Archives of Oral Biology 142 (2022) 105510

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

The protective efficacy of Quercetin and Naringenin against radiation-related submandibular gland injury in female rats: A histopathological, immunohistochemical, and biochemical study

Muhammed Sedat Sakat [a,*], Korhan Kılıç [a], Abdulkadir Sahin [a], Hilal Kiziltunc Ozmen [b], Serkan Yıldırım [c], Ahmet Kiziltunc [d], Seda Askin [d], Yavuz Selim Saglam [c]

[a] Ataturk University, Faculty of Medicine, Department of Otorhinolaryngology, Erzurum, Turkey
[b] Ataturk University, Faculty of Medicine, Department of Radiation Oncology, Erzurum, Turkey
[c] Ataturk University, Faculty of Veterinary, Department of Pathology, Erzurum, Turkey
[d] Ataturk University, Faculty of Medicine, Department of Biochemistry, Erzurum, Turkey

ARTICLE INFO

Keywords:
Quercetine
Naringenin
Radiation
Submandibular gland

ABSTRACT

Objective: In this study, we aimed to reveal the effectiveness of Quercetin and Naringenin in preventing radiotherapy-associated submandibular gland injury.
Design: The study was conducted using 48 adult female Sprague Dawley rats. The rats were randomly assigned into six groups of eight animals each. Group 1 represented the control group. The rats received only Naringenin was regarded as Group 2, received only Quercetine was regarded as Group 3. The rats exposed to radiotheraphy at a dose of 15 Gy was regarded as Group 4. Rats in group 5 were received Naringenin at a dose of 50 mg/kg daily for one week prior to radiotheraphy exposition while rats in group 6 was received Quercetine at a dose of 50 mg/kg daily for one week prior to radiotheraphy. Rats were sacrificed after radiotheraphy and submandibular glands were dissected for biochemical and immunohistochemical evaluations.
Results: Quercetin and Naringenin were found to have protective effect against radiation-induced damage. Naringenin and Quercetin increased the levels of Superoxide dismutase, Catalase, Glutathione Peroxidase, Glutathione and Total antioxidant status while decreasing the levels of Myeloperoxidase and Total oxidant status. Also, these agents inhibited the expression of Tumor Necrosis Factor-α and 8-hydroxy 2-deoxyguanosine immunohistochemically.
Conclusions: With their powerful antioxidant and anti-inflammatory effects, Naringenin and Quercetin exhibit histopathological, immunochemical, and biochemical protection against radiation-related submandibular gland injury. In addition, Quercetin was found to be superior to Naringenin in terms of this efficacy.

1. Introduction

Head and neck cancers represent 4% of all body cancers (Gatta et al., 2015). Radiotherapy has become increasingly important in the treatment of these cancers in recent years. Radiotherapy acts against the tumor by causing direct DNA injury or by increasing the production of reactive oxygen species (ROS) (Ozgur et al., 2019). However, since this is not specific to the tissue concerned, surrounding normal tissues are also affected. In addition to the direct injury caused by radiotherapy, inflammation is also responsible for damage arising in normal tissues. This restricts the use of radiotherapy, and may also sometimes lead to complications necessitating discontinuation of treatment. The most common of these phenomena include oral complications such as xerostomia, mucositis, tooth decay, esophagitis-related dysphagia, and oral infections (Lalla et al., 2017). The main cause of these complications is damage arising in the salivary glands due to radiosensitivity. This damage derives from the irreversible death of acinar and stem cells in the salivary glands in association with radiotherapy (De la Cal et al., 2012). Irreversible damage in these cells is associated with apoptosis as a result of oxidative stress. The resulting decrease in saliva secretion is

Abbreviation: CAT, Catalase; GPx, Glutathione Peroxidase; GSH, Glutathione; MPO, Myeloperoxidase; OSI, Oxidative stress index; ROS, Reactive oxygen species; SOD, Superoxide dismutase; TAS, Total antioxidant status; TOS, Total oxidant status; TNF- α, Tumor Necrosis Factor-α; 8-OHdG, 8-hydroxy 2-deoxyguanosine.

* Correspondence to: Ataturk University, Faculty of Medicine, Department of Otorhinolaryngology, Erzurum 25240, Turkey,
E-mail address: sedat.sakat@atauni.edu.tr (M.S. Sakat).

https://doi.org/10.1016/j.archoralbio.2022.105510
Received 22 May 2022; Received in revised form 11 July 2022; Accepted 12 July 2022
Available online 14 July 2022
0003-9969/© 2022 Elsevier Ltd. All rights reserved.

thus responsible for the emergence of oral complications (Liu et al., 2020). Various agents have been employed to prevent this damage that may occur in the salivary glands. Only amifostine has entered into clinical use following such research. However, amifostine use is limited by its toxic effects (Rosen et al., 2015). Novel, natural radioprotective agents with minimal side-effects are therefore being sought.

Quercetin is a polyphenolic flavonoid found in several fruits and vegetables. It has been the focus of several studies because of its anti-carcinogenic, anti-inflammatory, antiviral, antioxidant, and psychostimulant characteristics. Quercetin has been found to exhibit its antioxidant efficacy through its free radical-scavenging and endogenous antioxidant level-increasing properties (Li et al., 2016).

Naringenin is a natural flavonoid with wide biological and pharmacological activities. It possesses anti-carcinogenic, anti-inflammatory, anti-diabetic, and antioxidant efficacy. The antioxidant effectiveness of Naringenin is particularly prominent. It exhibits this through the inhibition of pro-oxidant enzymes such as lipoxygenase, nicotinamide adenine dinucleotide phosphate oxidase, cyclo-oxygenase, and xanthine oxidase and its powerful free radical-scavenging property. Additionally, Naringenin increases the physiological levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Joshi et al., 2018).

The purpose of this study was to employ biochemical, histopathological, and immunohistochemical methods to reveal the effectiveness of Quercetin and Naringenin, with their powerful antioxidant characteristics, in preventing radiotherapy-associated submandibular gland injury.

2. Methods

Approval for the study was granted by the Ataturk University experimental animals' ethical committee at 14.04.2021 with number 2100107031. The experiments were then conducted using 48 Sprague Dawley rats weighing 250-300 g obtained from the Ataturk University Medical Application and Research Center. Throughout the study the rats were housed in steel cages at 23±2 °C and 45±10% relative humidity in a 12-h dark:light cycle, with no food restriction. The study was performed in compliance with the rules for the care and use of animals set out in the Declaration of Helsinki.

2.1. Power analysis

Using G power software, the power of the study was calculated for an effect size of 0.72 and an alpha error of 0.05, as 91 with 8 animals in each group.

2.2. Study design

The adult female rats were randomly assigned into six groups of eight animals each.

Group 1: The Control group. The eight rats in this group received physiological saline solution at a dosage of 8 ml/kg by intragastric gavage once daily for seven days.

Group 2: The Naringenin group. The eight rats in this group received 50 mg/kg Naringenin by the intragastric route once daily for seven days. The dose of naringenin used in our study was adjusted according to previous studies in the literature (Haider et al., 2020).

Group 3: The Quercetin group. The rats in this group received 50 mg/kg Quercetin by the intragastric route once daily for seven days. The dose of quercetin was adjusted according to the doses used in previous studies in the literature (Gorbenko et al., 2021).

Group 4: The Radiotherapy group. The rats in this group were exposed to single dose 15 Gy radiation including the head and neck region. The dose of radiation was adjusted according to the doses used in previous studies in the literature (Liu et al., 2021).

Group 5: The Radiation+Naringenin group. The eight rats in this group received 50 mg/kg Naringenin by the intragastric route once daily for seven days. Next, 24 h after the final dose, they were exposed to single-dose 15 Gy radiation including the head and neck region.

Group 6: The Radiation+Quercetin group. The eight rats in this group received 50 mg/kg Quercetin by the intragastric route once daily for seven days. Next, 24 h after the final dose, they were exposed to single-dose 15 Gy radiation incorporating the head and neck region.

All intragastric applications were performed at 8 a.m.

Naringenin and Quercetin were obtained from Sigma-Aldrich Chemical Co, dissolved in saline before application. The rats were sacrificed by cervical dislocation under general anesthesia with intraperitoneal administration of ketamine (Ketalar ampoule, Pfizer, Istanbul) at a dose of 40 mg/kg and xylazine hydrochloride (Rhompun vial, Bayer, Istanbul) at a dose of 10 mg/kg. Both submandibular glands were then removed through a vertical incision through the midline of the neck. In the study, the submandibular gland was preferred because it may be more sensitive to radiation damage due to its superficially location, and surgical removal is easier. The rat right gland was set aside for histopathological examination, while the rat left gland was used for biochemical analysis.

2.3. Radiation application

Prior to irradiation, the rats were exposed to general anesthesia using ketamine hydrochloride (Ketalar ampoule, Pfizer, Istanbul) at a dose of 40 mg/kg with xylazine hydrochloride (Rhompun vial, Bayer, Istanbul) at a dose of 10 mg/kg administered via the intraperitoneal (i.p) route. Once the rats to be irradiated were anesthetized, they were fixed in a supine position. The beam was prepared with the rats in groups of four, in a supine position, targeting a 40 cm×17.5 cm area of the head and neck region, using a 1 cm bolus with the SAD technique. A total of 15 Gy photon (X-ray) radiation was applied at a dose rate of 600 mu/min at a 0° gantry with 6 MV energy and a single fraction using a 13.6 Version Varian Trilogy (Serial No: 6196 USA) brand linear accelerator device.

2.4. Biochemical assay

Tissue specimens from right submandibular glands were extracted and immediately frozen at −80 °C prior to use during biochemical analyses. In the following stage, 0.1 g of tissue was homogenized with 900 μL of ice-cold phosphate-buffered saline at pH 7.4 (10% w/v). This was then centrifuged for 15 min at 4000 rpm at 4 °C. The resulting supernatants were next stored at −80°C until being used for the calculation of myeloperoxidase (MPO), glutathione (GSH) and superoxide dismutase (SOD) levels. The Bradford method was applied to determine the protein content of the supernatants, with bovine serum albumin being employed as standard (Bradford, 1976).

2.4.1. Myeloperoxidase assay

The method previously described by Bradly et al. was applied to measure MPO activity in the supernatant. Briefly, to 0.1 ml of supernatant was added 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.0005% hydrogen peroxide and 0.167 mg/ml O-dianisidine dihydrochloride. The resulting change in absorbance was determined using a spectrophotometer at 460 nm. MPO enzyme activity was expressed in the form of U/mg protein in the sample (Bradley et al., 1982).

2.4.2. Superoxide dismutase assay

: SOD activity was determined using modified method of (Sun et al., 1988). The principle of this method is based on the reduction of nitroblue tetrazolium (NBT) by the xanthine-xanthine oxidase system, a superoxide producer. Activity was based on measuring the increase in absorption at 560 nm due to the eventual reduction of NBT to $NBTH_2$ at the end of 20 min. One unit of SOD activity was defined as the amount of enzyme protein causing a 50% inhibition of the speed of reduction to $NBTH_2$. The results were expressed as U\mg protein.

2.4.3. Glutathione peroxidase (GPx) assay

GPx activity was investigated using the method described by Paglia and Valentine, 1967. The oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) is catalyzed by GSH-Px while the reduction of GSSG to GSH is catalyzed by glutathione reductase and NADPH in the presence of hydrogen peroxide. GSH-Px activity was calculated by reading the decrease in absorbance at 340 nm during the oxidation of NADPH to NADP+. The results were expressed as IU \ mg protein.

2.4.4. Catalase (CAT) assay

CAT activity was studied using the method described by Beers and Sizer, 1952. CAT assay was performed by measuring the decrease in absorption due to H_2O_2 consumption at 240 nm. The absorbance difference (ΔA 240) is a measure of CAT activity per unit of time. The results were expressed as U\mg protein.

2.4.5. Glutathione assay

GSH levels were measured using the method described by Beutler (1984). Glutathione reductase catalyzes the reduction of GSSG to GSH by NADPH. The GSH level is determined by measuring the absorbance difference of NAD(P)H, which is oxidized during the reaction, at 37 °C and at a wavelength of 340 nm. The results were expressed as mmol\mg protein.

2.4.6. Total antioxidant status (TAS) and total oxidant status (TOS) assay

The TOS test was performed in line with the manufacturer's protocol (Rel Assay Diagnostics, Gaziantep, Turkey, catalog no. AK181020). The results are expressed in units of mmol H_2O_2 equivalent/L. The TAS test was also performed in line with the manufacturer's kit protocol (Rel Assay Diagnostics, catalog no SR16067A). The results are expressed in units of mmol Trolox-equivalent/L. The ratio of TOS to TAS was determined as the oxidative stress index (OSI).

2.5. Histopathological analysis

Tissue specimens from salivary glands collected for histopathological examination following necropsy were fixed in 10% formalin solution for 48 h, then embedded in paraffin blocks. Sections 4 μm in thickness were taken from each block. The preparates were than stained with hematoxylin-eosin (HE) and examined under a light microscope (Leica DM 1000, Germany). Depending on their histopathological findings, such as degeneration, necrosis and atrophy in glandular epithelium and vascular congestion, sections were graded as none (-), mild (+), moderate (++), or severe (+++). Degeneration and necrosis in glandular epithelia were defined as a degenerated or necrotic cell number 0 (-), 2-3 (+), 4-7 (++) and more than 8 (+++). Atrophy in glandular epithelia was defined as atrophic cell number 0 (-), 2-3 (+), 4-7 (++) and more than 8 (+++). Vascular congestion was classified as number of congested vessels 0(-), 3-5 (+), 6-9 (++) and more than 10 (+++). Histopathological findings in gland tissues were scored from images obtained by digital camera from five different regions at 40x magnification. (Ahlner et al., 1994) All analyzes were performed blindly.

2.6. Immunohistochemical analysis

All sections placed onto adhesion (poly-L-lysine) for immunoperoxidase examination were passed through xylol and alcohol series. Endogenous peroxidase inactivation was established by placing the sections into 3% H_2O_2 after washing with PBS. Following treatment with antigen retrieval solution at 500 W for 2×5 min in a microwave to release the antigens in tissues, these were left to cool. The tissues were than incubated with Tumor Necrosis Factor-α (TNF- α) (Catalog no. sc-52746, diluent ratio: 1/100 Santa Cruz, EU) at 37° C for 30 min to determine inflammation and with 8-hydroxy 2-deoxyguanosine (8-OHdG) (Catalog no. sc-52746, sc-66036, diluent ratio: 1/100 Santa Cruz, EU) at 37° C for 60 min to determine DNA injury. The procedures were conducted in line with the immunohistochemistry kit protocol (AbcamHRP/DAB Detection IHC kit). 3-3' Diaminobenzidine (DAB) was used as a chromogen. Background staining was applied with hematoxylin. In order to determine the intensity of positive staining from the pictures obtained as a result of the dyeing; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program. The data were expressed as nm.

2.7. Statistical analysis

Statistical analyses were carried out on SPSS 20.0 software. The Shapiro Wilk test was applied to check distribution in the analysis. Since distribution was normal, the biochemical and immunochemical analyzes were performed using the One-Way ANOVA test and post-hoc Tukey test. Data were defined as mean and standard deviation (mean ±SD).

Intergroup differences in data obtained semiquantitatively at histopathological examination were analyzed using the non-parametric Kruskal-Wallis test, while the Mann Whitney U test was employed for two-group comparisons. As a result of all tests, a P value of <0.05 was considered significant.

3. Results

3.1. Biochemical results

One-Way ANOVA test and post-hoc Tukey test was used for the analyses of biochemical data. Once the rat submandibular gland tissues had been processed, the following biochemical data were obtained (Table 1). Significantly higher MPO levels were determined in the submandibular glands of rats exposed to radiotherapy than in the control group. In addition, the activities of the antioxidant enzymes SOD, GSH, GPX, and CAT also decreased significantly in these rats compared to the control group. This shows that the oxidative balance in submandibular gland tissue is compromised in favor of oxidative stress following radiotherapy. MPO levels were significantly lower in the rats receiving Quercetin and Naringenin compared to the radiation group, while significant elevation was observed in SOD, GPx, GSH, and CAT activities. This showed that Quercetin and Naringenin protect the oxidative balance by preventing oxidative stress. The activities of SOD, GSH and PON was significantly higher in rats received quercetin compared to the rats received naringenin while the level of MPO was significantly lower. Additionally, no significant difference was determined in gland SOD, GPX, and CAT activities between rats administered Quercetin and the control group. This shows that Quercetin bestows more effective protection than Naringenin.

TOS levels and OSI values were significantly higher while TAS levels were significantly lower in rats exposed to RT compared to the control group. However, TOS and OSI values were significantly lower in the rats receiving Quercetin and Naringenin compared to those exposed to radiotherapy, while TAS values were significantly higher. This also shows that Quercetin and Naringenin administration protects the oxidative balance in submandibular gland tissue. In addition, no difference was found in terms of TAS, TOS, and OSI values between the rats receiving Quercetin and the control group.

3.2. Histopathological findings

Semiquantitative histopathological analyses were performed by using Mann Whitney U test. The salivary glands from the rats of Control, Naringenin and Quercetin groups showed normal histological appearance. (Fig. 1-A, B, C). Examination of the salivary glands from the rats in radiation group revealed severe dark eosinophilic cytoplasmic degeneration in the epithelia, necrosis, and in some glands, atrophy. Mononuclear cell infiltration into interstitial spaces, dilated parenchymal vessels, and severe congestion were also observed (Fig. 1-D).

Table 1
The oxidant and antioxidant parameters of the groups.

	CONTROL	RT		RT+QUE		RT+NAR	
		Mean±SD	P (vs Control)	Mean±SD	P (vs RT)	Mean±SD	P(vs RT)
MPO	14.98±4.2	54.07±2.3	<0.0005*	26.86±2.7	<0.0005*	42.91±2.7	<0.0005*
SOD	56.37±1.4	31.56±5.3	<0.0005*	50.15±1.5	<0.0005*	39.01±1.5	0.033*
GSH	16.6±1.2	4.89±0.3	<0.0005*	13.42±1.1	<0.0005*	7.18±0.3	<0.0005*
GPX	14.09±1.3	6.96±0.7	<0.0005*	12.54±0.9	<0.0005*	9.91±1	0.002*
CAT	32.92±2.9	14.84±1.7	<0.0005*	28.55±1.5	<0.0005*	22.85±2.8	0.001*
TAS	7.1±1.1	4.43±0.3	0.001*	6.6±0.8	0.009*	6.05±0.2	<0.0005*
TOS	16.6±1.8	38.84±1.1	<0.0005*	18.18±1	<0.0005*	21.17±2.4	<0.0005*
OSI	42.83±5.5	11.43±1.1	0.001*	36.44±5.4	0.002*	28.85±3.1	0.001*

MPO: Myeloperoxidase, SOD: Superoxide dismutase, GSH: Glutathione, GPX: Glutathione peroxidase, CAT: Catalase, TAS; Total antioxidant status, TOS: Total oxidant status. OSI: Oxidative stress index
All biochemical data were analyzed parametrically with one-way ANOVA and post-hoc Tukey test
*: Statistically significant ($p<0.05$)

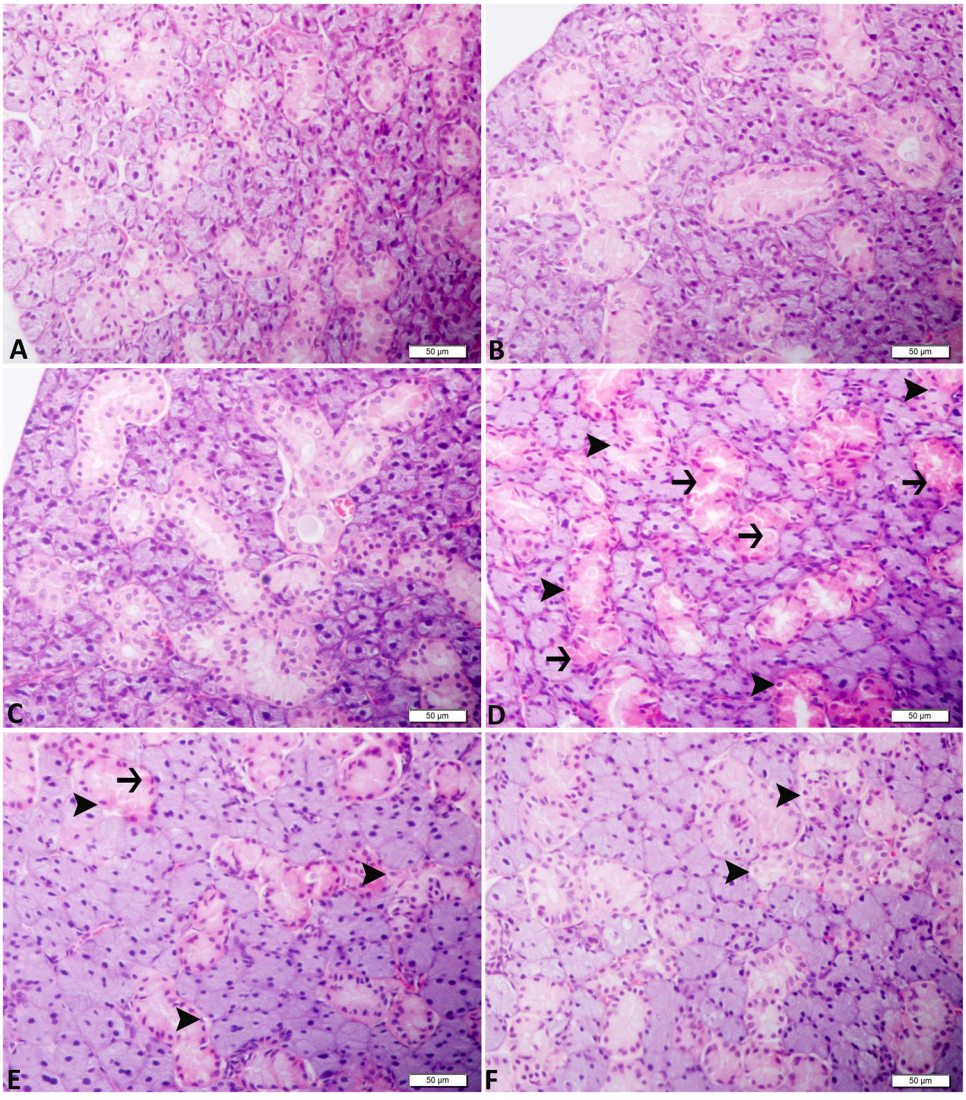


Fig. 1. : Salivary gland, Control, Nar, and Quer groups, normal histological appearance (A, B, C); RT group glandular epithelial cytoplasm with severe degeneration (arrow heads), necrosis (arrows) (D); RT+Nar group, moderate degeneration in the glandular epithelia (arrow heads), moderate necrosis (arrow) (E)l RT+Nar group, mild degeneration in glandular epithelia (arrow heads) (F), H&E, Bar:50 µm.

Examination of the salivary glands from the rats in Radiation+Naringenin group revealed moderate degeneration in the glandular epithelia and mild necrosis, and vascular dilation and congestion (Fig. 1-E). Significant prevention of salivary gland injury was observed compared to the RT group ($p<0.05$). Also, examination of the salivary glands from the rats in Radiation+Quercetine group revealed mild degeneration and vascular congestion in the glandular epithelia (Fig. 1-F). Significant prevention of salivary gland injury was observed

compared to the radiotherapy group ($p<0.05$). The histopathological findings are summarized in Table 2.

3.3. Immunohistochemical findings

One-Way ANOVA test and post-hoc Tukey test was performed to compare positive immunoreactive cells and immunopositive stained areas. Immunohistochemical examination of the rat salivary glands in Control, Naringenin, and Quercetin groups revealed negative TNF-α and 8-OHdG expression (Figs. 2–3–A, B, C). Examination of the rat salivary glands from Radiation group revealed strong TNF-α expression around the vessels, in the interstitial spaces, and in the cytoplasms of mononuclear cells in this region (Fig. 2-D). Also, strong cytoplasmic 8-OHdG expression was observed in the acinar and ductal epithelia (Fig. 3-D). Rat salivary glands from Radiation+Naringenin group revealed moderate TNF-α expression in the interstitial spaces, around the vessels, and in mononuclear cell cytoplasm (Fig. 2-E). Moderate cytoplasmic 8-OHdG expression was also detected in the acinar and ductal epithelia (Fig. 3-E). A significant difference was determined compared to the radiotherapy group ($p<0.05$). Rat salivary glands from Radiation+Quercetin group revealed mild TNF-α expression in mononuclear cell cytoplasm in interstitial spaces (Fig. 2-F). Mild cytoplasmic 8-OHdG expression was also observed in the acinar and ductal epithelia (Fig. 3-F). A significant difference was determined compared to the radiotherapy group ($p<0.05$). The immunohistochemical findings are summarized in Tables 2–3. As shown, quercetin exhibited more effective protection against radiation injury compared to Naringenin.

4. Discussion

Radiotherapy is a therapeutic modality frequently applied in the treatment of head and neck cancers. While it represents the primary option in some head and neck cancers, it can be employed as an adjuvant in others. The most important factor limiting this treatment is xerostomia developing in association with injury forming in the salivary glands. Xerostomia, characterized by a decrease in salivary secretion, causes significant morbidities by impairing such basic physiological functions as speech, mastication, and swallowing. Xerostomia has been shown to largely develop in association with degeneration occurring in acinar cells (Acauan et al., 2015; De la Cal et al., 2012). Histopathological examination of the submandibular gland following single dose 15 Gy external radiotherapy in the head and neck region in the present study revealed severe degeneration with dark eosinophilic cytoplasm in the glandular epithelium, necrosis and atrophy in some glands, mononuclear cell infiltration in the interstitial spaces, dilatation in the parenchymal vessels, and severe congestion. No side-effects sufficiently severe as to leave any rat outside the scope of the study were observed after 15 Gy radiotherapy.

These adverse effects of radiotherapy on the submandibular gland result directly in DNA injury and indirectly in oxidative stress. Studies have shown an increase in oxidative stress parameters and a decrease in antioxidative parameters in the submandibular gland after radiotherapy. (Ozgur et al., 2019) examined the submandibular glands of rats exposed to radiation and showed a significant decrease in Total Antioxidant Status levels in gland tissue and a significant increase in Total Oxidant Status. Another similar study showed that malondialdehyde, Total Oxidant Status, and Oxidative Stress Index values in the parotid gland increased significantly following radiotherapy, while levels of the antioxidant enzymes Glutathion Peroxidase and Catalase decreased significantly. No significant change was reported in Superoxide Dismutase levels after radiotherapy (Cakmak et al., 2016). However, studies examining levels of the important antioxidant Superoxide Dismutase following radiotherapy have also reported a decrease in enzyme levels (Koc et al., 2003). Similarly, in the present study, Myeloperoxidase levels increased in submandibular gland tissue after radiotherapy, while significant decreases were observed in Superoxide Dismutase, Glutathion Peroxidase, Catalase, and Glutathion levels. In addition, Total Antioxidant Status levels in submandibular gland tissue decreased following radiotherapy, while Total Oxidant Status and Oxidative Stress Index values increased.

8-OHdG is a biomarker that has been the subject of frequent interest in showing radiotherapy-related oxidative DNA injury in recent years. Fardid et al. (2017) showed that 8-OHdG expression in pulmonary tissue increased following radiotherapy. The present study also showed an increase following radiotherapy in the expression in the submandibular gland of 8-OHdG, of critical importance in showing DNA injury caused by Reactive Oxygen Species production. The study findings also show that the application of Quercetin and Naringenin prevented impairment of the oxidative balance after radiotherapy.

Radiotherapy also causes inflammation in tissues. Radiation has been shown to modulate the immune system by upregulating inflammatory mediators. Radiation-dependent cytokine activation is of particular, critical importance. TNF-alpha is an important cytokine used by the immune system for cell signaling. TNF-alpha is released by macrophages in case of inflammation and plays a critical role in stimulating other immune system cells. Several studies have shown that TNF alpha production increases after radiation (Jelonek et al., 2017). In the present study, TNF-alpha was investigated using immunocytochemical methods in order to show inflammation in salivary gland tissue and to reveal the protective effectiveness of the agents applied. Strong TNF-alpha immunopositivity was observed in submandibular gland tissue exposed to radiation, while this immunopositivity decreased significantly in the submandibular gland tissues of rats given Quercetin and Naringenin.

Due to the key roles of oxidative stress and inflammation in the damage occurring in the salivary glands, several studies have used agents with antioxidant and anti-inflammatory characteristics to prevent tissue damage. Similarly, Naringenin and Quercetin, both with antioxidant and anti-inflammatory efficacy, were similarly employed in the present study.

Naringenin is a flavonoid with antioxidant, anti-inflammatory, and cardiovascular effectiveness fund in citrus fruits. It exhibits its antioxidant effects through metal ion chelation and free radical scavenging properties by inhibiting pro-oxidant enzymes. It also increases the levels of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase (Joshi et al., 2018). In a rat study of the radioprotective efficacy of Naringenin, (Zhang et al., 2018) showed that it exhibited a protective effect against radiation-induced lung damage. They also found that Naringenin not only ameliorated acute radiation pneumonia and chronic radiation fibrosis, but also improved radiation-associated

Table 2
Salivary gland histopathological and immunohistochemical findings.

	Degeneration in glandular epithelia	Necrosis in glandular epithelia	Atrophy in glandular epithelia	Vascular congestion	Tnf-α	8-OHdG
Control	-	-	-	-	-	-
RT	+++	+++	+++	+++	+++	+++
Nar	-	-	-	-	-	-
Quer	-	-	-	-	-	-
RT+ Nar	++	+	++	+++	++	++
RT+ Quer	+	-	+	++	+	+

none (-), mild (+), moderate (++), severe (+++)

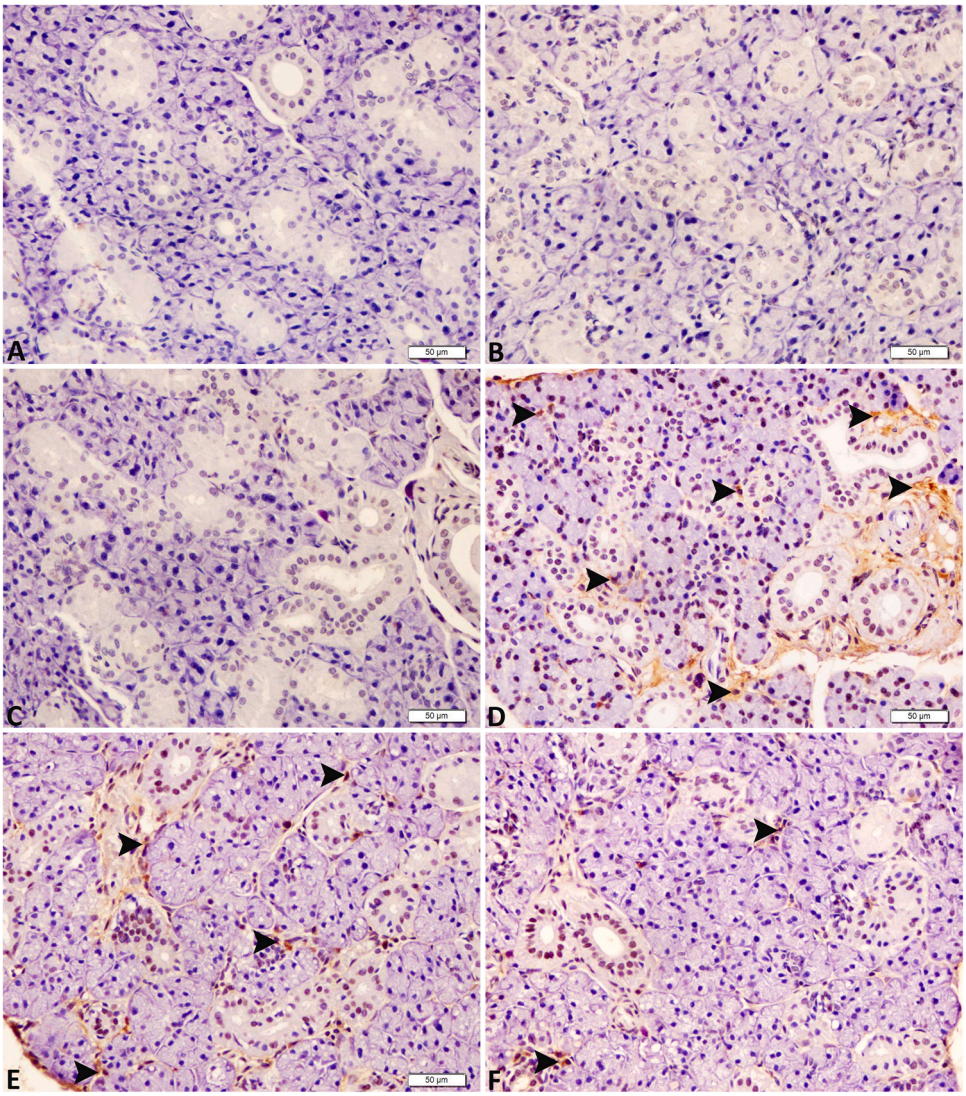


Fig. 2. : Salivary gland, Control, Nar, and Quer groups, negative TNF-α expression (A, B, C); RT group, severe TNF-α expression in interstitial spaces, around the vessels, and in mononuclear cells (arrow heads) (D), RT+Nar group, moderate TNF-α expression in interstitial spaces, around the vessels, and in mononuclear cells (arrow heads) (E), RT+Nar group, mild Tnf-α expression in mononuclear cells (arrow heads) (F), IHC-P, Bar:50 µm.

inflammatory hemostasis. In another study, Naringenin was found to exhibit protective activity against radiation-induced DNA, chromosome, and membrane damage in mice by inhibiting the Nuclear Factor kappa B (NF-kB) pathway and reducing apoptotic proteins Kumar and Tiku, 2016. The present study investigated the protective efficacy of Naringenin against radiation-related submandibular gland injury using biochemical, histopathological, and immunohistochemical methods. To the best of our knowledge, this study involving Naringenin is the first of its kind in the literature.

Quercetin is a flavonoid found in several fruits and vegetables. It exhibits its antioxidant effectiveness through enzymatic activities, signal transduction pathways, and Reactive Oxygen Species production. Through these properties, it exhibits powerful antioxidant efficacy by establishing oxidative balance (Xu et al., 2019). In a study investigating the protective effects of Quercetin against radiation-induced brain injury, (Kale et al., 2018) showed that Quercetin reduced malondialdehyde levels in blood and tissue while increasing Total Antioxidant Status values. They also showed that it protected brain tissue histopathologically against the deleterious effects of radiation. The authors concluded that Quercetin exhibited neuroprotective efficacy against radiation-associated brain damage. (Takahashi et al., 2015) reported that Quercetin increased saliva secretion in radiation-related salivary gland injury. The present study through biochemical, histopathological, and immunohistochemical analyses, also shows that Quercetin exhibits efficacy against radiation-related submandibular gland injury. To the best of our knowledge, this study involving Quercetin is also the first of its kind on this subject.

In the study, the submandibular gland was preferred because it may be more sensitive to radiation damage due to its superficially location, and surgical removal is easier. The principal limitation of this study is that saliva production was not investigated using scintigraphic or quantitative methods. Second limitation is that the agents employed were not applied at different doses. Third limitation was the use of single-sex rats and that was a minor weakness of our study.

5. Conclusion

Submandibular gland damage developing in association with radiation is principally linked to oxidative stress and inflammation. With their powerful antioxidant and anti-inflammatory effects, Naringenin and Quercetin exhibit protection against radiation-related submandibular gland injury. In addition, Quercetin was found to be superior to Naringenin in terms of this efficacy. Further prospective studies are now needed to investigate the clinical use of these two agents.

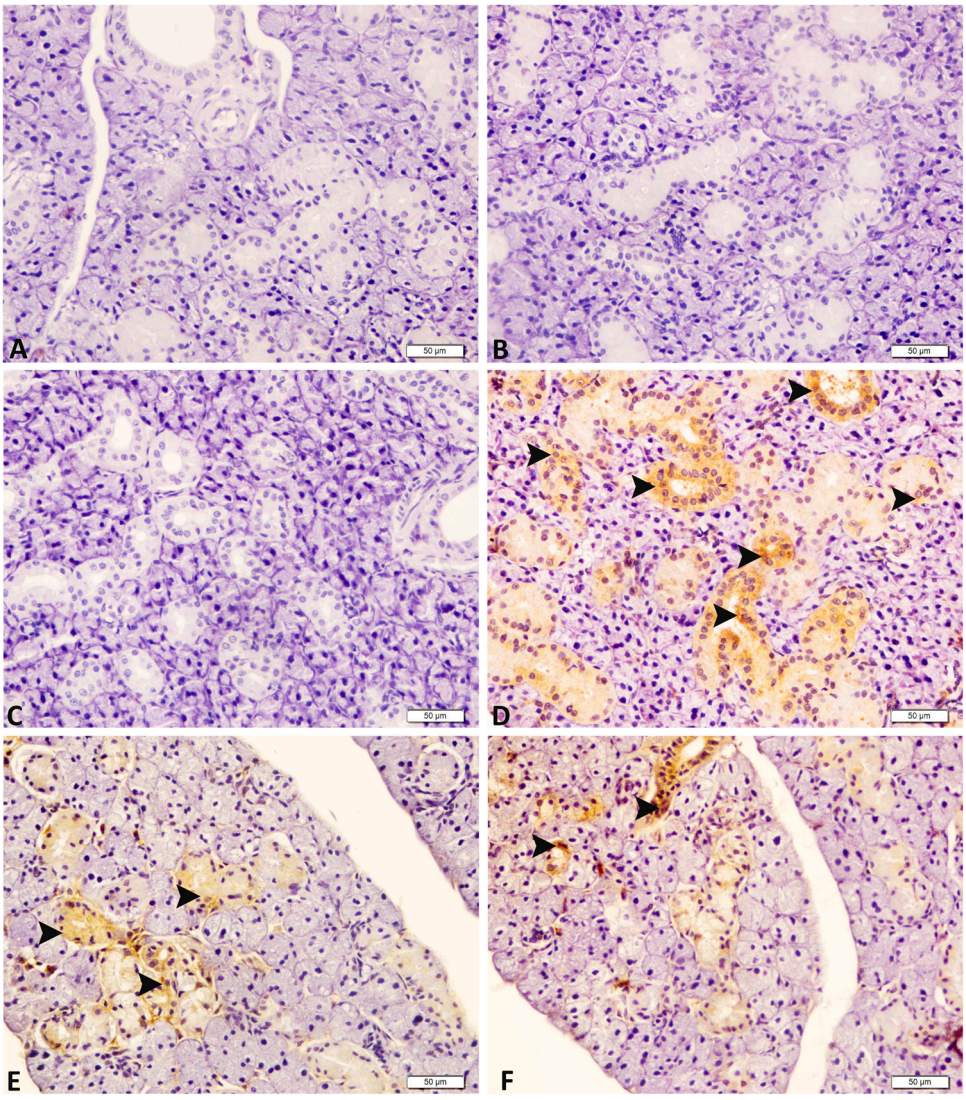


Fig. 3. : Salivary gland, Control, Nar, and Quer groups, negative 8-OHdG expression (A, B, C); RT group, strong 8-OHdG expression in the acinar and ductal epithelia (arrow heads) (D); RT+Nar group, moderate 8-OHdG expression in acinar and ductal epithelia (arrow heads) (E), RT+Nar group, mild 8-OHdG expression in acinar and ductal epithelia (arrow heads) (F), IHC-P, Bar:50 μm.

Table 3
Salivary gland immunohistochemical findings.

	Tnf-α (nm) (Mean±SD)	8-OHdG (nm) (Mean±SD)
Control	23,41±5,42[a]	25,95±4,12[a]
RT	83,26±2,22[b]	81,41±3,62[b]
Nar	22,77±1,37[a]	26,80±5,41[a]
Quer	22,25±4,55[a]	25,81±3,62[a]
RT+ Nar	60,61±2,43[c]	52,41±3,22[c]
RT+ Quer	42,15±6,52[d]	39,85±4,10[d]

a,b,c,d, Different letters in the same column were considered statistically different. ($p<0.05$)

Funding

This work was supported by Research Fund of the Ataturk University, Erzurum, Turkiye with . Project Number: TAB-2021-9219.

Ethical approval

This study was performed in accordance with the PHS Policy on Human Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Animal Experiments ethical committee of Ataturk University.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

CRediT authorship contribution statement

MSS, KK, SY, HKO and AS contributed the conception and design of the study. MSS, KK, SY, YSS, SA, AK and AS contributed in analysis and interpretation of data. HKO performed the radiotheraphy. AK and SA carried out the biochemical studies while SY and YSS performed the histopathological experiments. MSS, KK, SY, YSS, SA, AK, HKO and AS contributed in drafting the article. All authors read and approved the final manuscript.

References

Acauan, M. D., Figueiredo, M. A., Cherubini, K., Gomes, A. P., & Salum, F. G. (2015). Radiotherapy-induced salivary dysfunction: Structural changes, pathogenetic mechanisms and therapies. *Archives of Oral Biology, 60*(12), 1802–1810.

Ahlner, B. H., Hagelqvist, E., & Lind, M. G. (1994). Influence on rabbit submandibular gland injury by stimulation or inhibition of gland function during irradiation: Histology and morphometry after 15 gray. Annals of Otology. *Rhinology & Laryngology, 103*(2), 125–134.

Beers, R. F., & Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry, 195*(1), 133–140.

Beutler, E. (1984). Red Cell Metabolism. In *A manual of biochemical methods* (3th ed.„ *72-73* pp. 105–106). Orlando: Grune & Stratton.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 7*(72), 248–254.

Bradley, P. P., Priebat, D. A., Christensen, R. D., & Rothstein, G. (1982). Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology, 78*(3), 206–209.

Cakmak, K. I., Simsek, G., Yildiz, A., Vardi, N., Polat, A., Tanbek, K., … Parlakpinar, H. (2016). Melatonin's protective effect on the salivary gland against ionized radiation damage in rats. *Journal of Oral Pathology Medicine, 45*(6), 444–449.

De la Cal, C., Fernández-Solari, J., Mohn, C., Prestifilippo, J., Pugnaloni, A., Medina, V., & Elverdin, J. (2012). Radiation produces irreversible chronic dysfunction in the submandibular glands of the rat. *The Open Dentistry Journal, 6*, 8–13.

Fardid, R., Salajegheh, A., Mosleh-Shirazi, M. A., Sharifzadeh, S., Okhovat, M. A., Najafi, M., … Abaszadeh, A. (2017). Melatonin ameliorates the production of COX-2, iNOS, and the formation of 8-OHdG in non-targeted lung tissue after pelvic irradiation. *Cell Journal, 19*(2), 324–331.

Gatta, G., Botta, L., Sánchez, M. J., Anderson, L. A., Pierannunzio, D., & Licitra, L. (2015). Prognoses and improvement for head and neck cancers diagnosed in Europe in early 2000s: The EUROCARE-5 population-based study. Eurocare Working Group *European Journal of Cancer, 51*(15), 2130–2143.

Gorbenko, N. I., Borikov, O. Y., Kiprych, T. V., Ivanova, O. V., Taran, K. V., & Litvinova, T. S. (2021). Quercetin improves myocardial redox status in rats with type 2 diabetes. *Endocrine Regulations, 55*(3), 142–152.

Haider, S., Liaquat, L., Ahmad, S., Batool, Z., Siddiqui, R. A., Tabassum, S., Shahzad, S., Rafiq, S., & Naz, N. (2020). Naringenin protects AlCl3/D-galactose induced neurotoxicity in rat model of AD via attenuation of acetylcholinesterase levels and inhibition of oxidative stress. *PLoS One, 15*(1). Article e0227631.

Jelonek, K., Pietrowska, M., & Widlak, P. (2017). Systemic effects of ionizing radiation at the proteome and metabolome levels in the blood of cancer patients treated with radiotherapy: the influence of inflammation and radiation toxicity. *International Journal of Radiation Biology, 93*(7), 683–696.

Joshi, R., Kulkarni, Y. A., & Wairkar, S. (2018). Pharmacokinetic, pharmacodynamic and formulations aspects of Naringenin: An update. *Life Science, 15*(215), 43–56.

Kale, A., Piskin, Ö., Bas, Y., Aydin, B. G., Can, M., Elmas, Ö., & Büyükuysal, Ç. (2018). Neuroprotective effects of Quercetin on radiation-induced brain injury in rats. *Journal of Radiation Research, 59*(4), 404–410.

Koc, M., Taysi, S., Buyukokuroglu, M. E., & Bakan, N. (2003). Melatonin protects rat liver against irradiation-induced oxidative injury. *Journal of Radiation Research, 44*(3), 211–215.

Kumar, S., & Tiku, A. B. (2016). Biochemical and molecular mechanisms of radioprotective effects of naringenin, a phytochemical from citrus fruits. *Journal of Agricultural and Food Chemistry, 64*(8), 1676–1685.

Lalla, R. V., Treister, N., Sollecito, T., Schmidt, B., Patton, L. L., Mohammadi, K., Hodges, J. S., & Brennan, M. T. (2017). Oral complications at 6 months after radiation therapy for head and neck cancer. OraRad Study Group *Oral diseases, 23*(8), 1134–1143.

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M. T., Wang, S., Liu, H., & Yin, Y. (2016). Quercetin, Inflammation and Immunity. *Nutrients, 8*(3), 167.

Liu, Q., Wang, Y., Cui, Z., Ma, X., Shi, H., & Zhang, W. (2020). Erythropoietin plays a protective role in submandibular gland hypofunction induced by irradiation. *Journal Oral Maxillofac Surgery, 79*(6), 1373–1383.

Liu, X., Subedi, K. P., Zheng, C., & Ambudkar, I. (2021). Mitochondria-targeted antioxidant protects against irradiation-induced salivary gland hypofunction. *Science Reports, 11*(1), 7690.

Ozgur, A., Terzi, S., Özdemir, D., Tümkaya, L., Rakıcı, S., Mercantepe, T., … Dursun, E. (2019). Protective effect of whortleberry extract on salivary gland damage induced by neck irradiation in rats. *Ear Nose Throat Journal, 98*(6), E64–E69.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory Clinical Medicine, 70*(1), 158–169.

Rosen, E. M., Day, R., & Singh, V. K. (2015). New approaches to radiation protection. *Frontiers in Oncology, 20*(4), 381.

Sun, Y., Oberley, L. W., & Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry, 34*(3), 497–500.

Takahashi, A., Inoue, H., Mishima, K., Ide, F., Nakayama, R., Hasaka, A., Ryo, K., Ito, Y., Sakurai, T., Hasegawa, Y., & Saito, I. (2015). Evaluation of the effects of quercetin on damaged salivary secretion. *PloS one, 10*(1). Article e0116008.

Xu, D., Hu, M. J., Wang, Y. Q., & Cui, Y. L. (2019). Antioxidant activities of quercetin and its complexes for medicinal application. *Molecules, 24*(6), 1123.

Zhang, C., Zeng, W., Yao, Y., Xu, B., Wei, X., Wang, L., … Liang, W. (2018). Naringenin ameliorates radiation-induced lung injury by lowering IL-1β Level. *The Journal of pharmacology and experimental therapeutics, 366*(2), 341–348.