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Melatonin's protective effect on the salivary gland against ionized radiation damage in rats

Isil Cakmak Karaer[1], Gokce Simsek[2], Azibe Yildiz[3], Nigar Vardi[3], Alaadin Polat[4], Kevser Tanbek[4], Simay Gurocak[5], Hakan Parlakpinar[6]

[1]*Department of Otolaryngology, Malatya State Hospital, Malatya, Turkey;* [2]*Department of ENT, School of Medicine, Kirikkale University, Kirikkale, Turkey;* [3]*Department of Histology and Embryology, School of Medicine, Inonu University, Malatya, Turkey;* [4]*Department of Physiology, School of Medicine, Inonu University, Malatya, Turkey;* [5]*Department of Radiation Oncology, School of Medicine, Inonu University, Malatya, Turkey;* [6]*Department of Pharmacology, School of Medicine, Inonu University, Malatya, Turkey*

OBJECTIVES: The aim of this study was to examine the effects of melatonin on ionized radiation-induced salivary gland damage using an experimental model.

MATERIALS AND METHODS: Thirty-two rats were randomized into four groups: (i) the control group (C, *n* = 8) that received intraperitoneal (i.p.) 0.9% NaCl; (ii) the melatonin group (M, *n* = 8) that received i.p. 5 mg/kg melatonin; (iii) the radiotherapy group (RT, *n* = 8) that underwent irradiation; (iv) the melatonin plus radiotherapy group (M+RT, *n* = 8) that received i.p. 5 mg/kg of melatonin, followed by irradiation 30 min later; and (v) the radiotherapy plus melatonin group (RT+M, *n* = 8) that received irradiation followed by i.p. 5 mg/kg of melatonin 30 min later. The medications and irradiation were administered for 5 days and the salivary glands of the rats were excised 10 days later; the histopathological changes in the salivary glands were assessed and biochemical analyses were conducted (tissue levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI)).

RESULTS: Regardless of whether melatonin was administered before or after radiotherapy, melatonin decreased the radiation-induced parotid and submandibular histological damage. In addition, regardless of whether administration occurred before or after radiotherapy, melatonin decreased oxidative stress markers, such as MDA, TOS, and OSI. On the contrary, levels of antioxidative markers, such as CAT and GPx, were increased by melatonin.

CONCLUSIONS: Melatonin may have a significant protective effect on salivary gland damage secondary to ionizing radiation.

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Correspondence: Isil Cakmak Karaer, Department of ENT, State Hospital, 44330 Malatya, Turkey. Tel: +904445634, Fax: +904223410728, E-mail: drisil_cakmak@yahoo.com
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Introduction

Radiotherapy (RT) has been widely used as both primary and adjuvant treatment for head and neck cancer. In addition to its curative effects, some side effects have been noted with RT. The deleterious effect of radiation occurs from the production of free radicals and reactive oxygen species (ROS) (1). ROS, notably hydroxyl radicals, cause membrane lipid peroxidation, oxidation of cell proteins, damage within the deoxyribonucleic acid (DNA) helix and cell death, and also initiate an inflammatory response cascade (2). The dead cells, which release ROS, pro-inflammatory, and anti-inflammatory cytokines, as well as endothelial and macrophage-like cells, activated by ionic reactions can lead to this inflammatory response that can last a long time and cause irreversible tissue damage (3). However, the radiation-induced injury to the major salivary glands is believed to be mediated by free radicals (4).

Melatonin (N-acetyl-5-methoxytryptamine) is considered a hormone and is synthesized by the mammalian pineal body, as wells as by extrapineal tissues such as the retina, harderian gland, gastrointestinal tract, testes, and lymphocytes (5–7). Melatonin has immunomodulatory, anti-inflammatory, and antitumor effects (8). It is the major element of the defence system against both ROS and reactive nitrogen species (9–11). Melatonin and its metabolites are powerful antioxidants. They reduce oxidative stress by several mechanisms, including direct free radical scavenging actions. First, melatonin selectively and efficiently detoxifies highly reactive oxidants by donating an electron (12). Melatonin also improves mitochondrial homeostasis and gene regulation by increasing the expression of antioxidant enzymes, while suppressing pro-oxidant enzymes (13). Melatonin protects cells by supporting endogenous antioxidant enzymes such as superoxide dismutase and glutathione

peroxidase. Moreover, melatonin stimulates the production of reduced glutathione, which is another endogenous antioxidant and substrate for glutathione peroxidase that prevents the inactivation of catalase.

The purpose of this study was to examine the protective effects of intraperitoneal melatonin supplementation on salivary gland damage occurring in rats exposed to ionized radiation.

Materials and methods

Care and treatment of animals

This study was carried out in the Experimental Research Laboratory of the Inonu University Faculty of Medicine. The study was approved by the Malatya Animal Ethics Committee and carried out within the guidelines for the care and use of experimental animals. Thirty-two female Wistar albino rats, each weighing between 250 and 300 g, were used in this study. The animals were ranked by weight at the beginning of the study to ensure similar starting body mass weights between groups. The health of the rats were maintained according to the standard guidelines.

Drug preparation and treatment

The 32 rats were randomized (using random number tables) into four groups: (i) the control group (C) (n = 8); (ii) the melatonin group (M) (n = 8); (iii) the radiotherapy group (RT) (n = 8); and (iv) the radiotherapy and then melatonin group (RT+M) (n = 8). In the control group C, the rats received intraperitoneal (i.p.) 0.9% NaCl. In the RT group, rats underwent irradiation treatment without any medication. In the M group, 5 mg/kg of melatonin (Sigma Chemical, St Louis, MO, USA) was injected i.p. The rats in the RT+M group were treated with melatonin i.p., 5 mg/kg 30 min after irradiation. Because the half-life of melatonin is very short and it is only active for about 20–30 min (14), we chose 30 min as the duration between RT and administration of the drug. Whereas the rats in the C and M groups received no irradiation, the rats in the RT and RT+M groups underwent irradiation for 5 days. The dose of 5 mg/kg of melatonin and the duration of five consecutive treatment days were chosen based on previous biological studies that related to the antioxidant effects of melatonin in an experimental rat model (15, 16).

Irradiation

Ionized radiation was delivered from anterior–posterior parallel fields at SAD: 80 cm, with the bodies of the rats positioned radially and their heads placed centrally to rule out the influence of a possible dose loss at the periphery. In the RT group and the RT+M group, 33 Gy was applied to the total cranium in five fractions of 6.6 Gy/day for five successive days. The calculated (α/β = 3.5) biological effective dose of fractionated irradiation was equal to 60 Gy conventional fractionation. All of the rats in the groups were anesthetized i.p. with 20–30 mg/kg of ketamine hydrochloride (Rotex, Trittau, Germany) before irradiation. In the RT group, one animal died within 72 h of undergoing irradiation. Ten days after radiation exposure, all rats were sacrificed with high doses of the anesthesia mixture (ketamine hydrochloride and xylazine). The right-sided salivary glands were carefully dissected from the surrounding tissues, quickly removed, and used for biochemical analysis. The left-sided salivary glands of each animal were also removed for histopathologic investigation.

Histological analysis

The histological analysis was performed by Nigar Vardi, histologist, who was blinded to which group each sample belonged to. Parotid gland samples were fixed in 10% formalin and were embedded in paraffin. Paraffin-embedded specimens were cut into 5-μm thick sections and mounted on slides. For light microscopic evaluation, they were stained with hematoxylin–eosin and toluidine blue. Histological evaluation was performed using the Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd, Cambridge, UK).

Parotid tissue was evaluated according to the disruption in the structures of acini, the loss of density of acinar cell cytoplasm, the swelling of acinar cells and perinuclear vacuolization, and the number of mast cells in acinar cells. These changes in the parotid tissue were graded as follows: 0: normal; 1: damage involving <25% of the total area; 2: damage involving 25–50% of the total area; and 3: damage involving >50% of the total area. The histological changes were measured in 10 different fields for each specimen. Mast cells were counted in 20 random areas of each specimen under an ×40 objective.

Tissue preparation for biochemical analyses

Two hundred milligrams of frozen parotid gland tissue specimens were cut into pieces on dry ice, homogenized in 1.15% KCl buffer (1:9, w/v) using a manual glass homogenizer for approximately 5 min, and flushed with centrifugation for approximately 10 s to remove large debris. The supernatant was used for analysis.

Determination of tiobarbituric acid reactive substance (TBARS) test representing Malondialdehyde content

The tiobarbituric acid reactive substance (TBARS) contents of the homogenates were determined by TBARS (17). The results were expressed in nanomoles per gram (nmol/g tissue) according to a standard graph, which was prepared from the measurements of standard solutions (1,1,3,3-tetramethoxypropane).

Determination of Superoxide dismutase

Total (Cu–Zn and Mn) superoxide dismutase (EC 1.15.1.1) activity was determined based on the method by Sun et al. (18). Activity was expressed as units per milligram protein (U/mg protein).

Determination of Catalase

Catalase (EC 1.11.1.6) activity was determined with respect to Aebi's method (19). Results were expressed as kilograms per gram protein (kg/g protein).

Determination of Glutathione peroxidase

Determination of glutathione peroxidase activity (EC 1.6.4.2) was measured by the method of Paglia and

Valentine (20). Activity was given in units per gram protein (U/g protein).

Determination of Glutathione
The glutathione content in the lung tissue as nonprotein sulfhydryls was analyzed following a previously described method (21). The absorbance values were extrapolated from a glutathione standard curve and expressed as glutathione (in micromoles per g of tissue).

Measurement of Total oxidant status
Total oxidant status was determined using a novel automated measurement method, developed by Erel (22).

Measurement of Total anti-oxidant status
Total anti-oxidant status levels were determined using a novel automated colorimetric measurement method developed by Erel (23). The results were expressed as millimoles of trolox equivalent per liter.

Measurement of oxidative stress index (OSI)
The percentage ratio of TOS to TAS yields the OSI, an indicator of the degree of oxidative stress calculated as OSI (arbitrary unit)=TOS (mmol H_2O_2 equivalent/l)/TAS (mmol trolox equivalent/l) (23).

Statistical analysis
To detect even minor effects, the required sample sizes used in this experiment were identified using statistical power analysis. The sample sizes necessary for a power of 0.80 were estimated using NCSS software. Data were analyzed using the SPSS software program for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). The normality of the distribution was confirmed using the Kolmogorov–Smirnov test. The variables that did not have normal distribution were presented as median and minimum/maximum, whereas those with normal distribution were presented as mean ± standard deviation (SD). According to the results obtained from the normality test, one-way analysis of variance (ANOVA) and the Kruskal–Wallis H test were used for the statistical analysis as appropriate. After a significant Kruskal–Wallis H test, a Conover test was also carried out. Multiple comparisons were conducted using Tukey's test (for homogeneous variances) after the ANOVA test. Results were regarded as statistically significant when P values were less than 0.05.

Results

Histopathological Results
The histopathological results of the groups are outlined in Table 1.

Melatonin group
The melatonin and control groups were similar in respect to the disruption in the structures of acini, loss of density in acinar cell cytoplasm, swelling of acinar cells, perinuclear vacuolization and polymorphic nuclei in acinar cells, and the number of mast cells ($P > 0.05$) (Fig. 1A,D).

Radiation group
When comparing the histological score of the RT and control groups, the disruption in structures of acini [1.0 (0.0–3.0) vs. 0.0 (0.0–2.0), respectively], loss of density of acinar cell cytoplasm [1.0 (0.0–3.0) vs. 0.0 (0.0–1.0), respectively], swelling of acinar cells, [2.0 (0.0–3.0 vs. 0.0 (0.0–0.0), respectively], perinuclear vacuolization [3.0 (1.0–3.0) vs. (0.0 (0.0–1.0), respectively] and polymorphic nuclei in acinar cells [2.0 (1.0–3.0) vs. 0.0 (0.0–1.0), respectively] were found to be statistically significantly greater among the RT group than among controls (Fig. 1B) ($P < 0.01$). However, the difference in the number of mast cells was not statistically significant between the two groups [1.0 (0.0–10.0) vs. 0.0 (0.0–7.0), respectively; $P > 0.05$] (Fig. 1E).

Radiotherapy then melatonin group
When comparing the histological score of the RT+M group and the RT group, loss of density of acinar cell cytoplasm [1.0 (0.0–2.0) vs. 1.0 (0.0–3.0), respectively; $P < 0.01$], swelling of acinar cells [1.0 (0.0–3.0) vs. 2.0 (0.0–3.0), respectively; $P < 0.01$], perinuclear vacuolization [1.0 (0.0–3.0) vs. 3.0 (1.0–3.0), respectively; $P < 0.01$] and polymorphic nuclei in acinar cells [1.0 (0.0–3.0) vs. 2.0 (1.0–3.0), respectively; $P < 0.01$] were found to be significantly different (Fig. 1C). By contrast, the number of mast cells [0.0 (0.0–9.0) vs. 1.0 (0.0–10.0), respectively; $P > 0.05$] and disruption in structures of acini [0.0 (0.0–2.0) vs. 1.0 (0.0–3.0), respectively; $P > 0.05$] were not statistically significantly different between the two groups (Fig. 1F).

Oxidative stress parameters
The oxidative stress parameters of the groups are outlined in Table 2.

Melatonin group
Overall, the oxidative stress parameters in the melatonin group and the control group were similar. However, the glutathione peroxidase level was decreased in the melatonin group compared to the control group [351.15 + 94.72 vs. 447.76 + 95.45 U/mg, respectively; $P = 0.05$] (Table 2).

Radiation group
When comparing the oxidative stress parameters of the RT group and the control group, there was an increased malondialdehyde level [282.65 ± 60.15 nmol/g vs. 135.3 ± 24.96, respectively; $P < 0.01$]. By contrast, glutathione peroxidase levels and catalase activity were statistically significantly decreased in the RT groups compared to the control group [glutathione peroxidase: 192.99 ± 41.52 vs. 447.76 ± 95.45 U/mg, respectively; $P < 0.01$ and catalase activity: 2.94 ± 0.59 vs. 4.18 ± 0.87 kg/g protein, respectively; $P = 0.05$]. No significant difference was found between the groups regarding the superoxide dismutase and glutathione levels ($P > 0.05$) (Table 2).

The level of TOS in the salivary glands was increased by radiotherapy when compared to the controls (5.77 ± 1.77 vs. 3.96 ± 0.79 mmol H_2O_2 equivalent/l, respectively; $P = 0.02$). Radiotherapy caused no change in the TAS level [1.08 ± 0.50 vs. 1.10 ± 0.57 mmol trolox equiv/l,

Table 1 The comparasion of severity of parotid histological damage among groups and number of mast cells

	Control	*M*	*RT*	*Radiotherapy then melatonin*
Distruption in structures of acini	0.0 (0.0–2.0)	0.0 (0.0–1.0)	1.0 (0.0–3.0)[a]	0.0 (0.0–2.0)
Loss density of acinar cells cytoplasm	0.0 (0.0–1.0)	0.0 (0.0–1.0)	1.0 (0.0–3.0)[a]	1.0 (0.0–2.0)[b]
Swelling of acinar cell	0.0 (0.0–0.0)	0.0 (0.0–1.0)	2.0 (0.0–3.0)[a]	1.0 (0.0–3.0)[b]
Perinuclear vacuolization	0.0 (0.0–1.0)	0.0 (0.0–1.0)	3.0 (1.0–3.0)[a]	1.0 (0.0–3.0)[b]
Polymorphic nuclei in acinar cells	0.0 (0.0–1.0)	1.0 (0.0–2.0)	2.0 (1.0–3.0)[a]	1.0 (0.0–3.0)[b]
Number of mast cells	0.0 (0.0–7.0)	0.0 (0.0–6.0)	1.0 (0.0–10.0)	0.0 (0.0–9.0)

[a]Significantly increased when compared with Control group ($P < 0.05$).
[b]Significantly decreased when compared with RT group ($P < 0.05$).

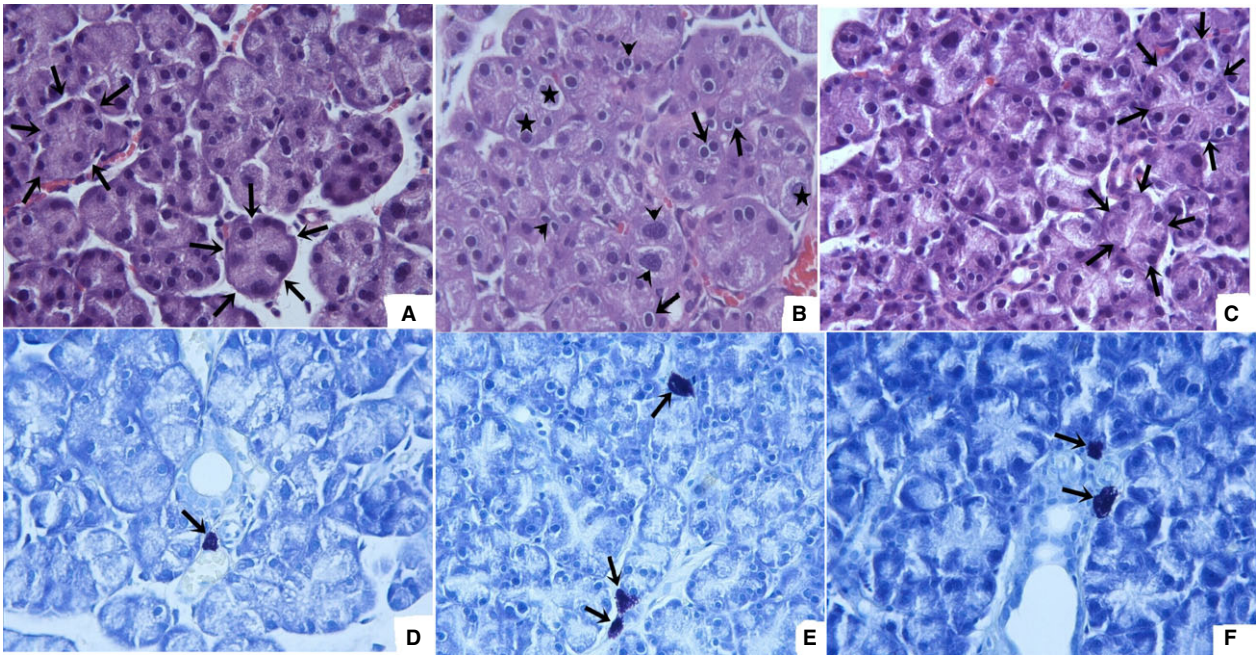

Figure 1 (A) Normal histological appearance of acini, H–E × 40. (B) Perinücleer vacuolization condenced-polymorphic nuclei and swelling in the aciner cells in the RTgroups. (C) RT+M groups show less perinuclear vacuolization and polimorphic nuclei. (D) Mast cells are observed around excretory ducts and blood vessels in the normal histological appearance. TB ×40. (E) Mast cells are observed in interstitial tissue after ionize-radiation. (F) Mast cells in interstitial tissue have remained after administration melatonin in the RT+ M groups.

respectively; $P = 0.05$]. OSI was increased in the RT group when compared to the control group [5.34 ± 1.74 vs. 3.58 ± 0.71 arbitrary unit, respectively; $P < 0.01$].

Radiotherapy then melatonin group

When comparing the oxidative stress parameters of the RT+M group and the RT group, tissue malondialdehyde levels were significantly reduced in the RT+M group [118.76 + 28.06 nmol/g vs. 282.65 + 60.15 nmol/g, respectively; $P < 0.01$]. By contrast, glutathione and catalase activity were significantly higher in the RT+M group than in the radiotherapy group. [glutathione: 53.69 ± 11.12 vs. 41.18 ± 6.80 U/mg, respectively; $P = 0.02$ and catalase: 4.95 ± 1.82 vs 2.94 + 0.59 nmol/g, respectively; $P = 0.02$]. No significant difference was found between the groups when considering the superoxide dismutase and glutathione peroxidase levels ($P > 0.05$) (Table 2).

Both tissue TOS and TAS levels were reduced in the RT+M group when compared to the RT group [TOS: 2.43 ± 1.02 vs. 5.77 ± 1.77 mmol H_2O_2 equivalent/l, respectively; $P < 0.01$, and TAS: 1.01 ± 0.08 vs. 1.08 ± 0.50 mmol trolox equiv/l, respectively; $P = 0.03$]. OSİ levels were significantly decreased in the RT+M group when compared to the RT group [2.47 ± 1.29 vs. 5.34 ± 1.74 arbitrary unit, respectively; $P < 0.01$].

Discussion

In this study, we found that melatonin reduces the salivary gland damage caused by irradiation. When melatonin was administered after radiotherapy, melatonin decreased the radiation-induced salivary gland damage histologically. In addition, administration of melatonin after radiotherapy decreased oxidative stress markers, such as malondialdehyde, TOS and OSI, and increased catalase and glutathione levels.

An important limitation of this study is that we did not examine the efficiency of melatonin at different dosages. On the other hand, to our knowledge, this is the first study examining the protective effects of melatonin on irradiation-induced damage to the salivary glands.

Table 2 The comparison of selected oxidative stress paramaters among groups

	Control n = *8*	*M* n = *8*	*RT* n = *7*	*Radiotherapy then melatonin* n = *8*
MDA (nmol/g)	135.3 ± 24.9	126.95 ± 45.68	282.65 ± 60.15[a]	118.76 + 28.06[b]
GPx (U/mg)	447.76 ± 95.4	351.15 ± 94.72[a]	192.99 ± 41.52[a]	280.13 + 16.95[b]
GSH (μmol/g tissue)	36.01 ± 13.5	41.50 ± +11.42	41.18 ± 6.80	53.69 + 11.12[b]
SOD (U/mg protein)	1.11 ± 0.16	1.18 ± 0.26	0.98 ± 0.22	1.20 + 0.38
CAT (k/g protein)	4.18 ± 0.87	3.90 ± 1.07	2.94 ± 0.59[a]	4.95 + 1.82[b]
TOS (mmol H2O equiv/l)	3.96 ± 0.79	4.62 ± 1.36	5.77 ± 1.77[a]	2.43 + 1.02[b]
TAS (mmoltrolox equiv/l)	1.10 ± 0.57	1.11 ± 0.92	1.08 ± 0.50	1.01 + 0.08[b]
OSI (arbitrary unit)	3.58 ± 0.71	4.18 ± 1.22	5.34 ± 1.74[a]	2.47 + 1.29[b]

All values are expressed as mean + SD.
[a]Statistically significant difference when compared with the control group.
[b]Statistically significant difference when compared with the RT group.

In this study, radiation caused significant salivary gland injury as demonstrated by findings in the RT group, such as acinar shape defects, a partial loss of apical cytoplasmic material, considerable swelling and perinuclear vacuolization, and condensed-polymorphic nuclei in the acinar cells. These findings seem to be related to the induction of gland injury, consistent with other studies that utilized radiation-induced rat models (24–26). Moreover, increased malondialdehyde and TOS levels in the salivary glands were observed after 10 days of radiation administration. In addition, OSI, which is a novel and important index of oxidative stress, was higher in the RT group. However, radiotherapy caused a decrease in the glutathione peroxidase and catalase levels. When compared with the control group, in the RT group, there was no change in glutathione and superoxide dismutase levels.

In this study, we observed that melatonin administration after radiotherapy caused a decrease in the malondialdehyde level, which had increased after radiotherapy. These results demonstrate that melatonin clearly decreases lipid peroxidation in the salivary glands induced by irradiation and decreases the level of malondialdehyde. In agreement with our study, Jang et al. (27) reported that melatonin administered 2 weeks prior to irradiation causes a marked decrease in malondialdehyde in lung tissue. Likewise, there are other studies reporting that melatonin administration leads to a decrease in malondialdehyde levels in various tissues (28–30).

Also in this study, we found that melatonin administration after radiotherapy leads to an increase in both catalase and glutathione levels. Catalase participates in the removal of hydrogen peroxide (H_2O_2) produced by the dismutation of superoxide anion radicals. The activities of this enzyme are reduced under highly elevated oxidative stress conditions, as a result of damage to the molecular mechanism that is required to induce these enzymes. Melatonin has been shown to increase the activity of these antioxidant enzymes under basal oxidative stress conditions and to prevent the reduction in their activity caused by elevated oxidative stress (31, 32). Similarly, there are studies showing an increase in the glutathione levels following melatonin administration (33).

In our study, we found no increase in superoxide dismutase levels. Among the endogenous antioxidants, superoxide dismutase has been widely studied. Superoxide dismutase catalyzes the dismutation of superoxide anion to hydrogen peroxide and oxygen, but hydrogen peroxide can still cause oxidative injury in the liver. However, a few studies have reported that superoxide dismutase activity is not affected by melatonin administration, but rather that this enzyme is a less sensitive predictor of oxidative stress (34, 35). Nevertheless, there are studies reporting an increase in superoxide dismutase activity before melatonin administration and radiotherapy (36, 37).

In this study, we found that melatonin administration caused a decrease in TOS and OSI values, which were increased by radiotherapy. In line with this study, melatonin supplementation over 3 months caused a marked decrease in TOS levels in women with multiple sclerosis (38). Although it has been claimed that melatonin increases TAS, we did not find this expected beneficial effect. In agreement with our study, another experimental study reported that melatonin did not affect blood plasma TAS level *in vitro* (39). On the contrary, Benot et al. (40) found that melatonin administration increased TAS levels.

Conclusions

This study has shown that the damage to salivary glands caused by radiotherapy via the oxidative pathway can be reduced by melatonin. Clinically, melatonin may be used to prevent radiation-induced dysfunction in salivary glands after radiotherapy. Melatonin is widely available in pure form and can be self-administered via multiple routes, e.g., orally, sublingually, transdermally, as a suppository or as a nasal spray. The side effects are negligible over a wide range of doses, and melatonin is routinely used chronically by many individuals as an antioxidant and to correct sleep-related disorders. Melatonin also has an extensive shelf life and is inexpensive. Prospective clinical studies on humans might elucidate the role of melatonin in the protective effect on salivary gland damage secondary to ionizing radiation.

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Conflict of interest statement

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