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Micro CT assessment of the effect of single and fractionated X radiation doses in rats tibias

Avaliação microtomográfica do efeito de doses únicas e fracionadas de radiação X em tibias de ratos

Evaluación por Micro CT del efecto de dosis de radiación X fraccionadas y únicas en tibias de ratas

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Abstract
The aim of this study was to evaluate the effect of X radiation in single and fractionated doses in tibias of rats by micro-computer tomography (µCT) analysis. The sample was consisted by 20 male rats, divided into 3 groups: Control, Single dose and Fractionated dose. The rats were submitted to a radiation X exposure on lower limbs. The single dose group was exposed to single radiation of 15 gray (Gy), while the fractionated group was submitted to three irradiation sessions of 5 Gy each, totaling 15 Gy. After 24 hours and 25 days, the rats were euthanized; the tibias were removed and scanned using a µCT unit, SkyScan 1174 Compact Micro-CT (Kontich, Bélgica). The parameters total bone area (Tt.Ar) , cortical bone area (Ct.Ar) , total cross-sectional bone area ratio (Ct.Ar / Tt.Ar) e cortical thickness (Ct.Th) for cortical bone, e bone volume ratio (BV / TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) e trabecular separation (Tb.Sp), for trabecular bone analysis were evaluated. Data were submitted to one-way ANOVA and Tukey's test ($\alpha = 0.05$). The µCT evaluation showed significant differences on Tt.Ar and Tb.Sp parameters ($p<0,05$). It was observed a lower Tt.Ar in the fractionated group compared to control, and higher Tb.Sp in the group receiving a single dose when compared to the control and fractionated groups. It is concluded, in relation to bone microarchitecture, that the radiation X in fractionated doses presents more deleterious effects on the cortical bone and when in singe doses, more damage on trabecular spaces, leading to a higher porosity.
Keywords: X-Ray Microtomography; X-Ray Therapy; Dose Fractionation, Radiation; Radiation Dosage; Radiation, Ionizing.

Resumo
O objetivo deste estudo foi avaliar o efeito da radiação X em doses únicas e fracionadas em tíbias de ratos por análise de microtomografia computadorizada (µCT). A amostra foi composta por 20 ratos machos, divididos em 3 grupos:

Controle, Dose Única e Dose Fracionada. Os ratos foram submetidos à exposição da radiação X nos membros inferiores. O grupo dose única foi exposto a radiação única de 15 gray(Gy), enquanto o grupo fracionado foi submetido a três sessões de irradiação de 5 Gy cada, totalizando 15 Gy. Após 24 horas e 25 dias, os ratos foram eutanasiados; as tíbias foram removidas e digitalizadas usando uma unidade µCT, SkyScan 1174 Compact Micro-CT (Kontich, Bélgica). Foram avaliados os parâmetros área transversal total (Tt.Ar) , área do osso cortical (Ct.Ar) , razão total da área óssea transversal (Ct.Ar / Tt.Ar) e espessura cortical (Ct.Th) para osso cortical, e proporção de volume ósseo (BV/TV), número trabecular (Tb.N), espessura trabecular (Tb.Th) e distância trabecular (Tb.Sp), para análise de osso trabecular. Os dados foram submetidos a ANOVA one-way e teste de Tukey ($\alpha = 0,05$). A avaliação do µCT mostrou diferenças significativas nos parâmetros Tt.Ar e Tb.Sp ($p<0,05$). Observou-se menor Tt.Ar no grupo fracionado em relação ao controle, e maior Tb.Sp no grupo que recebeu dose única quando comparado aos grupos controle e fracionado. Conclui-se, em relação à microarquitetura óssea, que a radiação X em doses fracionadas apresenta mais efeitos deletérios na cortical óssea e quando em doses únicas, mais danos nos espaços trabeculares, levando a uma maior porosidade.
Palavras-chave: Microtomografia por Raio-X; Terapia por Raios X; Fracionamento da Dose de Radiação; Doses de Radiação; Radiação Ionizante.

Resumen
El objetivo de este estudio fue evaluar el efecto de la radiación X en dosis únicas y fraccionadas en tibias de ratas mediante análisis microtomografía computarizada (µCT). La muestra estuvo constituida por 20 ratas macho, divididas en 3 grupos: Control, Dosis Única y Dosis Fraccionada. Las ratas fueron sometidas a una exposición a radiación X en las extremidades inferiores. El grupo de dosis única fue expuesto a sola radiación de 15 gray (Gy), mientras que el grupo fraccionado fue sometido a tres sesiones de irradiación de 5 Gy cada una, totalizando 15 Gy. Después de 24 horas y 25 días, las ratas fueron sacrificadas; las tibias se extrajeron y escanearon usando una unidad µCT, SkyScan 1174 Compact Micro-CT (Kontich, Bélgica). Se evaluaron los parámetros área total de la sección transversal (Tt.Ar) , área del hueso cortical (Ct.Ar) , relación total del área ósea de la sección transversal (Ct.Ar / Tt.Ar) y grosor cortical (Ct.Th) para hueso cortical, e relación de volumen óseo (BV / TV), número trabecular (Tb.N), espesor trabecular (Tb.Th) y distancia trabecular (Tb.Sp), para análisis de hueso trabecular. Los datos se sometieron a ANOVA unidireccional y prueba de Tukey ($\alpha = 0,05$). La evaluación µCT mostró diferencias significativas en los parámetros Tt.Ar y Tb.Sp ($p<0,05$). Se observó una menor Tt.Ar en el grupo fraccionado en comparación con el control, y una mayor (Tb.Sp) en el grupo que recibió una dosis única en comparación con los grupos control y fraccionado. Se concluye, en relación a la microarquitectura ósea, que la radiación X en dosis fraccionadas presenta más efectos deletéreos sobre el hueso cortical y cuando en dosis únicas, más daño sobre los espacios trabeculares, lo que conduce a una mayor porosidad.
Palabras clave: Microtomografía por Rayos X; Terapia por Rayos X; Fraccionamiento de la Dosis de Radiación; Dosis de Radiación; Radiación Ionizante.

1. Introduction

X-radiation is an ionizing radiation routinely used for diagnostic and interventional purposes, as well as an adjuvant treatment of several malignancies (Hamilton et al., 2006; Baxter et al., 2005). Exposure of cells to ionizing radiation generates reactive ions by ionizing and exciting molecules, causing oxidative damage to DNA, lipids, and proteins, which influence important cellular functions, including proliferation, differentiation, and apoptosis (Reisz et al., 2014).

Despite the relevance on the subject, the result of the levels of ionizing radiation is not yet fully defined. Yet, recently, there are responses of cells and organisms exposed to low doses, as routinely happen with humans exposed to natural radiation, low doses of internal radiation and internal radioactive isotopes (Guéguen et al., 2018). Other studies indicate that such responses affects the regulation of the cell cycle and of protein kinases linked to DNA (Nenoi et al., 2014). The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) defines low doses as values below 100mGy (Guéguen et al., 2018).

Constantly proliferating cells are more radiosensitive than non-proliferating cells, as well as less differentiated cells are more prone to radiation damage than highly differentiated cells. Based on this radioinduced cytotoxicity, local therapies against malignant neoplasms with the use of ionizing radiation were developed (Hutchinson, 1966).

In bone tissue, radiation alters the remodeling process, leads to the death of endothelial cells, decreased vascularization and metabolism, thus altering the entire bone matrix (Rocha et al., 2017). Even if the target is not bone,

ionizing radiation can generate deleterious effects in the skeletal system in the irradiated area (Mitchell and Logan, 1998; Lima et al., 2017), being able to reduce cell activity, blood supply and partial pressure of oxygen in the bone, which has consequences for its quality and quantity (Ma & Shen, 2012).

Frequent or excessive exposure of the skeletal system to X-rays leads to side effects that are closely related to the radiation dose, such as growth retardation, skeletal deformities, bone loss, and hematologic abnormalities (Mitchell & Logan, 1998; Williams & Davies, 2005). Other deleterious effects of radiotherapy related to the bone tissue are often observed (e.g. osteoradionecrosis and pathologic fracture) and they can be limited by administering fractional doses and restricting the radiation field (Mitchell & Logan, 1998).

An experimental methodology that has been widely used to evaluate the bone cell/radiation therapy interaction is the rat and rabbit tibia model. It consists of a well-established model capable of evaluating the main biomechanical and biological characteristics of bone, which could suffer consequences due to exposure to large doses in radiation therapy (Barth et al., 2011).

In the literature, the effects of single doses, whether low or high, are well established, as seen in several studies (Barth et al., 2011; Lima et al., 2017; Soares et al., 2018), however, there is a paucity of studies comparing the effects of fractioning the dose with the already established effects of the single dose. Therefore, the aim of this study was to evaluate the effect of X radiation in single and fractionated doses in tibias of rats by microtomography (µCT) analysis.

2. Methodology

After approval of this study by the Ethics Committee in Animal Research of School of Dentistry – University of Campinas (FOP-UNICAMP) (protocol number 2673-1), twenty adult male *Rattus norvegicus, albinus, Wistar* were obtained through the University of Campinas bioterium. The animals were placed in cages on a 12-hour light-dark schedule, average temperature of 23ºC and were fed standard rat chow and water ad libitum. They were randomly assigned in 3 experimental groups: Control (n=4), Single dose (n=8) and Fractionated dose (n=8).

Radiation

The rats were anesthetized with a Ketamine/Xylazine combination and positioned (8 per exposure) in a linear accelerator. By regulating the collimation device in an area of 18 x 30 cm, only the lower limbs region was irradiated (Figure). The single dose group was exposed to a single irradiation of 15 gray (Gy) of X radiation on lower limbs, while the fractionated group was submitted to three irradiation sessions of 5 Gy each, during three consecutive days, totaling 15 Gy.

Sacrifice

The animals were euthanized 24 hours and 25 days after irradiation procedure. Four rats of Single and Fractioned groups were euthanized 24 hours after, and the twelve remaining rats of Control, Single and Fractioned were euthanized after 25 days. The tibias were removed and stored in formalin.

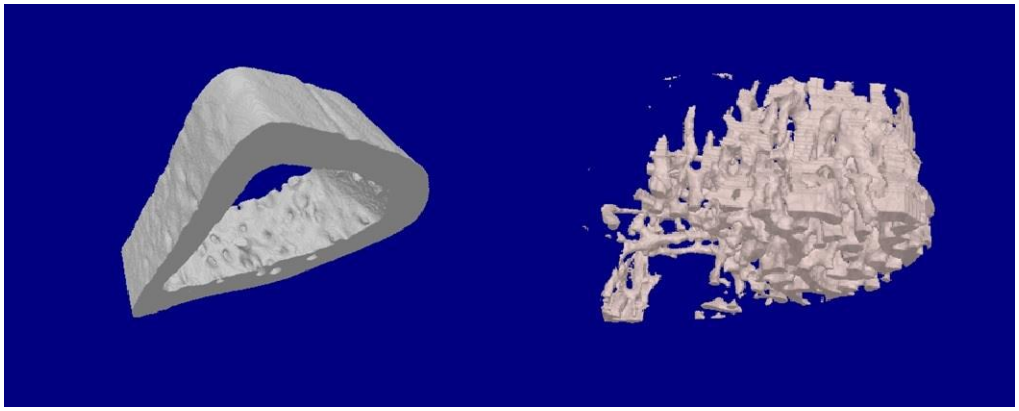
Micro CT scanning

For the acquisition of the µCT scans, a SkyScan 1174 Compact Micro-CT unit (Kontich, Bélgica) was used operating at 50 kVp and 795µA.s; a 0,5 mm aluminum filter was used. Scans were performed with an isotropic matrix size of 6,6µm and images were reconstructed in 1,024x1,024 pixel matrices.

After the reconstruction of the images on NRecon software, the dataset was defined on CtAn software. For trabecular bone analysis, a dataset of 150 slices had the trabecular region of interest defined manually, saved as a new image for posterior

analysis in the software. For cortical bone, a new dataset of 100 slices with the cortical region of interest defined manually was saved. The three dimensional reconstruction of the region of interest can be seen in Figure 1.

Figure 1. Three dimensional Reconstruction of µCT images.



Source: Authors.

Micro CT parameters

The parameters Tissue Volume (TV), Bone Volume (BV), Volume of medullary bone (TV-BV), Intersection Surface (IS), Bone surface (BS), IS/BS, Closed Porosity (PoCl), Open Porosity (PoOp), Total Porosity (PoTot), Separation of pores (Tb.Sp), Connectivity (Conn) and Degree of Anisotropy (DA) [Tt.Ar , Ct.Ar , Ct.Ar / Tt.Ar e Ct.Th] for cortical bone were analyzed. And the evaluated parameters for trabecular bone were Bone volume fraction (BV / TV), Trabecular number (Tb.N), Trabecular thickness (Tb.Th) and Trabecular separation (Tb.Sp). Data were submitted to one-way ANOVA and Tukey's test ($\alpha = 0.05$).

3. Results

The µCT evaluation showed significant differences on Tt.Ar and Tb.Sp parameters ($p<0,05$). It was observed a lower total transverse area (Tt.Ar) in the fractionated group compared to control, and higher distance between trabecular space (Tb.Sp) in the group receiving a single dose when compared to the control and fractionated groups. All results of the µCT evaluation can be seen in the Tables 1 and 2.

Table 1. Cortical bone analysis (Different letters in the same line mean statistically significant difference. Tukey's test $p \leq 0.05$).

	Control	**Single**	**Fractioned**
Ct.Ar	243087.75 a	267159.34 a	270631.63 a
Tt.Ar	53764.66 a	59668.66 ab	62547.50 b
Ct.Ar/Tt.Ar	4.51 a	4.47 a	4.31 a
Ct.Th	37.68 a	37.94 a	41.71 a

Source: Authors.

Table 2. Trabecular bone analysis (Different letters in the same line mean statistically significant difference. Tukey's test $p \leq 0.05$).

	Control	**Single**	**Fractioned**
BV/TV	8.38 a	5.22 a	8.16 a
Tb.N	0.007 a	0.004 a	0.006 a
Tb.Th	10.81 a	10.97 a	12.20 a
Tb.Sp	59.37 a	91.68 b	62.51 a

Source: Authors.

4. Discussion

The ionizing radiation is capable of causing damage to the cells of the organism through the interaction of DNA with reactive oxygen species, which results in cell death (Willey et al., 2011). It is the stochasticity of this interaction that is linked to the unpredictability of the clinical response to radiotherapy for years. The clarification of this topological and temporal relationship at the cellular, tumor and organism levels is the key to the advancement of treatments that use radiation. Currently allowing its biological analysis, thanks to biomedical technologies (Furdui, 2014).

In an environment of hypocellularity, hypovascularity and tissue hypoxia induced by radiotherapy, apoptosis of osteoblasts, osteocytes and osteoprogenitor cells is certain, leading to a progressive hyalinization of the medullary spaces (Zhang et al., 2010). Thus impairing bone formation after radiation therapy by reducing the number of cells present in the bone marrow, and osteoblast precursors (Willey et al., 2011).

In addition, ionizing radiation causes an early increase in osteoclasts, however there is a depletion of osteoclast progenitor cells, which leads to the long-term decline of these cells (Oest et al., 2015). Thus, it is capable of generating deleterious changes in the skeletal system even if the target of ionizing radiation is not directly the bone (Mitchell & Logan, 1998; Lima et al., 2017). It may have consequences in bone quality and quantity by reducing bone density, cellular activity, blood supply and partial pressure of oxygen in bone (Ma & Shen, 2012).

In general, oncologists use conventional fractionation, dividing a total of 65 to 72 Gy of radiation by 7 weeks (Lieshout & Bots, 2013). The literature states that cellular damage occurs proportionally to the applied radiation dose, with no resorption of cortical bone at 10 to 20 Gy exposures, but with a significant reduction in bone neoformation, with an almost total inhibition when 22 to 25 Gy are used. From 30 Gy onwards, apoptosis of osteocytes is predicted in the cortical bone and at doses between 45 and 90 Gy there is reduced periosteal apposition and the opposite in endosteal apposition (Lucatto et al., 2011).

Concerning the tissue response to irradiation, it has been shown that some inflammatory genes can be rapidly induced, between 1 and 6 hours, after exposure to ionizing radiation, and the use of fractional radiation maintains an upregulation of the expression of the cytokine gene for a longer period than a single dose, suggesting that there are differences in tissue responses to exposure to fractional doses versus single doses of radiation (Hong et al., 1999). In the present study, significant differences were observed between exposure to a single dose (15 Gy) and fractionated dose (three doses of 5 Gy) for some bone parameters analyzed, both for cancellous bone and cortical bone, suggesting the existence of differences in the bone tissue response with the different types of radiation exposure evaluated.

In an in vitro study, a reduction in the number of cells and in the differentiation of osteoprogenitor cells to osteoblasts was demonstrated after exposure to a single dose of 0.5-5 Gy (Chen et al., 2014), however there is still no consolidated evidence available that demonstrate the corresponding changes in cancellous bone microarchitecture (Lima et al., 2017). In

rodent studies, a significant reduction in cancellous bone volume (BV/TV) was observed on the third day after exposure to gamma radiation of 2 Gy (Kondo et al., 2009). In another mouse study, the animals were exposed to 2 Gy gamma radiation, protons, carbon and iron, and a significant reduction in BV/TV and trabecular numbers was observed at day 110 after irradiation (Hamilton et al., 2006).

Lucatto et al., 2011, compared the effects of ionizing radiation on bone neoformation of the tibiae of rats with a single dose of 30 Gy in relation to the contralateral side that received a secondary dose of 7 Gy. They came to the conclusion that the tibias that received the 30 Gy dose showed greater cellular damage, thus suggesting that the damage to bone neoformation is proportional to the magnitude of the radiation dose. The 30 Gy irradiation can also cause changes in the trabecular arrangement, causing stagnation of the repair process of newly formed bone and a misfit of ostocytes and collagen (Mendes et al., 2019).

The behavioral, genetic and biological characteristics of rats are similar to the humans, enabling the replication of human conditions and their clinical analysis (Trejo-Iriarte et al., 2019). In this sense, the tibiae of rats were used in this study for a comparative analysis of the effect of x-radiation in single and fractional doses.

In clinical practice, the way the mechanical and biological behavior of bone occurs allows the observation of local and systemic factors, helping to better understand the bone regeneration process. μCT has been an essential tool in this assessment, both in animal and human samples, determining bone quality (Sinibaldi et al., 2017). μCT allows different types of image scans using principles of computed tomography, producing high resolution images of small samples, whithout destrucion of the samples, beng also possible to generate three-dimensional reconstructions (Fernandes, Appoloni & Fernandes, 2016; Baird & Taylor, 2017). This technique has been widely used for ex-vivo bone assessment of rats and small animals in order to evaluate morphology and microarchitecture (Bouxsein et al., 2010; Irie et al., 2018).

A set of factors is necessary to determine the morphometry of the trabecular bone, they are characteristics such as the number, thickness and trabecular separation, (Tb.N, Tb.Th and Tb.Sp) respectively, in addition to the measurement of bone volume (BV) (Bouxsein et al., 2010). Yet, cortical bone requires an evaluation of different parameters such as porosity (Ct.Po) and thickness (Ct.Th) (Zebae & Seeman, 2014).

Some studies advocates the use of ionizing radiation in a fractional way per day, making a parallel with its use in the clinical treatment of oncological patients, others bring the total application in the form of a single dose. This divergence in the way of application of radiation implies in several controversial findings in the scientific area (Miranda et al., 2021).

In the present study, the application of μCT in the microstructural analysis of bone architecture showed notable differences regarding the application of the ionizing radiation. When 15 Gy of radiation was divided into three doses, more deleterious effects were observed in the cortical bone, with a smaller total cross-sectional area compared to the control group. When the same 15 Gy dose was applied in a single manner, a greater distance between the trabecular space was observed. Evidencing important differences in the parameters Tt.Ar and Tb.Sp ($p<0.05$).

5. Conclusion

Ionizing radiation (x-rays) applied in a fractional manner cause more deleterious effects in the microarchitecture of the cortical bone and when in singe doses, more damage on trabecular spaces, leading to a higher porosity.

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