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The role of local irradiation timing on the osseointegration process in implants

O papel do tempo de irradiação local no processo de osseointegração em implantes

El papel del tiempo de irradiación local en el proceso de osteointegración en implantes

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Luana Marotta Reis de Vasconcellos
ORCID: https://orcid.org/0000-0003-4344-0578
Paulista State University, Brazil
E-mail: luana.marotta@unesp.br
Leonardo Silva Gomes Kamezawa
ORCID: https://orcid.org/0000-0002-0546-7000
Paulista State University, Brazil
E-mail: leonardo.kamezawa@unesp.br
Bruno César Almeida Costa
ORCID: https://orcid.org/0000-0003-4995-2216
Paulista State University, Brazil
E-mail: profbrunobio@gmail.com
Ana Luisa Theodoro
ORCID: https://orcid.org/0000-0002-7822-0893
Paulista State University, Brazil
E-mail: ana.theodoro@unesp.br
Camila Duarte da Silva
ORCID https://orcid.org/0000-0003-2419-069X
Paulista State University, Brazil
E-mail: camila.duarte@unesp.br
Estela Kaminagakura Tango
ORCID: https://orcid.org/0000-0003-2456-4516
Paulista State University, Brazil
E-mail: estela.tango@unesp.br
Leonardo Alvares Sobral Silva
ORCID: https://orcid.org/0000-0002-6743-4423
Paulista State University, Brazil
E-mail: leeo.sobral_@outlook.com
Claudio Antonio Federico
ORCID: https://orcid.org/0000-0002-8263-0270
Paulista State University, Brazil
E-mail: claudiofederico@ieav.cta.br
Rubens Nisie Tango
ORCID: https://orcid.org/0000-0003-0946-1826
Paulista State University, Brazil
E-mail: rn.tango@unesp.br

Abstract

Purpose: The objective of this study was to evaluate the effects of Gamma irradiation by simulating a total dose of conventional head and neck cancer radiotherapy on bone remodeling and osseointegration of implants in femurs of rats.*Materials and Methods:* Sixty rats received threaded titanium (Ti6Al4V) implants in both femurs. The animals were divided: No-Irradiation (N-Ir): N-Ir group with implant solely, Early-Irradiation (E-Ir): implant + irradiation after 24hrs.; Late-Irradiation (L-Ir): implant + irradiation after 4 weeks and Previous-Irradiation (P-Ir): irradiation + implant after 4 weeks. Irradiation was fractioned in two sessions of 15 Gy each, totaling 30 Gy. The animals were euthanized at 3 days, 2 and 7 weeks after treatment, which were chosen from previous studies. Runx2 immunohistochemical markers and Picrosirius red stain, histomorphometric analysis (BIC and BAFO) and reverse torque test were used to evaluate the implant/bone interface and surrounding bone. Data of Runx-2 (%), picrosirius red stain (%), BIC and BAFO (%), and reverse torque (N/cm) was submitted to ANOVA and Tukey tests for comparison among groups, both with α=0.05. *Results:* The results showed that Runx-2 expression was different in irradiated groups when compared to N-Ir group. In general, the collagen fiber analyses in the irradiated groups differed from the N-Ir group. Regarding BIC, BAFO, and the reverse torque test, the L-Ir group demonstrated results similar to the N-Ir group, while the others groups were impaired by radiotherapy. *Conclusions:* Ionizing radiation negatively affected bone remodeling, and its effect depends on when radiotherapy is applied, with a delay of osseointegration when applied previously or close to surgery.

Keywords*:* Osseointegration; Radiotherapy; Immunohistochemistry; Torque.

Resumo

Objetivo: O objetivo deste estudo foi avaliar os efeitos da irradiação gama simulando uma dose total de radioterapia convencional em câncer de cabeça e pescoço na remodelação óssea e osseointegração de implantes em fêmures de ratos.*Materiais e Métodos*: Sessenta ratos receberam implantes de titânio rosqueado (Ti6Al4V) em ambos os fêmures. Os animais foram divididos: Sem Irradiação (N-Ir): Grupo N-Ir com implante somente; Irradiação Precoce (E-Ir): implante + irradiação após 24h; Irradiação Tardia (L-Ir): implante + irradiação após 4 semanas e Irradiação Prévia (P-Ir): irradiação + implante após 4 semanas. A irradiação foi fracionada em duas sessões de 15 Gy cada, totalizando 30 Gy. Os animais foram sacrificados aos 3 dias, 2 e 7 semanas após o tratamento. Marcadores imuno-histoquímicos Runx2 e coloração Picrosirius red, análise histomorfométrica (BIC e BAFO) e teste de torque reverso foram utilizados para avaliar a interface implante/osso e osso circundante. Os dados de Runx-2 (%), coloração picrosirius red (%), BIC e BAFO (%) e torque reverso (N/cm) foram submetidos aos testes ANOVA e Tukey para comparação entre grupos, ambos com α=0,05.*Resultados:* Os resultados mostraram que a expressão de Runx-2 foi diferente nos grupos irradiados quando comparados ao grupo N-Ir. Em geral, as análises de fibras colágenas nos grupos irradiados diferiram do grupo N-Ir. Em relação ao BIC, BAFO e teste de torque reverso, o grupo L-Ir apresentou resultados semelhantes ao grupo N-Ir, enquanto os demais grupos foram prejudicados pela radioterapia.*Conclusões:* A radiação ionizante afetou negativamente a remodelação óssea, e seu efeito depende de quando a radioterapia é aplicada, com retardo da osseointegração quando aplicada previamente ou próximo à cirurgia.

Palavras-chave: Osseointegração; Radioterapia; Imuno-histoquímica; Torque.

Resumen

Propósito: El objetivo de este estudio fue evaluar los efectos de la radiación Gamma mediante la simulación de una dosis total de radioterapia convencional para el cáncer de cabeza y cuello en la remodelación ósea y la osteointegración de implantes en fémures de ratas.*Materiales y Métodos:* Sesenta ratas recibieron implantes roscados de titanio (Ti6Al4V) en ambos fémures. Los animales se dividieron: Sin Irradiación (N-Ir): grupo N-Ir con implante solo, Irradiación Temprana (E-Ir): implante + irradiación después de 24 h; Irradiación tardía (L-Ir): implante + irradiación después de 4 semanas e Irradiación previa (P-Ir): irradiación + implante después de 4 semanas. La irradiación se fraccionó en dos sesiones de 15 Gy cada una, totalizando 30 Gy. Los animales fueron sacrificados a los 3 días, 2 y 7 semanas después del tratamiento. Se utilizaron marcadores inmunohistoquímicos Runx2 y tinción roja de Picrosirius, análisis histomorfométrico (BIC y BAFO) y prueba de torsión inversa para evaluar la interfaz implante/hueso y el hueso circundante. Los datos de Runx-2 (%), tinción roja de picrosirius (%), BIC y BAFO (%) y torque inverso (N/cm) se sometieron a las pruebas de ANOVA y Tukey para la comparación entre grupos, ambas con α=0,*05. Resultados:* Los resultados mostraron que la expresión de Runx-2 fue diferente en los grupos irradiados en comparación con el grupo N-Ir. En general, los análisis de fibra de colágeno en los grupos irradiados diferían del grupo N-Ir. Con respecto a BIC, BAFO y la prueba de torsión inversa, el grupo L-Ir mostró resultados similares al grupo N-Ir, mientras que los otros grupos se vieron afectados por la radioterapia.*Conclusiones:* Las radiaciones ionizantes afectan negativamente al remodelado óseo, y su efecto depende del momento de aplicación de la radioterapia, con un retraso de la osteointegración cuando se aplica de forma previa o próxima a la cirugía.

Palabras clave: Osteointegración; Radioterapia; Inmunohistoquímica; Torsíon.

1. Introduction

The estimated number of new cases of head and neck cancer in 2018, worldwide, was about 890,000, and within them squamous cell carcinoma (SCC) is one of the most common malignancies, resulting in 396,147 deaths per year (Globocan, 2020). Radiotherapy has been an important adjuvant and neoadjuvant therapeutic modality, especially with advanced clinical stage SCC's (Adelstein, et al., 2003). However, when ionizing radiation reaches the deepest tissues, it can trigger mechanisms that result in cell death, due to the incapacitation of cell reproduction or inactivation of their vital systems (Hubenak, et al., 2014; Aline, et al, 2020).

Patients undergoing cancer treatment for head and neck neoplasms show increased rates of edentulism in need of prosthetic rehabilitation (Quispe, et al., 2018; Volsseman, et al, 2021). These patients are often unable to receive conventional dental prostheses due to changes in the anatomical structures of the oral cavity (Pace-Balzan, et al., 2000; Schoen, et al., 2007), and due to the presence of mucositis and xerostomia as in result of cancer therapy, which compromises the stability and retention of prostheses (Sroussi, et al., 2017). In this situation, osseointegrated implants are an alternative, with greater functional and esthetic benefits (Nelson, et al., 2007; Mancha de la Plata, et al., 2012; Fenlon, et al., 2012; Fareen, et al, 2021).

Patients undergoing implant retained dental rehabilitation have reported increased solid food intake (Nelson, et al., 2007; Mancha de la Plata, et al., 2012; Fenlon, et al., 2012), improvement in esthetics with a positive impact on their social life, and an immediate positive impact on their quality of life (Dholam, et al., 2016). Nevertheless, there are several radiation side-effects on bone tissue characterized by the loss of osteoblastic activity, the increased production of adipose tissue in the bone marrow, and decreased microvascularization (Williams & Davies, 2006). There is also radio-induced fibrosis, in which endothelial cells in the radiation field undergo direct and indirect damage by reactive oxygen species, i.e., free radicals (Lyons & Ghazali, 2008). Radiation affects bone cellular activity, mainly in osteoblasts that are more sensitive to radiation than osteoclasts (Vissink, et al., 2003). Runx-2 regulates the differentiation of pluripotential cells into preosteoblasts which differentiate into mature osteoblasts (Tang, et al., 2011).

In this context, current studies address the ideal moment for the installation of implants in cancer patients (Nooh, et al., 2013; Claudy, et al., 2015; Wetzels, et al., 2017; Woods, et al., 2019; Alberga, et al., 2020a; Alberga, et al., 2020b; da Cruz Vegian, et al., 2020; Soares, et al, 2020). The installation of implants in humans between 6 and 12 months after radiotherapy is associated with a 34% higher risk of osseointegration failure, and it has been suggested that it is better to wait for periods longer than 12 months after radiotherapy for implant surgery (Claudy, et al., 2015). Other reports suggest that the longer the time interval between radiotherapy and implant placement, the worse will be the prognosis due to a progressive decrease of bone repair capacity after radiotherapy (Granström, 2000; Granström, et al., 1994). Due to these controversies and the lack of studies that can clearly guide the ideal time for rehabilitation of these patients with implants, the aim of this study was to test different radiotherapy / implant surgery intervals in a murine model, and their influence on collagen fiber maturation Runx-2 expression and reverse torque testing.

2. Methodology

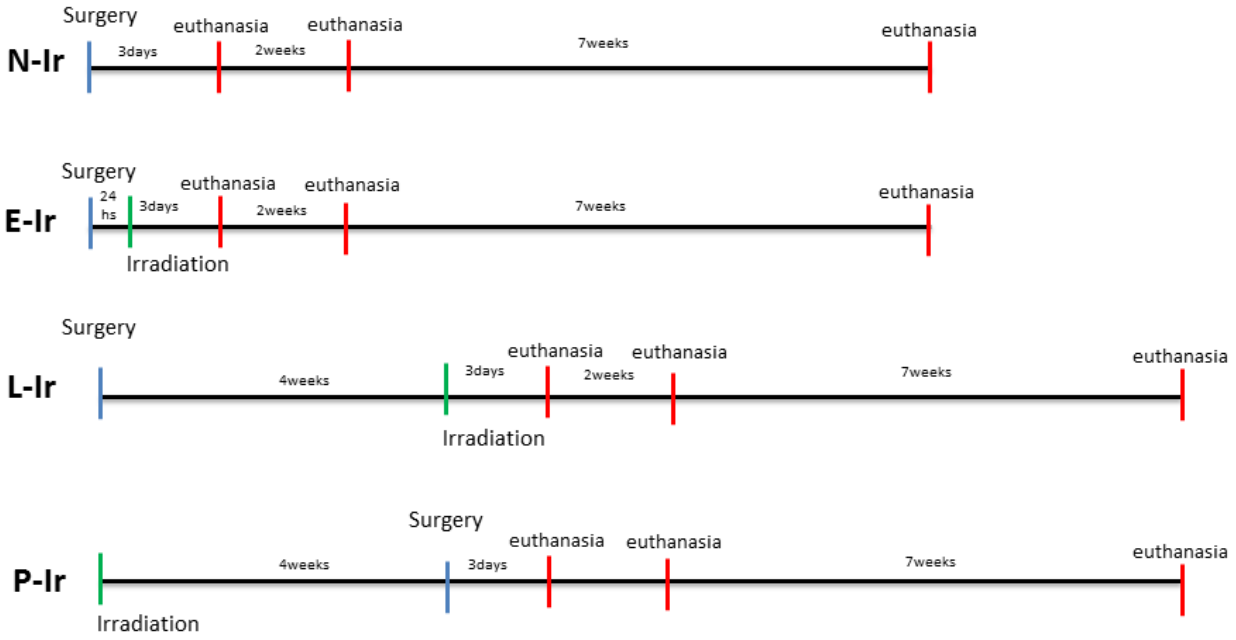
2.1 Surgery procedures

The animal experimental protocol was approved by the Animal Ethics Committee (CEUA 003/2016) of the Institute of Science and Technology at Sao Jose dos Campos/UNESP, and was carried out in accordance with the ethical principles of the Brazilian National Animal Care Ethical Council (CONCEA). The sample size was based on previously published papers (Vasconcellos, et al., 2014; Vasconcellos, et al., 2016; da Cruz Vegian, et al., 2020), and the guidelines of the Animal Research: Reporting In Vivo Experiments (ARRIVE) were followed (Percie, et al., 2020).

Sixty Wistar rats weighing about 300g were collected from the Unesp Central Biotery and remained in cages (20°C and humidity at 55%, n = 5) where they received water and feed *ad libitum*.

After 30 days of adaptation to the environment, with 12/12hrs. day and night cycles, this experiment was started. The animals were divided according to the timing of radiotherapy (Fig. 01): N-Ir group (N-Ir): implant surgery (healthy tissue without irradiation); Early irradiation group (E-Ir): implant surgery and initiation of irradiation after 24hrs.; Previous irradiation group (P-Ir): irradiation and implant surgery after 4 weeks; Late irradiation group (L-Ir): implant surgery and irradiation after 4 weeks. All periods of euthanasia and irradiation were based on a previous study that correlated the age of adult rats to humans (Quinn, 2005). The irradiated groups also were performed to evaluate the effects of radiotherapy in different moments (Figure 1).

Figure 1. Timeline of study.



Subtitle: N-Ir=No-Irradiation group; E-Ir=Early-Irradiation group; L-Ir=Late-Irradiation group; P-Ir=Previous Irradiation group.
Source: drawn up by the author.

For surgical procedures, the animals were anesthetized with xylazine hydrochloride (Anasedan - Vetbrands, Jacareí, SP, Brazil) and ketamine (Dopalen - Agibrands Brasil Ltd., Paulínia, SP, Brazil). Threaded Grade V titanium implants measuring 2.5 mm in diameter by 6.0 mm in length, produced by Emfils (Itu, São Paulo, Brazil) were installed in the right and left femurs (Vasconcellos, et al., 2014). After surgery, animals received an intramuscular injection with 0.1 mg/ kg of phenyl dimethyl pyrazolone (Algivet, Vetnil, Louveira, SP, Brazil).

2.2 Irradiation protocols

For irradiation, the animals were anesthetized and immobilized, two by two, in an apparatus to standardize the irradiation field. The irradiation was performed in two sessions with a total dose of 30 Gy, under a 60 Co gamma radiation telethermotherapy irradiator (Eldorado 76, Atomic Energy of Canada, Chalk River, Ontario, Canada).

After surgery and irradiation sessions, the animals were placed in cages, according to subgroups, and were monitored until euthanasia was carried out at 3 days, 2 and 7 weeks. The times for the euthanasias were chosen according to the analysis of osseointegration in the short, medium and long term in previous studies (Cunha et al., 2007; Luccato et al., 2011; Nunes, et al., 2020; Verdonck, et al., 2008) and also based on the recent study conducted by our research group (da Cruz-Vegian, et al., 2020). Nunes, et al. (2020) evaluated osseointegration in 5 different periods (03, 07, 21, and 45 days), thus we confirmed that the short period of 3 days was adequate. The period of 2 weeks, considered an average time of repair in which osseointegration is already observed, was based on the literature of Luccato, et al. (2011). The period of 7 weeks was based on the average of the periods presented in different previous studies that evaluated long-term osseointegration, such as 6 weeks (Cunha, et al., 2007) and 8 weeks (Verdonck, et al., 2008; Luccato, et al., 2011).

2.3 Immunohistological analyses

After euthanasia, the bone fragments were fixed in 4% paraformaldehyde solution for at least 48 hrs., and were decalcified in 10% ethylenediaminetetraacetic acid solution (EDTATitriplex III, EMD Millipore, MA, USA) for 5 weeks.

After demineralization, the blocks were embedded in paraffin with histotechnology equipment (Leica TP1020, Wetzlar, Germany) and longitudinal sections of the implant/bone interface with 3.5 μm and 4.5 μm thickness were obtained for immunohistochemical and histological analysis, respectively.

For immunohistochemical processing, the sections were incubated with the primary antibody Runx-2 (1:100, AbCam, Cambridge, UK). Negative N-Irs were obtained from the histological sections of the respective groups. The slides were scanned (Scanner Panoramic Desk, 3DHistech, Budapest, Hungary) and analyzed with Pannoramic Viewer 1.15.4 for Windows (3DHistech). Measurement of immunolabeled cells for Runx-2 antibody (%) was performed throughout the entire implant/bone contact region (da Cruz Vegian et al., 2020).

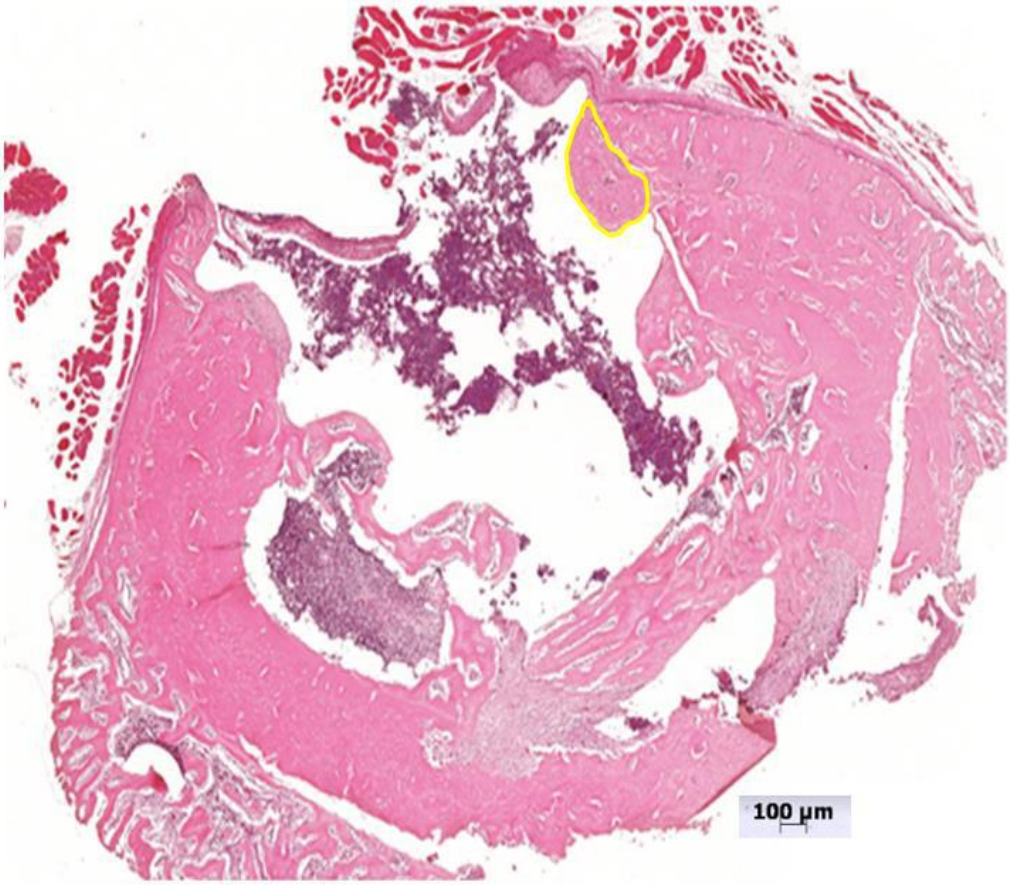
The qualitative and quantitative analyses were made under blindfold test conditions by the same evaluator as follows: based on the staining of immunolabeled cells for each antibody: negative (−), positive (+), superpositive (++), and hyperpositive (+++); and based on the conversion of the scores into percentages: 0, 20, 60, and 90%, respectively (dos Santos, et al., 2016, dos Santos, et al., 2013, Queiroz, et al., 2008; Esteves, et al., 2013).

2.4 Histological analyses and collagen fibers

Seven semi-serial histologic sections were obtained from the same blocks for analysis. Five and two sections were stained with picrosirius and hematoxylin/eosin, respectively. The collagen analysis with the picrosirius red stain was conducted by two calibrated blindfold examiners. Immature and mature collagen were quantified (%) on five randomized areas of the implant/bone interface. Images were captured with 10x magnification by polarized light microscopy (Eclipse Ti-Series Nikon, Tokyo, Japan). Collagen analysis of each area was performed using the Las Phase program (Leica Application Suite, Wetzlar, Germany). A threshold was chosen for both the red and green images separately (except the blue channel), and then the greenish/reddish birefringence was measured.

The sections hematoxylin/eosin stained were used to assess the bone formation in the implant/bone interface (Figure 2).

Figure 2. Cross section of the femur with region analyzed for bone formation (bounded area in yellow).



Source: Drawn up by the author.

2.5 Histomorphometric analyses

For the histomorphometric analysis of bone neoformation the slides were captured with an Axiocam MRC 5 camera (Zeiss, Carl Zeiss, Oberköchen, Germany) coupled to an optical microscope (Zeiss Axiophot 2, Carl Zeiss, Oberköchen, Germany), at 20x magnification, with fixed focus. Two fields of each slide corresponding to the mesial and distal interfaces of the implant were subjected to histomorphometric analysis (Image J, version 1.34, Image Processing and Analysis in Java, NIH, Bethesda, MD, USA) to calculate the bone area formation (BAFO), bone implant contact (BIC) of the internal region of a thread (%).

2.6 Reverse torque test

The animals euthanized at 2 and 7 weeks were evaluated because 3 days was too short a time for this specific test. Femurs with implants (n=5) were preserved in Ringer solution at -20ºC until the biomechanical test was performed at room temperature. The fragments were embedded in methyl methacrylate, a 1.2mm implant key was adapted to the implant and peak load (N/cm²) of reverse torque was registered with a digital torque wrench (MGT12, MARK-10 Corporation, NY, USA) mounted in a torque wrench-mounting device to minimize non-axial forces during the test.

2.7 Statistical analysis

The sample size was calculated using the G* Power 3.112 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). Data of Runx2 expression (%), collagen maturation (%), BAFO and BIC (%), and reverse torque (N/cm^2) were submitted to 1-way ANOVA and Tukey tests for comparison among groups, both with $\alpha=0.05$. All graphs were rendered with GraphPad Prism (Version 7.0, San Diego, CA).

3. Results

3.1 Bone remodeling markers

In the quantification of Runx-2 marking, at 3 days, there was greater production in the N-Ir group, which differed statistically from the other groups ($p < 0.05$), which showed expression of this bone remodeling marker as less than 20%. Among irradiated groups the E-Ir and P-Ir groups did not differ from each other, and they exhibited a lower value of Runx2 ($p>0.05$), but they statistically differed from L-Ir ($p<0,05$). In the 2-week and 7-week periods, the P-Ir group showed the lowest values and a difference with the other groups was observed ($p<0.05$) (Fig. 3).

3.2 Analysis of collagen fibers

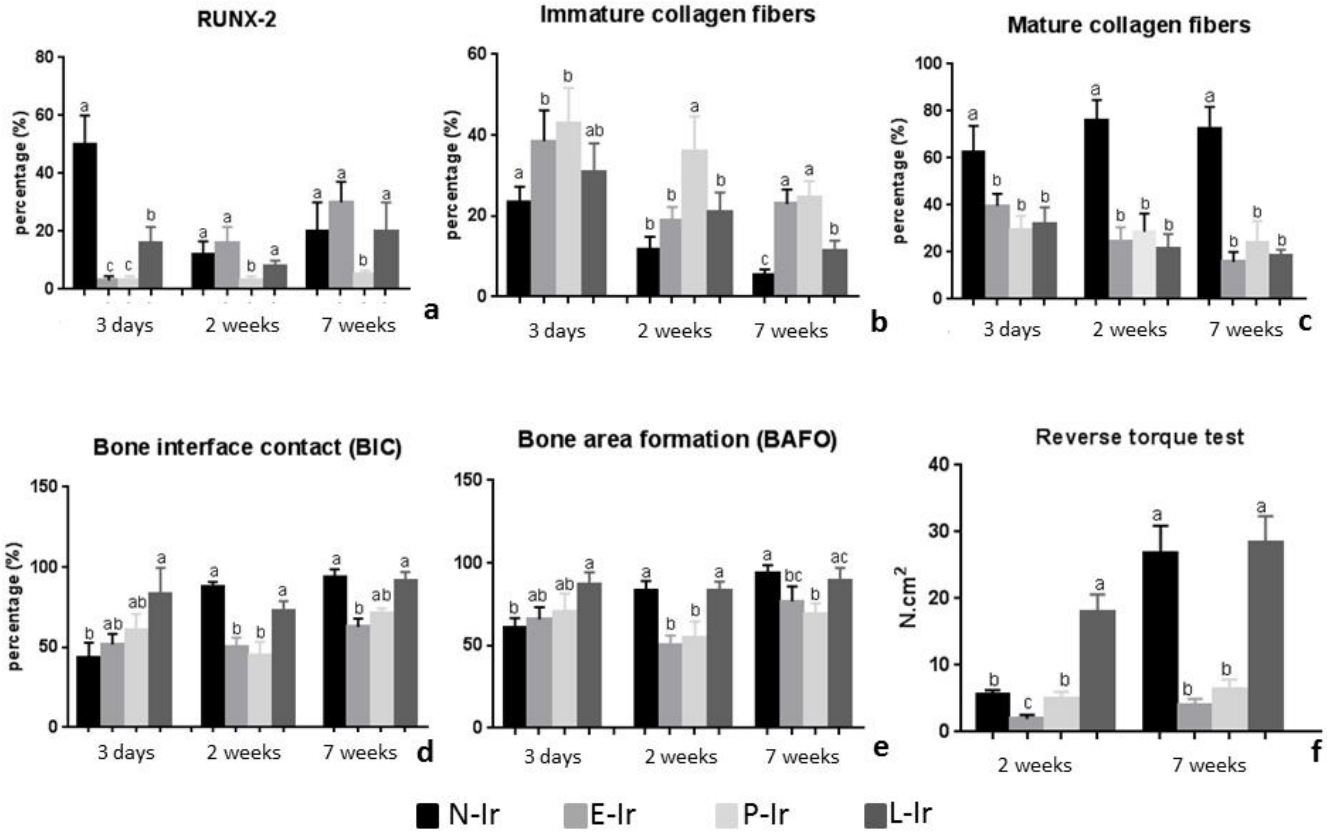
The highest quantity of mature collagen (about 70%) was observed in the N-Ir group in all periods of this study, and a statistical difference between this group and the irradiated groups was observed ($p<0.05$) (Fig. 3). There was no difference between the irradiated groups ($p>0.05$).

Regarding immature collagen fibers, the N-Ir group always showed the lowest values, and sometimes exhibited a statistically significant difference with the irradiated groups. At 3 days, significantly higher amounts of immature collagen were observed for the irradiated group than in the N-Ir, and a statistical difference was observed ($p<0.05$), with the exception of the L-Ir group ($p>0.05$). In the 2-week period, the P-Ir group showed the highest value and differed ~~of~~ from all other groups ($p<0.05$). The P-Ir group maintained the highest value at 7 weeks and showed no difference from the E-Ir group ($p>0.05$), but it differed from the N-Ir and L-Ir groups ($p<0.05$), which also differed from each other ($p<0.05$) (Fig. 3).

3.3 Histological and histomorphometric analyses

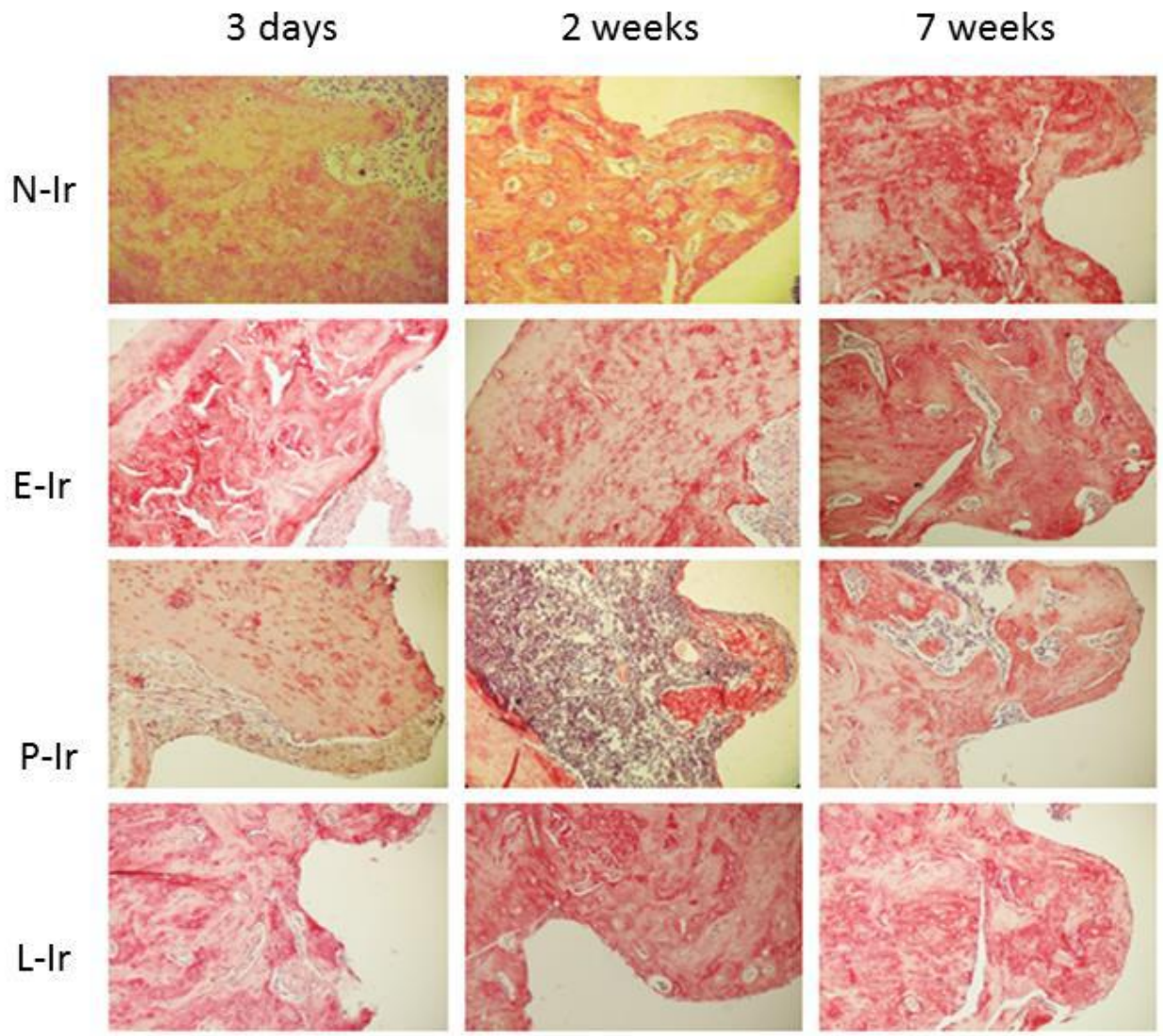
At 3 days, in the N-Ir group many cells were observed at the implant/bone interface, while few cells were observed in the irradiated groups. At 2 weeks, the area of the implant thread space in the N-Ir group was filled with bone exhibiting wide medullary spaces, and in the irradiated groups, little bone neoformation was observed. After 7 weeks, lamellar bone was observed at the implant/bone interface, while the irradiated groups showed immature bone only with lamellar bone areas. Representative images of these results are shown in Figure 4.

Figure 3. Graph of mean values and standard deviation (±) for different tests.



Subtitle: Analysis of: a) percentage of immunolabelled cells for antibody Runx2; b) the amount of mature collagen (reddish birefringence); c) the amount of immature collagen (greenish birefringence); d) bone interface-contact (BIC); e) bone area formation (BAFO), f) reverse torque test on No-Irradiation (N-Ir), Early-Irradiation (E-Ir), Previous Irradiation (P-Ir) and Late-Irradiation (L-Ir), at 3 days, 2 and 7 weeks. Different letters indicate a statistical difference ($p<0,05$).
Source: Drawn up by the author.

Figure 4. Photomicrography of bone neoformation in screw thread area for each group and period.



Subtitle: No-Irradiation (N-Ir), Early-Irradiation (E-Ir), Previous Irradiation (P-Ir) and Late-Irradiation (L-Ir), in 3 days, 2 and 7 weeks. Source: Drawn up by the author.

In general, bone remodeling differed statistically from the N-Ir group with irradiated groups ($p<0.05$), except with L-Ir. The mean values of BIC and BAFO at 3 days, 2 weeks and 7 weeks are presented in Figure 3. The BIC (%) values of the N-Ir group were statistically higher than E-Ir at 2 and 7 weeks ($p<0,05$) and P-Ir group at 2 weeks only ($p<0,05$). On the other hand, at 3 days the BIC of the N-Ir group was lower than E-Ir and P-Ir, but no statistical difference was observed ($p>0.05$). The L-Ir group exhibited behavior different from the other groups, since it presented a higher value than N-Ir at 3 days, and a statistical difference was observed ($p<0,05$). At 2 and 7 weeks, L-Ir and N-Ir showed no statistical difference ($p>0,05$).

At 2 and 7 weeks, the BAFO (%) values of N-Ir were statistically higher than E-Ir and P-Ir ($p<0,05$), but between the N-Ir and L-Ir groups no statistical difference ~~it~~ was observed in these periods ($p>0.05$). Similarly to BIC, at 3 days, the BAFO (%) value of N-Ir was lower than that of all groups, but only a statistical difference was observed with the L-Ir group ($p<0.05$).

3.4 Reverse Torque Test

The reverse torque resistance (N/cm^2) is presented in Figure 3. The results showed the significance of moments of irradiation. At 2 weeks, the L-Ir group presented the highest reverse torque compared to the other groups ($p<0.05$). In this period, the E-Ir group presented a lower value than N-Ir and P-Ir, and a statistical difference was observed ($p<0,05$), but the N-Ir and P-Ir groups did not differ between them ($p>0.05$). The strength of L-Ir and N-Ir at 7 weeks was statistically higher than the other groups ($p<0.05$), but they did not differ from each other ($p>0.05$).

4. Discussion

Although there are articles published since the 1990s correlating ionizing irradiation with osseointegration (Marx & Morales, 1998; Öhrnel, 1997; Niimi, 1998; Wagner, 1998), the survival of implants in irradiated patients is still controversial (Nobrega, 2016; Wetzels, et al., 2017; Ruiz, et al., 2018; Woods, et al., 2019; Alberta, et al., 2020a; Alberta, et al., 2020b; da Cruz Vegian, et al., 2020). The success of osseointegration depends on the influence of different variables related to irradiation, such as the time interval between radiotherapy and implant surgery, dose quantity and anatomical site (Ruiz, et al., 2018; Alberga, et al., 2020a; Alberga, et al., 2020b). Nowadays, most irradiation research is either clinical studies about the survival of implants in irradiated bone (Wetzels, et al., 2017; Ruiz, et al., 2018; Woods, et al., 2019; Alberga, et al, 2020b; Ettl, et al, 2020) or they are systematic reviews of the literature (Noberga, et al., 2016; Shugga, et al., 2016; Chambrone, et al., 2013; Alberga, et al., 2020a; Tonetti, et al, 2021). Thus, *in vivo* studies using animal models that evaluate the influence of timing of implant placement on the time of new bone formation (BAFO, BIC and collagen fiber maturation), cell differentiation marker expression for osteogenesis (Runx-2) and the biomechanics of osseointegration (torque removal test) in a single study are rare. The development of the present study is very important to compare these aspects in a single study, because comparison with the previous studies is difficult due to the variation of many parameters, such as: the animal model, the total irradiation dosage, and the irradiated anatomical site.

Previous studies have shown that implant survival is adversely affected by radiotherapy but that oral rehabilitation with implants is possible in irradiated patients (Chambrone, et al., 2013; Nobrega, et al., 2016, Shugaa, et al., 2016; Ruiz, et al., 2018; Alberga. et al., 2020a, Alberga, et al., 2020). However, strict monitoring is very important to prevent complications, and to reduce possible failures (Nobrega, et al., 2016). Currently, several authors are evaluating whether immediate or delayed implant placement in relation to ionizing irradiation interferes with implant survival rates (Alberga et al., 2020a, Alberga et al., 2020b; Chambrone et al., 2013; Nobrega et al., 2016), and the authors reported that both situations showed acceptable overall implant survival ratios (Shugaa et al., 2016; Wertezls et al., 2017; Woods et al., 2019; Alberga et al., 2020a Alberga et al., 2020b).

In the present study, the influence of ionizing irradiation on osseointegration at different times was considered in order to simulate the clinical situation reported in these previous articles, and the groups were named E-Ir and P-Ir, respectively. In this study, it was also concluded that the situations exhibited similar results, since in BAFO and BIC analyses, maturation of collagen fibers and in the osseointegration fixation strength test, no statistical difference was observed between them. However, these groups most often presented worse results than the N-Ir group, proving the negative effect of irradiation on osseointegration, as previously reported (Oliveira, et al. 2011, Nobrega, et al., 2016).

When the insertion of implants occurred after the ionizing irradiation, the P-Ir group showed results compatible with the previous literature, which presents high failure rates due to the changes in bone quality in the irradiated region (Brasseur, et al., 2006; Verdonck, et al., 2008; Shugaa, et al., 2016; Nobrega, et al., 2016; Ocana, et al., 2017; Woods, et al., 2019). Similar to our results, previous studies showed that osseointegration may be possible in irradiated bone tissue (Brogniez, et al., 2002; Brasseur, et al., 2006; Chambrone, et al., 2013; Shugaa, et al., 2016, Ruiz, et al., 2018), because its osteogenic potential and

bone vitality are not definitively impaired by irradiation (Brogniez, et al., 2002; Brasseur, et al., 2006), but the irradiated bone exhibits delay in the bone maturation process in osseointegration when compared to non-irradiated bone (Ocana, et al., 2016) and also presents altered vascularization (Verdock, et al., 2008).

The increased quantity of immature collagen fibers and the lower quantity of mature collagen fibers observed until the 7-week period in the P-Ir group, associated with the lower fixation strength of the implant to the bone in the reverse torque test, confirmed these aspects as reported in the previous studies. Additionally, the P-Ir group showed the worst results in relation to the Runx-2 marker in all periods, with a statistical difference from the N-Ir group ($p<0.05$). Similar to our values observed in BIC and BAFO, Alberga, et al., (2020b) recently reported that there is a tendency for lower bone formation in irradiated patients before implant placement than in those without radiotherapy. Thus, the decision on whether to install implants or not in already irradiated bone tissue must be made considering the risks and benefits to the patient in each case, because it can improve their quality of life and can facilitate social rehabilitation (Nobrega, et al., 2016). Further prospective long-term studies are necessary in order to evaluate the ideal waiting time required for implant placement (Brasseur, et al., 2006; Chambrone, et al., 2013; Ruiz, et al., 2018, Alberga, et al., 2020b).

The effect of radiotherapy during the early stage of healing of osseointegration, represented by the E-Ir group in this study, has been evaluated previously in few studies, both with *in vivo* animal models (Doh, et al., 2016; da Cruz Vegian, et al., 2020; Soares, et al, 2020) and clinical studies (Wetzels, et al., 2017; Woods, et al., 2019; Alberga, et al., 2020b). In this group, the aim was to show that irradiation affects osseointegration, since these implants were in the process of osseointegration when radiotherapy was performed. In general, the E-Ir group showed the lower quantitative values obtained in mature collagen fiber analysis, BIC and BAFO percentages, reverse torque test, and most of the time, a statistical difference with the N-Ir group was observed ($p<0.05$). These results can be explained by the characteristics of ionizing radiation that generates hypoxia and cellular deterioration due to vascular alteration (Ihde, 2009; Verdonck, et al., 2008), which are essential to the dynamic process of osseointegration. Additionally, Green, et al., (2014) reported that ionizing radiation can lead to bone marrow failure; and also promoting a negative influence on osseointegration.

Previous studies with a similar methodology of irradiation a short time after implant placement, reported that the process of angiogenesis and cell differentiation, events that start osseointegration were impaired (Doh, et al., 2016; da Cruz Vegian, et al., 2020) as also observed in the present study, since the Runx-2 marker was delayed at 3 days in the E-Ir group when compared to the N-Ir group. Although there is a negative influence of ionizing irradiation in the osseointegration process as described in our results and recently reported (Doh, et al., 2016; Wetzels, et al., 2017; Woods, et al., 2019; Alberga, et al., 2020b; da Cruz Vegian, et al., 2020), this procedure has been performed in clinical practice because it reduces costs and rehabilitation time (Wetzels, et al., 2017; Woods, et al., 2019; Alberga, et al., 2020b).

The influence of irradiation on osseointegrated implants was also the aim of the present study. The L-Ir group showed similar results to the N-Ir group at 7 weeks in most analyses, with the exception of the mature collagen fiber analysis. These implants were not in the process of osseointegration when radiotherapy was performed, and the osseointegration already established may have been fundamental for this success. Previous studies have also reported adequate osseointegration of implant placement before irradiation (Brogniez, et al. 2000; Brogniez, et al., 2002; Brausser et al., 2006; Doh et al., 2016), probably due to the balance between bone resorption and the osteogenesis starting to be restored in a few weeks (Brogniez, et al., 2002).

This bone remodeling was confirmed by Brausser, et al., (2006), who observed cortical bone remodeling. Our bone remodeling results, observed by means of BIC and BAFO, also confirmed the restoration of osteogenesis. However, the expression of marker Runx-2 at 3 days was different from the N-Ir group, suggesting a delay in cellular differentiation, but over time, this aspect became normal.

5. Conclusion

Within the limits of this study, the timing of irradiation in the early periods of the osseointegration process demonstrated more negative effects than when the irradiation was applied after implant placement. Therefore, the effect of irradiation ionization on implant survival rates remains a matter of debate.

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