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Cytoprotective Effect of Gallic Acid against Injuries Promoted by Therapeutic Ionizing Radiation in Preosteoblast Cells

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ABSTRACT

Gallic acid (GA) is a powerful antioxidant extracted from plants of the Brazilian Cerrado. Oxidative stress plays an important role in the occurrence of radiation-induced osteonecrosis in patients treated for head and neck cancer. There is a need to develop research aimed at developing complementary therapies to prevent or reverse bone damage. The aim of the present study was to investigate the effect of GA in preosteoblasts exposed to therapeutic ionizing radiation. MC3T3-E1 preosteoblast cells were treated with 10 µM GA and exposed to 6 Gy ionizing radiation. We performed *in vitro* assays of cell proliferation, oxidative stress analysis by detection of reactive oxygen species, and alkaline phosphatase assay. GA at lower concentrations was able to significantly increase proliferation and inhibit radiation-induced generation of reactive oxygen species in osteoblast precursor cells, despite ionizing radiation-induced injury. Furthermore, GA significantly increased alkaline phosphatase at a dose of 6 Gy. The findings suggested that GA could attenuate ionizing radiation-induced injuries in osteoblast precursor cells. Moreover, *in vivo* studies are needed to better investigate the role of GA in osteonecrosis, especially in cancer patients undergoing radiotherapy or taking antiresorptive drugs.

Keywords: Antioxidant, Gallic acid, ionizing radiation, osteonecrosis, osteoradionecrosis, radiotherapy

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Introduction

Radiotherapy is one of the treatment modalities for cancer patients (1). However, therapeutic radiation can irreversibly damage bone tissue in the irradiated area, leading to osteoradionecrosis (2,3), subsequent loss of bone mass, and increased risk of bone fracture (4).

Osteoradionecrosis is characterized at the cellular level by a reduced number of osteocytes and decreased vascularization and osteoblastic activity (5). Studies have shown that damage to osteoblasts after irradiation contributes to decreased bone mineral density, reduced osteoblast proliferation and differentiation, cell cycle arrest and even radiation-induced apoptosis (2,6). Excessive formation of reactive oxygen species (ROS) caused by increased oxidative stress is also described as a predominant mediator of ionizing radiation-induced damage (4).

The process of bone remodeling involves a constant balance between bone resorption by osteoclasts and regeneration by osteoblasts (7). It is assumed that the proliferation and differentiation of osteoblasts are essential processes for the continuation of bone healing after radiation-induced injuries (8).

Antioxidants, such as gallic acid, curcumin, lycopene, resveratrol, and lutein have been attributed to radioprotective effects in animal study models, especially on the hematopoietic system (9-12). Considering the role of radiation-induced oxidative stress on bone cells, one study investigated the effect of antioxidant supplementation to prevent radiation-mediated cell damage on MC3T3-E1 preosteoblast cells. The synthetic phenethylurea compound (E)-1-(3,4-dihydroxyphenethyl)-3-(3,4-dihydroxystyryl)-urea (DPDS-U) was shown to inhibit radiation-induced cell damage in osteoblasts, primarily due to its ability to eliminate intracellular ROS accumulation and DNA damage, in addition to restoring glutathione and superoxide dismutase levels and activating the Nrf2/HO-1 pathway in irradiated cells (4).

Gallic acid (GA) is a polyhydroxyphenol compound that is frequently found in various fruits and medicinal plants. It has antioxidant, anti-inflammatory and antineoplastic properties as well as therapeutic activities in gastrointestinal, neuropsychological, metabolic and cardiovascular disorders (13-16). However, its effects on bone cells have not yet been sufficiently investigated. In this context, the present study hypothesized that GA might protect bone cells from radiation-induced injury. Therefore, the current study investigated whether GA can affect cell proliferation, osteogenic differentiation, and oxidative stress in MC3T3-E1 preosteoblast cells exposed to therapeutic ionizing radiation.

Materials and methods

Chemical reagents

Gallic acid (GA; purity ≥98.0%), 2'7'-dichlorofluorescein diacetate (H2DCFDA), ethanol (EtOH) as well as β-glycerophosphate and L-ascorbic acid for the culture medium for osteogenic differentiation were purchased from Sigma-Aldrich (St. Louis, MO, USA). Minimum essential medium (MEM), antibiotic-antimycotic 100X, fetal bovine serum, trypsin-EDTA 0.25% and trypan blue 0.4% were from Gibco (Grand Island, NY, USA). A commercial kit was used (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil) for the analysis of alkaline phosphate.

Cell Culture and Gallic acid treatment

The osteoblastic cell line MC3T3-E1, derived from a C57BL/6 mouse calvaria (ATCC, CRL-2593)

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was cultured in MEM medium supplemented with 10% fetal bovine serum and antibiotics and maintained at 37ºC in a humidified atmosphere with 5% CO_2.

To investigate the protective effect of GA on irradiated MC3T3-E1 cells, a dose-response curve was tested with different GA concentrations (5, 10, 15, and 20 μM) for 21 days, as this is the previously described time to osteoblastic differentiation (2). The culture medium with fresh GA was renewed every day. The 0.01% ethanol vehicle in the culture medium was used as a control. Once a single GA concentration was defined that did not affect cell proliferation, cells were subjected to pretreatment and continued treatment with GA 10 μM and irradiation.

Irradiation and Cell proliferation assay

Similarly, a dose-response curve was constructed to test different doses of ionizing radiation, including 2, 4, and 6 Gy, at a dose rate of 0.5 Gy/min using an Elekta Synergy linear accelerator (Atlanta, GA) with a field-to-source distance of 97.5 cm. The single dose of 6 Gy, leading to a significant reduction in cell proliferation, was set to irradiate the MC3T3-E1 cells simultaneously with GA pretreatment and continued treatment for 21 days.

A large number of $3.5x10^4$ MC3T3-E1 cells were plated in 12-well plates and subjected to both experimental conditions, GA treatment and irradiation. After treatment, cell number was measured by trypan blue exclusion.

Reactive oxygen species Assay

The current study investigated the effect of GA on radio-induced ROS generation in MC3T3-E1 cells treated with GA after 72 hours of exposure. This time point (72 hours) was considered because radiation induces oxidative stress already in the first period after exposure (17). The groups treated with an osteogenic differentiation medium with or without GA were not relevant for this analysis, as the osteogenic differentiation medium exerts its effect approximately 21 days later (2). After the experimental treatments, MC3T3-E1 cells were incubated with 10 μM 2'7'-dichlorofluorescein diacetate (H2DCFDA, Sigma-Aldrich, USA) for 30 min at 37°C, washed twice with PBS buffer, and immediately photographed under a fluorescence microscope (Olympus, Center Valley, PA, USA) and quantified using Image J software. Cells treated with 100 μM H_2O_2 in 2% fetal bovine serum were used as an in-reaction control for ROS generation (18). Results are expressed as the mean of at least three hotspot fields with ROS-positive cells relative to total cells.

Osteogenic Differentiation and Alkaline phosphatase assay

Alkaline phosphatase (ALP) analysis was used as a marker to verify osteogenic differentiation. For osteoblastic cell lines, the use of a culture medium containing 10% FBS, 10 mM β-glycerophosphate and 50 μg/mL L-ascorbic acid is required for osteogenic differentiation (19).

After pretreatment with GA, MC3T3-E1 cells were irradiated and cultured in osteogenic differentiation medium (ODM) with or without GA for 7 days (2) to measure ALP. This was assessed by cell staining. In brief, ALP-positive cells were fixed in 4% formaldehyde and incubated in a solution containing naphthol AS-MX-PO4 and Fast Red (Sigma, St Louis, Missouri, USA). Following, we quantified the ALP-positive cells area (in pixels) and the results were expressed as the mean of each group in triplicate.

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Statistical analysis

Statistical analysis was performed using SPSS 20.0 software and GraphPad Prism software (version 6.0, GraphPad Software Inc., San Diego, CA, USA). For the normality test, the Shapiro-Wilk test was used to check the distribution patterns. One-way or two-way ANOVA tests with post hoc Tukey's multiple comparisons test were used to analyze phenotypic and molecular assays. Probability values $p<0.05$ were considered statistically significant.

Results

Gallic acid increased MC3T3-E1 viability when exposed to ionizing radiation

To determine whether GA influences the effect of ionizing radiation on MC3T3-E1 cells, we performed the cell proliferation assay. First, the isolated effect of each experimental condition (i.e., ionizing radiation, and GA treatment) was investigated in the current study. GA was able to significantly increase the proliferation of osteoblast precursor cells at 5 µM and 10 µM, indicating a cytotoxic effect at higher concentrations ($p <0.001$; Figure 1A). Exposure of the cells to the different radiation doses for 24, 48, and 72

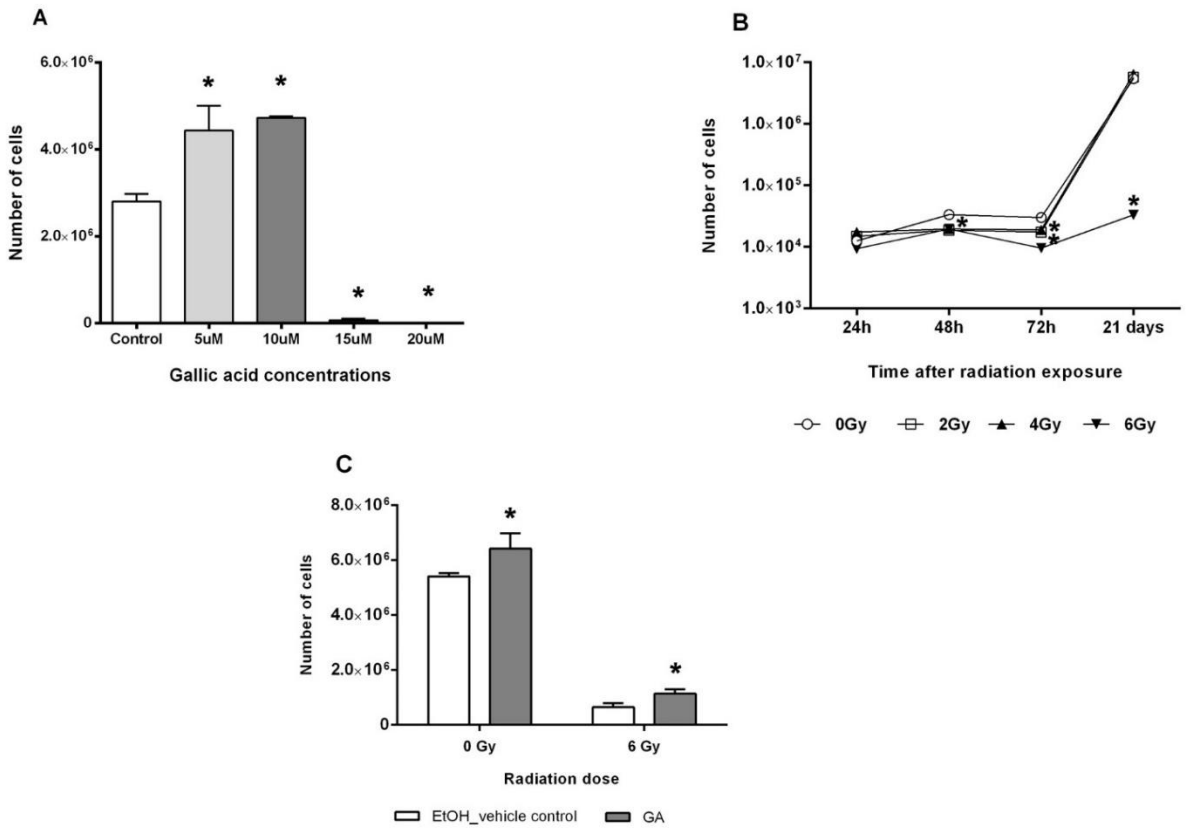

Fig. 1. Effects of gallic acid and ionizing radiation on the viability of MC3T3-E1 cells; (A) Changes in proliferation behavior of cells exposed to GA treatment at different concentrations, (B) Exposure of cells to the different ionizing radiations only, and (C) Treatment of cells with 10 µM GA and exposure to 6 Gy of irradiation. One-way and two-way ANOVA tests; Asterisks indicate statistical significance, $p<0.05$.

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hours, and 21 days revealed that the radiation impaired cell proliferation after 48 hours of exposure at all radiation doses. After 21 days of irradiation, the time required to induce osteoblastic differentiation, this result was even more pronounced at a dose of 6 Gy, significantly reducing the cell count ($p<0.001$; Figure 1B). When the simultaneous effect of GA treatment and irradiation on MC3T3-E1 cells was analyzed in the present study, the results suggested that GA was able to increase the proliferative activity of the cells despite the injuries caused by ionizing radiation. In line with this, GA exerted a cytoprotective effect on preosteoblast cells exposed to therapeutic ionizing radiation, as it reduced radiation-induced cell damage (Figure 1C).

Gallic acid inhibited radiation-induced ROS generation

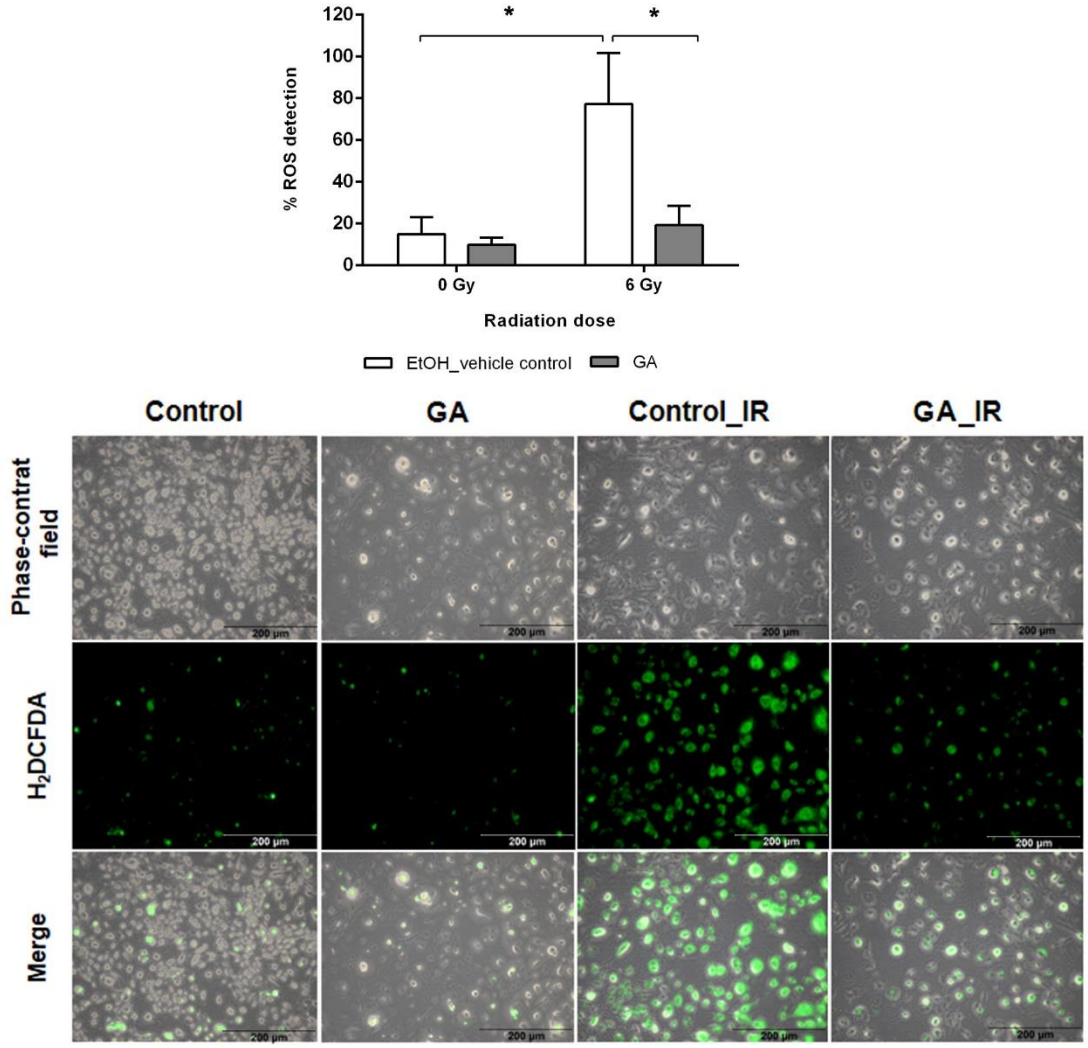

Fig. 2. Assay for reactive oxygen species in MC3T3-E1 cells; Cells were treated with 10 µM GA and irradiated with 6 Gy of ionizing radiation. GA: gallic acid, EtOH_vehicle: ethanol vehicle control, AG_IR: cells exposed to both experimental treatments, GA and irradiation. Results are expressed as the mean percentage of ROS-positive hotspots of the microscopic fields, taking into account the ratio fluorescent cells/ total cells. Two-way ANOVA test; Asterisks indicate statistical significance, $p<0.05$.

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Oxidative stress is an important pathway through which ionizing radiation exerts its effects. Based on these findings, the current study investigated the effect of GA on radiation-induced ROS formation in MC3T3-E1 cells by comparing EtOH vehicle control and GA groups at baseline. GA treatment modulated intracellular ROS accumulation, leading to a significant reduction in the percentage of ROS detection in the irradiated cells and playing a protective role against oxidative stress (Figure 2).

Gallic acid increased alkaline phosphatase in MC3T3-E1 irradiated, indicating osteogenic differentiation

To investigate the osteogenic differentiation capacity of the cells under the two experimental conditions, GA treatment and irradiation, the ALP analysis was assessed in the present study. As shown in Figure 3, it is first important to note that irradiation per se promotes a reduction in ALP expression, as demonstrated by the comparison between irradiated and non-irradiated ODM groups (without GA). Treatment with GA significantly increased ALP expression at a dose of 6 Gy. GA reversed the irradiation-induced effect on ALP and increased its expression, which may favor osteogenic differentiation.

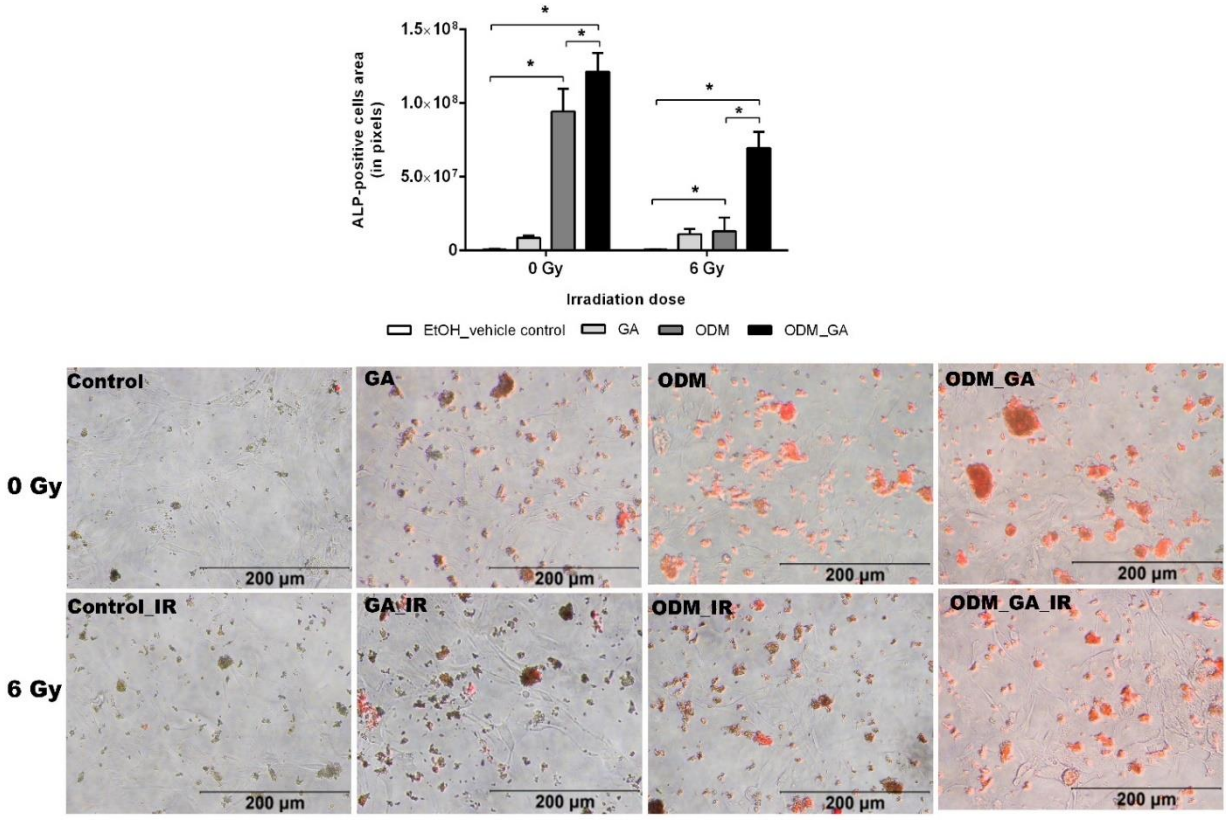

Fig. 3. Staining test for alkaline phosphatase (ALP) in MC3T3-E1 cells. Cells were treated with 10 µM GA and irradiated with 6 Gy of ionizing radiation. In addition to GA treatment, osteogenic differentiation medium (ODM) is required for the *in vitro* osteogenic differentiation assay. Two-way ANOVA test; Asterisks indicate statistical significance, $p<0.05$. GA: gallic acid, EtOH_vehicle: ethanol vehicle control. IR: exposure to ionizing radiation.

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Discussion

The development of complementary therapies that promote bone and soft tissue repair is critical to the treatment of patients undergoing radiation for head and neck cancer. Antioxidants are potential substances for this due to their effects on the cells, such as cell renewal, tissue repair, regulation of oxidative stress on osteoblast, and osteoclast metabolism (20-22). GA is an antioxidant that has been shown to regulate metabolism, has anti-inflammatory effects and has antineoplastic effects by suppressing the proliferation and invasion of cancer cells (13,16). However, its functions on bone cells and its pharmacological potential in bone repair are not yet well understood.

Damage to osteoblasts and osteocytes is thought to be one of the main factors in the reduction in bone mineral density observed after tissue irradiation (6). In this sense, this study has revealed a cytoprotective role of GA against radio-induced injuries, leading to the improvement of proliferation and activity of preosteoblasts and the regulation of increased oxidative stress, which can result in cell death by ionizing in irradiated cells (Figure 4).

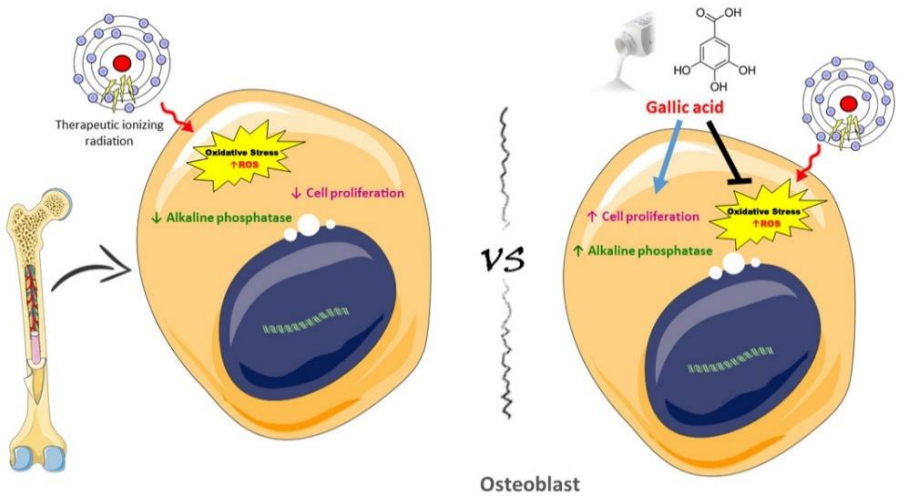

Fig. 4. Schematic representation of the cytoprotective effect of gallic acid against injury promoted by therapeutic ionizing radiation in preosteoblasts; GA at low concentrations significantly increased cell proliferation, decreased ROS formation, and increased alkaline phosphatase expression, to promote osteogenic differentiation.

The biological effects of irradiation on bone are subject to a number of controversies. Some studies have pointed to the harmful effects of ionizing radiation on bone tissue (8, 23). Irradiation has been shown to impair bone formation by interfering with the proliferation and differentiation of osteoblasts, inducing cell cycle arrest, reducing collagen production and increasing sensitivity to apoptotic agents (23). However, other studies describe the positive effects of low doses of radiation on bone cells and the expression of bone-related genes (8,12,24).

These contradictory results may be due to differences in the cell lines, radiation dose and duration of irradiation of the cells. Furthermore, the radiosensitivity of the cell differs depending on the type and stage of differentiation (8,25). In the present study, the proliferation behavior of MC3T3-E1 cells exposed to different doses of ionizing radiation at different times was examined. The cells were kept in culture for 21

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days after irradiation, as this is the time required for osteoblast differentiation (2). During this treatment period, the cells exposed to 2 and 4 Gy were able to significantly increase their cell proliferation. Nevertheless, proliferation activity was significantly lower with 21 days of 6 Gy irradiation than with the other doses.

Ionizing radiation has direct effects on cell proliferation and DNA damage (breaks in single and double strands and damage to the nitrogenous bases) that occur in response to radiation. However, high doses of radiation can cause extensive DNA damage, which is usually difficult to repair and can affect cell cycle progression and apoptosis (26). Irradiation with a dose of 6 Gy resulted in a decrease in the number of MC3T3-E1 cells in the control group, which does not contain gallic acid.

The current study found that treatment of MC3T3-E1 cells with GA at low concentrations significantly increased cell proliferation, while at higher concentrations it reduced cell viability. This result is consistent with a previous study that demonstrated an enhanced osteogenic effect of epigallocatechin-3-gallate at low concentrations in cells from the human alveolar bone, while at higher concentrations it prevents osteogenic differentiation of the cells (27). The results of the ongoing showed that GA at a concentration of 10 µM exerted a cytoprotective effect on preosteoblasts exposed to a single 6 Gy irradiation. It increases cell proliferation and also protects the cells from oxidative stress, as shown by the reduction in the percentage of ROS detection.

The differentiation of osteoblasts is a tightly controlled mechanism governed by specific regulatory factors of bone, and alkaline phosphatase is responsible for stabilizing the bone matrix (27). Despite the controversies about the effect of ionizing radiation on alkaline phosphatase (5, 28), the present study demonstrated that radiation affected the expression of alkaline phosphatase in MC3T3-E1 cells. However, GA acted as a potential inducer of osteoblast differentiation since it caused an increase in ALP.

In summary, the ongoing study highlights GA as an antioxidant that can protect preosteoblasts from injuries caused by ionizing radiation. These results are relevant in view of mainly the bone damage induced by radiotherapy in cancer patients or the use of antiresorptive drugs, which can lead to osteonecrosis. These results emphasize the need for new *in vivo* and clinical studies to better understand the mechanisms involved in the potential radioprotection by GA in osteonecrosis, especially in cancer patients undergoing radiotherapy or taking antiresorptive drugs.

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