demonstrate such alterations, as cortical bone matrix disorganization.

In irradiated group, the significant difference in bone matrix area and lacunarity probably represents alterations in micro-structure and morphological characteristics. These features were confirmed by differences in area and perimeter of bone channels that indicates a response to radiation effects, suggesting an unorganized bone remodeling process. The mean values calculated to standard deviation ROI area and perimeter were used to evaluate the size and shape variation on bone channel systems. The data indicate that γ radiation affects the general channels organization, which is evidenced by more expressive standard deviation in irradiated group, reflecting in the sizes and shape of bone channels.

The high values to lacunarity obtained in control group are related to an important characteristic of this mathematic measure: translational invariance. In a general sense, lacunarity is measure of the lack of rotational or translacional invariance in an image [18]. Therefore, the lacunarity in control group is related to a spatial homogeneity in the distribution of ROIs, while in irradiated group, these findings represent alterations on bone micro-structure, indicating substantial changes in the Havers systems and their respective bone channels. These alterations can suggest important implications on biomechanical and morphological properties, as demonstrated precisely in studies using conventional histomorphometric methods [20,26]. This study also counted the osteocytes. It has been suggested that osteocytes density regulate the morphometric parameters of predicted architecture and the remodeling process [2, 27]. Maeda et al. (1988) [20] demonstrated a reduction in the number of osteocytes, which is related to immediate effects of radiation. However, the osteocytes count did not change after radiotherapy, suggesting a recovery of the number of cells, probably related to the long period between the irradiation and sacrifice. Therefore, the absence of differences on osteocyte number did not exclude some changes in cells metabolism of irradiated bone, as alterations on growth factor [28]. In addition, it is important to emphasize that the same sections were used to osteocyte count and to lacunarity analysis, allowing several analytical opportunities to the better understanding of intricate organization of Harvesian system and bone behavior.

The results indicate that radiotherapy causes reduction of bone matrix and modifies the micro-structure of bone channels network, making it more heterogeneous and less organized, suggesting important implications on morphological and biomechanical cortical bone properties. All in all, this work described how concepts of complex network theory, used jointly with traditional morphometric parameters, can be effectively applied in order to appreciate the mechanisms associated to bone turnover, providing a precise representation of the connectivity of bone channels, improving the comprehension of bone vascularization [13,22]. Once the neo-vascularization is strictly related to regenerative properties and bone remodeling, the representation of channels network distribution and organization allows all the rich concepts regarding bone turnover to be applied [13-15,22]. Though the present study had evaluated irradiated bone, the mathematical model used could be an interesting methodology to further investigations of structural changes on cortical bone subject to orthodontic forces or the bone remodeling on implants subjects to early load. Hence, lacunarity is a complimentary research tool on bone biology and its association with others histomorphometric parameters shows great promise for improving the comprehension of bone metabolism.
References


