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Topically applied melatonin ameliorates radiation-induced skin fibrosis in mice

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► ***Original article***

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ABSTRACT

Background: We investigated whether topical administration of melatonin ameliorates radiation-induced skin fibrosis (RISF) and inhibits the expression of profibrogenic genes in mice. ***Materials and Methods***: Forty-eight female BALB/c mice were randomly divided into three groups: topically applied 5% ethanol (Control), topically applied 5% ethanol plus irradiation (IR), and topically applied melatonin plus irradiation (Mel+IR). The right hind legs of the IR and Mel+IR group mice were exposed to two fractions of electron beam radiation (20 Gy × 2). For 4 weeks, melatonin solution (10 mg/day) was topically applied to Mel+IR group mice. Fourteen days after IR, the relative levels of transforming growth factor (TGF)-β1 mRNA expression and TGF-β1 protein in skin specimens were analyzed by real-time quantitative PCR and immunohistochemical staining. Dermal thickness and tissue collagen accumulation were measured at 100 days post irradiation. ***Results:*** The Radiation caused a 2.2-fold increase in TGF-β1 mRNA expression relative to that in control group, which was decreased by 37% following melatonin treatment (P = 0.024). We also observed substantial reduction of TGF-β1 expression in immunohistochemical studies. The mean values of dermal thickness were 105 ± 11 μm (Control), 195 ± 21 μm (IR), and 148 ± 19 μm (Mel+IR). Dermal thickness and collagen accumulation, which increased in the IR group, was significantly reduced by topically applied melatonin. ***Conclusion:*** Topical administration of melatonin successfully attenuated RISF.

Keywords: *Melatonin, Radiation, Fibrosis, Transforming growth factor-β, Topical application.*

INTRODUCTION

Although radiotherapy (RT) plays an important role in cancer treatment, its toxicity towards adjacent normal tissues limits its efficacy. RT is associated with various late adverse effects caused by fibrosis and vascular damage, which are regarded as the main pathological processes [(1)]. Among them, fibrotic changes in the skin develop over the course of months to years and occur at doses greater than a single dose of 20–25 Gy or fractionated doses of 70 Gy or higher. These changes are characterized by excessive accumulation of extracellular matrix and the proliferation of fibroblasts in irradiated skin [(2)]. Pain, limited range of motion, and poor cosmetic appearance are well-known adverse events caused by radiation-induced skin fibrosis (RISF). Furthermore, RISF reduces the quality-of-life of

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cancer survivors who have received RT [3]. Thus, it is very important to prevent and alleviate this harmful complication.

Melatonin (*N*-acetyl-5-methoxytryptamine), an endogenous hormone produced mainly by the pineal gland, is well-known for its important functions in circadian rhythm and seasonal biorhythm regulation [4]. Furthermore, melatonin can directly scavenge free radicals such as reactive oxygen and nitrogen species or act indirectly by stimulating the activity of several anti-oxidative enzymes such as superoxide dismutase, glutathione peroxidase, and catalase [5,6]. Oxidative stress plays a key role in fibrinogenesis. Indeed, numerous studies have confirmed that melatonin reduces fibroblast proliferation and collagen synthesis in the liver and lung of fibrogenetic models [7,8]. However, the preventive effects of melatonin on RISF have not been investigated. Considering the differences in tissue composition and physiology, it is unclear whether the molecular mechanisms of skin, pulmonary, and hepatic fibrosis are identical.

Melatonin, which is highly lipophilic, can easily penetrate the skin and protect cellular structures from oxidative stress at sites where damage occurs [9]. Bangha *et al.* demonstrated the dose-dependent suppression of UV-induced erythema by topical melatonin treatment in a human study [10]. Melatonin emulsion significantly reduced radiation-induced dermatitis in patients treated with adjuvant breast radiation [11], but studies regarding local application of melatonin to attenuate fibrosis in the skin are lacking.

Among the profibrogenic cytokines, transforming growth factor (TGF)-β, which activates Smad2/3 proteins, is considered to play a central role in mediating radiation-induced tissue fibrosis. TGF-β is synthesized in several cell types and is known to mediate several aspects of the fibrotic process that induce fibroblast proliferation and transformation to myofibroblasts, leading to deposition of collagen and extracellular matrix protein [12]. Radiation can directly activate the TGF-β signaling pathway, and increased expression of TGF-β after irradiation (IR) was previously reported to be a predictor of skin fibrosis in a mouse model [13].

The anti-fibrotic role of melatonin against RISF is unknown. We predicted that melatonin mitigates skin fibrosis associated with IR possibly through its antioxidant effects. The current study evaluated the inhibitory effect of topical melatonin against RISF by measuring TGF-β1 expression levels and via histopathologic analysis of skin tissue in mice.

MATERIALS AND METHODS

Study design

This study protocol was approved by the Laboratory Animal Ethical Board of our hospital. A total of 48 female BALB/c mice (Central Laboratory Animal, Korea) (aged 7–8 weeks, weighing between 23 and 27 g) were used for all studies. During the study period, mice were housed in plastic cages under standard environmental conditions as follows: controlled humidity (40–50%) and temperature (22 ± 2°C) with a 12-h light-dark cycle, with free access to rodent chow diet and tap water.

The mice were assigned randomly to three groups: control (non-irradiated), IR, and IR with melatonin (Mel+IR), each consisting of 16 mice. The IR and Mel+IR group mice underwent IR. For mice in the control and IR groups, 5% ethanol was topically applied to the right hind legs, while mice in Mel+IR group were treated with melatonin at irradiated sites. All treatments were initiated 2 days before irradiation and continued for 4 weeks. At 14 and 100 days post irradiation, skin samples with a size of 5 x 5 mm from the right thigh were obtained from eight mice per group for each time point.

Drug administration

Melatonin (2 g) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 50 mL of ethanol. Subsequently, melatonin solution was diluted to a concentration of 2 g/L with distilled water. After shaving, this solution (10 mg of melatonin per day) was topically applied to right hind legs of the Mel+IR group mice once daily for 4 weeks, beginning 2 days before IR. For mice in

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the control and IR groups, 5 mL of 5% ethanol was topically applied for 4 weeks. Polyvinyl wrap was applied for 2 h after topical administration.

Irradiation

The right hind leg of each mouse received two weekly radiation doses of 20 Gy (dose rate of 3 Gy/min) using a linear accelerator 4-MeV electron beam (Versa HD, Elekta AB, Stockholm, Sweden). During IR, the mice were immobilized on an acryl plate by taping the extremities with plastic wrap under anesthesia with an intraperitoneal injection of 80 mg/kg ketamine and 16 mg/kg xylazine. The anteroposterior field was used with a compensator (0.5 cm thickness) for even radiation exposure. Treatment planning was performed based on CT images with Monte Carlo dose calculation algorithm (Monaco, Elekta AB). The radiation field extended from the femoral head to the tip of the extremity (figure 1).

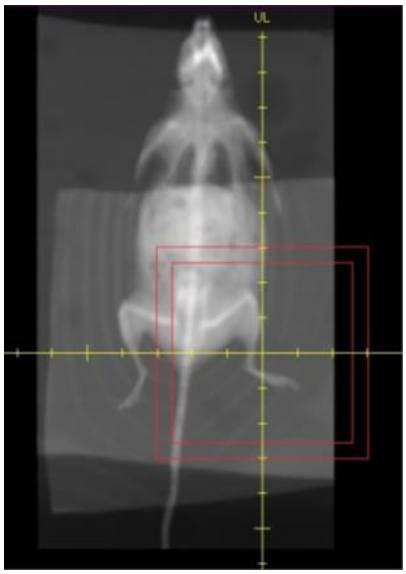


Figure 1. Anteroposterior irradiation field (field size, 5 × 5 cm) of right hind leg encompassed the entire thigh and tip of the foot.

Immunohistochemical staining

On day 14 post IR, skin tissues were prepared as 10% formaldehyde-fixed, paraffin-embedded, and 5–7-μm sections. The sections were incubated in 3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase activity after washing with PBS. Nonspecific binding was blocked with 5% normal serum for 30 min at room temperature. Subsequently, sections were incubated with a polyclonal TGF-β1 antibody (R&D Systems, Minneapolis, MN, USA) at 8 μg/mL overnight at 4°C. After washing with PBS, the sections were incubated for 30 min at room temperature with a biotinylated secondary antibody. After two washes with PBS, the sections were exposed to avidin-biotin complex for 30 min at room temperature and then washed with PBS. Finally, the slides were reacted with diaminobenzidine for 3 min and counterstained in hematoxylin.

RNA isolation and quantitative real-time PCR

Total RNA from the skin tissue was extracted using TRIzol® Reagent (Ambion, Austin, TX, USA) and complementary DNA (cDNA) was synthesized from the prepared RNA using AccuPower CycleScript RT PreMix (dT20) (Bioneer, Daejeon, Korea). Quantitative detection of TGF-β1 mRNA was performed with the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland) using a LightCycler instrument (Roche Molecular Systems, Pleasanton, CA, USA) for real-time PCR. The PCR primer pairs for cDNA amplification were as follows: (sense) 5′- CCACTGATAC-GCCTGAGTGGC-3′ and (antisense) 5′-GGTGCCGTGAGCT GTGCAGG-3′ for mouse TGF-β1, (sense) 5′-CTCTCTTCCAGCCTTCCTTCC-3′ and (antisense) 5′-CTCCTTCTGCATCCTGTCAGC-3′ for mouse β-actin. PCR amplifications performed in separate tubes for 50 cycles (10 sec at 95°C; 5 s at 57°C; 15 s at 72°C) using PCR master mixtures specific for TGF-β1 and the housekeeping gene β-actin, respectively. The PCR cycle number at which the fluorescence of the probe increased above the background is the cycle threshold (Ct). The Ct of each sample is directly proportional to the log of the amount of input cDNA. By plotting the Ct value of the study sample on the standard curve, the amount of target sequence in the sample was calculated.

Histologic analysis

One-hundred days after IR, skin specimens

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were fixed in 10% buffered formalin for 24 h and embedded in paraffin wax. Paraffin-embedded specimens were cut into 6-mm-thick sections, mounted on glass slides, and stained with hematoxylin and eosin (H&E) and Masson's trichrome for collagen. The dermal thickness from the basement membrane to the base of the dermis was measured in three separate microscopic fields. All measurements were carried out in a blinded fashion without knowledge of the study-group assignments.

Statistical analysis

Values were expressed as the mean ± standard deviation. One-way analysis of variance was performed to assess differences among means. Pairwise comparisons were carried out using Tukey's methods. Statistical analysis of data was performed using SPSS statistical software package v18.0 (SPSS, Inc., Chicago, IL, USA). A two-sided P-value < 0.05 was considered statistically significant.

RESULTS

Tolerance to irradiation

In the weekly assessment, there was no significant difference in body weight between the three groups. Typically, two weeks after the delivery of 40 Gy to the right hind leg, mice begin to develop alopecia, followed by erythema and dry desquamation in the irradiated field. There was no evidence of a difference in the onset, severity, or duration of acute skin reaction between the three groups of mice.

TGF-β1 mRNA expression and immunohisto-chemistry

As shown in figure 2, TGF-β1 mRNA expression in skin tissues measured by RT-PCR in the IR group was 2.2-fold higher than that in the control group (P = 0.003). In the melatonin-treatment group, TGF-β1 expression was substantially reduced to approximately two-thirds of that in the IR group (P = 0.024).

Consistent with the RT-PCR results, immunohistochemical staining showed that TGF-β1 expression was markedly augmented in the skin of mice exposed to radiation. In contrast to the results for the IR group, the skin tissue of melatonin-treated mice showed reduced TGF-β1 immunoreactivity. In the skin of the control group, epithelial and stroma cells expressed TGF -β1 at low levels (figure 3).

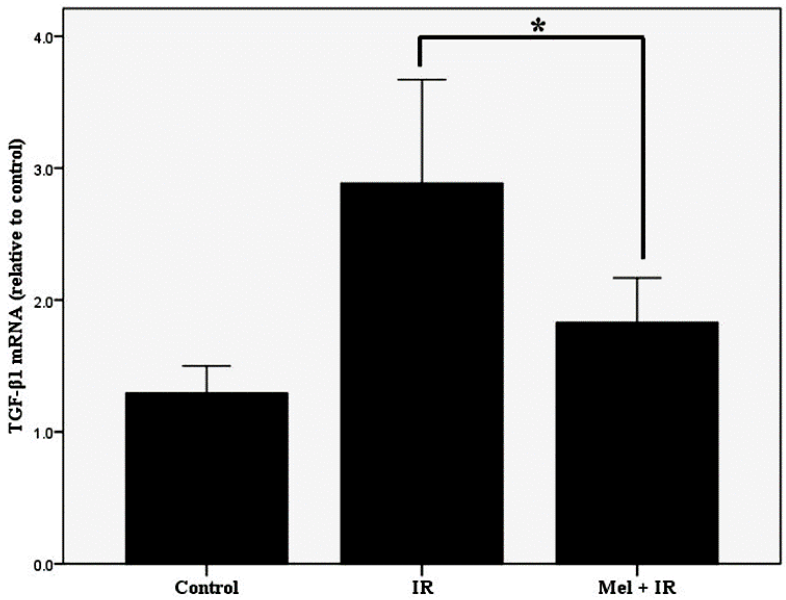


Figure 2. Effect of melatonin (Mel) on transforming growth factor (TGF)-β1 mRNA expression in mouse skin tissue 14 days after irradiation (IR). Values are expressed as the mean ± SD in each group (n = 8). *P < 0.05 between IR and Mel+IR groups.

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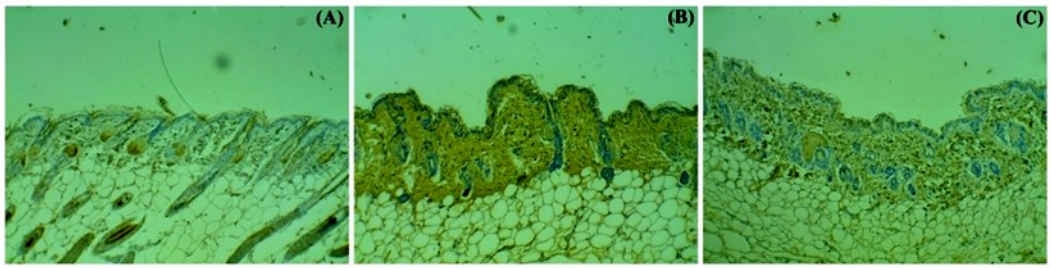


Figure 3. Immunohistochemical staining of transforming growth factor (TGF)-β1 in skin tissue. Weak TGF-β1 expression (brown color) in skin tissue of non-irradiated group (**A**, control group). TGF-β1 expression was present mainly in the epidermis and dermis of mice after receiving radiation (**B**, IR group) and treatment with melatonin markedly reduced this expression (**C**, Mel+IR group). Sections were photographed at 100X magnification.

Histopathological findings

Dermal thickness in the IR group increased by 1.8-fold compared to that in the control group (195 ± 21 μm vs. 105 ± 11 μm, $P < 0.001$). Topical treatment with melatonin reduced the thickness of the dermis to 148 ± 19 μm ($P = 0.019$) (figure 4). RISF was not completely ameliorated by melatonin based on the significant thickening of the epithelium in the Mel+IR group compared to that in the control group ($P < 0.001$).

There was marked hyperplasia of the epidermis, along with a dense, compacted dermis and loosening of the dermis-hypodermis interface in H&E-stained images of the IR group. Mel+IR group mice were relatively unaffected and the pathological changes were less pronounced compared to that in the IR group. In addition, skin appendages such as hair follicles and glands were not observed in the IR group, but were detected in the irradiated skin of melatonin-treated mice (figure 5A).

We also observed the histological features of skin tissue using Masson's trichrome staining. In the IR group, radiation caused a significant increase in dermal collagen accumulation compared to that in the control group. Topical melatonin treatment significantly prevented increases in dermal collagen contents produced by radiation at 100 days post IR (figure 5B).

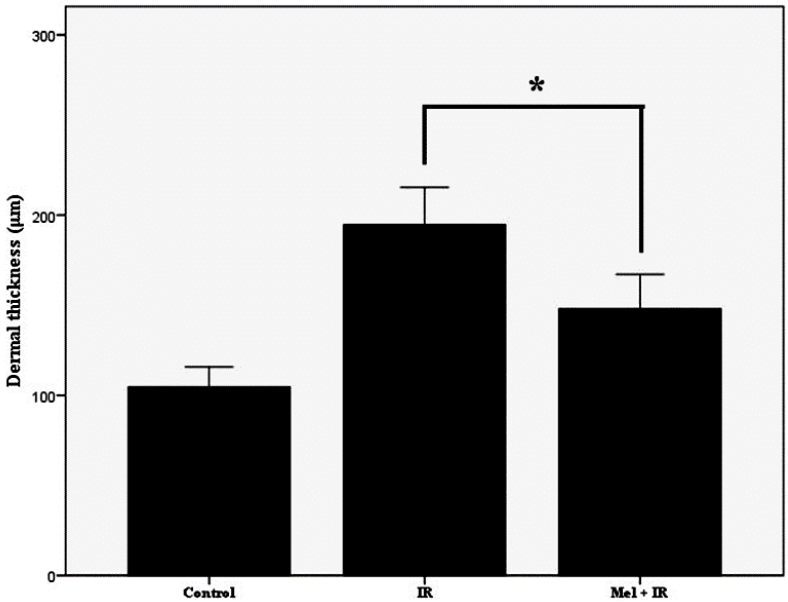


Figure 4. Levels of dermal thickness in the three groups at 100 days post irradiation. Values are the mean ± SD in each group (n = 8). *P < 0.05 between IR and Mel+IR groups.

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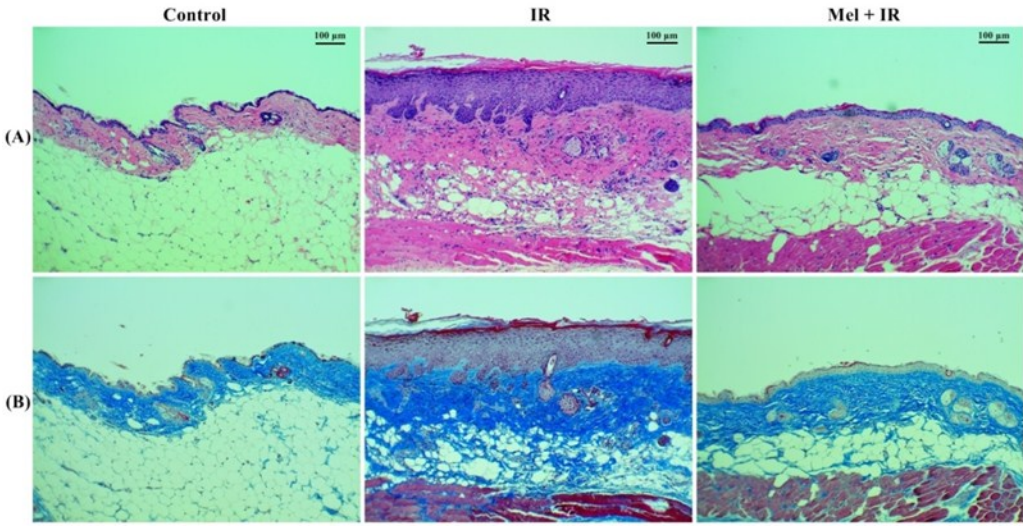


Figure 5. Histopathological features in skin tissue sampled 100 days after irradiation (IR). Upper row **(A)** shows H&E staining and lower row **(B)** shows Masson's trichrome staining of collagen (blue color). Marked prevention of dermal thickening and collagen accumulation were observed in melatonin-treated mice after irradiation (Mel+IR group) compared to mice that received radiation and 5% ethanol (IR group). (magnification X100).

DISCUSSION

In this study, administration of topical melatonin reduced RISF. Furthermore, the mRNA expression of TGF-β1 in skin tissue after IR decreased compared to that in non-melatonin treated mice. Although the beneficial effects of melatonin have been observed in radiation-induced hepatic and pulmonary fibrosis [7,8], this is the first study demonstrating that activation of the TGF-β signaling pathway in response to radiation, which is regarded as a major mechanism in the pathogenesis of RISF, was effectively controlled by topically applied melatonin, leading to the amelioration of RISF in a mouse model.

Radiation-induced fibrosis is a dynamic process characterized by long-term fibroblast activation via stimulation of various factors, such as cytokines and growth factors [2]. Among these pro-fibrogenic cytokines, TGF-β is a key signal in the development of radiation fibrosis. Increased expression of TGF-β after receiving radiation has been demonstrated in several types of human tissues. Binding of TGF-β to its receptor phosphorylates ligand-specific Smad proteins, which act as intracellular signal mediators and transcription factors for procollagen and other extracellular matrix genes [14,15]. For the time course of TGF-β1 expression after IR, previous studies reported increased expression after 1 week, maximum expression at 2–4 weeks, and decreased expression thereafter [16]. Thus, we measured TGF-β1 mRNA expression on post-radiation day 14.

Oxidative stress is often associated with the pathogenesis of fibrogenesis. Free radicals are likely to stimulate the activity of myofibroblasts, which are associated with organ fibrosis. Additionally, reactive oxygen species (ROS) and lipid peroxidation products release active TGF-β1 from its latent complex [2]. Indeed, cells generate ROS, and elevated ROS stimulate fibrogenesis by activating TGF-β, which stimulates collagen and plasminogen activator inhibitor-1 in irradiated tissues [17]. Moreover, proline hydroxylase (a key enzyme in collagen biosynthesis) uses superoxide anion (O_2^-) as a specific substrate. Melatonin's electron-rich structure permits it to donate electrons to hydroxyl radical (•OH). One melatonin molecule scavenges two •OH by electron donation, forming cyclic 3-hydroxymelatonin, an oxidative melatonin metabolite. Because of the antioxidant effects of metabolites produced during melatonin metabolism, one melatonin molecule scavenges more than four radicals [18]. The scavenging ability of melatonin against ROS

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including O_2^- would therefore reduce collagen synthesis [19].

The anti-fibrotic effect of melatonin has been reported in the liver and lung of animal models [7,8]. Furthermore, the toxicity of melatonin is very low. A daily dose of 1 g melatonin for 30 days had no observable side effects in human subjects [20]. The effect of exogenous melatonin depends on the administration route because of its short half-life of approximately 30 min in the serum and first-pass degradation in the liver [21]. Melatonin has a small molecular size and high lipid and water solubility, facilitating its translocation across the cellular membranes. Topical melatonin can penetrate the skin and form a depot in the upper layers of the epidermis. Next, it is gradually released into the dermis and blood vessels. Therefore, topically administered melatonin is expected to potently protect against radiation in skin tissues [22].

Previous studies demonstrated that melatonin can protect the skin against damage induced by UV radiation when administered before IR. Thus, melatonin should be present in skin tissues prior to radiation exposure [23]. In this study, melatonin was applied 30 min before IR because the antioxidant should be administered before radiation to effectively scavenge free radicals produced during IR.

Tumor cell killing after RT was mediated mainly by free radical-induced DNA strand breakage, so the anti-oxidative effect of melatonin may limit the anti-tumor effect of RT. However, previous animal studies reported that melatonin has antitumor activity, including apoptosis-inducing and antiangiogenic effects [24,25]. Therefore, melatonin treatment may yield enhanced tumor sensitivity to radiation as well as anti-fibrotic effects.

We examined the protective effect of topically administered melatonin against RISF. The design of this experiment is well-established and provides an appropriate means for reproducing radiation-induced skin damage expected from fractionated RT [13]. However, local application of melatonin resulted in partial relief of RISF. This suggests that other mechanisms are involved in the pathogenesis of RISF. Thus, combining melatonin with novel pharmacological agents targeting other pathways may benefit patients with RISF.

In summary, our results suggest that melatonin is an effective anti-fibrotic drug because of its free-radical scavenging activity. This is the first study to demonstrate the protective effect of topical melatonin against RISF. Although the exact mechanisms remain unclear, efficient protection can be achieved by application of topical melatonin. These protective actions of melatonin may reduce complications, particularly in patients who received RT for the head, neck, and breast. The optimal dose in relation to the route of administration and sedative side effects requires further investigation.

***Conflicts of interest*:** *Declared none.*

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