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periodontais em modelo de periodontite experimental - análise
por Micro-CT**

*Effect of ionizing radiation on periodontal tissue repair in an experimental
periodontitis model - Micro-CT analysis*

Trabalho de conclusão de curso
apresentado à Faculdade de Odontologia da
Universidade Federal de Uberlândia, como
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DEDICATÓRIA

Dedico este trabalho à Deus, minha família, meus amigos e a todos que de alguma forma contribuíram para eu chegar até aqui...

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***“Bom mesmo é ir à luta com determinação, abraçar a vida e viver com paixão,
perder com classe e vencer com ousadia, pois o triunfo pertence a quem se atreve...
E a vida é muito para ser insignificante.” (Charles Chaplin)***

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Effects of different doses of ionizing radiation on alveolar bone repair in post-extraction tooth socket: an experimental study in rats

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: This study was approved by the Ethics Committee on Animals Use of Federal University of Uberlândia (CEUA 103/19). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

ABSTRACT

Background: The aim of this study was to investigate the effects of ionizing radiation (IR) on periodontal repair after treatment of experimental periodontitis in rats. **Methods:** Twenty-eight male Wistar rats were randomly divided to 4 groups (n=7): control group (nPDnIr): non-ligated and nonirradiated; irradiated group (nPDIr): non-ligated and irradiated; periodontal disease group (PDnIr): ligated and nonirradiated; periodontal disease + irradiation group (PDIr): ligated and irradiated. Experimental periodontitis was silk ligatures-induced into the second mandibular molars for 7 days, the contralateral side served as a control, non-ligated. The ligatures were removed and the animals of the irradiated group received a single dose (30Gy) of ionizing radiation at 7 and 20 days after removal of the ligature. After 7 days of irradiation, the rats were sacrificed. Micro-computed tomography (micro-CT) was used to measure alveolar bone loss and hematoxylin and eosin staining for histological analysis. Statistical analysis was performed using the Shapiro–Wilk test, ANOVA and Tukey’s test for normal data and Kruskal-Wallis and Dunn’s test for non-parametric data ($p < 0.05$).

Results: The linear analyzes (alveolar bone loss), the experimental periodontitis groups showed higher values of distance between the cemento-enamel junction (CEJ) to bone crest (BC) regardless of the evaluated region and experimental period used ($p < 0.05$). In addition, the animals submitted to radiotherapy also showed higher values for CEJ-BC than the groups that did not undergo radiotherapy, regardless of the experimental period ($p < 0.05$). Bone volume fraction data were lower for the PDIr at 20 days group. The histological analysis, RT and PD significantly influenced in inflammation and fibrosis ($p < 0.05$).

Conclusions: Ionizing radiation modulated the extension of alveolar bone loss. In the long term (20 days), IR increased alveolar bone loss in an experimental periodontitis rat model, even after treatment of periodontal disease. Ionizing irradiation upregulates the inflammation and this effect may be related with the impairment of the periodontal repair.

Keywords: Ionizing radiation; X-Ray Microtomography; Experimental periodontitis; Inflammation; Alveolar bone loss; Periodontal disease.

INTRODUCTION

Periodontal disease (PD) is a chronic inflammatory condition caused by the colonization of highly complex subgingival biofilm on the tooth surfaces, which may affect gingival tissue, periodontal ligament, cementum, and alveolar bone [1,2]. Pathogenesis is considered a dysbiotic process resulting from an imbalance between periodontopathogens microorganisms of the biofilm and the host responses [3,4], and is modulated through multiple factors including genetics, smoking, general health, social variables, and nutritional status [5]. The pathophysiology of periodontitis is characterized by key molecular pathways. The proteinases produced by the host are activated, therefore, destruction of the periodontal ligament fibrils, as well as apical migration of the junctional epithelium, occur, which creates a favorable environment for the bacterial biofilm to spread along the root surfaces [6, 7].

The conventional treatment for periodontitis relies on the mechanical removal of bacterial plaque from the periodontal pockets by scaling and root planning, which are considered the conventional and essential procedures in periodontal therapy [8,9]. Several factors increase the risk of periodontal diseases. These risk factors, modifiable and non-modifiable, contribute toward the clinical significance of periodontal diseases, such as cigarette smoking; medications such as steroids and anti-epilepsy, cancer therapy drugs; systemic diseases such as diabetes mellitus, cardiovascular disease, osteoporosis and others [10].

According to the International Agency for Research on Cancer, the worldwide incidence of cancer is expected to reach 27.5 million by 2040 [11]. In head and neck cancer, adjuvant radiotherapy (RT) is often a critical component of treatment [12,13]. The delivery of that radiation is associated with devastating collateral effects on surrounding healthy tissues. These complications still persist despite advancements in administration protocols and technological advances in radiation oncology [14].

Several secondary effects related to RT can occur in the oral cavity, including progressive periodontal attachment loss, dental, soft tissue necrosis, and osteoradionecrosis (ORN) [15]. Patients who receive radiation in the head

and neck region have increased risk for periodontal disease, which may be associated with hyposalivation and oral microbiome modification [16,17], especially in patients with periodontitis [18]. The prevalence of PD in adults is frequent and is likely to get worse with oncology treatment. The effect of RT on the periodontium seems to depend on the dose and is associated with periodontal problems before radiotherapy initiation, therefore periodontal infection should be eliminated in the pre-radiotherapy period [17,18].

Numerous experimental studies have been conducted to determine the mechanism of destruction caused by periodontal disease, to prevent destruction, or to determine the ways to compensate for the consequences of this destruction [4, 7, 8, 19]. However, few efforts have been carried to assess the destruction caused by ionizing radiation (IR) in situations after periodontal treatment [18]. It is not known for sure how periodontium behaves when high dose fraction is used. Alterations in the cellularity, vascularity, and the repair potential of the periodontium has been shown, however the literature is scarce [17]. Therefore, the aim of this study was to investigate the effects of ionizing radiation on periodontal repair after treatment of experimental periodontitis in rats. The present study was designed to evaluate whether a single dose of ionizing radiation (30 Gy) influence the periodontal repair after ligature removal in animal model of periodontitis. The null hypothesis is that RT does not affect periodontal repair after treatment of experimental periodontitis in rats.

Materials and methods

Animals

The experimental protocol was approved by the Institution's Ethics Committee on the Use of Animals (CEUA 103/19) of the institution, according to the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The animals were conditioned in plastic boxes in groups of 4 in a controlled-environment room (temperature, 23 °C ± 3 °C; humidity, 25%; light/dark cycle, 12/12 h), with free access to rat food and water *ad libitum*. The study included 28 male rats (*Rattus norvegicus*, *albinus*, Wistar), aged approximately 9 weeks and weighing 275–325 g.

Ligation-induced experimental periodontitis

Experimental periodontitis was induced at the beginning of the experiment by the accumulation of bacterial biofilm through the ligature in the second lower molar [20]. The animals were submitted to intramuscularly anesthesia combining of 0.1 mL of ketamine hydrochloride (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brazil), along with 0.05 mL of xylazine hydrochloride (Rompum® Bayer SA, São Paulo, SP, Brazil) per 100 g of body weight. Ligatures (3–0 silk suture, Ethicon, Johnson & Johnson, Somerville, New Jersey) were placed submarginally [18, 21] around the second mandibular molar on the right side, in the most cervical position possible, by two nodes on the buccal side.

To facilitate ligature placement, a slightly separation in the interproximal area with the aid of a periodontal probe (Hu-Friedy, Chicago, Illinois) was performed. In all animals, the ligature was inspected every other day and repositioned if necessary, to maintain the ligature during the entire experimental period. For the ligation conference the animals were anesthetized with an inhalation method using Isoforine® (Cristália Prod. Quím. Farm. Ltda). Ligatures were maintained in position in order to allow biofilm accumulation during the 7 days of periodontal disease experimental induction [20, 22]. The second molars on the left side were left no-ligated so that they could be used as controls (periodontally healthy, nPDnIr).

Periodontal treatment

Periodontal treatment was performed under general anesthesia with the removal of the ligatures after 7 days. Subsequent to the development of the experimental periodontitis, ligatures were removed, and rats were randomized into four groups: 1- Control group (nPDnIr), rats were non-ligated and non-irradiated; 2- Irradiated group (nPDIr), rats were non-ligated and irradiated; 3- Periodontal disease group (PDnIr), rats were ligated and non-irradiated; 4- Periodontal disease + irradiated group (PDIr), rats were ligated and irradiated;

In addition, the groups were evaluated at two evaluation periods, with 7 and 20 days after periodontitis treatment (ligation removal) (n=7 each group)

(Figure 1). Removal of the ligatures allows investigation of the resolution of inflammation and the healing response [23].

Ionizing radiation

After 7 (nDPIr7d; DPIr7d) and 20 days (nDPIr20d; DPIr20d) of PD treatment by removing the ligature, the animals in the irradiated group received a single dose of 30 Gy of ionizing radiation in the mandible bilaterally through a medical linear accelerator 6MeV (Varian 600-C © Varian Medical System Inc Palo Alto, California, USA) with a source-skin distance of 60 cm and the field size was 15 x 15 mm [24]. The animals were submitted to general anesthesia, positioned with only their mandible region in the radiation field and immobilized with adhesive tape. A wax bolus measuring 1,5 cm was placed over the mandible to increase the thickness of the soft tissues and concentrate the maximal dose on bone. The radiation dose was selected based on previous studies by the research group. Animals of nonirradiated groups were also anesthetized (sham method).

Sample collection

After 7 days of radiotherapy procedure, all animals were submitted to euthanasia by intraperitoneal overdose of thiopental (150 mg/kg). The mandible from each animal was dissected and then hemisected. The hemimandibles were removed and immediately fixed in 4% phosphate-buffered paraformaldehyde solution during 48h and were then kept in ethanol (70%). The samples were first scanned using X-ray microcomputed tomography (micro-CT) for morphometric analysis of alveolar bone loss and subsequently decalcified in 4% EDTA for 8 weeks, under agitation at room temperature, dehydrated with graded ethanol and embedded in paraffin for histological analysis.

Microcomputed tomography (micro-CT) analysis

The samples were scanned using microcomputed tomography Skyscan 1272 microcomputed tomography (Bruker, Kontich, Belgium). Mandibles were scanned at 12- μ m resolution (X-ray source 80 kV, 125 μ A) using an aluminum filter (1 mm of thickness) and exposure of 400 ms with an angular increment of 0.4° and averaging of 2 frames. The reconstructions were performed using the software nRecon (version 1.6.10.1, SkyScan, Bruker, Belgium), following the parameters of smoothing (0), ring artifacts reduction (4) and beam-hardening (0%) for all samples. Three-dimensional (3D) volume viewing and analysis software (DataViewer, CT-volume and CT-analyser, SkyScan, Bruker, USA) were used to visualize and quantify 2D data. All images were reoriented to standard angles to view cemento-enamel junction and root apex in the same slices using Dataviewer software (Skyscan, Bruker-microCT).

The alveolar bone loss (ABL) was evaluated by single experienced operator, and the measurements were determined of distance between the cemento-enamel junction (CEJ) to bone crest (BC) [25, 26] in the mesial and distal root of the mandibular second molar teeth and the furcation ceiling (FC) to bone crest (BC) in the furcation region (Figure 2A,B) using software DataViewer (SkyScan, Bruker, USA). Mean values of measurements in millimeters were calculated for statistical analysis. The measurements were performed after intraexaminer calibration by evaluating 10 images. The examiner took the linear measurements twice within 48 hours.

For volumetric analysis, a standardized region of interest of was defined of 1,400 $\times$ 500 $\times$ 540 μ m (length $\times$ height $\times$ thickness), including the mesial, distal region and furcation of the second molar (Figure 2C) positioned on the images according to anatomical landmarks and a standardized gray-scale (63-255) value was used to visualize mineralized tissues only. Bone volume fraction (BV/TV in %), which means the percentage of the ROI filled with bone volume was calculated using CT Analyzer software (version 1.12.4.0 Bruker microCT, Skyscan, Belgium) by a trained examiner who was not aware of the experimental conditions of each sample.

Statistical analysis

The Jamovi software (Version 1.6, software obtained free of charge from <https://www.jamovi.org>.) was used to carry out the statistical analysis of this study. The effect of the independent variables of this study (Induction of experimental periodontitis, irradiation and experimental period) on the study outcomes (alveolar bone loss, BV/TV%) were evaluated using the Three-way Anova complemented by the Tukey test to compare the groups ($p < 0.05$). The results are expressed as mean $\pm$ standard deviation or percentage.

Results

Micro-CT analysis, it was found that the presence of periodontal disease, radiotherapy and the experimental period significantly influence the CEJ-BC data. Regarding data from linear analyzes (alveolar bone loss), groups with PD showed higher values of CEJ-BC regardless of the evaluated region and evaluation periods ($p < 0.05$). The animals submitted to RT also showed higher values for these parameters than the groups that did not undergo radiotherapy (nIr), regardless of the experimental period ($p < 0.05$), except in the furcation region. In the proximal and furcation region, there was a significant difference ($p < 0.05$) in relation to the experimental period for the group with irradiated periodontal disease (PDIr), with higher values of bone loss for the period 20 days (Table 1; Figure 3).

BV/TV% data were influenced by radiotherapy and periodontal disease induction. Regarding the BV/TV% data, it was verified that in the proximal regions, the PDIr group presented lower values in both evaluation periods than the control- nPDnIr group in both evaluation periods ($p < 0.05$) and that in the PDnIr group at 20 days ($p < 0.05$). Additionally, it was found that the nDPIr group had lower BV/TV% values in the proximal region than the control- nPDnIr group in the 20-day period ($p < 0.05$). Regarding the values of BV/TV% in the furcation region, it was verified that the PDIr20d group presented the lowest values of this parameter than the other groups ($p < 0.05$) (Table 2).

Discussion

Experimental animal models of periodontitis have provided important insights into the disease pathogenesis [20, 27, 28]. Among the various methods employed to mimic periodontitis in animals, the most commonly used model is ligature-induction [20]. The use of a ligature throughout the cervical region of teeth leads to accumulation of biofilm, bacterial colonization resulting in apical epithelial migration and bone loss, symptoms similar to those observed in a clinical situation. The connective tissue and bone loss predictably occur over a period of 7–15 days in rats [20, 29]. In the present study, inflammatory cells infiltrated into the connective tissue was observed histologically in rat with

experimental periodontitis. In addition, micro-CT results showed an increased CEJ-BC distance in the PD groups compared with the control group (nPD). Other studies also found gingival inflammation and alveolar bone resorption 7 days after ligation [20, 31, 30]. The results of the present study indicate that experimental periodontitis model was successfully established.

This study evaluated the role of irradiation in the alveolar bone loss. The animals submitted to RT showed higher values for bone loss than the groups that did not undergo RT (nIr), regardless of the experimental period. Moreover, the lowest bone mineralization (BV/TV) mean value was observed in interproximal and furcation region for the PDIr group at 20 days when compared to the other experimental and control groups. The results of this study corroborate with other findings, that RT increased periodontal attachment level and alveolar bone loss in irradiated rats with experimental periodontitis [18]. This can show the unwanted long-term effects caused by irradiation in the periodontium after PD treatment. Moreover, it is possible to observe the irradiation effects in bone remodeling. This may represent a potential negative effect on tissue repair or turnover [31] in experimentally induced periodontal disease in rats even after treatment with ligature removal. Thus, the null hypothesis that RT does not affect periodontal repair after treatment of experimental periodontitis in rats was rejected.

Moreover, no significant difference was observed for data obtained from BV/TV in the furcation region in the evaluated groups and their respective control at 7 and 20 days, except for the PDIr20d group. This result provides a comprehensive reading of the entire second molar region. The induction and treatment of PD in the evaluated experimental period, may have been sufficient to improvement of damage caused to bone tissue in furcation region [32].

Conclusion

Within its limitations, this study showed that the

- Ionizing radiation modulated the extension of alveolar bone loss in periodontitis induced in rats.
- In the long term (20d), ionizing radiation increased alveolar bone loss in an experimental periodontitis rat model, even after treatment of periodontal disease.

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Legend of figures

Fig 1. Fluxogram of the experiment. nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Fig 2. Reconstructed 3D micro-CT images. A. Linear measurements of alveolar bone loss (ABL) determined of distance between the cemento-enamel junction (CEJ) to bone crest (BC) in the mesial and distal root of the mandibular second molar teeth and the furcation ceiling (FC) to bone crest (BC) in the furcation region. (Green line: JCE, red line: CO, yellow line: distance (mm)); coronal plane measurements. B. Linear measurements in the sagittal plane. C. Coronal view, a rectangular region was selected as the ROI to evaluate the ratio of the bone volume fraction (BV/TV).

Fig 3. Alveolar bone loss in the mandible in the region of the mandibular second molar observed by micro-CT. The groups induced to experimental periodontitis and ionizing radiation showed higher values of CEJ-BC compared to the other experimental groups ($p < 0.05$).

Figures

Fig 1

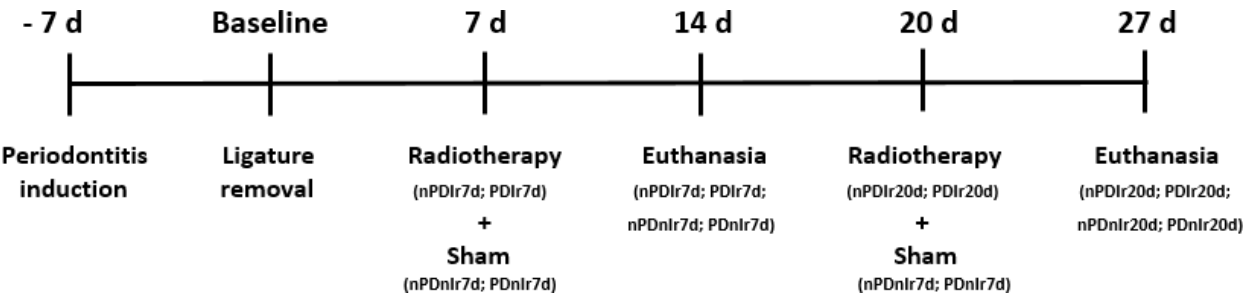


Fig 2

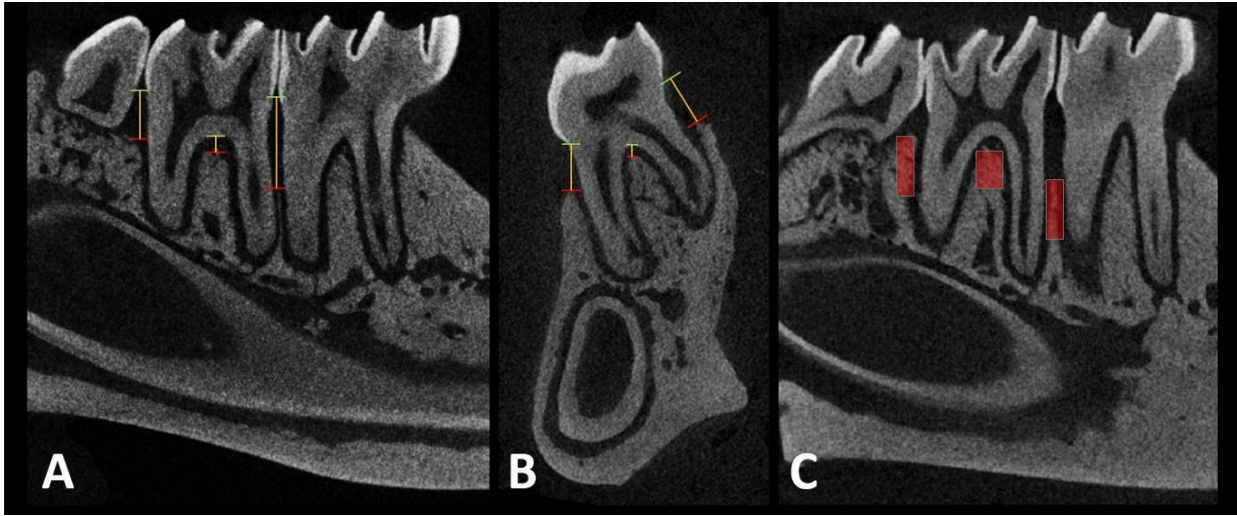


Fig 3

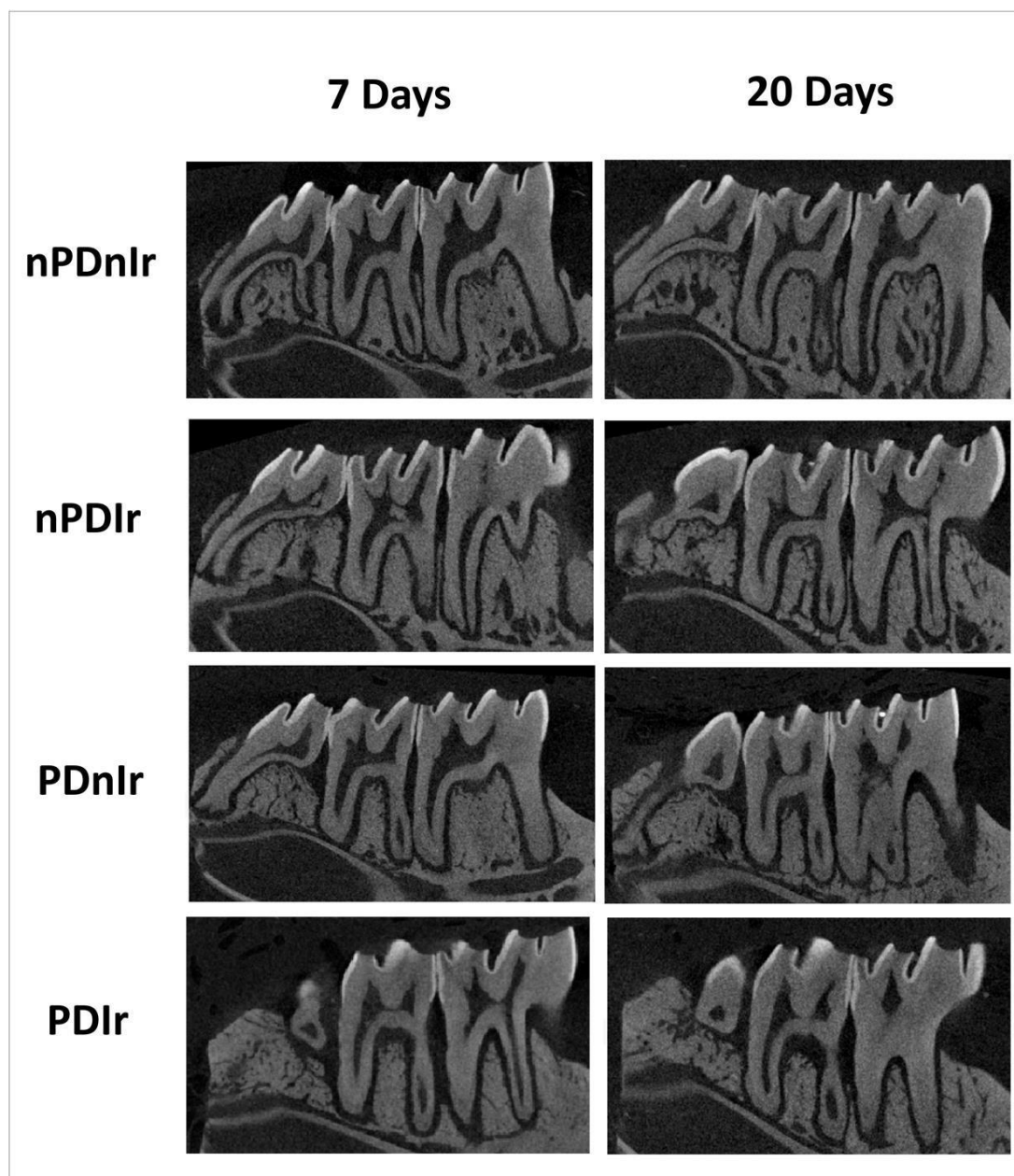


Table 1: Mean data and standard deviation of linear measurements by micro-CT performed in all groups and experimental periods.

Group	Experimental period	CEJ-BC	CEJ-BC	FC - BC
		Interproximal	Faces livres	
nPDnIr	7d	0.60 ± 0.08 ^a	0.47 ± 0.04 ^a	0.15 ± 0.05 ^a
	20d	0.41 ± 0.04 ^a	0.54 ± 0.04 ^a	0.13 ± 0.02 ^a
nPDIr	7d	1.02 ± 0.16 ^b	0.58 ± 0.06 ^b	0.18 ± 0.05 ^a
	20d	0.71 ± 0.13 ^b	0.53 ± 0.07 ^b	0.12 ± 0.02 ^a
PDnIr	7d	1.27 ± 0.11 ^c	1.00 ± 0.13 ^c	0.39 ± 0.14 ^b
	20d	1.17 ± 0.15 ^c	0.94 ± 0.12 ^c	0.30 ± 0.09 ^b
PDIr	7d	1.28 ± 0.18 ^c	1.11 ± 0.18 ^d	0.36 ± 0.11 ^b
	20d	1.64 ± 0.30 ^d	1.58 ± 0.33 ^d	1.01 ± 0.04 ^c

Different letters represent different levels of statistically significant differences. Three-way Anova complemented by Tukey. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days). Abbreviations: cemento-enamel junction (CEJ); bone crest (BC); furcation ceiling (FC).

Table 2: Mean and standard deviation data of BV/TV% values by micro-CT.

Group	Experimental period	BV/TV% Interproximal	BV/TV% Furcation
nPDnIr	7d	72.73 ± 1.77 ^a	79.56 ± 4.36 ^a
	20d	75.45 ± 8.01 ^a	79.70 ± 7.01 ^a
nPDIr	7d	66.96 ± 7.15 ^{a,b}	72.79 ± 10.10 ^a
	20d	62.67 ± 8.69 ^{b,c}	71.30 ± 6.17 ^a
PDnIr	7d	64.00 ± 6.03 ^{a,b}	69.12 ± 5.94 ^a
	20d	72.94 ± 6.10 ^{a,b}	72.02 ± 11.41 ^a
PDIr	7d	60.27 ± 9.31 ^b	68.91 ± 8.56 ^a
	20d	52.26 ± 9.59 ^c	48.46 ± 16.12 ^b

Different letters represent different levels of statistically significant differences. Three-way Anova complemented by Tukey. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

