NATHÁLIA RODRIGUES GOMES

COMPARAÇÃO ENTRE ANÁLISE FRACTAL E AVALIAÇÃO DA RADIOPACIDADE COMO INSTRUMENTOS PARA ESTUDO DO REPARO ÓSSEO EM SÍTIOS ENXERTADOS COM BIOMATERIAIS EM MODELO ANIMAL

Faculdade de Odontologia
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Monografia apresentada ao Curso de Especialização Radiologia Odontológica e Imaginologia da Faculdade de Odontologia da Universidade Federal de Minas Gerais como requisito parcial à obtenção do título de Especialista em Radiologia Odontológica e Imaginologia.

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Ata da Comissão Examinadora para julgamento de Monografia da aluna **NATHALIA RODRIGUES GOMES**, do Curso de Especialização em Radiologia Odontológica e Imaginologia, realizado no período de 13/02/2017 a 20/12/2018. Aos 18 dias do mês de dezembro de 2018, às 14:00 horas, na sala de Pós-Graduação (3403) da Faculdade de Odontologia, reuniu-se a Comissão Examinadora, composta pelos professores Cláudia Borges Brasileiro (orientador), Tania Mara Pimenta Amaral e Maurício Augusto Aquino de Castro. Em sessão pública foram iniciados os trabalhos relativos à Apresentação da Monografia intitulada **"Comparação entre análise fractal e avaliação da radiopacidade como instrumentos para o estudo do reparo ósseo em sítios enxertados com biomateriais em modelo animal"**. Terminadas as arguições, passou-se à apuração final. A nota obtida pela aluna foi 10,0 (dez) pontos, e a Comissão Examinadora decidiu pela sua aprovação. Para constar, eu, Cláudia Borges Brasileiro, Presidente da Comissão, lavrei a presente ata que assino, juntamente com os outros membros da Comissão Examinadora. Belo Horizonte, 18 de dezembro de 2018.

Prof. Cláudia Borges Brasileiro
Orientador

Prof. Tania Mara Pimenta Amaral

Prof. Maurício Augusto Aquino de Castro

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RESUMO

Objetivo: Avaliar a reparação de defeitos ósseos em modelo animal de rato por meio da análise fractal e radiopacidade em imagens radiográficas. Metodologia: 120 ratos Wistar tiveram seu primeiro molar extraído e foram divididos em quatro grupos (n=6/grupo) de acordo com o material de enxertia utilizado para preencher o defeito ósseo: Osso bovino mineralizado (OBM); Osso bovino desmineralizado (OBD); Coágulo sanguíneo como controle negativo (CN); Osso bovino Bio-Oss® como controle positivo (BO). Os animais foram sacrificados após 1, 7, 14, 21 e 49 dias e submetidos à análise radiográfica por dimensão fractal em um único ROI de 30x30 pixels e níveis de radiopacidade em três pontos (apical, médio e coronal) de 5x5 pixels. A avaliação histológica foi realizada como padrão ouro por meio da histomorfometria da neoformação óssea e maturação da matriz óssea. Resultados: A avaliação histomorfométrica sugere que o grupo OBD apresenta deposição mineral acelerada e um osso estatisticamente mais maduro aos 49 dias em relação ao CN. O grupo OBM apresenta características similares ao BO, porém, com menor percentual de deposição óssea. Em relação à maturação óssea, não houve diferença com significância estatística em nenhum momento da análise. A análise de radiopacidade mostra diferença com significância estatística entre OBD e o CN aos 49 dias. A análise fractal não mostrou diferenças estatísticas, mas seguiu padrão semelhante. Conclusão: A análise da radiopacidade mostrou-se mais efetiva na quantificação do reparo ósseo em relação à análise fractal no grupo desmineralizado. Não houve diferença entre os dois métodos no grupo mineralizado, concluindo que os dois métodos tem efetividade semelhante.

Palavras-chave: Fractais. Biomateriais. Radiografia.

ABSTRACT

Comparison between fractal analysis and radiopacity evaluation as a tool for studying repair of an osseous defect in an animal model using biomaterials

The aim of this study was to evaluate bone repair of an osseous defect in a rat animal model through fractal analysis and radiopacity analysis in radiographic images. Materials and methods: 120 Wistar rats were subjected to extraction of their first molar and were divided into four groups (n=6/group) according to the material used for bone grafting: mineralized bovine bone (MBB), demineralized bovine bone (DBB), blood clot (BC; as a negative control) or Bio-Oss (BO®; as a positive control). The animals were sacrificed after 1, 7, 14, 21 and 49 days and subjected to radiographic evaluation. For fractal analysis (FA), a rectangular ROI of 30x30 pixels was used, and radiopacity was measured as the mean gray scale (MGS) value for three points of 5x5 pixels in the apical, medial and coronal regions of the defect. Histomorphometric evaluation was realized as the gold standard for measuring bone neo-formation and the maturation of the new osseous matrix. Results: the histomorphometric evaluation suggested that DBB showed faster mineralized deposition and resulted in more mature bone at the final time point of evaluation. MBB and BO presented similar results. The mineralized groups did not show significant differences in bone maturation. The radiopacity analysis revealed a significant difference ($p<0.05$) between the DBB and BC groups at the final time point. FA did not show any significant differences at the final time point. Conclusion: Radiopacity analysis seemed to be more effective for the quantification of bone repair than fractal analysis in the demineralized group in this animal model. The results for the mineralized groups did not reveal a significant difference, leading to the conclusion that both methods are effective

Keywords: Fractals. Radiography. Biomaterials.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

MGS Mean Gray Scale

FA Fractal analysis

EDTA Ethylenediamine Tetraacetic Acid

BC Blood Clot (grupo controle)

BO Bio-Oss®

MBB Mineralized Criteria's Bovine Bone (Osso bovino mineralizado)

DBB EDTA-demineralized Criteria's Bovine Bone (Osso bovino desmineralizado)

FD Fractal Dimension

ROI Region of Interest

BMPs Bone Morphogenetic Proteins

SUMÁRIO

1 INTRODUÇÃO

Defeitos ósseos em maxila e mandíbula podem ocorrer devido perdas dentárias, trauma ou cistos e tumores odontogênicos (ZHAO *et al.*, 2013; BOROWSKA *et al.*, 2015; JUNIOR *et al.*, 2016). A reabilitação funcional e estética desses defeitos é essencial e tem sido alcançada em alguns casos através de enxertos ósseos e colocação de próteses suportadas por implantes (DE MOLON *et al.*, 2015).

Os enxertos ósseos podem ser realizados utilizando diferentes fontes doadoras. Essa fonte pode ser o próprio indivíduo receptor, caracterizando o enxerto autólogo, que tem a melhor compatibilidade, porém uma maior morbidade do sítio doador (ZHAO *et al.*, 2013). A enxertia pode também ser realizada com um biomaterial, chamado de enxerto xenogênico ou aloplástico, que pode ter origem animal ou sintética (BOROWSKA *et al.*, 2015).

A marca comercial mais utilizada e estudada em odontologia é o Bio-Oss®, um xenoenxerto derivado de osso bovino que se integra ao osso neoformado (GALINDO-MORENO *et al.*, 2010; PALACHUR *et al.*, 2014). O uso de um biomaterial como o Bio-Oss® não busca somente o preenchimento do espaço do defeito ósseo, mas também auxilia a neoformação óssea e a remodelação e cicatrização dos tecidos moles (KUMAR *et al.*, 2013). Um fator limitante no uso desse material é o seu alto custo.

Várias técnicas têm sido empregadas para avaliar a qualidade óssea e caracterizar as mudanças estruturais como a análise histológica, histomorfometria, radiografias, tomografia computadorizada de feixe cônico e análise de dimensão fractal (GALINDO-MORENO *et al.*, 2010; DE MOLON *et al.*, 2015). As imagens radiográficas permitem estimar a integralidade das estruturas mineralizadas (CHAKRAPANI *et al.*, 2013) e avaliar a regeneração óssea (AL-FOTAWEI *et al.*, 2014). Porém, os métodos radiográficos possuem limitações em relação a projeção, distorção e sobreposição de estruturas anatômicas na imagem (CHAKRAPANI *et al.*, 2013).

Métodos para avaliar o reparo do defeito ósseo através de radiografias têm sido estudados. O método mais usado tradicionalmente é a análise dos tons de cinza e, mais recentemente, tem se discutido o uso da análise fractal. A análise dos tons de cinza demonstra o valor médio dos tons de cinza de cada pixel de uma determinada imagem (CASTELLANO *et al.*, 2004; MUNDIM *et al.*, 2016). A análise fractal é um

método matemático que descreve o padrão estrutural do trabeculado ósseo. É um método quantitativo que mede a complexidade geométrica de estruturas que apresentam auto-similaridade em imagens. A complexidade das estruturas é expressa por um valor numérico representado pela dimensão fractal: valores maiores representam estruturas mais complexas (KOZAKIEWICZ *et al.*, 2013).

Uma das grandes vantagens da análise fractal é o uso de imagens radiográficas não padronizadas, já que pequenas variações de exposição, radiodensidade, alinhamento do trabeculado e projeção não afetam a análise (WOJTOWICZ *et al.*, 2003; AMER *et al.*, 2012). Dessa forma, a análise fractal é uma medida da complexidade da estrutura analisada. Sendo essa estrutura o trabeculado do tecido ósseo, um osso intacto terá uma estrutura mais complexa do que um osso neoformado (WOJTOWICZ *et al.*, 2003).

Estudos relacionam a análise fractal e regeneração óssea guiada, usando enxertos autógenos ou xenogênicos (KOZAKIEWICZ *et al.*, 2013; BOROWSKA *et al.*, 2015; DE MOLON *et al.*, 2015). Esse tipo de análise traz grande benefício para a condução desse tipo de tratamento, já que apenas a avaliação visual da formação óssea através da radiografia é uma avaliação muito subjetiva. Essa interpretação visual não é capaz de diferenciar a remodelação óssea adequada da falta de integração do material (KOZAKIEWICZ *et al.*, 2013), portanto, estudos de métodos alternativos para quantificação da remodelação óssea através de imagens radiográficas são necessários.

Diante dessas novas perspectivas de engenharia tecidual e análise do reparo ósseo, esse estudo avaliou a reparação óssea de um defeito produzido experimentalmente em ratos e preenchidos por biomaterias por meio da análise fractal e radiopacidade.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a reparação de defeitos ósseos em modelo animal de rato por meio da análise fractal e análise da radiopacidade em imagens radiográficas.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar a correlação da análise fractal em imagens radiográficas com a neoformação óssea avaliada pelo exame histomorfométrico (padrão ouro) nos defeitos ósseos de ratos enxertados com biomateriais.

Avaliar a correlação da análise fractal em imagens radiográficas com a maturação óssea avaliada pelo exame histomorfométrico pela coloração *Picro-Sirius* Red, que avalia a maturação das fibras colágenas, nos defeitos ósseos de ratos enxertados com biomateriais.

Comparar os resultados de análise fractal e radiopacidade e avaliar qual é mais coerente com a neoformação e maturação óssea.

3 RESULTADOS

Os resultados foram escritos em forma de artigo na língua inglesa e submetido ao periódico internacional Dentomaxillofacial Radiology.

3.1 ARTIGO

TITLE: Comparison between fractal analysis and radiopacity evaluation as a tool for studying osseous repair in animal model defect using biomaterials

KEYWORDS: fractals, radiography, biomaterials

INTRODUCTION

The evaluation of jaw bone quality and quantitative characterization of structural changes in the jaw bone after tooth loss have been performed via various techniques to plan proper rehabilitation with prosthetics and implants (1). The most accurate method, representing the gold standard, is histological analysis; however, this method is not applicable in routine clinical practice (2). The most common clinically used method is the analysis of radiographic images (periapical, panoramic and cone beam computed tomography) that allow estimation of the integrity of mineralized structures and evaluation of bone regeneration (3). However, periapical and panoramic radiography exhibit some limiting factors, such as different projections, distortion and superposition of images (4).

Methods for determining the healing of the jaw bone based on the radiographic aspects have been studied. The traditionally most commonly used of these techniques is mean gray scale (MGS) analysis, while more recently, fractal analysis (FA) has played a role in these evaluations. MGS provides the mean gray level of each pixel of a plain image and has been applied to oral radiographs to assess bone quantity at implant sites (5, 6). FA is a method that mathematically describes the structural pattern of trabecular bone, as the image of the bone is considered to exhibit self-symmetry and is considered a fractal image (7). This method has been widely used in the study of osteoporotic bone (8-10), and there are some reports of its application in the evaluation of bone quantity and quality at implant sites (11, 12) as well as after bone grafts (1, 13).

FA has been reported to present some advantages, as it is independent of radiodensity, geometrical projection and alignment of bone trabeculae (14). The method of box counting for bone analysis assesses the boundary between trabecular bone and marrow, meaning that a higher value indicates a more complex structure (15).

Studies have related FA and guided bone regeneration using autogenous or xenogenic bone grafts (1, 7, 16). This type of analysis would be beneficial for conducting this kind of treatment, as the visual assessment of bone roughness through radiographs alone is a subjective test. This visual interpretation cannot differentiate proper bone remodeling from a lack of material integration (16), which is why the study of alternative methods for bone quantification in radiological images is necessary.

This study aimed to evaluate bone repair in osseous defects in animals after bone grafting using two variables of bovine bone (mineralized and demineralized versions), through two methods of radiographic analysis: fractal analysis and mean gray level analysis. The efficacy of both methods was evaluated using the morphometric results as a reference. Therefore, it was possible to assess and clarify some aspects of FA in relation to its use for qualitative bone analysis.

METHODS

This study was approved by the local Animal Ethics Committee (CEUA/UFMG number 07/2015).

Biomaterial for bone grafting

The materials used for bone grafting were two different brands of bovine bone: Lumina-Bone® (Criteria, São Carlos, Brazil) and Bio-Oss® (Geistlich, Switzerland). The mineralized commercialized versions of both types were employed. Bio-Oss® was used as a positive control because of its consistently good results both in research and clinical applications. In addition to the mineralized versions, this study used a demineralized version to compare the influence of the organic matrix in bone grafts. The demineralized version was obtained by immersing a few blocks of Lumina-Bone® in 10% EDTA (ethylenediamine tetraacetic acid) for 72 h, which were then washed with water and kept in sterile PBS solution. Prior to the surgical procedures, the blocks were portioned into 1-2 mm fragments.

Surgical procedures

The animals used in this research were 120 male, adult Wistar rats (*Rattus norvegicus*), with body weights between 280 g and 350 g. The rats were evaluated in periods of 1, 7, 14, 21 and 49 days after graft surgery. Animals were anesthetized and positioned on a surgical table. The first left superior molar of the animals was extracted, and a defect was created with a cylindrical diamond drill, removing the remaining interradicular septum. The generated osseous defects were patterned to exhibit a diameter and profundity of 2.5 mm. The animals were divided into four groups (n=6/group/time) according to the material used to fill the cavity: blood clot (control group - BC), Bio-Oss® (BO), mineralized Criteria's bovine bone (MBB) and EDTA-demineralized Criteria's bovine bone (DBB).

Radiographic evaluation (Digital X-ray)

The jaws of the rats were fixed in 10% neutral buffered formalin for 72 h. After this period, the maxillae were cut in half along the median line of the palate, between the central incisors, using a diamond disc. The pieces were washed and kept in alcohol 70% for radiographic procedures. Only the hemi-maxilla with the osseous defect (left side) was subjected to radiography. Images were captured using a 3x4 cm phosphor plate (Durr Dental, Bietigheim, Bissingen, Germany) and a Gendex 756DC® (Pennsylvania, USA) radiographic device. The exposure parameters were as follows: 0,125 seconds exposure time, 65 kV, 7 mA and 10 cm focus/film distance. The plates were digitalized with a VistaScanPerio Plus® (Durr Dental, Bietigheim, Bissingen, Germany) scanner and processed using DBSWIN Imaging Software® (Durr Dental, Bietigheim, Bissingen, Germany). The images were converted to jpeg format with a 1080 dpi final resolution (Figure 1 – A).

Radiopacity evaluation (mean gray scale - MGS)

Using Adobe Photoshop CS5 software, three regions of interest (ROIs) of 5x5 pixels were determined in the apical, medial and coronal regions of the surgical site, 1 mm distant from the mesial root of the second molar (Figure 1 – B). For this purpose, a vertical line measuring 2.5 mm was positioned near the mesial root of the second molar, and a 1.0 mm horizontal line was then traced from the center of that first line. The end of that line defined the medial ROI and was used as a reference for determining the apical and coronal points, which were always at the limits of the bone defect. With the histogram tool, the gray scale was measured for each point, and the mean between the three points was calculated.

Fractal analysis

The fractal analysis, in which the results were expressed numerically as fractal dimensions (FDs), was realized based on the procedure described by White and Rudolph (1999) (10) using the box-counting method. The method for choosing the location of the ROI was as described for the MGS analysis, whereas for FA, the horizontal line measured 0.7 mm, and the end of the medial line defined the center of a square 30x30-pixel ROI (Figure 1 - C).

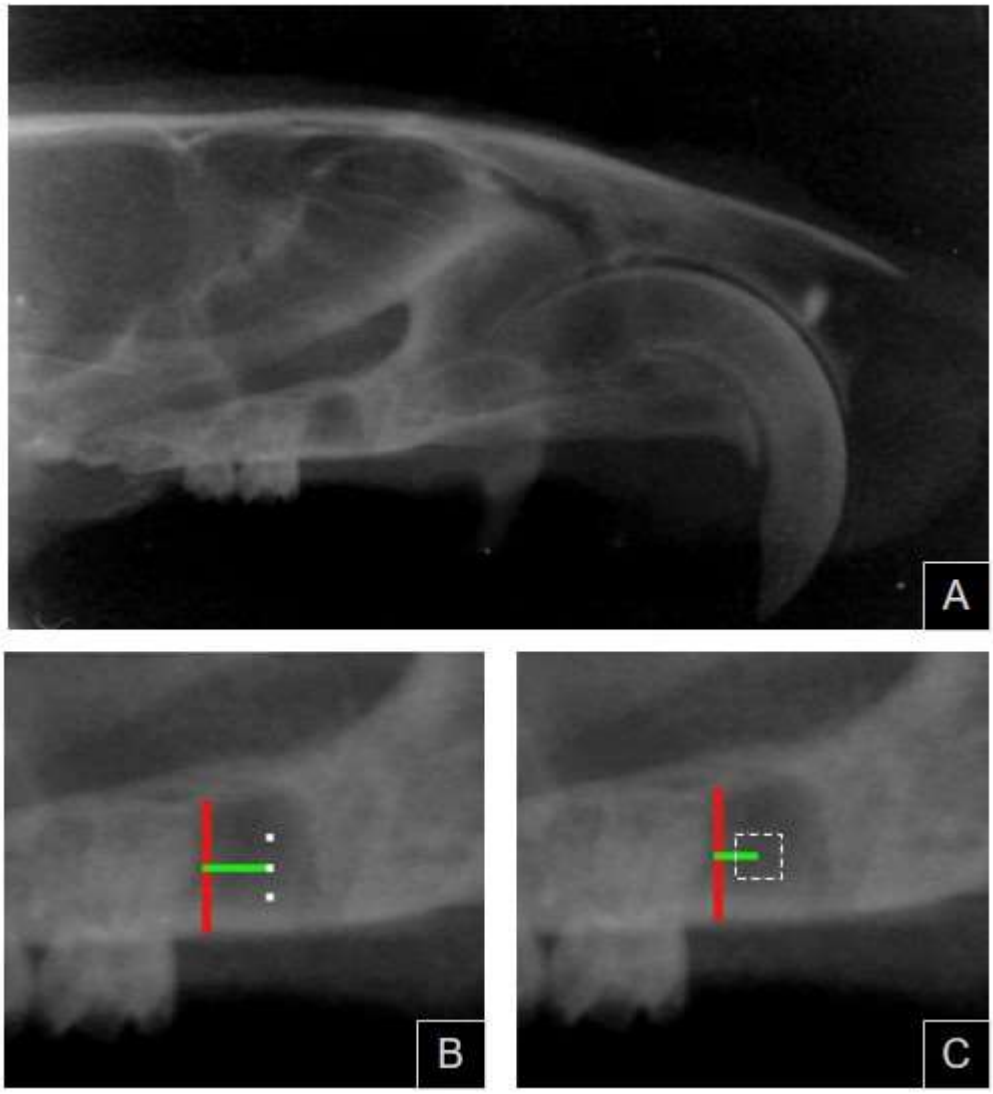


Figure 1: (A) radiographic image of a rat hemi maxilla of the blood clot group at time 0. (B) Vertical reference line in red and horizontal line in green. ROIs used for measuring MGS, located in coronal, medial and apical regions of the osseous defect (white squares). (C) Square ROI with the center placed by the end of the green line for FA.

Using ImageJ software, the ROI located in the center of the osseous defect was selected and blurred with a Gaussian filter (sigma = 35). This stage was applied to remove brightness variations due to overlaying soft tissues and variation in bone thickness. The blurred image was subtracted from the original, and a 128 gray value was added to each pixel to discriminate bone marrow spaces and trabeculae. After binarization, the components were segmented in an image that visually outlined the trabeculae from the bone marrow. The next steps, erosion and dilatation, are performed with the aim of eliminating image noise and emphasizing structures,

respectively. The last step, skeletonization, eroded the image until only the central line of pixels remained and prepared it for FA (10, 17). The box-counting method converts the image using a square grid of equally sized tiles and plots the number of counted tiles against the total number of tiles on a double logarithmic scale. Finally, the fractal dimensional values were calculated from the slope of the line.

Histological processing for histomorphometric analysis

Subsequent to radiography, the maxilla were demineralized in 10% EDTA, pH 7.2, then dehydrated with ethanol, diaphanized with xylol and embedded in paraffin. The blocks were sectioned at a 5 μm thickness along the frontal plane of the section and stained with hematoxylin and eosin, Masson's trichrome and *PicroSirius Red.*

Morphometric evaluation of osseous deposition was realized using ImageJ software. Three blinded and calibrated evaluators determined the percentage of the area occupied by newly formed bone, visualized as the trabeculae colored by Masson's trichrome. The histomorphometric measurements were realized in three antero-posterior sections of the defect (one mesial, one central and one distally located). The mean of the obtained values was subjected to statistical analysis.

The morphometric analysis using *PicroSirius Red* was conducted to investigate the organization and maturation of the new osseous matrix. Three sections of the paraffin block were again examined, and photographs were analyzed with polarized light, which enabled the study of collagen quality and organization. Collagen in the newly formed osseous matrix may form either finer fibers, exhibiting weaker green birefringence (type III fibers), or more organized and thicker fibers, visualized as yellow and red fibers (type I fibers). The red fibers show the maximum matrix maturation. ImageJ software was applied to determine the percentage of red fibers (more mature) using the canal colors tool in the images of the three slides, and the mean was used as the result of this analysis.

Statistical analysis

Statistics were plotted in Graph Pad Prism software using the t test and one-way ANOVA for parametric samples. The graphs were plotted using Microsoft Excel software.

RESULTS

Data obtained in the FA were compared with the results of the histomorphometric analysis of bone quantity and quality and with the radiopacity determined by MGS.

Comparisons were performed between the groups with mineralized bone grafts (BO and MBB) and between the demineralized bovine bone and blood clot groups (DBB and BC, respectively).

Blood clot x demineralized bovine bone

The histomorphometry results revealed that DBB accelerated the healing process, showing statistical superiority compared to the control group in the periods of 14 and 21 days (Graph 1). Despite these results, final bone repair at 49 days was similar in the two groups, without a significant difference.

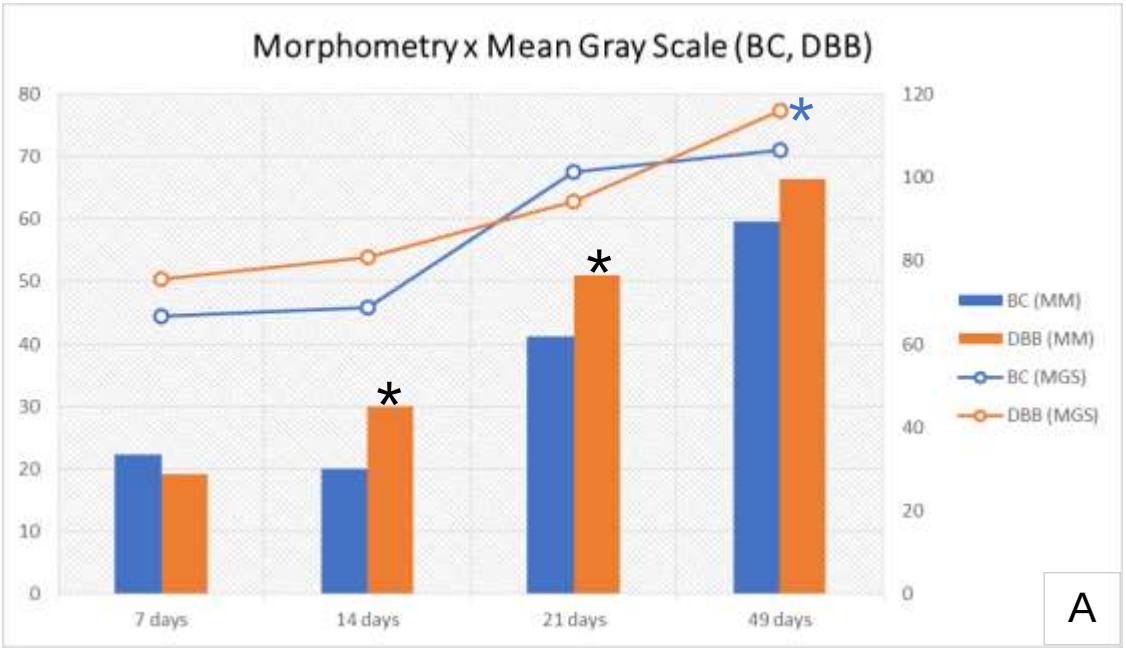
Regarding the maturation of collagen fibers in the new osseous matrix determined through *PicroSirius Red* analysis, the peak of maturation occurred from 21 to 49 days. Furthermore, at 49 days, the defects treated with DBB were considered to be significantly more mature than those of the BC group (Graph 2). When these results were compared with the histomorphometry results regarding osseous repair, 69,6% of the newly formed bone under DBB bone maturation consisted of mature collagen fibers, while this percentage was 52,2% in the BC group.

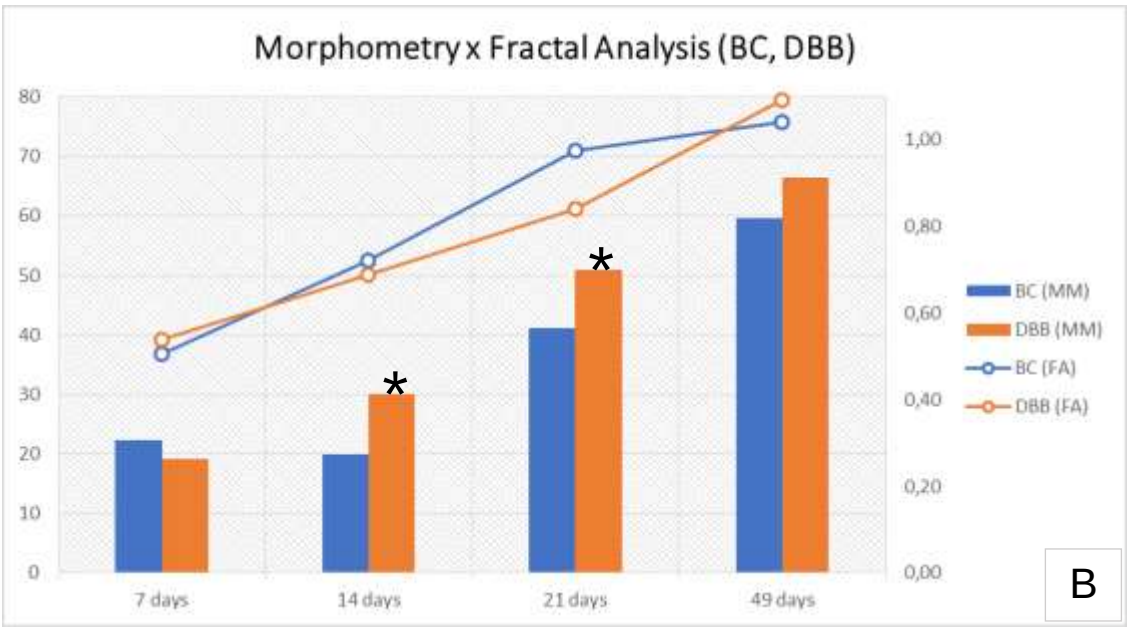
The mean FD and MGS values for all groups are shown in Table 1. Comparison of these histological evaluations with the radiographic methods revealed that radiopacity with MGS was the approach whose results were most closely related to the histomorphometry results. The MGS analysis of BC and DBB was consistent with bone repair at 14 and 49 days, and the DBB group even showed a significant difference compared with the BC group at the final time point of evaluation (Graph 1A), as observed in the analysis of the maturation of collagen fibers (Graph 2A).

However, FA did not reveal this pattern. Instead, a gradual increase in values was seen with time in both groups, without a significant significance. In contrast, the FD values of the BC group were larger than those of DBB at 14 and 21 days, as compared with the histomorphometric results (Graph 1B), which showed elevated bone deposition and more mature collagen fibers in the DBB samples (Graph 2B). Thus, it was noted that the results of MGS analysis presented more similarity to those of the histological evaluation than to those of FA.

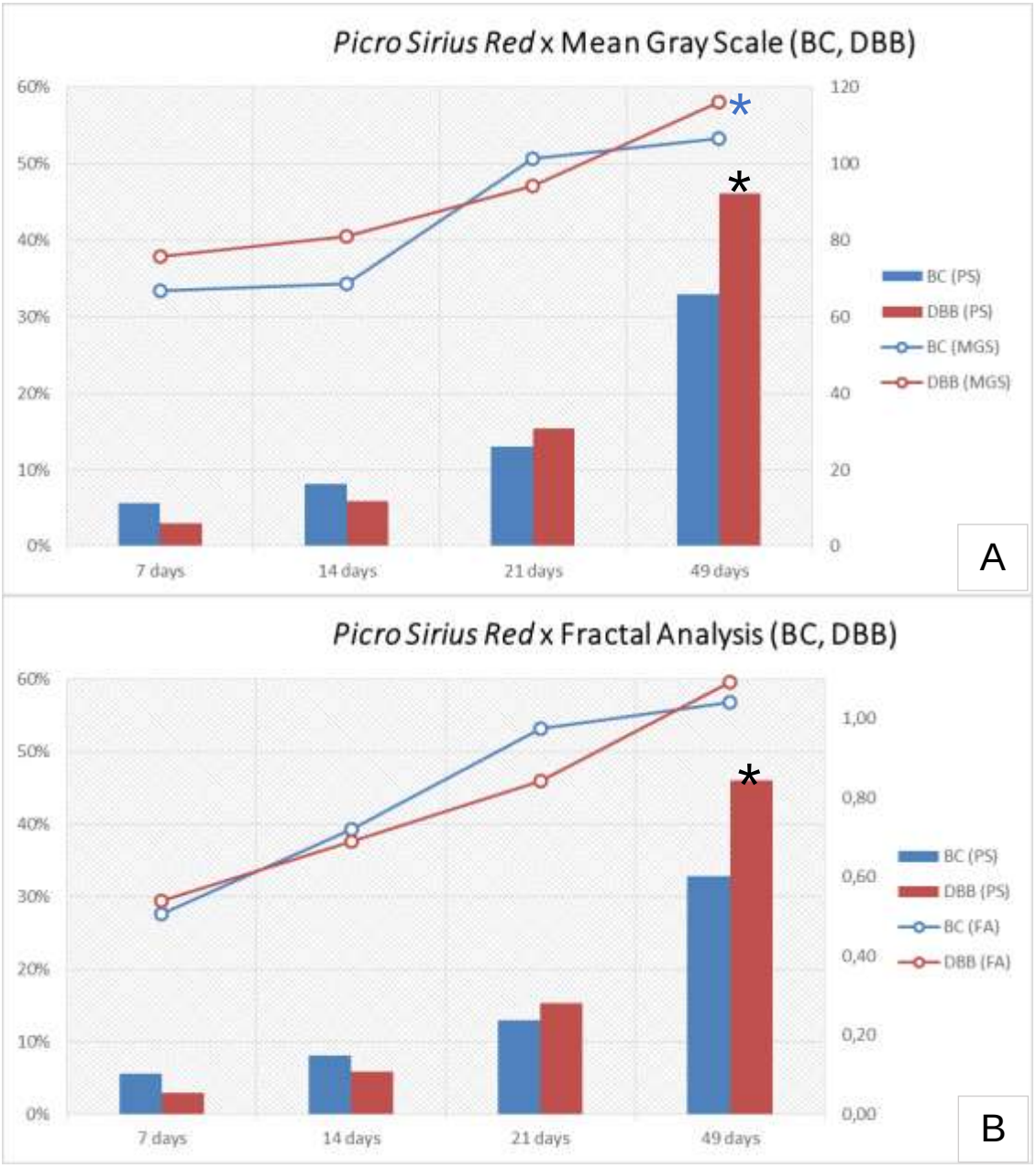
TABLE 1: Mean Values and standard deviation of FD and MGS for all evaluation times and study groups

		0	7	14	21	49
FD	**BC**	0,7779 (±0,09)	0,5055 (±0,18)	0,7209 (±0,27)	0,9755 (±0,25)	1,0426 (±0,12)
	DBB	0,6578 (±0,22)	0,5394 (±0,34)	0,6893 (±0,20)	0,8420 (±0,26)	1,0921 (±0,13)
	BO	0,8674 (±0,16)	0,9693 (±0,12)	0,9186 (±0,09)	0,7923 (±0,22)	0,9042 (±0,24)
	MBB	1,0208 (±0,13)	0,6887 (±0,15)	0,8192 (±0,08)	0,8339 (±0,26)	0,9096 (±0,24)
MGS	**BC**	84,89 (±4,82)	66,79 (±3,75)	68,68 (±11,24)	101,34 (±6,05)	106,61 (±7,59)
	DBB	85,92 (±12,96)	75,62 (±5,05)	80,95 (±11,39)	94,22 (±4,64)	116,17 (±6,50)
	BO	97,67 (±9,12)	109,56(±10,85)	96,77 (±5,39)	104,97 (±22,27)	106,05 (±5,16)
	MBB	99,30 (±9,91)	84,90 (±10,94)	76,29 (±9,00)	80,72 (±10,59)	105,71 (±4,10)





Graph 1: (A) The left axis represents the morphometric values (MM), and the right axis represents the MGS values. The columns show the results for bone formation determined through morphometric analysis, and the lines show the MGS results. Considering MGS, a significant difference was found between DBB and BC at the final time point ($p<0.05$) (t test). (B) The left axis represents the morphometric values, and the right axis represents the FA values. The columns show the results of bone formation determined through morphometric analysis, and the lines show the results of FA. The comparison between the groups according to the morphometric analysis showed a significant difference at 14 and 21 ($p<0.05$) days (t test). Under FA, no significant difference was found between the groups.



Graph 2: (A) The left axis represents the percentage of mature fibers acquired based on *PicroSirius Red* evaluation, and the right axis represents MGS values. The bone deposited in the DBB group was more mature by the final time point than that in the BC group, showing statistical significance ($p<0.05$) (t test). MGS also revealed this result, with statistical significance. (B) The left axis represents the percentage of mature fibers acquired based on the *PicroSirius Red* evaluation, and the right axis represents the FA values. The columns show the quantitative analysis of collagen fibers base on *PicroSirius Red* staining. The bone deposited in the DBB group was more mature by the final time point than that in the BC group, showing statistical

significance ($p<0.05$) (t test). FA also revealed this result, although the results were not consistent at all other times of evaluation, and it was not statistically significant.

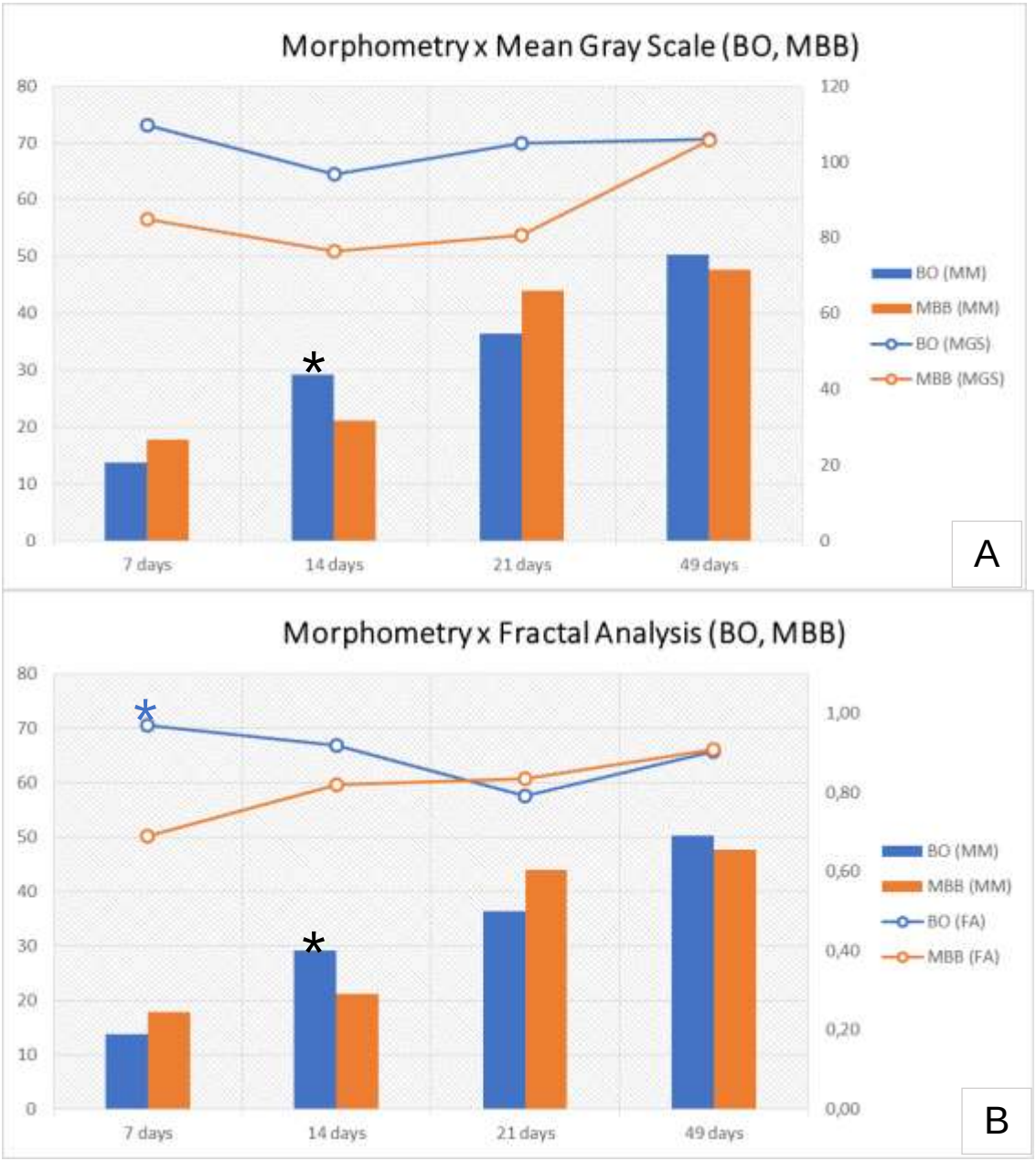
Bio-Oss® x mineralized bovine bone

Bone repair of defects with mineralized materials occurs along a different time curve in comparison with repair involving demineralized materials. When bone repair was compared between the BO and MBB groups, it was observed that BO exhibited significant bone formation at day 14. However, by 49 days, there was no significant difference between the groups (Graph 3).

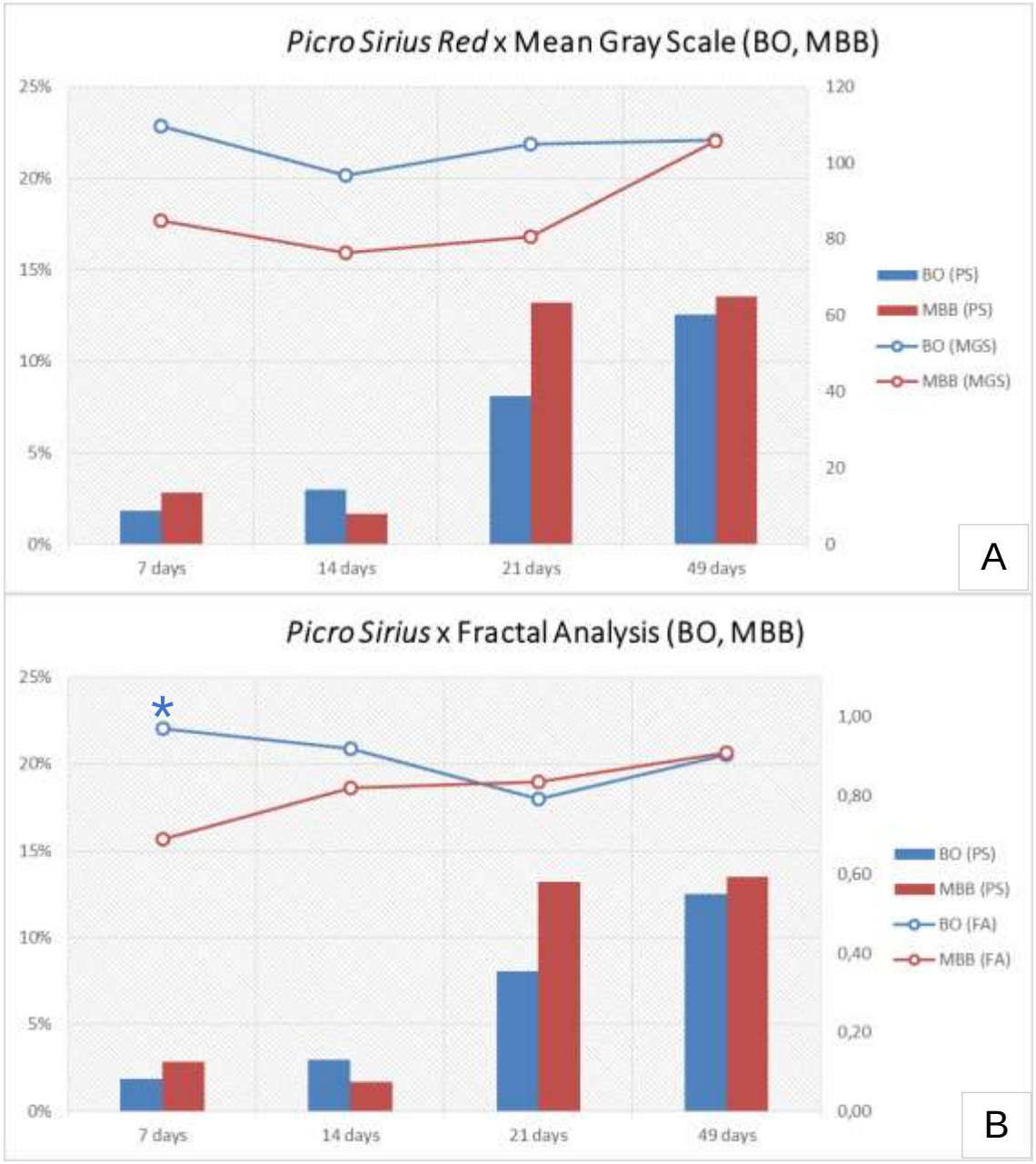
The *PicroSirius Red* analysis of BO and MBB did not shown any significant difference (Graph 4), although the percentage of red fibers was greater for the defects treated with MBB at 7, 21 and 49 days of evaluation. The final comparison of red fibers with newly formed bone showed that 28,4% of the bone in the MBB group and 23,8% in the BO group exhibited more mature fibers.

Although MGS revealed inverted absolute values in the evaluations performed at 7 and 21 days, no significant difference was found. Thus, the results of both FA and MGS analysis were similar to those of the histomorphometric evaluations (Graphs 3A and 3B). One exception was observed for the period of 7 days, when BO significantly outperformed MBB in the fractal analysis.

When analyzing the results of radiographic evaluations and bone maturation based on *PicroSirius Red* staining (Graphs 4A and 4B), FA was the method that best followed the pattern observed in the microscopic analysis, except for the period of 7 days. In contrast, MGS showed a continuous increase in values for both materials, with lower values obtained for MBB than BO, whereas the results for the two groups were similar at the final time point.



Graph 3: (A) The left axis represents the morphometric values, and the right axis represents the MGS values. No significant difference was found between the BO and MBB groups under MGS analysis; (B) the left axis represents the morphometric values, and the right axis represents the FA values. The columns show the results regarding bone formation determined through morphometric analysis, and the lines show the results of fractal analysis. The comparison between groups according to morphometric analysis showed a significant difference at 14 days (t test). A significant difference was found between the BO and MBB groups under FA in 7 days.



Graph 4: (A) The left axis represents the percentage of mature fibers acquired based on the *PicroSirius Red* evaluation, and the right axis represents the MGS values. The columns show the quantitative analysis of collagen fibers. MGS showed continuous growth of BO and MBB, with MBB presenting smaller values until the final time point, when the values were similar. This finding was only in accord with the maturation analysis at 14 and 49 days. (B) The left axis represents the percentage of mature fibers acquired based on *PicroSirius Red* evaluation, and the right axis represents the FA values. The bone deposited in the MBB group reached its peak of maturation before that in the BO group, at 21 days. However, there was no significant difference between the groups at the final time point. FA showed a significant difference

between groups only after 7 days of evaluation ($p < 0.05$) (t test), and these results were in accord with the observed bone maturation in the other times.

DISCUSSION

Since White and Rudolf (1999) (10) first described the use of the mathematical FA method for osteoporosis evaluation using radiographic images of the jaws, this method has been widely used in the detection of trabecular bone alterations in this region. Subsequently, studies using fractals for bone quantification and quality assessment started to draw attention toward this method (11, 14). As this approach is a recent type of evaluation, there are few studies (1) comparing the results of FA of images with those of histological analysis of the same site, as such comparisons are complicated *in vivo*.

One of the challenges in dentistry is the acceleration of osseous repair and substitution by biomaterials. Therefore, it is necessary to develop clinical methods for assessing these therapies. In the present study, bone repair of an intrabuccal osseous defect in rats was evaluated after the introduction of organic (DBB) and inorganic (BO and MBB) bone grafts. The bone grafts used in clinical dentistry are usually of an inorganic nature. In the present study, Bio-Oss® was chosen for this purpose because it is considered a gold standard material for bone-guided healing. Two versions of Lumina-Bone® were also employed: mineralized and demineralized. The use of a demineralized graft is based in the fact that inorganic components of the osseous matrix (such as BMPs, osteocalcin, osteopontin and collagen) retain osteogenic properties (18), conferring osteoinduction and osteoconduction properties to the graft. The present study focuses on the analysis of radiographic methods for clinically following bone repair with bone grafts. The advantages of radiographic evaluation of bone grafts with organic material include the fact that every change in radiopacity observed in images throughout the period of evaluation is considered to represent bone deposition and can be quantified. Radiographic evaluation of inorganic materials is more complex, as the radiopacity of the newly formed bone can be masked by the radiopacity of the biomaterials. Interestingly, the data presented in this report revealed that the results of radiographic evaluation by MGS were more closely related to the results of histomorphometry than those of FA.

Many reports indicate that FA is predictive of bone quality (11, 19, 20). Primarily, it is necessary to clarify the definition of bone quality, which was proposed

in 2000 by the National Institutes of Health (NIH) (21) as “the sum of all characteristics of bone that influence the bone’s resistance to fracture”. Therefore, bone quality cannot be defined only by bone density and image radiopacity, as it comprises a more complex combination of bone turnover, bone mineralization, micro-damage accumulation and bone architecture (22). In this study, the superposition of morphometric analysis using *PicroSirius Red* with Masson’s trichrome-stained samples produced a parameter indicative of the maturation of collagen fibers in the newly formed bone, which was considered a satisfactory qualitative evaluation approach for the bone for the purpose of the analysis. When those data were compared with the FA results for the radiographic images, no statistically significant correlation was found. Therefore, FA was not treated as being predictive of bone quality in the present study.

For the purpose of evaluation, radiopacity measured via MGS was more accurate than the FA for the prediction of bone repair in the demineralized and control groups. Many hypotheses can be formulated for this purpose. The first to be considered is the area of evaluation of each method. It was not possible to use the same acquisition method involving three points that was used in MGS analysis for FA, as no FA results was obtained with a ROI as small as five pixels. It has been reported that bone expresses self-similarity at sizes between 0.1 mm and 5 cm (23). Five pixels represented 0.12 mm in our images, which is too close to the value determined for the lack of these properties, which is probably why there were no results with a small ROI. The location of the three ROIs for MGS may also have played a role in the results, as alveolar repair occurs in an apical to coronal direction, and multiple points may provide a more detailed picture of the total repair than a single central ROI.

Another hypothesis is related to the use of blurs and filters in FA. In the description of this method from White and Rudolf (10), it is emphasized that the program is designed to remove large-scale variations in brightness in the image to reflect particular types of images (trabeculae and marrow spaces) and so that brightness levels that may cause individual variations in the image can be eliminated. A limitation of this type of analysis for newly formed bone is that it can cause clearance of incompletely matured trabeculae, as the step involving the Gaussian filter removes structures that are considered to be fine-scale and medium-scale structures, retaining only large variations in density.

Although the results of FA were not in accord with the observations of bone repair in the demineralized and control groups, they were similar to the bone repair process observed during the assessed period and with the MGS results at the final time point when the mineralized materials were compared. It is suspected that defects treated with mineralized and demineralized grafts exhibit differences in the progression of healing. MBB and BO are initially reabsorbed by osteoclasts before the healing process of new bone formation begins (24), which may explain the differences in the two groups in terms of bone repair. Additionally, the radiopacity and complexity of the materials may interfere in the evaluation of both methods, as these materials are not completely reabsorbed and cannot be differentiated from new bone in radiographic analysis.

Therefore, the use of FA for bone repair evaluation must be performed with caution. Many mathematical formulas, such as those of the power spectral density, triangular prism surface area, blanket method, intensity difference scaling, variogram analysis and the box-counting method, for the evaluation of trabeculae in X rays have been described (16). There is agreement in the literature that it is not possible to compare the results of different methods (16, 25). Molon *et al.* (2015) used the box-counting method and compared bone repair using autogenous bone grafts in sinus lifting with histomorphometric analysis. Although these authors did not observe significant differences between the fractal results for images and the results of microscopic methods, they found differences in fractals from the initial to final time points and this method to be reliable for the quantitative evaluation of bone (1). Although Kozakiewicz *et al.* (2013) did not perform a histomorphometric evaluation, they used the Fourier power spectrum method, which operates in the frequency domain (while other methods operate in the spatial domain), and the considered this approach to be effective for describing the dynamics of bone remodeling and useful as a quantitative indicator (7). In our study, the fractal dimension acquired via the box-counting method offered an illustration of bone repair, increasing with time, but the results were not equivalent to the histomorphometric results at all times of evaluation. This collection of literature shows that additional studies are necessary to compare different fractal methods and to determine which method is ideal for properly assessing bone repair.

Another concept that must be considered with caution regarding fractal dimensions is their capacity to measure bone quality. Many studies have associated

FA with bone mineral density and considered it to be predictive of quality (11, 26, 27). However, as discussed previously, this aspect should not be the only relevant factor in assessing bone quality. The present study used morphological methods to evaluate bone quality and did not find an association with FA.

While histomorphometric analysis considers the demineralized osseous matrix, radiographic analysis reads mineral deposition on that matrix. Therefore, histological analysis must be considered as a complementary evaluation that is still more reliable in terms of bone quantification, as it does not involve superposition of layers and is able to differentiate biomaterials from new trabeculae. Bone quality can be estimated based on the level of collagen fiber maturation, but it cannot be sufficiently determined based only on that criterion. In this context, radiography can be a useful tool. Radiographic evaluation presents limits as well, as it cannot differentiate biomaterials from osseous tissue during its formation, especially for mineralized forms, which present radiopacity similar to trabecular bone.

Although this study has some limitations, such as the use of an animal model, a small sample size, a brief period of evaluation, the small bone defect involved and small analysis' area, it provides a better comparison of FA and MGS considering morphometric evaluations. We conclude that although fractal analysis has been reported in the literature as a method for assessing bone repair, it did not exhibit significant differences compared to MGS, which is a simpler and more easily performed method for the same type of comparison. More studies are necessary to evaluate the use and benefits of FA in assessing bone repair, considering other experimental models and the different methods that can be used for this type of analysis.

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4 CONSIDERAÇÕES FINAIS

O estudo do uso da análise fractal para avaliação do reparo ósseo em modelos animais ainda tem muito a avançar. Apesar de ser uma técnica consolidada para avaliação da estrutura óssea em pacientes com osteoporose (WHITE and RUDOLPH, 1999), ainda são poucos e limitados os estudos sobre reparo ósseo, especialmente quando guiado por biomateriais.

Ao comparar o uso da análise fractal em imagens radiográficas de defeitos ósseos em ratos com o uso da radiopacidade e as análises histomorfométricas, conclui-se que a radiopacidade demonstrou padrão mais semelhante a histomorfometria do que a análise fractal no grupo dos desmineralizados. A análise final não demonstra diferenças significativas entre os dois métodos de análise radiográfica.

Devido às limitações do estudo, como tamanho da amostra, tamanho do ROI avaliado e tempo de avaliação, mais estudos sobre o uso da análise fractal para estudo da reparação óssea são necessários.

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ANEXOS

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o Protocolo nº. 7 / 2015, relativo ao projeto intitulado "Avaliação de enxertos ósseos bovinos, associados ou não à terapia celular no reparo de defeito ósseo intrabucal em ratos.", que tem como responsável Gerluza Aparecida Borges Silva , está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de 14/04/2015. Este certificado espira-se em 14/04/2020.

CERTIFICATE
We hereby certify that the Protocol nº. 7 / 2015, related to the Project entilted "Evaluation of bovine bone graft associated or not to cell therapy to repair intraoral bone defect in rats.", under the supervision of Gerluza Aparecida Borges Silva , is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in 14/04/2015. This certificates expires in 14/04/2020.

Cleuza Maria de Faria Rezende
Coordenador(a) da CEUA/UFMG
Belo Horizonte, 14/04/2015.

Atenciosamente.

Sistema CEUA-UFMG
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