UNIVERSIDADE FEDERAL DE UBERLÂNDIA

FACULDADE DE ODONTOLOGIA

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EFEITOS DA RADIAÇÃO IONIZANTE NA MICROARQUITETURA ÓSSEA CORTICAL: ALTERAÇÕES ESPECÍFICAS RELACIONADAS AO LONGO DO TEMPO

EFFECTS OF IONIZING RADIATION ON CORTICAL BONE MICROARCHITECTURE: SPECIFIC RELATED ALTERATIONS OVER TIME

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Trabalho de conclusão de curso apresentado à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Graduado em Odontologia.

Orientadora: Profª. Drª. Priscilla Barbosa Ferreira Soares

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SUMÁRIO

Effects of ionizing radiation on cortical bone microarchitecture: specific related alterations over time

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ABSTRACT

This study aimed to evaluate the cortical bone microarchitecture in rabbit tibias at intervals 7, 14 and 21 days after ionizing irradiation. Twelve adult male New Zealand rabbits were treated with a single radiation dose of 30Gy. The animals were randomly divided into 4 groups: Control (no radiation), Ir7, Ir14 and Ir21 days. Computadorized microtomography was used to analyze the microarchitecture of the cortical bone. The following parameters were used: cortical thickness (CtTh), bone volume (BV), total porosity (Ct.Po), intracortical porosity (CtPo (cl)), fractal dimension (FD) and degree of anisotropy (Ct.DA). One-way analysis of variance (ANOVA) was performed for all data followed by Tukey and Dunnet tests. The cortical thickness was different ($p <0.01$) between the control and irradiated groups, with thicker cortex to Ir 7 days. There was no difference between groups for total porosity, however, intracortical porosity revealed significance difference ($p <0.001$) between the irradiated groups and the control group, with a lower value for Ir7 days. The number of bone channels, fractal dimension and degree of anisotropy did not show significant difference between groups. The bone volume was lower in the Ir14 group in relation to control. In this way, the microarchitecture of the cortical bone can be affected by radiotherapy and the effects appear to be time-dependent. Cortical parameters found in the group Ir21 days were similar to the control group, suggesting that the cortical bone return to the regular conformation after 21 days.

Keywords: Radiotherapy; Cortical bone; Microarchitecture; Computadorized Microtomography

INTRODUCTION

Radiation Therapy (RT) in combination with surgical procedures are the main treatment modalities for bone and soft tissue malignant tumors [1]. Initial changes caused by radiotherapy affect directly the bone remodeling activity [2]. Therefore, high-dose irradiation causes irreversible side-effects on the tumor surrounding healthy tissue. These injuries lead to impairment in bone repair properties and complications such as infections, healing delay and osterradionecrosis [3].

The bone experience changes during RT, initially, with occurrence of a decrease in blood supply, caused by the increase of endothelial cell permeability. This event causes a perivascular edema, small vessel hemorrhage and decreased perfusion, eventually also occur fibrosis of vascular endothelium [2] [4] [5]. Bone tissue has a low proliferation velocity, so it has a relative condition to resist to radiotherapy. However, high and cumulative ionizing radiations doses have been reported to increase the risks of osteoradionecrosis [6].

Bones are generally divided into two distinct tissue areas, namely (1) an outer compact shell composed of cortical bone which surrounds (2) an inner lattice of trabecular bone [7]. Mostly studies have focused on trabecular changes [8] [9]. However, the cortex comprises the majority of the appendicular skeleton and it is the main involved in the bone loss later in life [10]. There is also a growing interest to investigate the vast osteocyte network and hence the role of cortical bone in physiology of the skeleton [7].

The sequence of histologic events in the irradiated cortical bone demonstrated in tibial shafts rabbits from 1 to 12 months after the single-dose radiation significant decrease in the vascular permeability and higher number of plugged channels, which is followed by bone resorption, neovascularization and then the second phase of remodeling showing bone formation [11]. The topology of the network of bone channels is of great importance in bone reconstitution capacity, being necessary to nourish the bone cells, which is done by blood vessels distributed within the channels [12].

The micro-CT is the gold standard to evaluate the microarchitecture and the morphology of cortical bone in small size animals. It allows to investigate the bone in a 3D image and does not cause any damage to the sample [13] [14]. Animal models have provided substantial data to investigate time and dose response of radiotherapy [11] [15]. Therefore, the aim of this study was to evaluate the cortical bone microarchitecture after single-dose radiation therapy in rabbits tibiaes 7, 14 and 21 days after irradiation using microtomography computadorized.

METHODS

Study design

A total of 12 male New Zealand rabbits (Oryctolagus cuniculus) with approximately 3000g (ranging from 3000 to 3500g) each were divided into four groups of three animals. Both legs from each animal were included in the study, totalizing 6 tibias per group (n=6). The animals were divided into groups: non-irradiated rabbits (control group – NIr), and irradiated rabbits, sacrificed at different times, as follows: group Ir7days, group Ir14days and group Ir21days, euthanized at 7 days, 14 days and 21 days after the radiation procedure, respectively. The Institutional Animal Care and Use Committee of the institution approved all procedures (CEUA-UFU; Protocol: 093/12).

Irradiation procedure

During the radiotherapy session, the animals (Ir groups) were under general anesthesia with an intramuscular injection of a combination of 0.25 mg of ketamine/kg of body weight (Ketamina Agener®; Agener União, São Paulo, SP, Brazil) and 0.5 mg of xylazine/kg of body weight (Rompum® Bayer, São Paulo, SP, Brazil). The left and right hind legs of each rabbit were subjected to a single dose of 30Gy. A dose of radiation was delivered with a source–skin distance of 60 cm and the field size was 15 x 15 mm with direct electron beam of 6 MeV electrons (Varian 600-C® Varian Medical Systems, Palo Alto, California, EUA). After radiation, the skin, hair, weight, and appetite of the rabbits were closely monitored by the responsible veterinarian. The animals were sacrificed by an overdose of anesthetic (0.75 mg of ketamine/kg of body weight). In the irradiated groups, the sacrifice was planned at 7, 14 and 21 days after radiation. The overlying soft tissues were removed, and the tibias were stored in phosphate buffered saline solution and frozen at -20 °C in plastic tubes.

Micro-CT analysis

The samples were stored frozen at -20 °C for 48 h. For assessing the tridimensional (3D) bone micro-architecture, the tibia diaphysis was examined using a desktop µCT system (SkyScan 1272, Bruker, Kontich, Belgium). During scanning, the tibia was placed in the polyethylene tube and immobilized inside the tubes by means of soft modeling clay. The scanning parameters were 15 µm pixel size, 50 kV X-ray voltage, 160 mA electric current and 0.5 mm Al filter. Subsequently, the reconstructed 3D data sets were obtained and quantified using NRecon and CTAn automated image analysis system. Cortical bone was segmented manually on a slice-by-slice basis (Fig. 1). After that, the volume-of-interest (VOI) for cortical analyses was selected and extending totally 300 slices (Fig. 2). The following parameters were measured in the cortices: mean thickness (Ct.Th, µm); total porosity (Ct.Po, %); closed porosity (just considering the intracortical pores, Ct.Po (cl), %); bone volume (BV, mm3); degree of anisotropy (Ct.DA,

#); and fractal dimension (FD, #), analyzed using CTAn software (Skyscan, Bruker, Belgium), with a global bone threshold (55 lower grey threshold and 255 upper grey threshold).

Statistical analysis

The mean thickness (Ct.Th), total porosity (Ct.Po), closed porosity (Ct.Po), pore number (Po.N), bone volume (BV); degree of anisotropy (Ct.DA) and fractal dimension (FD), data were tested for normal distribution (Shapiro-Wilk) and equality of variances (Levene's test), followed by parametric statistical tests. One-way analysis of variance (ANOVA) was performed for all data. Multiple comparisons were made using Tukey's test for comparison between irradiated groups on different periods and Dunnet test was used for comparison between control group and irradiated groups. Sample size was grounded on information from previous reports applying similar animal model. A post-hoc test was performed to define the minimum difference in the parameters assessed herein that would have been possible to detect applying a power of 80% and an alpha error of 0.05%. All tests employed $\alpha=0.05$ significance level and all analyses were carried out with the statistical package Sigma Plot version 13.1 (Systat Software Inc, San Jose, CA, USA).

RESULTS

Clinical evaluation revealed that irradiated rabbits developed alopecia due to radiotherapy. Concerning µCT results, cortical thickness was different ($p<0.01$) between control (1.01±0.04) and an irradiated group, with thicker cortices in the Ir7days group

(1.15±0.09). Among irradiated groups, the cortex was thicker at 7 compared to 14 days (1.01±0.05), but similar compared to 21 days (1.07±0.09) (Fig. 3a).

There was no difference for the total cortical porosity (Fig. 3b), however, the intracortical porosity (closed porosity), represented by intracortical channels, revealed significant difference ($p<0.001$), among the irradiated groups, with lower value for the Ir7days group (0.29±0.09) compared to Ir14 days (0.48±0.08) and Ir21days (0.44±0.06) (Fig. 3c). In addition, the intracortical porosity was also different ($p <0.001$) between Ir7days and control group (0.53±0.18) (Fig. 3c).

Fractal dimension (Fig. 3d) and degree of anisotropy (Fig. 3e) were not different between control and irradiated condition, and among the irradiated groups. Bone volume was lower ($p<0.001$) at 14 days (92.06±6.34) compared with: control group (108.16±8.95), and with both other irradiated groups, at 7 days (114.37±12.15) and 21 days (120.36±6.09) (Fig. 3f).

DISCUSSION

This study evaluated the cortical bone after single-dose radiation therapy (30Gy) in rabbits tibiaes at 7, 14 and 21 days after irradiation using a high resolution imaging technique. Few studies described changes in cortical bone after single dose of ionizing radiation. Cortical thickness presented an increase in the first period analyzed, then followed with a decrease after one week, and then follows similar to the first period at 7 days. Intracortical porosity decrease drastically in the first evaluation and then increase in the following periods. After irradiation, bone volume are decreased just in 14 days, different from 7 and 21 days.

Animal model has been used to study and evaluate the changes in soft and mineralized tissue after radiotherapy and to compare treatments [16]. Bone metabolism of rabbits is 3 to 4 times faster than human; therefore, they are very used as animal model to evaluate the effects of radiotherapy in bone tissue [11][15] [17]. It is well documented that not all animal models exhibits spontaneous cortical remodeling, like in mice and rats [18]. Aiming to analyse spontaneous remodeling, larger animal models, such as rabbits, sheep, dogs, are indicated [19]. By the difficulty inherent the study, the animals were exposed to just one dose of ionizing radiation. Fractionated doses also do not seem to have as much an effect on cortical bone strength as a single large dose [20].

It has been demonstrated a decreasing in cortical thickness after radiation procedement > 35 Gy [21] [22]. In contrast, we found higher values for cortical thickness in the Ir7d group. Zhang *et al*., (2017) [23] showed *in vitro* that radiation exposure (1 Gy) promoted spread and fusion of osteoclast, while higher dose (8 Gy) inhibited this process. Therefore, ionizing radiation might show dual effects on osteoclast differentiation. Lower doses promoted osteoclast formation, and higher doses had dramatically negative effects on osteoclastogenesis. It can explains the initial increase in cortical thickness that has probably occurred after the acute inhibition of the osteoclastogenesis. After 14 and 21 days after radiotherapy similar values to the control group was shown.

The relationship between high porosity and increased intracortical remodeling has been demonstrated [24] [25] [26]. The large resorption spaces associated associated with cortical turnover are well characterized by microCT [24]. Intracortical porosity was lower for Ir7d in our study. Buur *et al*., (1990) [26] detected elevated porosity in regions of the femoral cortex that also revealed increased osteon population density. Cortical porosity is important because of its impact on bone material and mechanical properties as well as its role in the remodeling process. It consists of a network of canal which provide space for the vasculature in the cortices, and also, to nourish the osteocytes [7]. Therefore, our finding suggests a decline in bone turnover after ionizing radiation, that are more pronounced in the initial period (7 days) after radiotherapy. Intracortical porosity tend to be closer to the control group in Ir14d and Ir21d groups suggesting that regular process of bone turnover began to resume. Michel *et al.* (2015) [21] also demonstrated the effect of radiation process (80 Gy) in both cortical and medullary vascularity showing reduced number of vessels, moreover, the vessels diameters was smaller in comparison with nonirradiated tibias.

The intercortical porosity of Ir7days was lower to other treatment groups and control group. This result suggests that the bone volume has increased, what is confirmed by the increase of bone thickness in Ir7day group. Although, there is no consensus about the effect of radiotherapy in the number and activity of osteoclasts, the data ranges to increase to decreased [4]. Again, in this study is possible that number and activity of osteoclasts decreased in this time interval.

Some studies have demonstrated that ionic radiation can cause a vascular occlusion [27] [28] and the time and severity of this event is dose and time-dependent, therefore the blood flow may eventually be restored over time [29]. These studies support that probably the dose and time of radiotherapy were low and allowed revascularization of this sites, what produced an increase of porosity in other treatment groups when compared to Ir7days group.

Lower values for bone volume was demonstrated at 14 days after 30 Gy irradiation compared to control group and other irradiated groups. Hamilton *et al.* (2006)[30], showed no significant differences in cortical porosity and bone volume after low dose (2 Gy) radiation. Nyaruba *et al*. (1998)[20], also found no difference in the total bone volume. On the other hand, a recent study [21] revealed substantial decreased in cortical thickness and bone volume after high doses of radiation (80 Gy). The authors demonstrated that bone microarchitecture was destroyed after irradiation leading to spontaneous fractures after 8 weeks. We suggest that considerable alteration on bone volume is noted after high doses of ionizing radiation as demonstrated by Michel *et al.*, (2015). Employing 30 Gy single-dose is not sufficient to alter permanently bone volume which returns to initial conformation at 21 days after radiation. These findings are in accordance with the hypothesis proposed by Barth *et al.,* (2011)[31] that permanent effects on biomechanics and bone volume occurs beyond 70 Gy irradiation.

Fractal dimension (FD) reflects the degree of complexity and the lacunarity quantifies the emptiness. Earlier studies in trabecular bone found a positive correlation between FD and mechanical properties. Failure loading increases according to FD by enhancing complexity of trabecular network with higher strength [32] [33] [34]. Rabelo *et al*. (2017) [35], showed lower cortical FD in osteoporotic fractures in human femoral neck when compared to hip osteoarthritis, showing that the decreased cortical complexity is one determinant of the bone fragility. Our findings showed no statistical difference between control and irradiated condition and among the irradiated groups. However, further studies are necessary in order to evaluate the effect of ionizing radiation on FD of trabecular and cortical bone comprising longer periods of evaluation. Assessment of different radiation doses effect is also important, since it was demonstrated a decrease in maximum load in rats tibias caused by irradiation (40 - 60 Gy) [20].

In conclusion, cortical bone microarchitecture can be affected by ionizing radiation and it appears to be time dependent. Cortical parameters (BV; Ct.Th; Ct.Po; Ct.Po(cl); Ct.Da; Ct.Fd found at Ir21d was similar to control group under 30 Gy irradiation, suggesting that cortical bone return to regular conformation after 21 days.

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FIGURE LEGENDS

Fig. 1 Cortical bone segmented slice-by-slice

Fig. 2 MicroCT image of bone structure

Fig. 3 Mean and standard deviation values of morphologic parameters measured by MicroCT for non-irradiated bone (control group) and irradiated bone from animals sacrificed after different periods (7, 14 and 21 d): **a** mean thickness - Ct.Th in µm; **b** total porosity - Ct.Po in %; **c** closed porosity - Ct.Po in %); **d** fractal dimension - FD; **e** degree of anisotropy - Ct.DA; **f** bone volume – BV in mm3, analyzed by Tukey and Dunnet test ($P < 0.05$)

Fig.1:

Fig. 2:

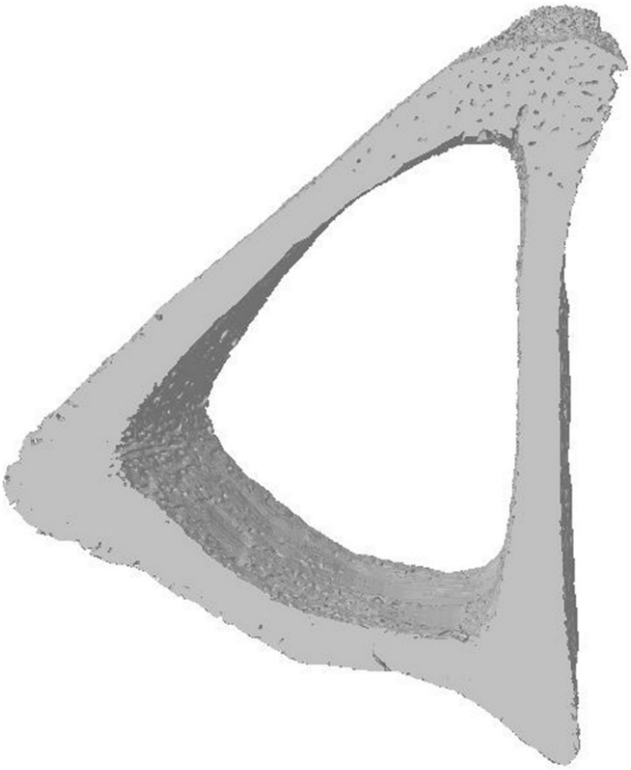
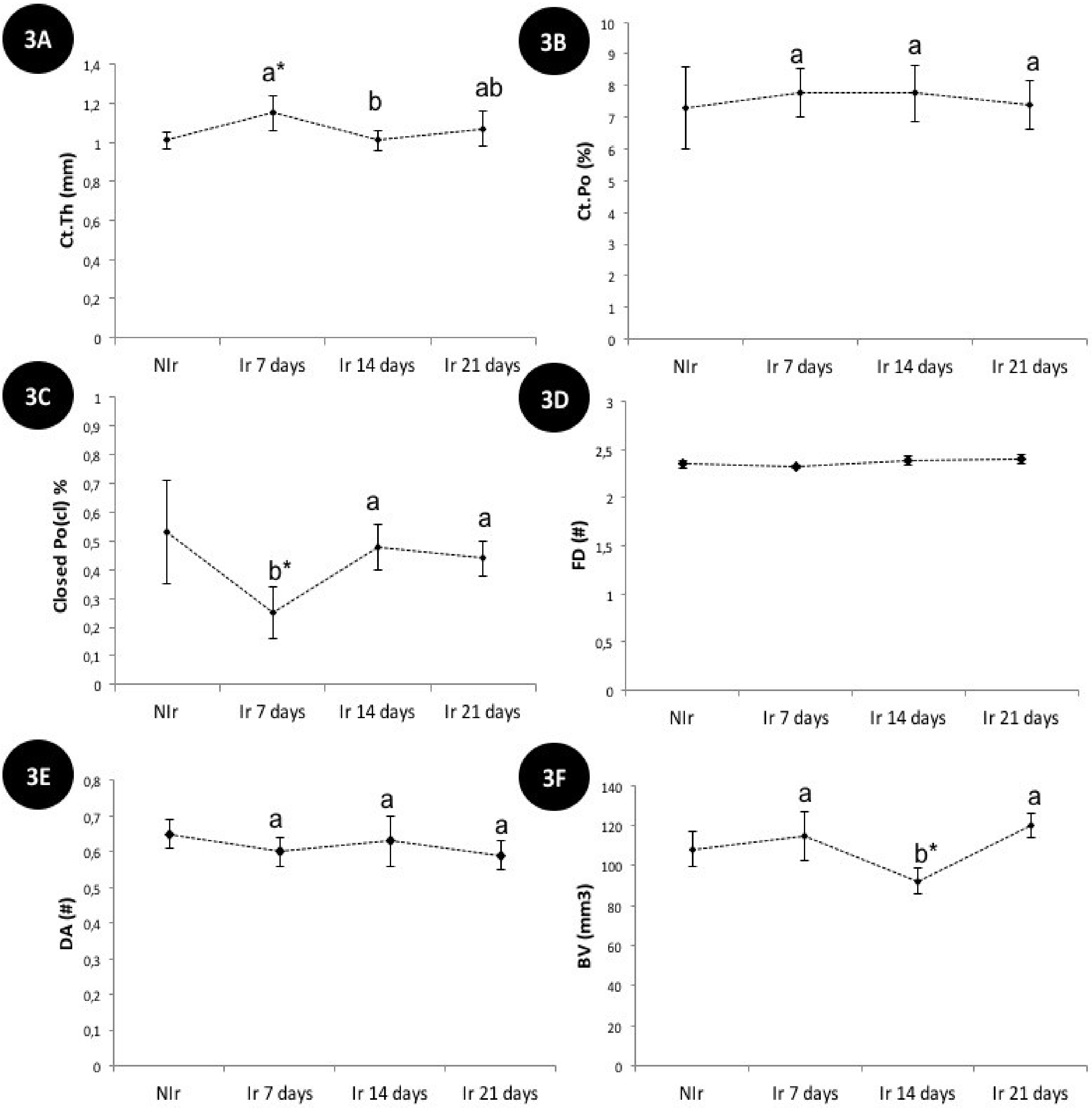


Fig.3:


3A
Ct.Th (mm)
0
0,2
0,4
0,6
0,8
1
1,2
1,4
a*
b
ab
NIr
Ir 7 days
Ir 14 days
Ir 21 days
3B
Ct.Po (%)
0
1
2
3
4
5
6
7
8
9
10
a
a
a
NIr
Ir 7 days
Ir 14 days
Ir 21 days
3C
Closed Po(cl) %
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1
b*
a
a
NIr
Ir 7 days
Ir 14 days
Ir 21 days
3D
FD (#)
0
0,5
1
1,5
2
2,5
3
NIr
Ir 7 days
Ir 14 days
Ir 21 days
3E
DA (#)
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
a
a
a
NIr
Ir 7 days
Ir 14 days
Ir 21 days
3F
BV (mm3)
0
20
40
60
80
100
120
140
a
b*
a
NIr
Ir 7 days
Ir 14 days
Ir 21 days