Melatonin enhancement of the radiosensitivity of human breast cancer cells is associated with the modulation of proteins involved in estrogen biosynthesis

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Abstract

Enhancing the radiosensitivity of cancer cells is one of the most important tasks in clinical radiobiology. Endocrine therapy and radiotherapy are two cancer treatment modalities which are often given together in patients with locally-advanced breast cancer and positive hormone-receptor status. Oncostatic actions of melatonin are relevant on estrogen-dependent mammary tumors. In the present study, we wanted to evaluate the effects of the combination of ionizing radiation and melatonin on proteins involved in estrogen biosynthesis in breast cancer cells. We demonstrated a role of melatonin in mediating the sensitization of human breast cancer cells to the ionizing radiation by decreasing around a 50% the activity and expression of proteins involved in the synthesis of estrogens in these cells. Thus, melatonin pretreatment before radiation reduces the amount of active estrogens at cancer cell level. Melatonin 1 nM induced 2-fold change in p53 expression as compared to radiation alone. The regulatory action of melatonin on p53 could be a link between melatonin and its modulatory action on sensitivity of breast cancer cells to ionizing radiation. These findings may have implications for designing clinical trials using melatonin and radiotherapy.

Key Words: Melatonin, breast cancer, MCF-7 cells, radiation, aromatase, sulfatase, 17β-hydroxysteroid dehydrogenase type 1.

1. Introduction

Melatonin is the main secretory product of the pineal gland that participates in the regulation of important physiological and pathological processes, such as the regulation of neoplastic cell growth. Oncostatic actions of melatonin are relevant on tumors corresponding to the mammary gland and especially on estrogen-dependent mammary tumors [1-6]. Evidence from *in vivo* studies on animal models and *in vitro* studies on breast cancer cell lines supports the hypothesis that melatonin's oncostatic effects on hormone-dependent mammary tumors are mainly dependent on its ability to interact with the estrogen-signalling pathway [5,7]. At the mammary tumor cell level, melatonin interferes with the estrogen receptor, modulates estrogen binding, DNA-binding, and transcriptional activity, and counteracts the effects of estrogens, thus behaving as a selective estrogen receptor modulator. Melatonin, at physiological concentrations, is also able to modulate the synthesis and transformation of androgens and biologically active estrogens in human breast cancer cells, through the inhibition of aromatase, sulfatase and 17β-hydroxysteroid dehydrogenase type 1 activity and expression, and the stimulation of sulfotransferase activity and expression, enzymes involved in the estradiol formation in breast cancer cells, thus behaving as a selective estrogen enzyme modulator [8-10].

The identification of estrogen's central role in mammary carcinogenesis has led to investigation of estrogen pathways as major targets for breast cancer therapy. Breast-conserving surgery, radiotherapy for the affected breast, and adjuvant hormone therapy using antiestrogens, like tamoxifen, or aromatase inhibitors, like letrozol, are standard treatments for postmenopausal early breast

cancer patients with hormone receptor-positive tumors [11,12]. Radiotherapy is considered a standard treatment option after surgery and adjuvant endocrine therapy is also universally used [11]. Timely initiation of radiation therapy is critical and contributes to a good recovery. Tamoxifen and letrozole are the current first-line endocrine therapy drugs. Tamoxifen, a selective estrogen receptor modulator, has provided a significant improvement in both local control and global survival rates [11,13]. Nowadays, it is estimated that tamoxifen may reduce the annual breast cancer death rate by 31% in estrogen receptor-positive mammary tumors [13,14]. *In vivo* studies have shown a synergistic effect of concurrent antiestrogen treatment and radiotherapy on tumor cells [12-14]. Aromatase inhibitors of third generation, which were introduced in the late 1990s, have proven to be superior to tamoxifen for postmenopausal patient [15]. Although limited data on the use of aromatase inhibitors with radiotherapy are available, Azria et al. [16] described that letrozole, a non-steroid selective aromatase inhibitor, sensitizes breast cancer cells to ionizing radiation and Ishitobi et al. [17] described that the administration of aromatase inhibitors after and concurrently with radiotherapy in patients with breast cancer are both reasonable treatment options.

Since melatonin has both antiestrogen and antiaromatase properties [4,5,8], we thought about the possibility that melatonin may regulate the sensitivity of human breast cancer cells to radiotherapy. Recently, our group demonstrated that melatonin pretreatment before radiation sensitizes breast cancer cells to the ionizing effects of radiation by decreasing cell proliferation, inducing cell cycle arrest and downregulating proteins involved in double-strand DNA break repair [18]. In the present study we investigated its effects on

radiated human breast cancer cells by regulating proteins involved in estrogen biosynthesis.

2. Materials and methods

2.1. Cells and culture conditions

MCF-7 human breast cancer cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). They were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France), penicillin (20 units/ml) and streptomycin (20 µg/ml) (Sigma-Aldrich, Madrid, Spain) at 37ºC in a humid atmosphere containing 5% CO_2.

2.2. Ionizing radiation treatment

MCF-7 cells were exposed to X irradiation using a model YXLON SMART 200 tube (Yxlon International, Hamburg, Germany) at room temperature. Based in a previous study [18], we used 8 Gy radiation as the optimal radiation dose. The radiation was delivered as a single dose of 8 Gy in an 11.5 cm x 8.5 cm field size, 96-multiwell plates, at a dose rate of 0.92 Gy/min. The source-half-depth distance was initially calculated to obtain a constant dose rate of 0.92 Gy/min. Control cells were removed from the incubator and were place for the same period of time into the irradiator but without radiation.

2.3. Measurement of cellular proliferation and cytotoxicity

Cells were initially cultured for 24 h in DMEM supplemented with 0.5% dextran-charcoal stripped FBS (csFBS) and then media were replaced by fresh ones with 10% FBS and containing either 1 mM, 10 μM or 1 nM melatonin (Sigma-Aldrich, Madrid, Spain) and/or vehicle (ethanol at a final concentration lower than 0.0001%). Plates were incubated at 37ºC for 1 week in these media. Then, cells were seeded into 96-multiwell plates at a density of 8 x 10^3 cells per well in DMEM supplemented with 10% FBS and incubated at 37ºC for 24 h to allow for cell attachment before irradiation. After irradiation cells were cultured for 6 days. Cell proliferation was measured by the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method [19], reading absorbance at 570 nm in a microplate reader (Labsystems Multiskan RC 351, Vienna, VA, USA). MTT was obtained from Molecular Probes Inc. (Eugene, OR, USA).

The effect of melatonin pretreatment before radiation on cellular survival was measured in a clonogenic assay. Control and melatonin (1 nM, 10 μM or 1 nM) pretreated (a week) MCF-7 cells were cultured in a bilayer soft-agar system modified from that described by Hamburger and Salmon [20,21]. Briefly, the bottom layer consisted of DMEM enriched with FBS, penicillin (20 units/ml) and streptomycin (20 μg/ml). A 3% suspension of hot agar (70-75ºC) was added to the enriched DMEM to make a final concentration of 0.5% agar. One ml of the resultant suspension (40-42ºC) was then dispensed into a 35-mm^2 petri dish and allowed to cool to room temperature. The upper layer consisted of the same medium as the bottom layer and 3% agar was added to make a final concentration of 0.6%. Plating was performed by aspirating 0.5 ml of the cell suspension (1.75 x 10^5 viable cells) followed by 0.5 ml of the upper layer

suspension (40-42ºC) and dispensing the 1 ml on top of the lower layer. Cells were then incubated at 37ºC with 5% CO_2 and 100% humidity. Then, cells were exposed to 8 Gy irradiation and colonies > 150 μm were stained with crystal violet and counted 10 days after plating. The survival fraction was calculated according to the percentage of survival fraction of control non-radiated cells.

2.4. Measurement of cellular aromatase activity

Aromatase activity in MCF-7 cells was measured by the tritiated water release assay which is based on the formation of tritiated water during aromatization of a labeled androgenic substrate such as [1β-3H(N)]-androst-4-ene-3,17-dione [22]. Control and melatonin (1 nM, 10 μM or 1 nM) pretreated (a week) MCF-7 cells were seeded into six-well plates at a density of (1.5×10^5 cells / well) in DMEM supplemented with 10% FBS, 20 units/ml penicillin and 20 μg/ml streptomycin and incubated at 37ºC for 24 h in a humid atmosphere containing 5% CO_2, to allow for cell attachment before irradiation. Then, cells were irradiated and after 24 hours, media were aspirated and changed to serum-free media supplemented with 0.5% csFBS and containing the labeled substrate 300 nM [1β-3H(N)]-androst-4-ene-3,17-dione (NEN Life Science Products, Boston, MA, USA) (75-80 Ci/nM). After 24 hours, six-well plates were placed on ice for 15 min to condense any water vapor and media were transferred to tubes containing 0.25 ml ice-cold 30% tricholoroacetic acid (w/v), vortexed and centrifuged at 1700 g for 20 min. The supernatants were extracted with chloroform, vortexed and centrifuged at 1700 g for 20 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal (Sigma-Aldrich, Madrid, Spain), vortexed, centrifuged at 1700 g for 20 min. and

the supernatants were added to vials with scintillation cocktail and counted in a beta counter (Beckman LS 6000 IC, Fulleton, CA, USA). The amount of radioactivity measured in [^{3}H]-water was corrected by substracting the blank values from each sample, obtained by incubating dishes containing medium with the tritiated androgen but no cells. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H]-water (NEN Life Science Products, Boston, MA, USA) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always higher than 85%.

2.5. Measurement of steroid sulfatase (STS) activity

STS activity in MCF-7 cells was assayed by the formation of estrone from a labelled substrate ([6,7-^{3}H(N)]-estrone sulfate ammonium salt) [23]. Control and melatonin (1 nM, 10 μM or 1 nM) pretreated (a week) MCF-7 cells were seeded into six-well plates at a density of (1.5 x 10^5 cells / well) in DMEM supplemented with 10% FBS, 20 units/ml penicillin and 20 μg/ml streptomycin and incubated at 37ºC for 24 h in a humid atmosphere containing 5% CO_2, to allow for cell attachment before irradiation. Then, cells were irradiated and after 24 hours, media were aspirated and replaced with fresh ones (1 ml per plate) containing 5 pM [6,7-^{3}H(N)]-estrone sulfate ammonium salt (NEN Life Science Products, Boston, MA, USA) (57.3 Ci / mM). After 24 h of incubation, culture dishes were placed on ice for 15 min to condense any water vapour and the media were transferred to tubes containing 5 ml of toluene, vortexed and centrifuged at 1000*xg* for 10 min. The resulting organic phase was added to

vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity measured in the [^{3}H]-toluene was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium but no cells with the tritiated estrone. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the procedure of incubation and processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H] estrone (NEN Life Science Products, Boston, MA, USA) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always higher than 92%.

2.6. Measurement of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) activity

Activity of 17β-HSD1 was assayed in MCF-7 cells by the formation of E_2 from a labelled substrate [2,4,6,7-^{3}H(N)]-estrone [24]. Control and melatonin (1 nM, 10 μM or 1 nM) pretreated (a week) MCF-7 cells were seeded into six-well plates at a density of (1.5 x 10^5 cells / well) in DMEM supplemented with 10% FBS, 20 units/ml penicillin and 20 μg/ml streptomycin and incubated at 37ºC for 24 h in a humid atmosphere containing 5% CO_2, to allow for cell attachment before irradiation. Then, cells were irradiated and after 24 hours, media were aspirated and changed for fresh ones (1 ml per plate) containing 2 nM [2,4,6,7-^{3}H(N)]-estrone (NEN Life Science Products, Boston, MA, USA) (100 Ci / mM). After 30 min of incubation, the media were transferred to tubes containing 4 ml of diethyl ether, vortexed and centrifuged at 800*xg* for 5 min. The aqueous phase was frozen and the resulting organic phase was decanted,

evaporated in tubes containing 50 μg of estradiol. The residue was resuspended in diethyl ether and separated by TLC using dichloromethane/ethyl acetate (4:1; v/v). Once the spots had been visualised, excised and eluted with methanol, they were counted in a liquid scintillation counter. Values were corrected for blanks and tritium recovery (higher than 82%) as described for STS activity.

2.7. Measurement of mRNA expression of STS, 17β-HSD1, aromatase, p53 and aromatase promoter regions pI.3, pII and pI.7 gene expression

Expression of the mRNA from different enzymes (aromatase, STS and 17β-HSD1), p53 mRNA expression and aromatase mRNA promoter regions pI.3, pII and pI.7 expression in MCF-7 cells were carried out by real-time reverse transcription (RT-PCR) after incubation of control and melatonin (1 nM, 10 μM or 1 nM) pretreated cells for 6 h after irradiation. The total cellular RNA was isolated from MCF-7 cells and purified with the Nucleospin RNA II kit (Macherey-Nagel. GmbH & Co. Düren, Germany) following the manufacturer's instructions. The absorbance ratio A260nm/A280nm was greater than 1.9. For cDNA synthesis, 1 μg of total RNA was denaturated at 65ºC for 10 min and reverse-transcribed for 30 min at 45ºC with the Tetro cDNA synthesis Kit (Bioline, London, UK) in a final volume of 20 μl in the presence of 500 ng of oligo (dT)12–18 primer. Real time PCRs were performed in an MX3005 (Agilent, CA, USA) using Brilliant® III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, CA, USA) following the manufacturer's instructions. The sets of human oligonucleotides (Sigma Genosys Ltd., Cambridge, UK) used as primers are indicated in table I. Ribosomal subunit (S14) mRNA

expression was used for normalization. Amplifications were initiated by 1 cycle at 95ºC for 3 min and then performed for 40 cycles for quantitative analysis using the following temperature profile: 95ºC for 20 sec (denaturation); 60ºC for 20 sec (annealing/extension), setting the instrument to detect and report fluorescence at each cycle during the 60ºC annealing/extension step. Each reaction was run in triplicate. Melting curves were performed by using dissociation curve to verify that only a single product with no primer-dimers was amplified. For the primers used there were no differences between transcription efficiencies, and the fold-change in each sample was calculated by the $2^{-\Delta\Delta}$Ct method [25].

2.8. Statistical analysis

The data are expressed as the mean ± standard errors of the mean (SEM) of six independent experiments. Statistical differences between groups were analyzed by using one way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. Results were considered as statistically significant at $p<0.05$.

3. Results

3.1. Effects of ionizing radiation and melatonin on cell proliferation and cytotoxicity

With radiation alone, the MCF-7 cell proliferation decreased in a dose-dependent manner and for 8 Gy radiation, the inhibition of cell proliferation was 40% with respect to control non-radiated cells (Fig. 1A). Treatment with physiologic concentrations of melatonin (1 nM) for 7 days before radiation led to

a significantly ($p<0.001$) higher decrease in cell proliferation in comparison with control radiated cells. Pretreatment with pharmacologic concentrations of melatonin (1 mM and 10 μM) also induced a marked decrease in cell proliferation but was less effective than pretreatment with physiologic concentrations (1 nM) (Fig. 1A).

Figure 1B shows the effect of ionizing radiation and melatonin pretreatment on cytotoxicity. The survival fraction after 8 Gy irradiation dose was reduced 84% with respect to non-radiated cells. Pretreatment with melatonin (1 mM, 10 μM and 1 nM) led to a significantly biggest decline in survival rates, with melatonin concentrations (1 mM) being the most effective in decrease the survival fraction (50 % of reduction when compared to control radiated cells).

3.2. Effects of ionizing radiation and melatonin on aromatase activity and expression and aromatase promoter regions pI.3, pII and pI.7 expression

With 8 Gy radiation alone, aromatase activity was significantly ($p<0.001$) decreased by 40%. Treatment with physiologic concentrations of melatonin (1 nM) for 7 days before radiation led to a significantly higher decrease in aromatase activity (Fig. 2A) (70% of reduction when compared to control non-radiated cells). Pharmacologic concentrations of melatonin (1 mM) were also effective to reduce ($p<0.001$) the aromatase activity of breast cancer cells (Fig. 2A). With the aim of determining whether the inhibitory effect of melatonin on aromatase activity is due to the downregulation of aromatase expression, we irradiated control and melatonin pretreated MCF-7 cells and after 6 h total RNA was isolated to perform real-time quantitative PCR with specific primers for

human aromatase. Radiation (8 Gy) alone significantly decreased ($p<0.001$) in a half aromatase RNA expression in MCF-7 cells (Fig. 2B). Physiologic concentrations of melatonin pretreatment before radiation resulted in a significantly higher decreased in aromatase mRNA expression (50% of control radiated cells). Very high pharmacologic concentrations of melatonin (1 mM) were also effective to reduce ($p<0.001$) the aromatase mRNA expression of breast cancer cells (Fig. 2B).

To determine the promoters involved in the melatonin control of aromatase expression, we used competitive RT-PCR to amplify each of the promoter-specific transcripts from RNA extracted from MCF-7 cells. As shown in Figure 3, melatonin 1 nM exerted a significant ($p<0.001$) inhibition in aromatase expression that is specific to the aromatase promoters I.3, pII and pI.7. Higher concentrations of melatonin were also effective to reduce ($p<0.001$) the aromatase expression specific to the aromatase promoters I.3, pII and pI.7 (Fig. 3).

3.3. Effects of ionizing radiation and melatonin on STS activity and expression

In a second set of experiments, the effects of melatonin treatment before radiation on the STS activity and mRNA expression were studied. With radiation alone a significant ($p<0.001$) decrease in STS activity (30% less activity), the enzyme responsible for the conversion of estrogens to the less potent sulfate conjugated forms, was observed (Fig. 4A). When MCF-7 cells were pretreated with physiologic concentrations of melatonin (1 nM) the inhibition of STS activity was 50% with respect to control radiated cells. Pretreatment with higher

concentrations of melatonin was also effective to reduce ($p<0.001$) the STS activity (Fig. 4A).

Radiation alone significantly decreased the expression of STS by 25%. Treatment with melatonin for 7 days before radiation led to a significantly greater decrease in STS mRNA expression compared with radiation alone and doubled the inhibition (Fig. 4B).

3.4. Effects of ionizing radiation and melatonin on 17β-HSD1 activity and expression

Next, the effects of melatonin treatment before radiation on the 17β-HSD1 activity and mRNA expression were studied. With radiation alone a significant ($p<0.001$) decrease in 17β-HSD1 activity, the enzyme responsible for the conversion of the relatively inactive estrone to the most potent 17β-estradiol, was observed (Fig. 5A). When MCF-7 cells were pretreated with physiologic concentrations of melatonin (1 nM) the inhibition of 17β-HSD1 activity was twice as much as that of control radiated cells. Pretreatment with higher concentrations of melatonin (1 mM) was even more effective to reduce ($p<0.001$) the 17β-HSD1 activity (Fig. 5A).

Radiation alone significantly also decreased by 20% the expression of 17β-HSD1. Treatment with melatonin for 7 days before radiation led to a significantly greater decrease in 17β-HSD1 mRNA expression compared with radiation alone (Fig. 5B), with physiologic melatonin concentrations (1 nM) being the most effective in decrease the 17β-HSD1 mRNA expression (a half of expression than radiated cells).

3.5. Effects of ionizing radiation and melatonin on p53 mRNA expression

With 8 Gy radiation alone, the expression of mRNA p53 increased two-times ($p<0.001$). Pretreatment with physiologic concentrations of melatonin (1 nM) for 7 days before radiation led to a significantly higher increase in p53 mRNA expression, three-times as big as control non-radiated cells (Fig. 6). Higher concentrations of melatonin (10 μM) were also effective to increase ($p<0.001$) p53 mRNA expression of breast cancer cells (Fig. 6).

4. Discussion

Endocrine therapy and radiotherapy are two cancer treatment modalities which are often given together in patients with locally-advanced breast cancer and positive hormone-receptor status. Enhancing the radiosensitivity of cancer cells whilst preserving the health of normal cells is one of the most important tasks in clinical radiobiology. Preclinical models have described changes in tumor cell kinetics and reduced tumor cell death with concurrent antiestrogen treatment and radiotherapy [12]. Preliminary results from simultaneous treatment with aromatase inhibitors and radiation also indicate that this combination of endocrine and radiation therapy could enhance cytotoxicity and improve tumor response [12,13]. Melatonin, the principal hormone synthesized and produced in the pineal gland, has been extensively reported as an important inhibitor of neoplastic proliferation, especially on tumors of the mammary gland, most of which are estrogen-responsive [1-4,26-28]. At cancerous cellular level, melatonin, on one hand, interferes with the estrogen-dependent pathways and partially abolishes the effects of estrogens, thus behaving as a selective estrogen receptor modulator and, on the other hand, is also able to modulate

both the catalytic activity and gene expression of some enzymes participating in the local synthesis of estrogens in human breast cancer and also in proximal vascular endothelial and adipose cells, thus behaving as a selective estrogen enzyme modulator [1,7,27-33]. Our group have described that melatonin, *in vitro,* could act as a radiosensitizer. Treatment for one week before radiation with melatonin, enhances the sensitivity of breast cancer cells to the ionizing effects of radiation by decreasing cell proliferation, inducing cell cycle arrest and downregulating proteins involved in double-strand DNA break repair [18]. In the present study, we wanted to evaluate *in vitro* the effects of the combination of ionizing radiation and melatonin on proteins involved in estrogen biosynthesis in malignant epithelial cells. Our results demonstrate that a combined treatment with melatonin and ionizing radiation could result in cooperative enhancement of cytotoxic and radiosensitizing effects in human breast cancer cells and that this enhancement of the radiosensitivity is associated with the modulation of enzymes involved in estrogen synthesis. Pretreatment of human breast cancer cells with melatonin one week before radiation led to a significantly higher decrease of MCF-7 cell proliferation (MTT assay) and survival fraction (clonogenic assay) compared with radiation alone. Those findings confirm previous results of our group [19] suggesting that the addition of melatonin promotes inhibition of cell proliferation and enhances the effects of radiation. Similarly to our findings, the sensitivity and dose-response characteristics of breast cancer cells to the inhibitory effect of melatonin are altered by the growth of cells in an anchorage-dependent culture as compared to anchorage-independent growth [1,2,21]. In proliferation assays (monolayer cultures) melatonin concentrations close to 1 nM, similar to those found in the serum of

most mammals during the night, are the most effective [1,2]. However, in the clonogenic assay, in which cancer cells grow suspended in a semisolid medium without attaching to the plate, pharmacological concentrations produce a maximal inhibition [21]. Cell attachment modify the sensitivity of MCF-7 cells towards melatonin. In addition, ionizing radiation decreased aromatase activity and expression, the enzyme that transform androgens into estrogens in malignant cells. Melatonin pretreatment before radiation induced a higher decrease in aromatase activity and expression. In disease-free breast tissue, aromatase is primarily expressed in adipose stromal fibroblasts via the relatively weak promoter I.4 [34,35]. However, in breast cancer tissue, aromatase promoters I.3 and II are activated, leading to a marked increase in aromatase expression in malignant epithelial cells and adipose fibroblasts. Both promoters are considered the major drivers of aromatase expression in breast cancer and surrounding adipose tissue, accounting for 80-90% of total aromatase expression [34,35]. The present study demonstrates that melatonin-dependent regulation of aromatase gene expression occurs mainly through a downregulation of gene expression of the both major specific aromatase promoter regions pII and pI.3 in breast cancer cells. Ionizing radiation inhibited aromatase promoter I.3 and II mediated expression and melatonin pretreatment enhanced the down-regulatory action on aromatase promoters I.3 and II induced by radiation. Aromatase pII was the most abundantly expressed compared to pI.3 and melatonin inhibitory action was more potent on this aromatase promoter region pII. Also, melatonin further enhanced the decrease triggered by radiation of the aromatase promoter region pI.7 expression, a novel breast cancer-associated aromatase promoter mainly active in vascular

endothelial cells but also present in malignant epithelial cells [36]. Besides this melatonin down-regulatory effect on aromatase activity and expression and on the two main promoters driving aromatase expression in breast cancer, in our study, melatonin also modulates the activity and expression of the STS and 17β-HSD1. The estrone sulfatase pathway is the major route of estrogen formation in breast tumors and the estrogen sulfatase activity is 40-500 times higher than aromatase activity in breast cancer tissues [37,38]. Radiation alone decreased STS and 17β-HSD1 activity and expression and melatonin pretreatment for one week before radiation enhanced the decrease in STS and 17β-HSD1 activity and expression induced by ionizing radiation. Thus, melatonin pretreatment by down-regulating aromatase, STS and 17β-HSD1 might reduce active estrogens levels at tumoral cell level.

The tumour suppressor p53 is a central factor for the maintenance of genome stability and, consequently, for the suppression of cancer [39]. p53 is upregulated and activated by genotoxic stress, like ionizing radiation, and induces a transcriptional program with effectors promoting apoptosis, cell cycle arrest, senescence and DNA repair [40,41]. There is growing evidence for a direct role of p53 in the fidelity control of DNA repair to ensure error-free DNA double-strand breaks repair and, therefore, to suppress detrimental genome rearrangements that drive cancer [39,42]. p53 modulates DNA repair and may assist in the down-regulation of inappropriate recombination in particular through direct interaction and inhibition of repair proteins such as RAD51 [43]. In addition it has been recently demonstrated that p53 is a negative regulator of aromatase in the breast [44]. Radiation alone increased p53 expression. Pretreatment of breast cancer cells with physiologic concentrations of melatonin

before radiation led to a greater increase of p53 expression. The up-regulation of the expression of p53 and p21WAF1 proteins induced by melatonin has been suggested as an important mechanism through which this indolamine produces antiproliferative effects in human breast cancer cells [45]. Since melatonin pretreatment before radiation sensitizes breast cancer cells to the ionizing effects of radiation by decreasing cell proliferation, inducing cell cycle arrest, downregulating proteins involved in double-strand DNA break repair and estrogen biosynthesis, and p53 promotes cell cycle arrest, DNA repair and aromatase down-regulation, the regulatory action of melatonin on p53 could be a link between melatonin and its modulatory action on sensitivity of breast cancer cells to the ionizing radiation.

In summary, the findings present herein suggest that melatonin enhancement of the radiosensitivity of human breast cancer cells is associated with the modulation of proteins involved in estrogen biosynthesis.

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Figure captions

Fig. 1. Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on MCF-7 cell proliferation and survival fraction. (A) Control and melatonin pretreated MCF-7 cells for a week were seeded into 96-multiwell plates in DMEM supplemented with 10% FBS and incubated at 37ºC for 24 h. Then, cells were irradiated, cultured for 6 days and cell proliferation was measured by the MTT method. (B) Control and melatonin pretreated MCF-7 cells for a week were cultured in a in a bilayer soft-agar system in DMEM supplemented with 10% FBS and agar. After 24 h of incubation at 37ºC, cells were irradiated, cultured for 10 days and colonies > 150 µm were stained and counted. Survival fraction was calculated as described in material and methods. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.001$ *vs* 1 mM melatonin; d, $p<0.001$ vs 10 µM melatonin.

Fig. 2. (A) Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on aromatase activity of MCF-7 cells. Control and melatonin pretreated MCF-7 cells for a week were seeded into six-well plates in DMEM supplemented with 10% FBS. After 24 h cells were irradiated and 24 h later media were aspirated and changed to serum-free media supplemented with 0.5% csFBS and tritiated androstenedione. Aromatase activity was determined after 24 h of incubation, as described in Material and methods. (B) Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on the expression of mRNA aromatase in MCF-7 cells. Aromatase mRNA expression was carried out by RT-PCR after incubation of control and melatonin (1 nM,

10 μM or 1 nM) pretreated cells for 6 h after irradiation. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.001$ *vs* 1 mM melatonin.

Fig. 3. Effects of melatonin pretreatment (1 mM, 10 μM or 1 nM) and ionizing radiation on aromatase promoter regions pI.3 (A), pII (B) and pI.7 (C) expression in MCF-7 cells. Control and melatonin pretreated cells were cultured for 6 h after irradiation. Total mRNA was extracted from MCF-7 cells and reverse transcribed. cDNA was subjected to PCR using specific primers for aromatase promoter I.3, II and I.7 or S14. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.01$ *vs* control radiated cells; d, $p<0.001$ vs 1 mM melatonin; e, $p<0.001$ *vs* 10 μM melatonin.

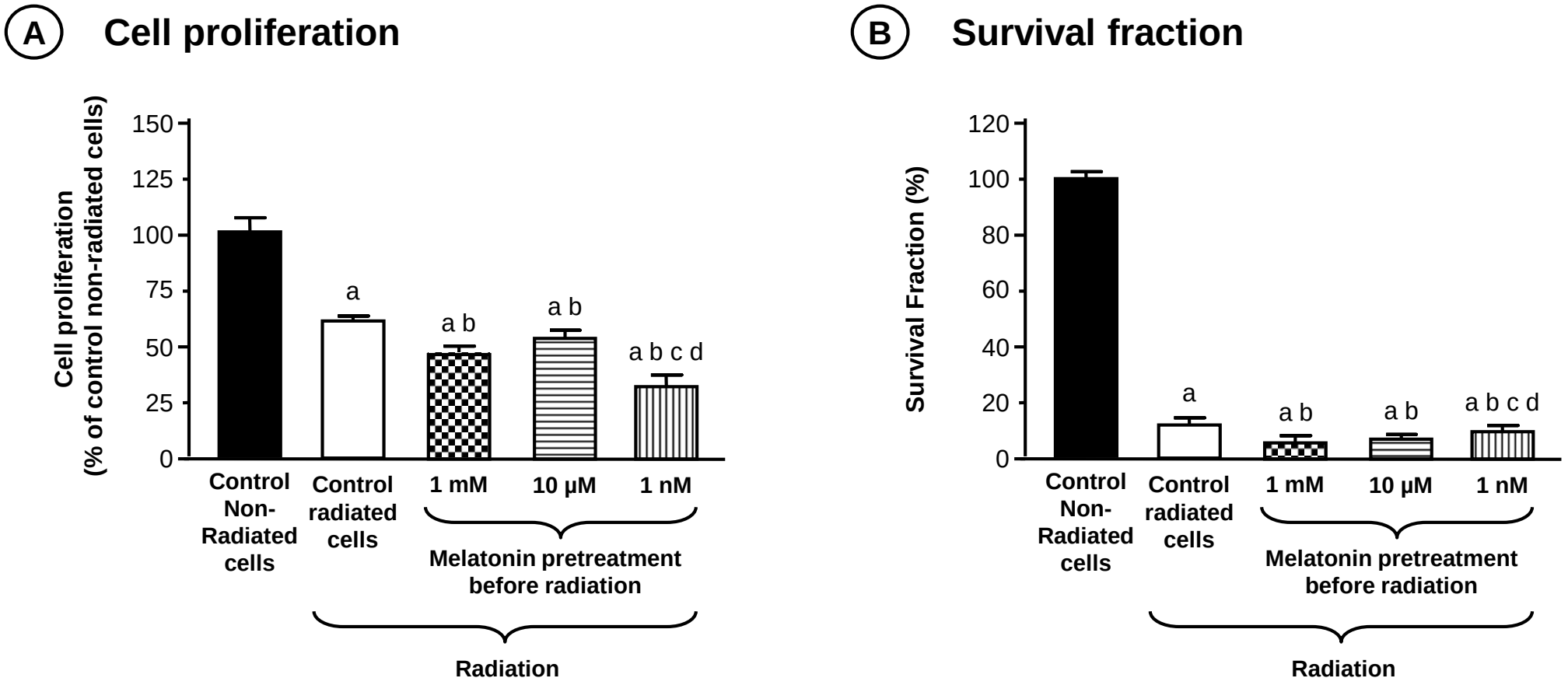
Fig. 4. (A) Effects of melatonin pretreatment (1 mM, 10 μM or 1 nM) and ionizing radiation on sulfatase activity of MCF-7 cells. Control and melatonin pretreated MCF-7 cells for a week were seeded into six-well plates in DMEM supplemented with 10% FBS. After 24 h cells were irradiated and 24 h later media were aspirated and changed to serum-free media containing tritiated estrone sulfate ammonium salt. Sulfatase activity was determined after 24 h of incubation, as described in Material and methods (B) Effects of melatonin pretreatment (1 mM, 10 μM or 1 nM) and ionizing radiation on sulfatase mRNA expression in MCF-7 cells. Control and melatonin pretreated cells were cultured

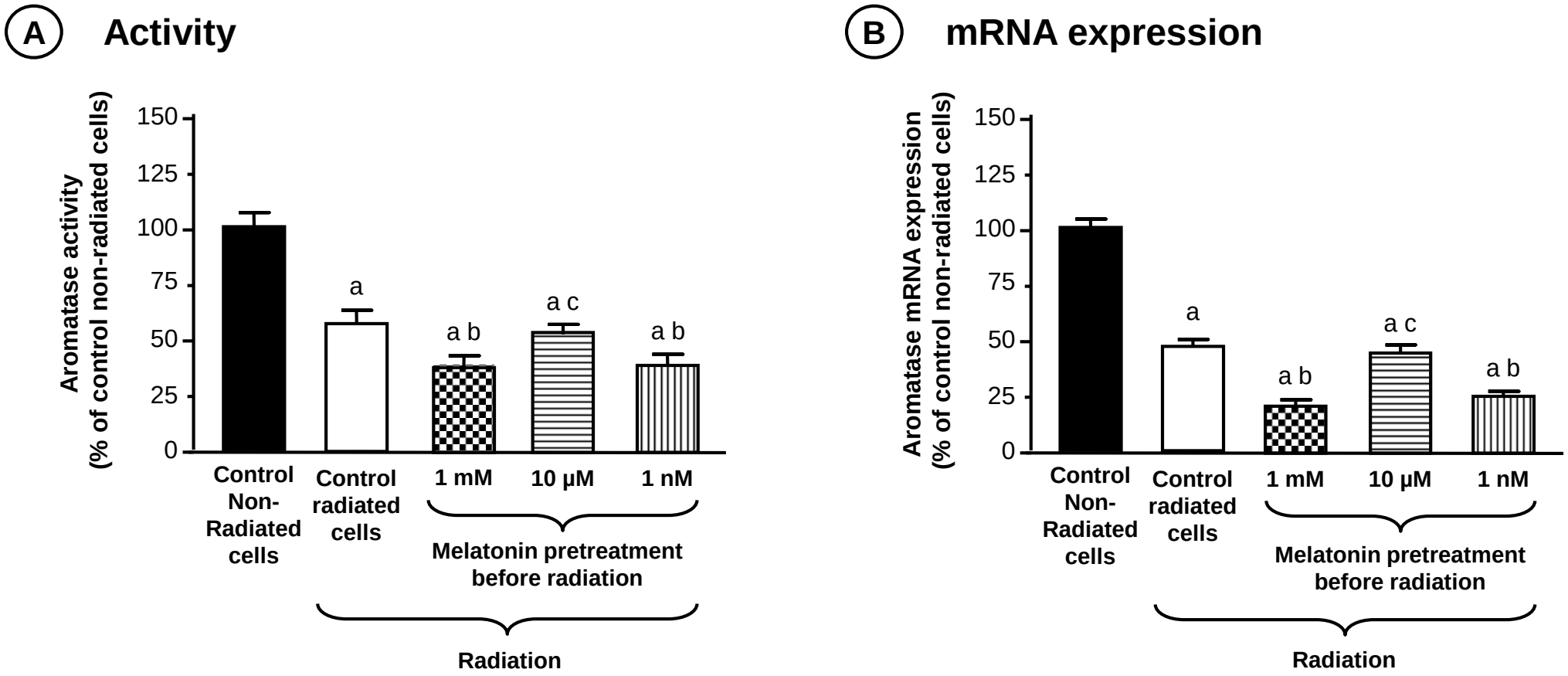
for 6 h after irradiation and total mRNA was isolated from cells and reverse transcribed. cDNA was subjected to PCR using specific primers for sulfatase or S14. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.01$ *vs* 1 mM melatonin; d, $p<0.001$ *vs* 10 µM melatonin.

Fig. 5. (A) Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on 17β-HSD1 activity in MCF-7 cells. Control and melatonin pretreated MCF-7 cells for a week were seeded into six-well plates in DMEM supplemented with 10% FBS. After 24 h cells were irradiated and 24 h later media were aspirated and changed to serum-free media containing tritiated estrone. 17β-HSD1 activity was determined after 30 min. of incubation, as described in Material and methods (B) Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on 17β-HSD1 mRNA expression in MCF-7 cells. Control and melatonin pretreated cells were cultured for 6 h after irradiation and total mRNA was isolated from cells and reverse transcribed. cDNA was subjected to PCR using specific primers for 17β-HSD1 or S14. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.001$ *vs* 1 mM melatonin.

Fig. 6. Effects of melatonin pretreatment (1 mM, 10 µM or 1 nM) and ionizing radiation on p53 mRNA expression in MCF-7 cells. Control and melatonin pretreated cells were cultured for 6 h after irradiation. Total mRNA was extracted from MCF-7 cells and reverse transcribed. cDNA was subjected to

PCR using specific primers for p53 or S14. Data are expressed as the percentage of the control non-radiated group (mean ± SEM). a, $p<0.001$ *vs* control non-radiated cells; b, $p<0.001$ *vs* control radiated cells; c, $p<0.001$ vs 1 mM melatonin; d, $p<0.001$ *vs* 10 µM melatonin.


A
Cell proliferation
Cell proliferation
(% of control non-radiated cells)
150
125
100
75
50
25
0
a
a b
a b
a b c d
Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation
B
Survival fraction
Survival Fraction (%)
120
100
80
60
40
20
0
a
a b
a b
a b c d
Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation


A
Activity
Aromatase activity
(% of control non-radiated cells)
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125
100
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25
0
a
a b
a c
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Control Non-Radiated cells
Control radiated cells
1 mM
10 µM
1 nM
Melatonin pretreatment before radiation
Radiation
B
mRNA expression
Aromatase mRNA expression
(% of control non-radiated cells)
150
125
100
75
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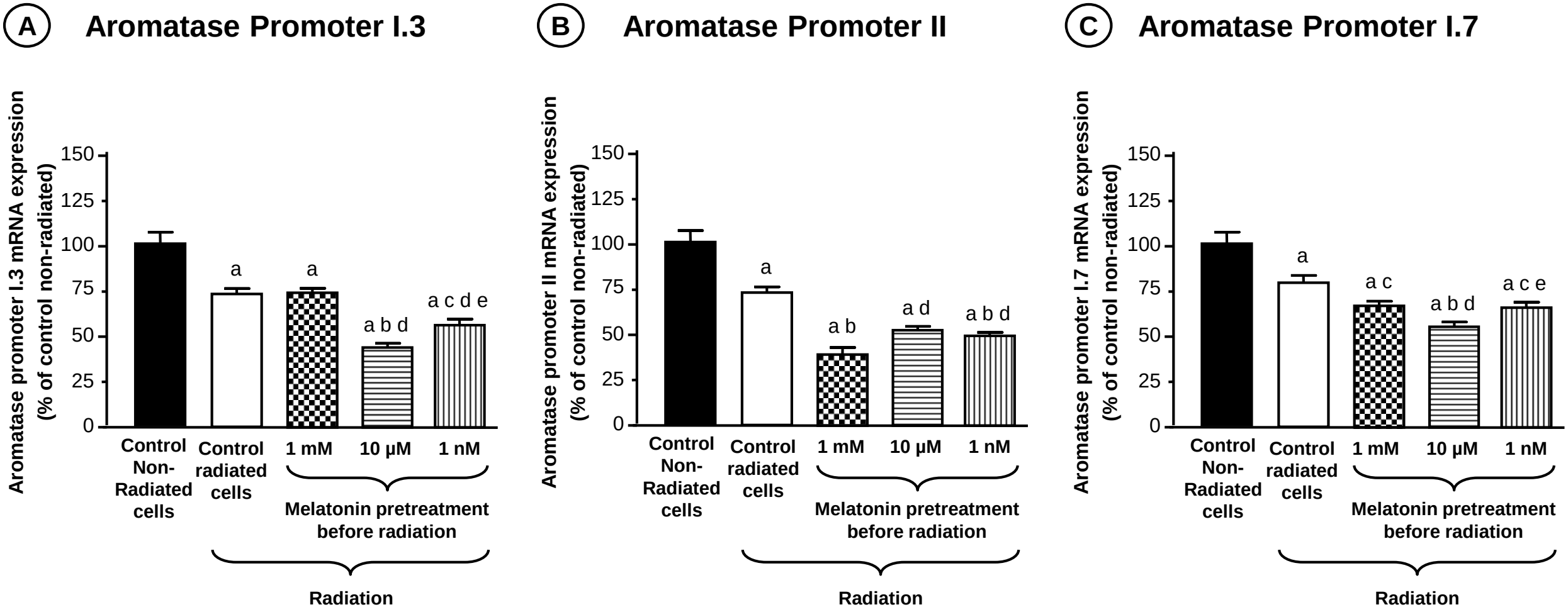
a b
a c
a b
Control Non-Radiated cells
Control radiated cells
1 mM
10 µM
1 nM
Melatonin pretreatment before radiation
Radiation


A
Aromatase Promoter I.3
Aromatase promoter I.3 mRNA expression
(% of control non-radiated)
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Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation
B
Aromatase Promoter II
Aromatase promoter II mRNA expression
(% of control non-radiated)
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Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation
C
Aromatase Promoter I.7
Aromatase promoter I.7 mRNA expression
(% of control non-radiated)
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100
75
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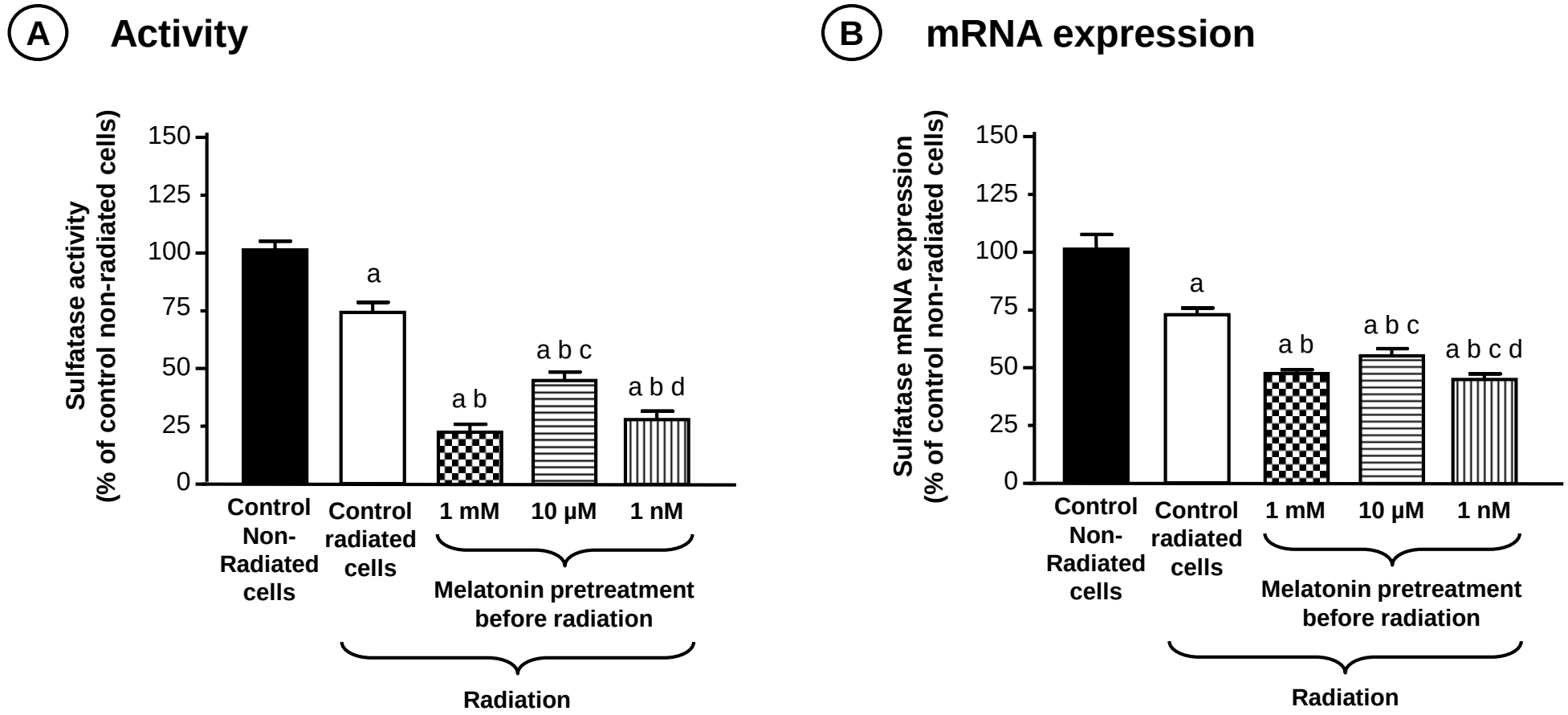
Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation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Activity
Sulfatase activity
(% of control non-radiated cells)
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Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation
B
mRNA expression
Sulfatase mRNA expression
(% of control non-radiated cells)
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a b
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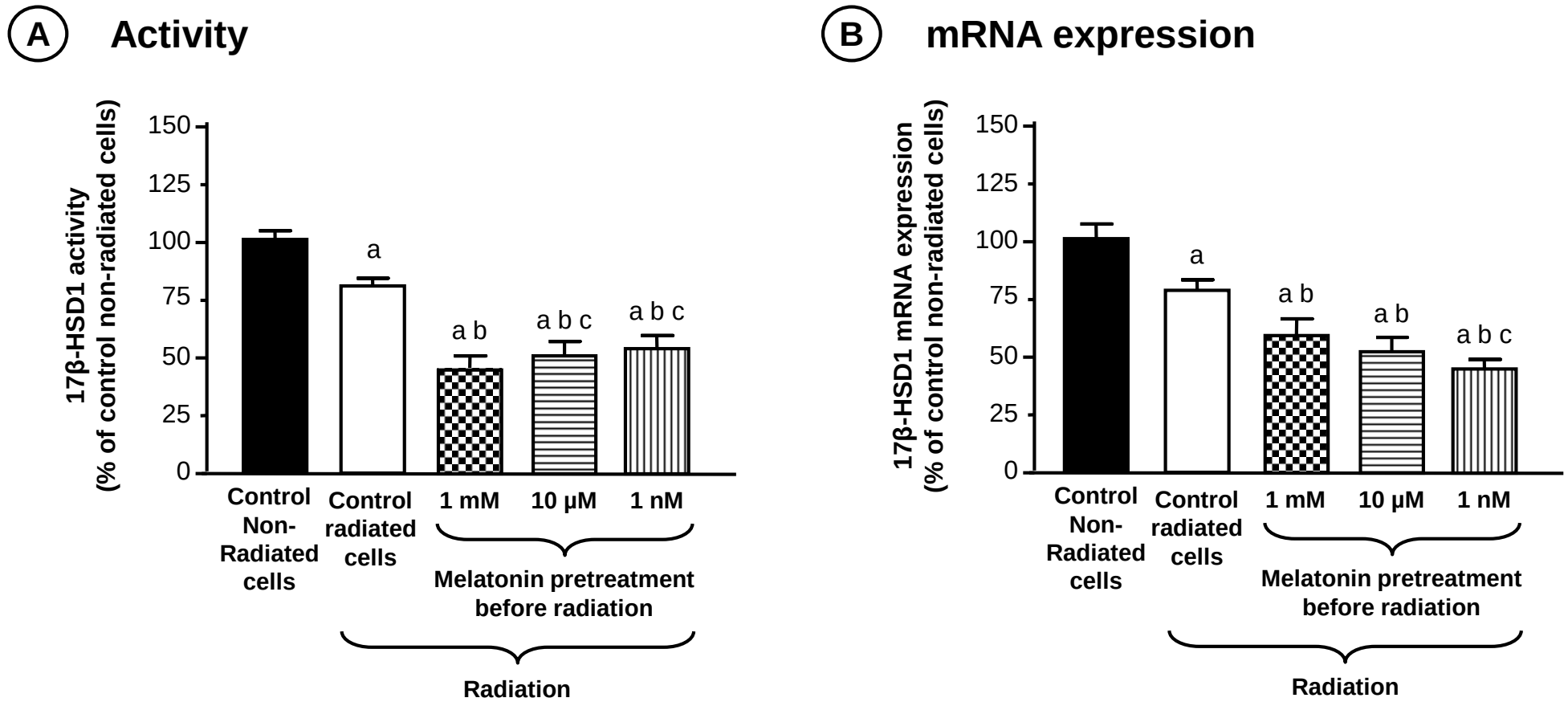
Control
Non-
Radiated
cells
Control
radiated
cells
1 mM
10 µM
1 nM
Melatonin pretreatment
before radiation
Radiation


A
Activity
17β-HSD1 activity
(% of control non-radiated cells)
150
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a
a b
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a b c
Control Non-Radiated cells
Control radiated cells
1 mM
10 μM
1 nM
Melatonin pretreatment before radiation
Radiation
B
mRNA expression
17β-HSD1 mRNA expression
(% of control non-radiated cells)
150
125
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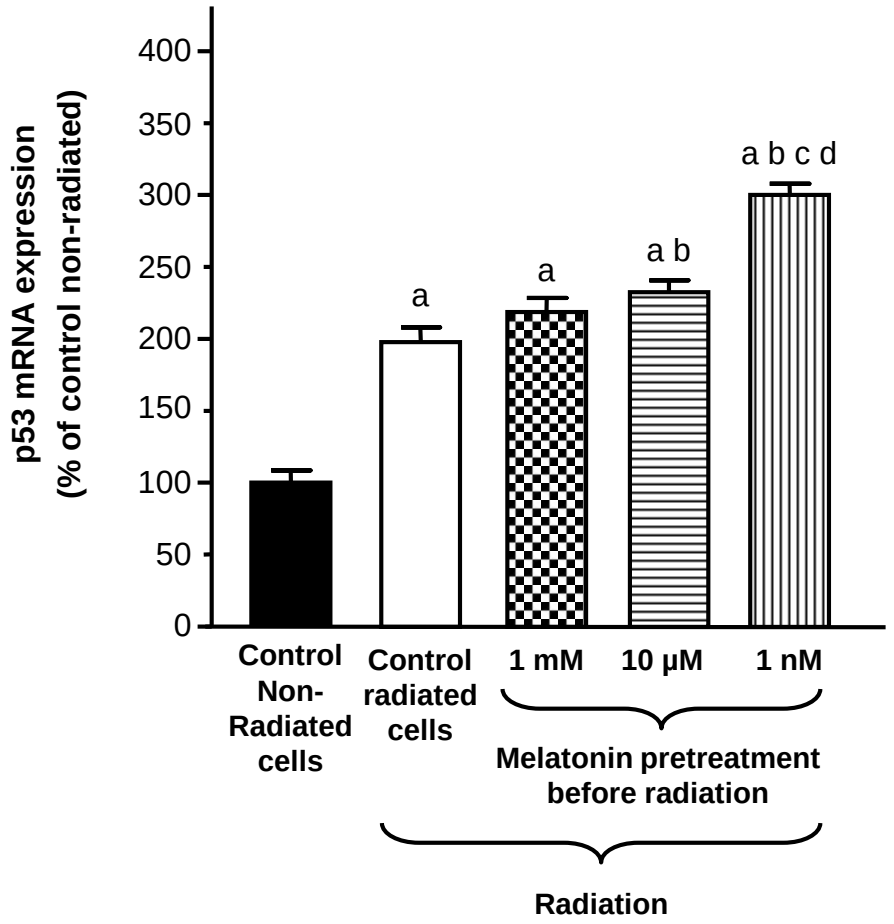
a b c
Control Non-Radiated cells
Control radiated cells
1 mM
10 μM
1 nM
Melatonin pretreatment before radiation
Radiation


p53 mRNA expression
(% of control non-radiated)
400
350
300
250
200
150
100
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a
a
a b
a b c d
Control Non-Radiated cells
Control radiated cells
1 mM
10 µM
1 nM
Melatonin pretreatment before radiation
Radiation