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Author(s)	Sakurai, Tomonori; Ito, Michitoshi; Mikamoto, Takahiro; Ohshio, Ryoussuke; Miyakoshi, Junji
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Ionizing irradiation-induced inhibition of differentiation of C3H10T1/2 cells to the osteoblastic lineage

Tomonori Sakurai¹⁾, Michitoshi Ito²⁾, Takahiro Mikamoto²⁾, Ryouusuke Ohshio²⁾, and Junji Miyakoshi¹⁾

*1) Laboratory of Applied Radio Engineering for Humanosphere,
Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Japan*

*2) Department of Radiological Life Sciences, Division of Medical Life Sciences,
Graduate School of Health Sciences, Hirosaki University, Hirosaki, Japan*

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Address correspondence to: Junji Miyakoshi, Professor
Laboratory of Applied Radio Engineering for Humanosphere
Research Institute for Sustainable Humanosphere, Kyoto University
Gokasho, Uji, Kyoto, 611-0011, Japan.

Phone & FAX; +81-774-38-3872

E-mail. miyakoshi@rish.kyoto-u.ac.jp

Address correspondence to: Tomonori Sakurai, Associate Professor
Laboratory of Applied Radio Engineering for Humanosphere
Research Institute for Sustainable Humanosphere, Kyoto University
Gokasho, Uji, Kyoto, 611-0011, Japan.

Phone; +81-774-38-3829

E-mail. sakurai@rish.kyoto-u.ac.jp

Abstract

Purpose: Previous studies using mouse osteoblast derived MC3T3-E1 and mouse myoblast derived C2C12 cells have not completely explained the mechanisms responsible for osteoradionecrosis. Thus, the aim of this study was to advance the *in vitro* experimental approaches for investigations of osteoradionecrosis.

Materials and Methods: The pluripotent stem cell line, mouse embryo derived C3H10T1/2, was treated with all-*trans*-retinoic acid after irradiation (1, 3 and 6 Gy), and cell growth, cell cycle distribution, apoptosis, and alkaline phosphatase (ALP) activity were assessed.

Results: We demonstrated that ionizing radiation inhibited the growth and decreased ALP activity in C3H10T1/2 cells. The decrease in cell growth was not due to apoptosis but was due to cell cycle delay. The decrease in ALP activity persisted in cells that were induced to an osteoblastic lineage 24 h after irradiation.

Conclusions: Our results suggested that C3H10T1/2 cells are suitable for investigating the effects of ionizing irradiation on osteoblast precursor cells.

Introduction

Therapeutic irradiation causes bone damage and may increase the risk of fracture. Osteoradionecrosis of the mandible has been reported as a common late effect during the treatment for cancer of the oral cavity (Glanzmann and Grätz 1995; Schultze-Mosgau et al. 2005). Pelvic irradiation has been reported to increase the risk of pelvic fracture in older women (Baxter et al. 2005). The recent use of magnetic resonance imaging (MRI) for follow-up in patients treated for nasopharyngeal carcinoma revealed that osteoradionecrosis is a more frequent complication than had been thought previously (King et al. 2009). For the treatment and prevention of osteoradionecrosis, hyperbaric oxygen has been used (Marx et al. 1985; Chavez and Adkinson 2001) because tissue hypoxia, hypovascularity, and hypocellularity have been considered as potential causes (Marx 1983). However, the benefit of hyperbaric oxygen has not been proven (Annane et al. 2004). A new theory has been recently proposed (Lyons and Ghazali 2008). A relationship between osteoradionecrosis and osteoblasts has also been suggested (Matsumura et al. 1996; Dare et al. 1997; Dudziak et al. 2000; Szymczyk et al. 2004). Consequently, the precise mechanism underlying the effect of radiation on bone formation has not yet been fully elucidated.

In *in vitro* studies, radiation-induced decreases in DNA content and cell growth were reported, whereas the alkaline phosphatase (ALP) activity, a typical marker of osteoblast differentiation, was increased in the mouse-derived osteoblast cell line, MC3T3-E1 (Matsumura et al. 1996; Dare et al. 1997; Dudziak et al. 2000). The radiation-induced decreases in DNA content and cell growth support the occurrence of osteoradionecrosis. However, the increase in ALP activity does not fully explain the occurrence of osteoradionecrosis, since information regarding osteoblast differentiation from osteoblast precursor cells is lacking. The number of osteoblast cells may increase due to an increase in differentiation from osteoblast precursor cells. A mouse-derived C2C12 myoblast cell line

was used to study the effects of ionizing irradiation. The cell line can be trans-differentiated into osteoblasts by treatment with bone morphogenic protein-2 (BMP-2). Irradiation reduced ALP activity when cells were treated with BMP-2 6 h before or after irradiation (Pohl et al. 2003) and decreased the cell number, expression of ALP and collagen type I mRNA and ALP activity (Sakurai et al. 2007), although radiation effects were not detected 24 h and immediately after irradiation (Ikeda et al. 2000; Pohl et al. 2003). This limited time course for the detection of the effects of ionizing radiation does not fully explain the occurrence of osteoradionecrosis, since the interval between radiation therapy and occurrence of osteoradionecrosis varies.

To resolve these experimental limitations, we investigated the C3H mouse embryo derived cell line, C3H10T1/2, which is known to exhibit pluripotent activity. Treatment with all-*trans*-retinoic acid (RA) or BMP-2 induces osteoblast differentiation (Katagiri et al. 1990; Gazit et al. 1993; Murata et al. 2003; Chan et al. 2003), and is an excellent model for studying osteoblast differentiation, because the expression of ALP activity is obvious (Katagiri et al. 1990). C3H10T1/2 cells have been used to study the effects of ionizing radiation under conditions that do not induce osteoblast differentiation (Miller et al. 2000; Bettega et al. 1998; Pazzaglia et al. 1996). However, the effects of ionizing irradiation in C3H10T1/2 cells under differentiation to the osteoblastic lineage have not yet been investigated.

In this study, we investigated the effects of ionizing radiation on osteoblast differentiation of C3H10T1/2 cells. We directed C3H10T1/2 cells into an osteogenic lineage after ionizing irradiation at 1, 3 and 6 Gy, and evaluated cell growth and osteoblast differentiation based on ALP activity.

Materials and Methods

Cell culture and osteoblast differentiation

C3H10T1/2 cells (clone 8; American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's Basal medium (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; BioWest, Miami, FL, USA). For the osteoblast differentiation study, cells were seeded at a density of 1×10^4 cells/cm² on 6-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA), and cultured for 24 h. Cells were then irradiated with X-rays at 1, 3 or 6 Gy. Immediately or 24 h after X-ray irradiation, the culture medium was changed to Eagle's Basal medium supplemented with 10% FBS and 10^{-6} mol/L RA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to induce osteoblast differentiation. Osteoblast differentiation was allowed to continue for up to 7 days with the medium changed on days 3 and 5.

X-ray irradiation

X-ray irradiation was performed using an X-ray generator (MBR-1520R; Hitachi Medical Corporation, Tokyo, Japan) operating at 150 kVp and 20 mA with 0.5-mm aluminum and 0.1-mm copper filters. The upper surface of the culture medium was placed 50 cm from the X-ray source, and the exposure dose was 1 Gy/min.

Cell number

Cells were seeded at a density of 1×10^4 cell/cm² on 12-well culture plates (Becton Dickinson), and cultured for 24 h. After X-ray irradiation, cells were induced to the osteoblastic lineage and incubated for up to 7 days with the medium changed 3 and 5 days after irradiation. Cells were then washed with calcium and magnesium-free phosphate buffered saline (PBS(-); Takara Bio, Shiga, Japan) and collected by trypsinization. Cells were

counted using an automated cell counter (Countess™; Invitrogen, Carlsbad, CA, USA).

Cell cycle analysis

Cells were seeded at a density of 1×10^4 cell/cm² on 100 mm dishes (Asahi Glass Co., Ltd, Hunabashi, Japan), and cultured for 24 h. After X-ray irradiation, cells were induced to the osteoblastic lineage and incubated for 6 or 24 h before being collected by trypsinization and centrifugation, and then fixed in 70% ethanol (Wako Pure Chemical Industries, Ltd.)/PBS(-) at -20 °C. On the day of the flow cytometric analysis, fixed cells were washed with PBS(-), treated with 250 µg/mL of RNase A (Roche Diagnostics, Basel, Switzerland) at 37 °C for 15 min, and stained with 10 µg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 15 min on ice. The DNA content was analyzed using a flow cytometer (EPICS XL; Beckmann Coulter Inc., Fullerton, CA, USA). More than two thousand cells were analyzed per sample using FlowJo software version 7.6 (Tree Star, Inc., Ashland, OR, USA).

Apoptosis detection using enzyme-linked immunosorbent assay (ELISA)

Apoptosis analysis was performed using an apoptosis ELISA Kit (ApoStrand™; Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA) according to the manufacturer's instructions. The kit is based on the sensitivity of DNA in apoptotic cells to formamide denaturation. Formamide is a gentle denaturing reagent, which denatures DNA in apoptotic cells, but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis (Frankfurt and Krishan 2001). Briefly, cells were seeded at a density of 1×10^4 cell/cm² on 60 mm dishes (Asahi Glass Co., Ltd), and cultured for 24 h. After X-ray irradiation, cells were induced to the osteoblastic lineage and incubated for 72 h before they were collected by trypsinization and centrifugation, and fixed with 14% methanol (Wako Pure Chemical Industries, Ltd.)/PBS(-) at -20 °C. On the day of the apoptosis analysis, 10,000

fixed cells for each group were aliquotted in duplicate onto a 96-well plate (Asahi Glass Co., Ltd), treated with formamide (included in the kit) for 10 min at room temperature followed by 30 min at 56 °C, and incubated with blocking solution (included in the kit) for 1 h. Cells were then incubated with a peroxidase-conjugated antibody to single-strand DNA (included in the kit) followed by incubation with peroxidase substrate (included in the kit). The optical density was recorded using a microplate reader (Sunrise™ Basic; Tecan, Zurich, Switzerland) at a wavelength of 450 nm (620 nm reference).

ALP activity

After differentiation into osteoblasts, the cells were collected using a cell scraper (Asahi Glass Co., Ltd) and extracted with CellLytic™ M cell lysis reagent (Sigma-Aldrich) according to the manufacturer's instructions. The cell extracts were frozen and thawed, and ALP activity was measured using a LabAssay™ ALP kit (Wako Pure Chemical Industries, Ltd.) according to the manufacturer's instructions. The optical density was recorded using a microplate reader (Sunrise™ Basic) at a wavelength of 405 nm (620 nm reference). The total protein content was measured by a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical analysis

The data were analyzed at each time point using Tukey's test for multiple comparisons. Each experiment was repeated independently at least three times, and the data are expressed as the mean±standard deviation (SD).

Results

The effects of ionizing radiation on cell proliferation

X-ray irradiation caused a significant decrease in cell number under osteoblastic differentiation conditions (Figure 1). Three days after irradiation, a dose-dependent decrease in cell number was observed. The cells in the non-irradiated group became confluent at 5 days after irradiation. At this time point, the cell numbers of the 1 or 3 Gy X-ray-irradiated group recovered nearly to the level of the non-irradiated group (1 Gy, $89\pm 15\%$ of the non-irradiated group; 3 Gy, $72\pm 12\%$ of the non-irradiated group; p =not significant). However, cells irradiated with X-rays at 6 Gy did not proliferate as much ($33\pm 3\%$ of the non-irradiated group at 7 days after irradiation).

<Figure 1>

Cell cycle distribution after ionizing radiation

To better define the mechanisms underlying the decrease in cell growth, we evaluated the cell cycle distribution by flow cytometric analysis, 6 and 24 h after ionizing radiation under osteoblastic differentiation conditions. A significant dose-dependent increase in G₂/M phase and a dose-dependent decrease in S phase were induced 6 h after X-ray irradiation (Figure 2A). At 24 h after irradiation, the cell cycle distributions in cells with 1 or 3 Gy X-rays were restored to that observed in non-irradiated cells, whereas the significant increase in G₂/M phase remained in cells exposed to 6 Gy X-rays (Figure 2B).

<Figure 2>

The effects of ionizing radiation on Apoptosis

To more closely investigate the decrease in cell number induced by irradiation, we assessed apoptotic cell death. Preliminary experiments revealed that X-ray-induced apoptotic cells were mostly detected at 72 h after irradiation (data not shown) and that apoptotic cells were detected significantly in cells exposed to 6 Gy X-ray irradiation in the non-differentiation stage, compared with non-irradiated cells (0 Gy absorbance, 0.37 ± 0.03 ; 6 Gy, 0.52 ± 0.07 ; $p=0.002$, Figure 3 RA (-)). Based on these findings, we analyzed apoptotic cells, 72 h after exposure in about 10,000 cells per experimental condition using ELISA. The number of apoptotic cells increased slightly as the irradiation dose increased, however, these differences were not significant (Figure 3).

<Figure 3>

The effects of ionizing radiation on osteoblast differentiation

We evaluated the effects of ionizing radiation on osteoblast differentiation by measuring ALP activity, a typical marker of osteoblast differentiation. The ALP activity was significantly reduced by ionizing radiation in a dose-dependent manner. The reduction was observed until at least day 7, and became more obvious as the duration of differentiation became longer. Since ALP activity was evaluated as "ALP activity/mg protein", and the total protein/well was also reduced in a dose-dependent manner by X-ray irradiation (data not shown), the reduction of ALP activity was clearly caused by a reduction of ALP activity in the cells (Figure 4A). To evaluate the effects of the interval between X-ray irradiation and induction to the osteoblastic lineage, which was reported to affect ALP activity in C2C12 cells (Pohl et al. 2003), we induced osteoblast differentiation 24 h after irradiation. The significant reduction of the ALP activity was still observed in dose-dependent manner at least until day 7 (Figure 4B).

<Figure 4>

Discussion

In this study, we demonstrated that ionizing radiation reduced cell growth (Figure 1) and osteoblastic differentiation in C3H10T1/2 cells (Figure 4). We detected that exposure to 1 Gy X-ray-induced a decrease in cell growth and osteoblast differentiation. This high sensitivity of C3H10T1/2 cells to X-ray irradiation was reported under non-osteoblast differentiation conditions (Calini et al. 2002). The decrease in cell growth was mainly due to cell cycle delay. Altered cell cycle distribution at 6 h after irradiation indicated that G₂ arrest was induced by X-ray irradiation (Figure 2A). The arrest was recovered within 24 h after irradiation at 1 and 3 Gy, but not 6 Gy (Figure 2B). These findings were reflected in cell growth (Figure 1). Three days after irradiation, the dose-dependent decrease in cell number was observed due to G₂ arrest. However, 5 and 7 days after irradiation at 1 or 3 Gy, the decrease in cell number was not clear because cells reached near confluence. At 6 Gy, cell growth was hardly observed.

Ionizing radiation decreased ALP activity in a dose-dependent manner (Figure 4A). The decrease in ALP activity was observed after considering the impact of the radiation-induced decrease in cell number on the ALP activity data. This suggested that the radiation-induced decrease in ALP activity in living cells contributed to radiation-induced decrease in ALP activity. The decrease in ALP activity was observed in cells induced to the osteoblastic lineage 24 h after irradiation (Figure 4B). This finding is a critical difference between our study and the previous study using C2C12 cells (Pohl et al. 2003), and suggested a potential explanation for the varied intervals between the occurrence of osteoradionecrosis after radiation therapy and the suitability of C3H10T1/2 cells for investigation, as an osteoblast precursor cell line. Significant repair of DNA strand breaks was reported 24 h after irradiation (Calini et al. 2002), and cell cycle distributions were restored to those of non-irradiated cells, within 24 h after X-ray irradiation under non-differentiating conditions (data not shown). However, our ALP activity data suggested that the effects of radiation remained in irradiated

cells. Furthermore, no decrease in cell number was observed in C2C12 cells despite the fact that they were irradiated at 20 Gy (Pohl et al. 2003). The main reason for these differences is thought to be the differences in the differentiation state between the 2 cell lines. C3H10T1/2 cells are more immature than C2C12 cells. The increased radiation-sensitivity of C3H10T1/2 compared with C2C12 is consistent with Bergonie-Tribondeau's law.

Many *in vitro* studies of osteoradionecrosis have used MC3T3-E1 cells (Matsumura et al. 1996; Dare et al. 1997; Dudziak et al. 2000; Szymczyk et al. 2004). However, studies using this cell line have only indicated that X-ray irradiation enhanced late differentiation of osteoblast cells, and did not indicate the mechanisms of reduction of bone cells. Our study showed that radiation decreased the growth of pluripotent osteoblast precursor cells and their differentiation to osteoblast cells. In addition to studies using MC3T3-E1 cells, our results provide an advanced experimental approach to investigate osteoradionecrosis *in vitro*.

The mechanisms of osteoblastic differentiation of C3H10T1/2 cells treated with RA have recently been reported (Wiper-Bergeron et al. 2007). They demonstrated that RA stimulates the dissociation of CCAAT/enhancer binding protein (C/EBP) β and promotes expression of runt-related gene 2 (Runx2). Since retinoic acid receptors α and γ have been suggested to mediate the effects of RA, it is important to evaluate the effects of ionizing radiation on the expression and binding activity of retinoic acid receptors and the expression of Runx2 to further understand the effects. These could be the targets of future investigations.

In this study, we demonstrated that X-ray radiation decreased cell growth and ALP activity in C3H10T1/2 cells induced to the osteoblastic lineage immediately and 24 h after irradiation. Our approach is suitable for studies of the effects of radiation on osteoblast precursor cells, which reinforces the *in vitro* experimental approach of studying osteoradionecrosis. Further studies of the molecular mechanisms are ongoing.

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Figure legends

Figure 1. The effects of ionizing irradiation on cell growth. Osteoblast differentiation was induced after exposure to ionizing irradiation at 1, 3, and 6 Gy and cells were subsequently cultured for 3, 5, and 7 days. The number of cells was then counted using Countess. Data are expressed as the mean±SD for n=4 independent experiment. ** $p < 0.01$ to 0 Gy.

Figure 2. The effects of ionizing irradiation on cell cycle distribution. Cell cycle distributions were analyzed 6 (A) and 24 h (B) after exposure to ionizing irradiation at 1, 3 and 6 Gy. The results of cell cycle analysis by FlowJo software are shown. Data are expressed as the mean±SD for n=4 independent experiment. * $p < 0.05$, ** $p < 0.01$ to 0 Gy.

Figure 3. The effects of ionizing irradiation on apoptosis. Osteoblast differentiation was or was not induced (RA (+) vs. (RA (-), respectively) after ionizing irradiation at 1, 3, and 6 Gy and cells were subsequently cultured for 3 days. Apoptotic cells were analyzed using an ELISA method, as described in the *Materials and Methods*. Data are expressed as the mean±SD for n=3 independent experiment. ** $p < 0.01$ to 0 Gy.

Figure 4. The effects of ionizing irradiation on alkaline phosphatase (ALP) activity in cells induced to the osteoblastic lineage immediately (A) and 24 h (B) after irradiation. Osteoblast differentiation was induced after ionizing irradiation at 1, 3, and 6 Gy and subsequent culture for 3, 5, and 7 days. Data are expressed as the mean±SD for n=3 independent experiment. * $p < 0.05$, ** $p < 0.01$ to 0 Gy.

Figure 1

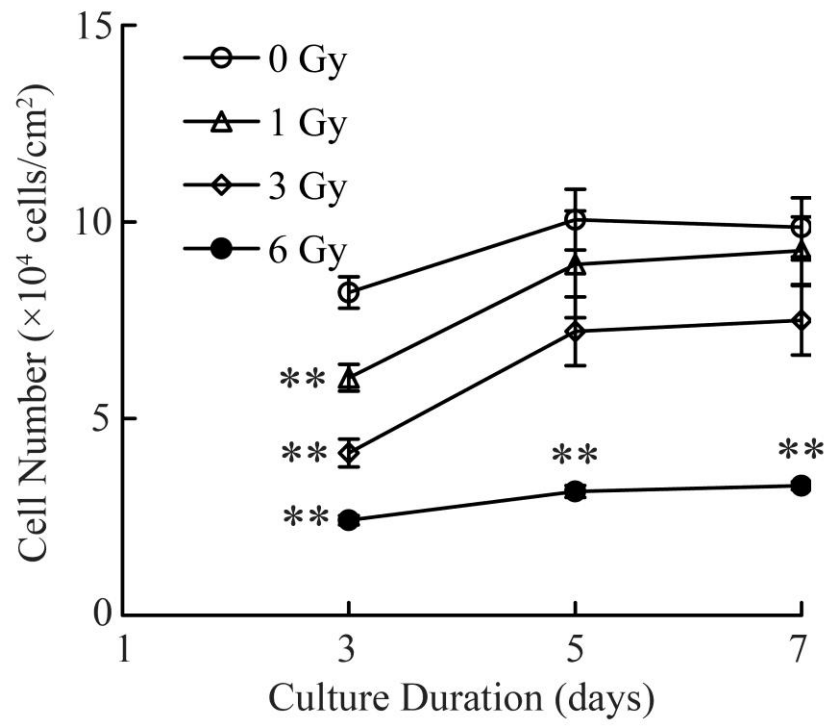


Figure 2

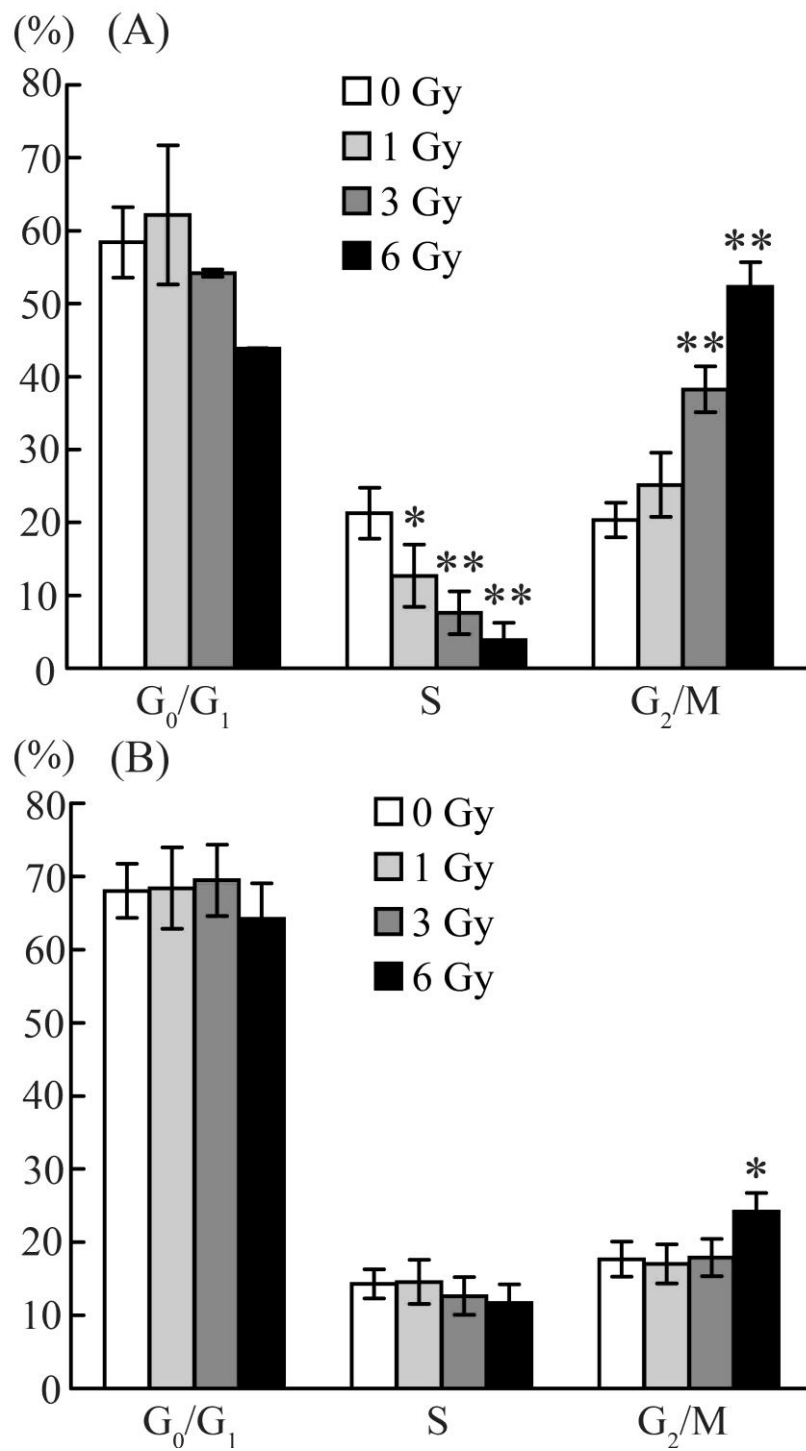


Figure 3

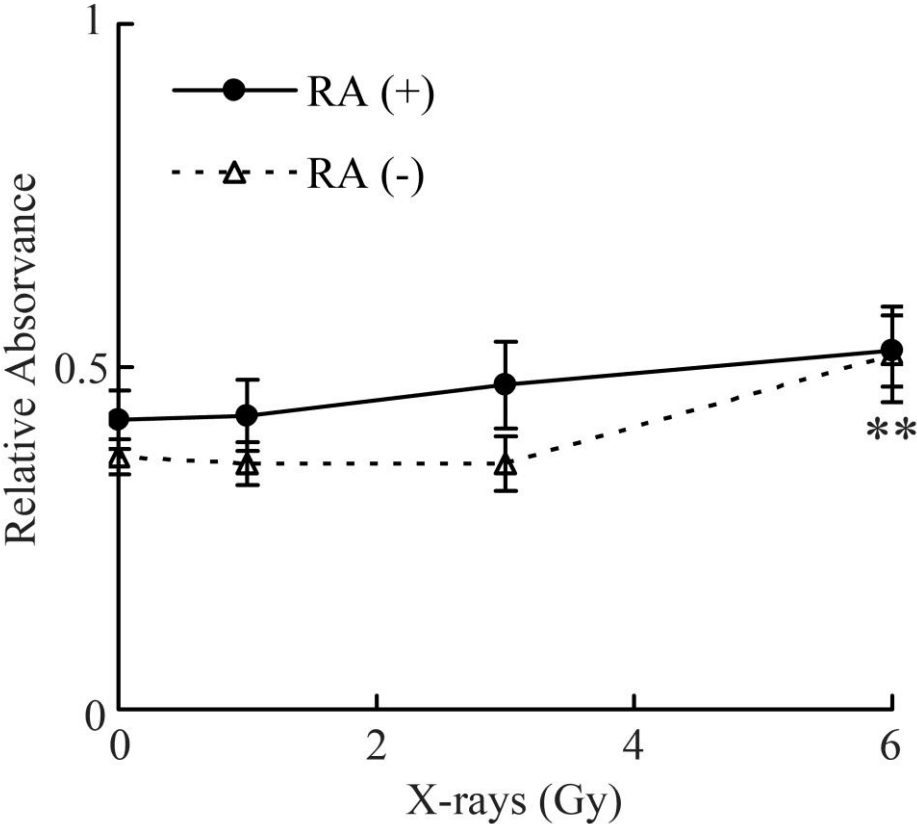
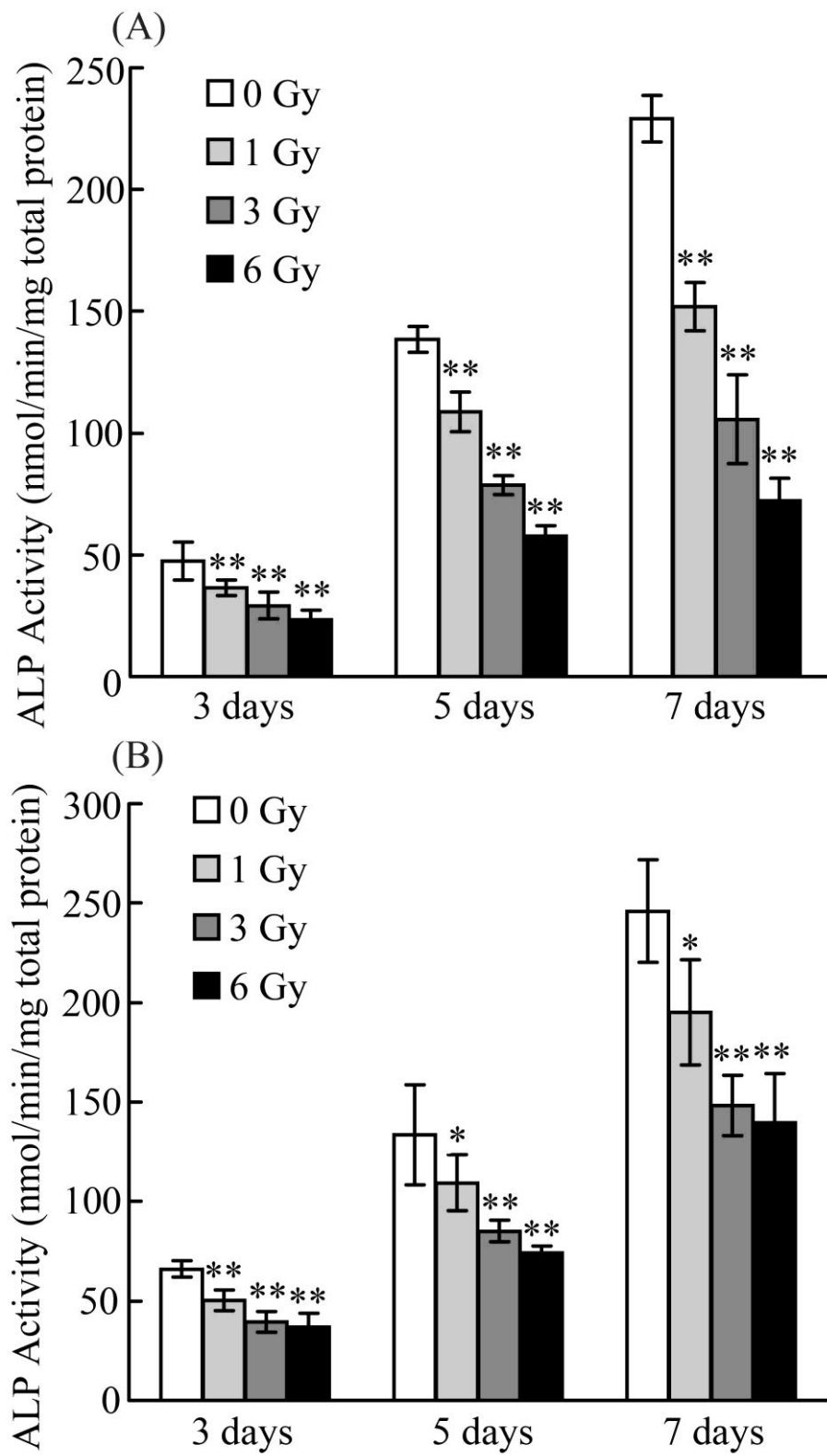


Figure 4



Declaration of Interest

The authors report no declaration of interest.