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ORIGINAL ARTICLE

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Effect of radiotherapy on the differentiation and osteogenic activity of mesenchymal stem cells on dental implants

Efeito da radioterapia na diferenciação e atividade osteogênica de células-tronco mesenquimais em implantes dentários

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ABSTRACT

Objective: to evaluate the differentiation and gene expression of transcripts related to osteogenesis in a primary culture of Mesenchymal Stem Cells (MSCs) derived from rat femurs submitted to radiotherapy and the installation of pure titanium implants. **Material and Methods:** fifty-four rats received titanium implants in both femurs and were divided into three groups: Control: implant surgery (C); Implant + immediate irradiation (IrI), and Implant + late irradiation (IrL). Euthanasia occurred 3, 14, and 49 days after surgery. The bone marrow MSCs from the femurs were isolated and cultivated. The cell viability, total protein content, alkaline phosphatase (ALP) activity, and the formation of mineralization nodules and cellular genotoxicity were analyzed. The gene expression of *Alkaline Phosphatase (phoA), Collagen 1 (COL1), Runt-related transcription factor 2 (RUNX2), Osterix (OSX), Osteopontin (OPN), Integrin β_1 ($ITGB_1$), Bone Sialoprotein (BSP), Osteonectin (SPARC), Osteocalcin (Bglap), Transforming Growth Factor β-type (TGF-β), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Interleukin-6 (IL-6), Apolipoprotein E (APOE)* and *Prostaglandin E_2 synthase (PGE_2)* were evaluated by qRT-PCR. **Results:** ionizing radiation suppresses the gene expression of essential transcripts for bone regeneration, as well as cellular viability, as observed in the IrI and IrL groups. **Conclusion:** although this can lead to the loss of osseointegration and failure of the implant, the MSCs showed more activity at 49 days than at 3 and 14 days.

KEYWORDS

Cancer treatment protocol; Osseointegration; Osteogenesis; Bone regeneration; Dental implants.

RESUMO

Objetivo: avaliar a diferenciação e expressão gênica de transcritos relacionados à osteogênese em cultura primária de MSCs derivadas de fêmures de ratos submetidos à radioterapia e instalação de implantes de titânio puro. **Material e Métodos:** cinquenta e quatro ratos receberam implantes de titânio em ambos os fêmures e foram divididos em três grupos: Controle: cirurgia de implante (C); Implante + irradiação imediata (IrI) e Implante + irradiação tardia (IrL). A eutanásia ocorreu 3, 14 e 49 dias após a cirurgia. As MSCs de medula óssea dos fêmures foram isoladas e cultivadas. Foram analisadas a viabilidade celular, teor de proteína total, atividade da fosfatase alcalina (ALP), formação de nódulos de mineralização e genotoxicidade celular. A expressão gênica de *Fosfatase Alcalina (phoA), Colágeno 1 (COL1), fator de transcrição relacionado a Runt 2 (RUNX2), Osterix (OSX),*

Osteopontina (OPN), Integrina β1 (ITGB1), Sialoproteína Óssea (BSP), Osteonectina (SPARC), Osteocalcina (Bglap), Fator de Crescimento Transformador tipo β (TGF-β), Fator Estimulante de Colônia de Granulócitos-Macrófagos (GM-CSF), Interleucina-6 (IL-6), Apolipoproteína E (APOE) e Prostaglandina E2 sintase (PGE2) foram avaliados por qRT-PCR. **Resultados:** a radiação ionizante suprime a expressão gênica de transcritos essenciais para a regeneração óssea, bem como a viabilidade celular, como observado nos grupos IrI e IrL. **Conclusão:** embora isso possa levar à perda da osseointegração e falha do implante, as MSCs apresentaram maior atividade aos 49 dias do que aos 3 e 14 dias.

PALAVRAS-CHAVE

Protocolo de tratamento do câncer; Osseointegração; Osteogênese; Regeneração óssea; Implantes dentários.

INTRODUCTION

Ionizing radiation is commonly used in the treatment of head and neck cancer. However, bone regeneration becomes compromised after irradiation because of the reduction of cellular activity, vascular supply, and local oxygenation [1]. The main alterations after radiation occur in osteoblasts, resulting in the alteration of bone matrix formation capacity [1].

Mesenchymal stem cells (MSCs) are fundamental in bone regeneration after irradiation [2]. They exhibit a high proliferative rate and high differentiation potential; however, the irradiation can alter the differentiation of osteoblasts [3]. This potential of MSCs to repair the damage resulting from ionizing radiation and to repopulate the medullary compartment is one of the signs in irradiated patients for verifying their tolerance to radiotherapy [3,4]. The number of osteoblasts decreases, and consequently collagen production and the porosity of the cortical bone increases [5].

Although radiation decreases the vitality of tissues, oral implants have been used for patient rehabilitation [6] to reestablish mastication and to provide fixation for maxillo-facial prostheses, with improvements in phonetics, esthetics, patient comfort, and quality of life [7]. In addition, the presence of implants can change the local radiation doses and distribution [6,8] and can alter the therapeutic scheme.

The aim of this study was to evaluate the effects of radiotherapy on the viability, genotoxicity, differentiation, and gene expression profile of related transcripts to early and late osteogenesis in the primary culture of MSCs from rat femurs submitted to the installation of titanium implants before radiotherapy.

MATERIAL AND METHODS

Animals

Fifty-four male Wistar rats (*Rattus norvegicus* albinus) weighing about 300 g were maintained in cages (n = 6) and given water and feed *ad libitum*. This experiment was approved by the Research Ethics Committee of Unesp No. 003/2016 and followed all the recommendations of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines for the execution and submission of studies on animals [9].

The 54 rats were subdivided into 3 groups (n = 18): Group Control (C); Implant Group IrI: Implant + immediate irradiation after 24 hours; and Group IrL: Implant + late irradiation after 4 weeks. They were each submitted to the installation of pure titanium implants, (5 mm in length and 3.5 mm in diameter) in both femurs and were subsequently euthanized. In groups C and IrI, euthanasia was performed 3, 14, and 49 days after implant surgery. In group IrL, euthanasia was performed 3, 14 and 49 days after radiotherapy.

Ionizing irradiation procedure

The delivered radiation doses were traceable to the national metrological standard by means of a farmer-type ionization chamber and electrometer, and a batch of dosimeter, TLD-800 (lithium borate manganese Li2B4O7: Mn), was used (Thermo Scientific Inc.) to continuously monitoring the radiation incidence during each procedure according to thermoluminescent dosimetry methodology. The parameters for obtaining the pre-established luminescence curve, as used by the IEAv Aerospace Dosimetry Laboratory (LDA) for irradiations performed in the ^{60}Co gamma beam, were produced by

Eldorado 78 irradiator teletherapy from Atomic Energy of Canadian Limited, located in the IEAv Ionizing Radiation Laboratory (LRI). The animals were anesthetized and positioned in immobilizers previously developed to reproduce the irradiation site [10]. The femurs were irradiated in two steps within 24 hours, at a mean source-object distance of 48 cm from the rats, with dose rates of 17.6 Gy/h in the tissue (femur) and over an area of 2 cm, according to Cunha et al. (2007) [11].

Analysis of changes in animals

The animals were monitored for clinical changes such as eating habits, hair loss, surgical wound infection, and weight loss. At the beginning of the experiment and immediately prior to euthanasia, all animals were weighed on a semi-analytical scale. The weight changes were recorded as a percentage, and a weight loss of less than 20% of body weight was considered acceptable [12].

Primary osteoblast culture

The femur was used in the primary cultures of osteoblasts, which were isolated from cells by washing the bone marrow with osteogenic culture medium according to Rosa et al. (2008) [13]. Subsequently, these cells were distributed in culture flasks of 75 cm^2 (Nunc, Denmark) and incubated at 37 ºC with 5% CO_2 (Ultrasafe HF 212 UV Incubator). The culture medium was replaced every three days, and the culture progression evaluated by inverted-phase microscopy (Carl Zeiss Microscope - Axiovert 40C, Germany). Two flasks were grown per group and period, and, after confluency, each one was plated with a total of 2×10^4 viable cells in each well of a 24-well plate (Nunc, Denmark) to evaluate cell viability, alkaline phosphatase activity, the formation of mineralization nodules, and cellular genotoxicity.

In another flask, after confluence, the culture medium was removed, and 3 mL of Trizol Reagent (Ambion, Life Technologies Corporation, Van Allen Way, Carlsbad, California, USA) was added. The cells were lysed, and their contents were collected and stored in a freezer at -80 °C according to the manufacturer's guidelines.

MTT assay

To evaluate cell viability, the cells were cultured in the wells and evaluated after 48 hours and 7 days. The cells were incubated for 4 hours with MTT dye (3-4,5-dimethylthiabromide), (Sigma Aldrich Inc, Darmstadt, Germany) in the proportion of 10 μL of 5 mg/mL of MTT dissolved in PBS for each well, followed by spectrophotometric analysis of the incorporated dye using colorimetric measurement on a microplate reader at a wavelength of 570 nm (Biotek EL808IU).

Alkaline phosphatase (ALP) activity

To evaluate ALP activity, the cells were cultured in the wells and evaluated after 48 hour and 7 days. ALP activity was determined by the release of thymolphthalein by hydrolysis of the thymolphthalein monophosphate substrate using a commercial kit according to the manufacturer's instructions (Labtest Diagnostica). The absorbance was measured with a spectrophotometer (Micronal AJX 1900) using a wavelength of 590 nm, and ALP activity was calculated from the standard curve using thymolphthalein on a scale of 0.012 to 0.4 μmol of thymolphthalein/hour/μg protein.

The level of ALP was normalized against the total protein concentration. The total protein was estimated from each individual sample [14].

Mineralization assay

The cells were incubated at 37 °C/5%CO_2 for 14 days after which the formation of mineralization nodules was quantified using Alizarin S 2% red staining (Sigma Aldrich Inc, Darmstadt, Germany), which consists of fixing the cell culture using 4% paraformaldehyde, exposure to alizarin S red dye for 30 min at room temperature, and washing with phosphate-buffered saline. The extraction of the nodules by acetic acid was measured in the spectrophotometer at 405 nm [15].

Genotoxicity test

Cells were fixed in 4% formaldehyde on the seventh day after plating, and fluoroshield 4- 6-diamidine-2-phenylindole solution (DAPI; Sigma-Aldrich) was added to each well. The cells were photographed using a digital camera (Sony F828 digital, Cyber-Shot, 8.0 megapixels; Sony Corporation, Tokyo, Japan) in an inverted light microscope. At least 10 images per well were obtained, and the genotoxicity analysis was performed from a total of 20,000 cells using Image J software (National Institutes of Health, Bethesda, MD).

Evaluation of gene expression by qRT-PCR

cDNA synthesis was performed by reverse transcription reactions according to the instructions from SuperScript III First-Strand Synthesis Supermix Kit, (Invitrogen Life Technologies Corporation-Van Allen Way, Carlsbad, California, USA). cDNA specimens were stored at -20 ºC. The cDNA was then used for RT-PCR with the Step One Plus Time PCR System (Thermofisher Scientfic Inc Waltham, Massachusetts, USA) using the SYBR Green Platinum System qPCR SuperMix-UDG (Invitrogen Life Technologies Corporation-Van Allen Way, Carlsbad, California, USA). Specific primers and 2 μL of cDNA were used in each reaction. The primers are listed in Table I. The specimens were submitted to real-time polymerase chain reaction (50 °C for 2 minutes followed by initial denaturation at 95 °C for 2 minutes and a further 40 cycles alternating 95 °C for 15 seconds and 60 °C for 40 seconds).

Table I - Descriptions of forward and reverse sequences, fragment length (pair of bases), and NCBI of genes related to early and late phase of osteogenesis and osteoclastogenesis

Gene	Sequence Forwad (F)/ Reverse (R)	Fragment length (pb)	NCBI
Genes related to early osteogenesis			
Alkaline phosphatase (phoA)	F TATGTCTGGAACCGCACTGAAC R CACTAGCAAGAAGAAGCCTTTGGG	192	XM_006239136.3
Collagen-1 (COL1)	F CCAACGAGATCGAGCTCAGG R GACTGTCTTGCCCCAAGTTCC	101	NM_053304.1
Runt-related transcription factor 2 (RUNX2)	F GCCGGAATGATGAGAACTA R GGACCGTCCACTGTCACTTT	200	NM_001278483.1
Osterix (OSX)	F CAAGAGTCGGATTCTAGGATTGGAT R CAAACTTGCTGCAGGCTGCT	208	NM_001037632.1
Osteopontin (OPN)	F ATCTGATGAGTCCTTCACTG R GGGATACTGTTCATCAGAAA	151	NM_012881.2
Integrin β1 (ITGB1)	F GGAGAAAACTGTGATGCCATACAT R TGGGCTGGTACAGTTTTGTTCA	85	NM_017022.2
Genes related to late osteogenesis			
Bone Sialoprotein (BSP)	F CTACTTTTATCCTCCTCTGAAACGGTT R GCTAGCGGTTACCCCTGAGA	202	NM_012587.2
Osteonectin (SPARC)	F CTCCCATTGGCGAGTTTG R TGTAGTCCAGGTGGAGCTTGTG	129	NM_012656.1
Osteocalcin (Bglap)	F GAGGGCAGTAAGGTGGTGAA R CGTCCTGGAAGCCAATGTG	154	NM_013414.1
Transforming Growth Factor β-type (TGF-β)	F GGACTCTCCACCTGCAAGAC R CTCTGCAGGCGCAGCTCTG	63	NM_021578.2
Genes related to Osteoclastogenesis			
Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)	F CCGACACAGGCTCTTCTATTCAG R CAGCCAGCAAGACTAGGATGA	84	XM_008761428.2
Interleukin-6 (IL-6)	F TCCTACCCCAACTTCCAATGCTC R TTGGATGGTCTTGGTCCTTAGCC	79	NM_012589.2
Apolipoprotein E (APOE)	F TGTTGGTCCCATTGCTGACAGGAT R TGGTGTTTACCTCGTTGCGGTACT	382	NM_001270681.1
Protaglandin E2 synthase (PGE2)	F CATGATCTACCCTCCCCACG R CAGACCAAAGACTTCCTGCCC	67	NM_017232.3
Reference gene			
β-actin	F GCAGGAGTACGATGAGTCCG R ACGCAGCTCAGTAACAGTCC	74	NM_031144.3

NCBI: Reference Sequence National Center for Biotechnology Information.

The Ct (Threshold Cycle) values of the specimens were used in the following calculations [16]. The mean of the biological triplicates of each gene was calculated for all specimens, and the Ct of the constituent gene was subtracted: ΔCt = Ct (gene) -Ct (constitutive). The ΔCt was subtracted from the calibrator specimen, resulting in ΔΔCt: ΔΔCt = ΔCt-ΔCt (calibrator). Relative Quantification (RQ) was then calculated for each gene in each specimen according to the equation:

$$RQ = 2^{-\Delta\Delta Ct} \quad (1)$$

Statistical analysis

Data from the MTT assay, alkaline phosphatase activity and mineralization nodules formation were submitted to ANOVA, and the Tukey tests for comparison among the groups. The Spearman correlation test was used to evaluate the gene expression. All tests were performed using GraphPad Prism 7.03 software (GraphPad Software INC, La Jolla, CA, USA). The level of significance was 5%.

RESULTS

Fifty-four animals were operated on, of which 6 were lost (11.1%); 2 died in the postoperative period of implant installation, and 4 others from the effects of irradiation. However, other alterations were observed in some animals, especially in the groups that remained for longer periods between irradiation and euthanasia, such as the animals in the 49-day groups that experienced hair loss, skin wounds, surgical site infections, and tissue necrosis.

MTT assay

Concentration absorbance values were significant in all groups ($p < 0.005$). Absorbance values were lower than 50% in the IrI and IrL groups ($p = 0.012$ and $p = 0.027$, respectively).

Alkaline phosphatase (ALP) activity

In the IrI and IrL groups, the ALP activity was detected at day 3, 14 and at day 49, the higher rate was observed, reaching up to 58% ($p < 0.005$).

Mineralization formation

Nodular structure was observed in all groups as shown in Figure 1A-1C. In the IrI group, the coloration of mineralized nodules in all periods of time decreased compared with the control group. However, at 49 days, the mineralization nodule formations in the IrI and IrL groups increased, with a statistically significant difference ($p = 0.017$ and $p < 0.005$, respectively). In the IrL group, the increase reached 63.9%, with a significant difference in all periods of time.

Genotoxicity (micronuclei formation)

In group C, the nuclei were similar to normal. In the 3-day test, a total of 475 cells was counted and, over time, that number declined, whereas at 14 days the mean number of cells was 271 and at 49 days 211. Despite the decline in the number of cells, no atypia or presence of micronuclei were observed in any of the specimens.

In the IrL group, at 3 days, a reduced number of stained nuclei was observed, and all presented an aberrant appearance. At 14 and 49 days, the cells were apparently recovering and multiplying but still with aberrant DNA. In the IrI group, they proliferated, however, aberrant DNA was present at 3 days to 49 days.

Effect of radiotherapy on mesenchymal cell's gene expression

Early phase of osteogenesis

In the present animal study, the mesenchymal cells presented similar behavior after implant

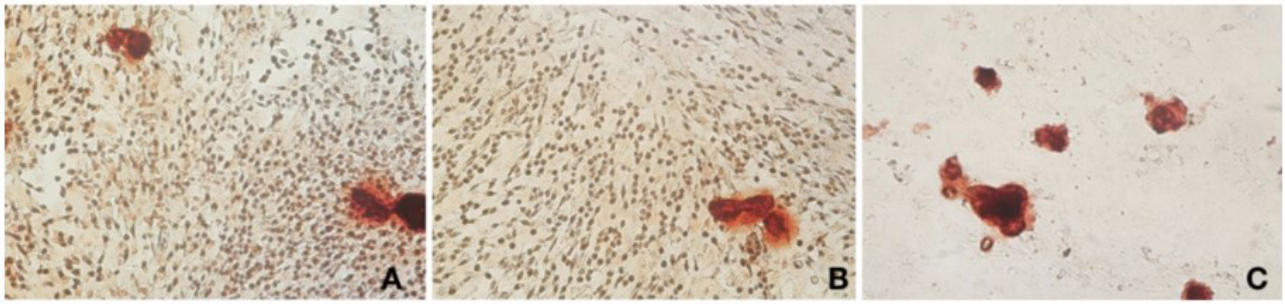

Figure 1 - Photomicrographs of mineralization nodule at 21 days. (A) Control group showing a large number of cells around the osteo-like nodules; (B) IrI group has an intermediate number of cells but fewer mineralization nodules; (C) IrL group has a smaller number of cells but shows a mineralization nodule. Alizarin S 2% red stain. 40× magnification.

installation and at different irradiation intervals. The genes *OSX, phoA,* and *COL1* related to the initial osteogenesis phase were downregulated in the 3 periods evaluated. However, at 49 days, more *phoA* RNAm transcripts were observed in the IrI group compared with 3 and 14 days ($p < 0.05$ and $p = 0.001$, respectively). In the IrL group, *phoA* RNAm transcripts were downregulated in all periods.

The *RUNX2* gene expression was irregular in all irradiated groups at 3 and 14 days, in the IrL group, at 3 days it was hyperregulated ($p < 0.0001$), and its expression was reduced at 14 to 49 days ($p = 0.0001$ and $p = 0.001$ respectively).

ITGB1 was downregulated in the 2 groups irradiated at 3 days ($p = 0.023$ and $p = 0.013$), but its transcripts were found at the subsequent periods in the IrI ($p = 0.103$ and $p = 0.045$) and IrL ($p = 0.045$ and $p = 0.114$) groups. *OPN* was hyperexpressed in the IrI group at 3 and 14 days ($p = 0.043$, $p = 0.141$) but, at 49 days, it was downregulated ($p = 0.042$). In the IrL, its expression was similar to that of the control group at 3 days ($p = 0.124$), but, at 14 days ($p = 0.042$), it was hyperregulated until 49 days ($p = 0.043$).

In the IrI group, the *phoA* expression was directly correlated to *RUNX2* ($p = 0.016$) and *OSX* ($p = 0.033$) expression, with a significant difference. The *RUNX2* and *OSX* transcripts tended to correlate to *ITGB1 and OPN*, but these differences were not statistically significant. In the IrL group, no significant correlation was found among different gene expressions.

Late phase of osteogenesis

The genes *Bglap, SPARC,* and *TGF-β* e *BSP* are expressed in the late phase of osteogenesis. The *Bglap, SPARC*, and *BSP* transcripts were downregulated in the IrI and IrL groups in all periods analyzed.

The *TGF-β* genes were hyperregulated in the irradiated group. MSCs from the IrI group, hyperexpressed this gene at 3 and 49 days ($p = 0.013$, $p = 0.0004$, respectively) compared with the control. Similar results were found in IrL group, these transcripts were higher at 3 ($p = 0.042$), 14 ($p = 0.012$), and at 49 days ($p < 0.0001$). There was correlation between *BSP* and *OSX* expression ($p = 0.2$); however, there was not significance in the IrI group.

The genes *APOE, PGE2, IL-6* e *GM-CSF* were evaluated as well. At day 3, 14 and 49, its *APOE* expression in the IrI group was similar to that in the control group ($p = 0.053$, $p = 0.107$, $p = 0.0727$, respectively). In the IrL group, RNAm transcripts were similar to those in the control group at day 3 ($p = 0.063$), but at 14 and 49 days, it was hyperregulated ($p = 0.0001$ and $p = 0.0002$, respectively).

The *PGE2* RNAm was overexpressed in the IrI group at 3 and 49 days ($p = 0.023$ and $p = 0.019$, respectively). In the IrL group, the expression was similar to that in the control group at 3 and 49 days ($p = 0.140$ and $p = 0.129$, respectively).

The *IL-6* was hyperregulated in the IrI group at 3 days ($p = 0.029$), but subexpressed at 14 ($p = 0.0007$) and 49 days ($p = 0.019$). In the IrL group, it was hyperexpressed at 3 ($p = 0.042$) until 14 ($p = 0.016$) days when it started to decline until 49 days ($p = 0.0192$). No difference was found between *GM-CSF* expression at 3 and 14 days ($p = 0.840$ and $p = 0.157$, respectively) in the IrI group, being overexpressed at 49 days in the IrI and IrL groups ($p = 0.0015$ and $p = 0.0057$, respectively). A correlation was found between *GM-CSF* and *PGE2* expression ($p = 0.174$) and *GM-CSF* between *Il-6* ($p = 0.23$) but, there was no significant difference from the IrI group. Figure 2 summarizes these gene expressions.

DISCUSSION

Ionization irradiation causes local infection, suture dehiscence, and mucositis [12,17,18]. In the present study, alopecia, ulceration and radiodermatitis were observed. We investigated two different periods of implant installation prior to the radiotherapy: IrI that mimicked oral cavity management for preradiotherapy treatment, the advantages of which include early oral rehabilitation that improves oral function such as speech and swallowing [6,19]; and IrL that evaluated the effects of radiotherapy on previously osseointegrated dental implants. The radiation dose at the bone implant interface is increased from scattering by the dental implants when they are installed in the radiation field and can alter both soft and hard tissues [20]. In both situations, the dose influences osseous tissue integrity and can lead to an imbalance in osteoblast and osteoclast activity. The increase

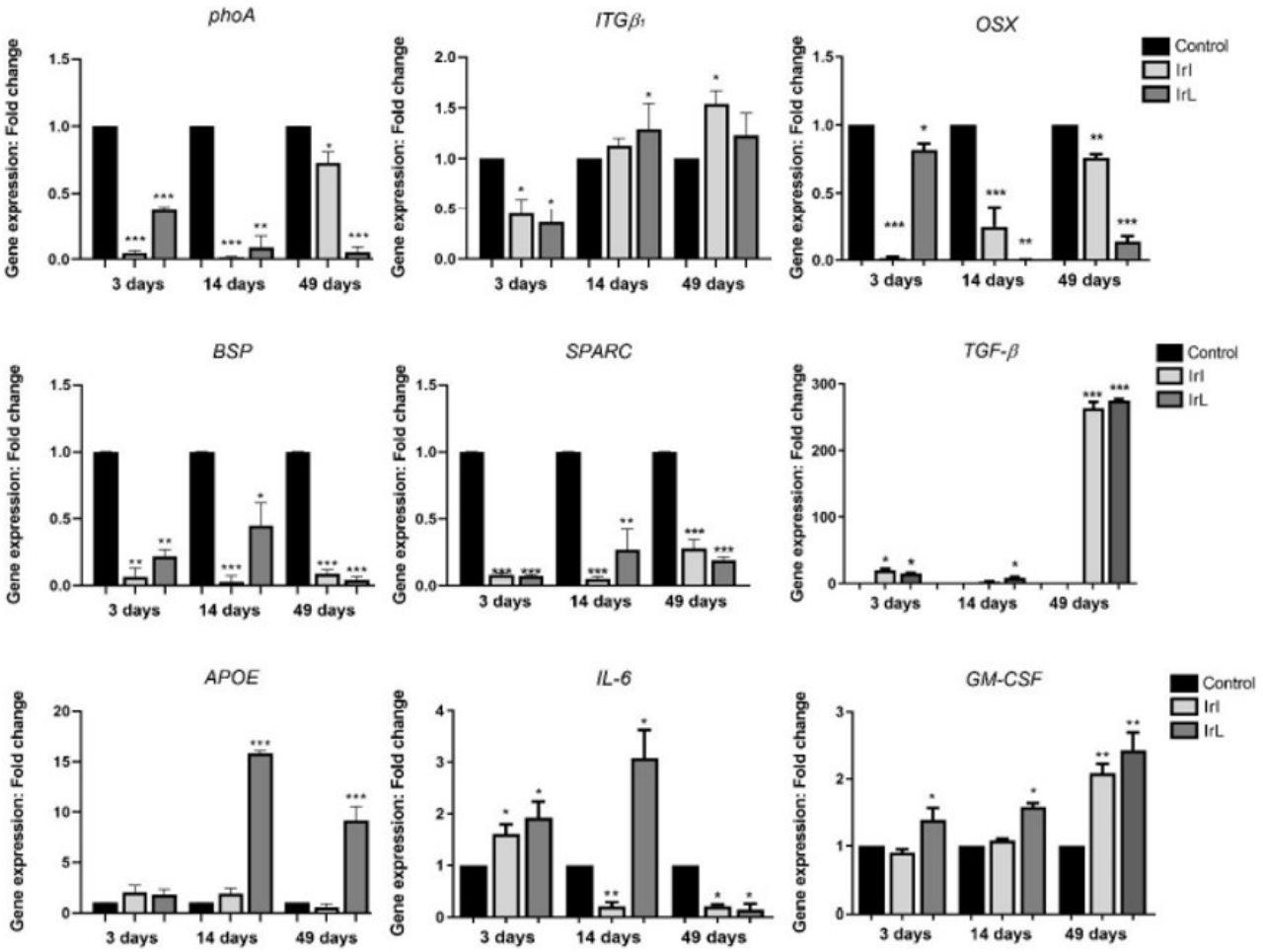

Figure 2 - Expression of transcripts essential for differentiation into osteoblast-like cells at 3, 14, and 49 days. Significant difference *p ≤ 0.05; **p≤ 0.005; ***p ≤ 0.0005.

in the concentration of osteoclasts and the decrease in the osteocytes and osteoblasts after irradiation [21], favors osteonecrosis and impairs the osseointegration process.

A cell viability test by MTT assay demonstrated that γ-ray exposure significantly reduced cell population in the IrI and IrL groups. In our study, the number of viable cells in the IrI group in the 48-hour period was similar to that of the control group, but, at 7 days, a significant decrease was found in the number of cells, and the DNA of these cells was aberrant. At 49 days, the cells were larger in number compared with at 3 and 7 days, and the DNA was again aberrant. The irradiated groups presented irregular ALP activity when compared with the control group; however, in all groups, mineralization nodules were observed. The number of mineralization nodules were inversely proportional to the dose received [22]. As MSC can resist radiation as a response to stress [3,4,23], these cells, when submitted to ionizing radiation, exhibit a higher antioxidant capacity in order to eliminate reactive oxygen species [23]. In our study, despite the presence of total protein, alkaline activity, and mineralization nodules in the irradiated groups at the different periods, the quantity and quality of the bone were low around the implants in an *in vivo* model (unpublished data).

Upon radiation, the *RUNX2* gene expression was affected in all groups at 3 and 14 days, and its expression started reducing at 14 to 49 days. RUNX2 plays an essential role in differentiating pluripotential cells into preosteoblasts. In the next phase, preosteoblasts differentiate into mature osteoblasts, a process in which OSX plays a critical role [24]. RUNX2 is downregulated by OSX [21]. The latter is required for osteoblast differentiation and bone formation. *OSX* knockout mice lacked bone completely [24]. Inhibition of osteoblast differentiation and the high number of immature osteoblasts in adult mice $Osx^{flox/-}$;Col1a1-Cre resulted in delayed trabecular bone and osteopenic cortical bone formation [21].

The *OSX* gene inactivation decreased *Bglap* expression and the accumulation of *OPN*-positive cells [21], similar to our results. In addition, the *OPN* regulation depends on mechanisms involving the intersection of three pathways, RUNX2, Vitamin D receptor, and Notch signaling, in osteoblastic cells [25].

OSX is required for the expression of the osteoblast's markers such as *COL1, Bglap, BSP, SPARC,* and *OPN* [24]. In our study, the genes *OSX, phoA,* and *COL1,* related to the initial osteogenesis phase, were downregulated. The irradiation decreases collagen production, increases the number of empty lacunae in the cortical bone [5], and leads to less hydroxyapatite deposition [26].

ITGB1 was downregulated in the IrI and IrL groups irradiated at 3 days, but their transcripts were found to be similar to those of the control group at the subsequent periods. Integrins have an essential role in cell–cell and cell–extracellular matrix adhesion, in osteoblast function and survival and, in osteoclast activity. The ablation of integrin regulator integrin β1-binding protein 1 (ICAP1) in osteoblasts results in defective osteoblast proliferation, differentiation and function, decreased fibronectin and *COL1* deposition, and delayed bone formation in mice [27,28].

All genes normally expressed in the late phase of the osteogenesis evaluated in the present study were downregulated, except for *TGF-β*, which was hyperregulated in the irradiated group in almost all periods. This result was comparable with 2-week *in vivo* results that reported that the early irradiated group exhibited higher TGF-β protein values than the control group, probably stimulated by the surgical procedures and irradiation trauma [10].

The genes *PGE2, GM-CSF*, and *IL-6* were upregulated in our study. The *IL-6* transcripts were upregulated at 3 up to 14 days in the IrI and IrL groups. IL-6 was secreted by hMSCs at day 1 after irradiation and started declining at day 7 until day 21, regardless of the dose and implant surface [29]. The release of IL-6 and GM-CSF contributes to pathological bone resorption by stimulating the differentiation of mononuclear phagocyte osteoclast precursors into mature bone-resorbing cells [10,30]. PGE2 signaling can protect cells from apoptosis and initiate stem cell self-renewal, and it is possible that up-regulation of PGE2 synthesis is an endogenous mechanism for radioprotection. In bone marrow, PGE2 production has been reported not to reach a maximum until several days after irradiation [31] as we demonstrated. A viable stem cell will naturally produce high levels of immune-response factors after any trauma or external influence, such as irradiation [29].

Interestingly, the *APOE* in the IrL group, at 14 and 49 days, was hyperregulated, suggesting that cells in this group could be quiescent [32,33]. In contrast, the APOE deletion in mouse models improves bone fracture healing by increasing bone formation and matrix mineralization [33].

Limitations of the current study include its *in vitro* design with a lack of microenvironment interaction. The bone vasculature can be damaged by direct radiation action [6] or indirectly by free radical production that deteriorates bone marrow blood vessels [26]. The MSCs can partially recover their activity in rats after 49 days of radiotherapy in contrast with the findings of Cao et al. (2011) [26]. In the preclinical model, irradiation is related to the bone marrow MSCs dysfunction that impairs bone formation and increases osteoclast activity, contributing to a rapid collapse of bone quantity and quality [34,35].

CONCLUSION

Based on our results, the majority transcripts of genes in the early and late phase of osteogenesis were downregulated. The radiation led to an osteogenesis and osteoclastogenesis imbalance; however, MSCs activity is partially recovered in rats after approximately 49 days.

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Author's Contributions

FHC, LMRV, MRCV, CDS, RFP, RNT, EK: Experimental Design. FHC, LMRV, MRCV, CDS,

RFP, RNT, EK: Laboratory Processes. FHC, LMRV, MRCV, CDS, RFP, RNT, EK: Sample Collection. FHC, LMRV, MRCV, CDS, RFP, RNT, EK, MJD, HFSS, CF: Formal Analysis. FHC, LMRV, MRCV, CDS, RFP, RNT, EK, MJD, HFSS, CAF: Writing – Review & Editing.

Conflict of Interest

No conflicts of interest declared concerning the publication of this article.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the guidelines and policies of the Brazilian National Animal Care Ethical Council (CONCEA), and the animal experimental protocol was approved by the Animal Ethics Committee (CEUA 003/2016) of the Institute of Science and Technology at São José dos Campos/ UNESP.

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