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Effects of thymoquinone in the lungs of rats against radiation-induced oxidative stress

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Abstract. – OBJECTIVE: Radiotherapy is an important treatment for a wide variety of malignancies, although many cancer patients who receive radiotherapy suffer from serious side effects during and after their treatment. Thymoquinone (TQ), the main active ingredient of Nigella sativa, has been reported to have various pharmacological properties, such as antioxidant, hepatoprotective, neuroprotective, antidiabetic, anti-inflammatory, nephroprotective, anticarcinogenic in many pharmacological and toxicological studies. In this study, we aimed to investigate whether there is a radioprotective effect of TQ on the lung tissue of rats exposed to ionizing radiation.

MATERIALS AND METHODS: This study was designed as a prospective, placebo-controlled study. A total of 40 Sprague-Dawley rats were divided into four groups to test the radiation-protective effectiveness of TQ administered by intraperitoneal injection. Biochemical parameters were studied to assess the radiation-protective effects of TQ.

RESULTS: Oxidative stress parameters, such as oxidative stress index (OSI), lipid hydroperoxide (LOOH) and total oxidant status (TOS), in lung tissue of the rats treated with TQ, were found to be lower than in received irradiation alone. Anti-oxidative parameters, such as total antioxidant status (TAS) level and paraoxonase (PON) activity, were statistically higher in the TR (IR plus TQ group) group compared with other groups.

CONCLUSIONS: Findings show that TQ clearly protects lung tissue from radiation-induced oxidative stress and can be used as a radioprotective agent.

Key Words:
Thymoquinone, Ionizing radiation, Lung damage, Oxidative stress, Antioxidants, Free radicals.

Introduction

Cardiovascular disease and cancer are the leading causes of death in Turkey and worldwide[1,2]. Cancer has been one of the worst diseases of the 20th century, and its incidence is increasing alarmingly due to changes in people's lifestyles and habits. It is spreading even further in the 21st century with continuity and increasing incidence. The situation is so worrying that one in four people has a lifetime risk of cancer[1-4].

Radiation therapy (RT) occupies an important place in the treatment of cancer[5]. Ionizing radiation causes oxidative/nitrosative stress by increasing free radical production[6]. This situation plays an important role in the pathogenesis of lung injury. During irradiation, the normal tissues surrounding the tumor entering the radiation field may cause symptomatic injury. Such damage to the airway epithelium causes radiation pneumonia (RP), which is a major dose-limiting toxicity in thoracic RT[7,8]. RP usually occurs within 6 months of RT, which might require considerable supportive measures, including steroids, oxygen supplementation, or even mechanical ventilation. RP has the potential to be fatal, even with treatment, in rare instances[9]. Therefore, the role of natural compounds that protect tissues and organs from the harmful effects of radiation and at the same time do not have a protective effect against cancer cells is very important in clinical RT[10-12].

Efforts to reduce the toxicity of radiation to normal tissues, organs, and cells and the search for cytoprotective agents have become important areas of interest today. Investigating the possible radioprotective properties of non-toxic antioxidants such as propolis, caffeic acid phenethyl ester (CAPE), and thymoquinone (TQ) has been an important research topic, as most radiation protectors have toxic side effects that limit their role in medical therapy[13-15].

The main active ingredient of *Nigella Sativa* seed – TQ – is a natural antioxidant molecule that draws attention with its free radical scavenging properties and important aspects that stimulate the antioxidant defense system[16].

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In the present study, we studied the effects of TQ on radiation-induced oxidative stress in lung tissues of rats by examining total antioxidant status (TAS), total oxidant status, and oxidative stress index (OSI). We also studied the possible mechanisms involved in the radiation-protective ability of TQ, which were their radical scavenging activities and their effects on levels of lipid hydroperoxides (LOOH) and antioxidant systems including enzymes such, as paraoxonase (PON) and arylesterase (ARE), low-molecular-weight free radical scavengers, such as total sulfhydryl (-SH) groups, and proteins, such as ceruloplasmin (Cp).

Materials and Methods

Animals

The experiment used 40 Sprague-Dawley rats. Rats were kept in the same laboratory conditions throughout the study. The rats were quarantined for at least seven days before irradiation, housed ten to a cage in a windowless laboratory room with automatic temperature (22 ± 1°C) and lighting controls (12 h light/12 h dark), and fed with standard laboratory chow and water. That were 10-12 weeks old and weighed 200 ± 25 g at the time of irradiation. All procedures involving the Sprague-Dawley rats adhered to the ARVO Resolution on the Use of Animals in Research. This study was designed as a prospective, placebo-controlled study.

Chemicals

Dimethyl sulfoxide (DMSO) and thymoquinone were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and reagents were obtained from the store of the Department of Medical Biochemistry, Gaziantep University, School of Medicine.

Experimental Protocol

The rats were quarantined for at least 1 week before gamma irradiation and fed by standard laboratory chow and water *ad libitum*. The rats were divided into four equal groups by simple random sampling method. Group CN (normal control group) did not receive TQ, or irradiation.

Group CS (sham control group) did not receive TQ or irradiation but received sham irradiation and intraperitoneal (ip) dimethyl sulfoxide (DMSO) injections at a volume equal to that of TQ dissolved for group TR, respectively.

Irradiation group (IR) received 5 Gy of gamma irradiation as a single dose to total thorax. Group TR (TQ plus group R) received both 5 Gy of gamma irradiation as a single dose to total thorax and TQ (50 mg kg^{-1} day^{-1}) daily by ip injection starting 30 min before the irradiation dose and subsequently daily for 10 days after irradiation. TQ was dissolved in DMSO just before giving to the rats. The final concentration of DMSO was 0.1%.

All of the rats were anesthetized with 80 mg kg^{-1} ketamine HCl (Pfizer Ilac, Istanbul, Turkey) before undergoing total thorax irradiation and were placed on a tray in the prone position.

The rats in the CR-IR and the TR groups received irradiation, using a Cobalt-60 teletherapy unit (Picker, C9, Maryland, NY, USA), from a source-to-surface distance of 80 cm by 5x5 cm anterior fields, with the thorax gamma irradiation as a single dose of 5 Gy. The dose rate was 0.49 Gy per min. The central axis dose was calculated at a depth of 1 cm.

Biochemical Measurements

On the 10^{th} day of the study, the rats were sacrificed, and lung tissue samples were taken. The lung tissues were homogenized in physiological saline solution (IKA-NERKE, GmBH & CO. KB D-79219, Staufen, Germany). The homogenate was centrifuged at 10,000 g for 1 h to remove debris. The clear upper supernatant was collected, and all assays were carried out on this fraction. All of the procedures were performed at 4°C.

Measurement of Total Antioxidant Status (TAS)

Tissue TAS was determined as described by Erel et al[17]. The TAS measurement was carried out using a fully automated TAS kit (Rel Assay DC, Gaziantep, Turkey) in a Beckman Chemistry Coulter AU480 autoanalyzer. Results are expressed in mmol Trolox Eq /L.

Measurement of Total Oxidant Status (TOS)

Total oxidant status was determined as described by Erel et al[18]. TOS measurements were carried out using a fully automated TOS kit (Rel Assay DC, Gaziantep, Turkey) in an autoanalyzer (Beckman Coulter Chemistry Analyzer AU480, Brea, CA, USA). Results are expressed in μmol H_2O_2 Eq/L.

The oxidative stress index (OSI) was calculated using TAS and TOS values (Table I). First, the

Table I. Oxidant parameters measured in the lung tissue of rats.

	CN	CS	IR	TR
TOS (μmol H_2O_2 equivalent/g protein)	4.36 ± 0.68[a]	4.72 ± 0.39[a]	5.97 ± 0.43	4.25 ± 0.67[a]
OSI (Arbitrary unit)	1.03 ± 0.29[a]	1.05 ± 0.41[a]	1.60 ± 0.69	0.64 ± 0.17[a]
LOOH (μmol/g protein)	1.08 ± 0.07[a]	1.13 ± 0.08[a]	1.33 ± 0.05	1.05 ± 0.05[a]

[a]: $p < 0.001$ *vs*. IR group. TOS, total oxidative status; OSI, oxidative stress index; LOOH, lipid hydroperoxides; CN, normal control group; CS, control shame group; IR, irradiation-alone group; TR, irradiation plus TQ group.

TAS unit was converted to μmol/L. Then, TOS values were divided by TAS values and multiplied by 100. The resulting ratio was expressed as OSI. OSI (arbitrary unit) = [TOS (μmol H_2O_2 equivalent/gr protein)/ TAS (μmol Trolox equivalent/gr protein)] × 100.

Measurement of Ceruloplasmin (Cp) and Total Sulfhydryl Groups (-SH)

The Ceruloplasmin measurement was performed using the Erel method[19]. Total Sulfhydryl Groups were measured using Ellman's method[20] as modified by Hu's method[21]. For Cp, the results are expressed in terms of mg/gr protein, and for the -SH groups, the results were expressed by mmol/gr protein (Table II).

Measurement of Arylesterase (ARE) and Paraoxonase-1 Activities (PON-1)

Phenylacetate was used as a substrate to measure ARE activity by monitoring the increase in absorbance at 270 nm at 37°C. The activity was calculated from the molar absorptivity coefficient of the produced phenol[22], 1,310 M^{-1} cm^{-1}. One unit of ARE activity was defined as 1 μmol phenol generated/min under the aforementioned conditions and expressed as U/gr protein.

PON-1 activity was measured in the basal activity. The rate of paraoxon hydrolysis was measured by monitoring the increase in absorbance at 412 nm at 37°C. The amount of generated p-nitrophenol was calculated from the molar absorptivity coefficient at pH 8[23], which was 17,000 M^{-1} cm^{-1}. PON-1 activity was expressed as U/gr protein.

Measurement of Lipid Hydroperoxides (LOOH) Levels

Tissue LOOH levels were measured using the ferrous ion oxidation-xylenol orange (FOX-2) method, as previously described[24]. The results are expressed as μmol/gr protein.

Statistical Analysis

Data analysis was performed using the Statistical Package for Social Sciences version 18 (SPSS Inc.; Chicago, IL, USA) software package. Data were analyzed with One-way analysis of variance followed by a post-hoc test (LSD alpha) for multiple comparisons. Data were expressed as mean ± standard deviation (SD) and p-values < 0.05 were considered to be statistically significant.

Results

Oxidative Parameters

Oxidative parameters of the four groups are shown in Figure 1. There was a statistically significant difference between the IR group and other groups. The TOS, LOOH, and OSI levels in the IR

Table II. Antioxidant parameters measured in the lung tissue of rats.

	CN	CS	R	TR
TAS (mmol Trolox equivalent/g protein)	0.44 ± 0.09[b]	0.52 ± 0.21[b]	0.43 ± 0.18[b]	0.69 ± 0.11
Cp (U/g protein)	94.1 ± 4.7	98.3 ± 6.1	91.9 ± 2.8	95.2 ± 11.4
ARE (U/g protein)	8.9 ± 0.4	8.8 ± 0.4	8.5 ± 0.7	9.1 ± 0.7
PON (U/g protein)	1.13 ± 0.24[a]	1.07 ± 0.18[a]	1.01 ± 0.17[a]	1.56 ± 0.19
–SH (mmol/g protein)	0.61 ± 0.045	0.57 ± 0.051	0.58 ± 0.04	0.59 ± 0.06

[a]: $p < 0.01$, [b]: $p < 0.001$ *vs*. TR group. TAS, total antioxidative status; Cp, ceruloplasmin; ARE, arylesterase; PON, paraoxonase; -SH, sulfhydryl.

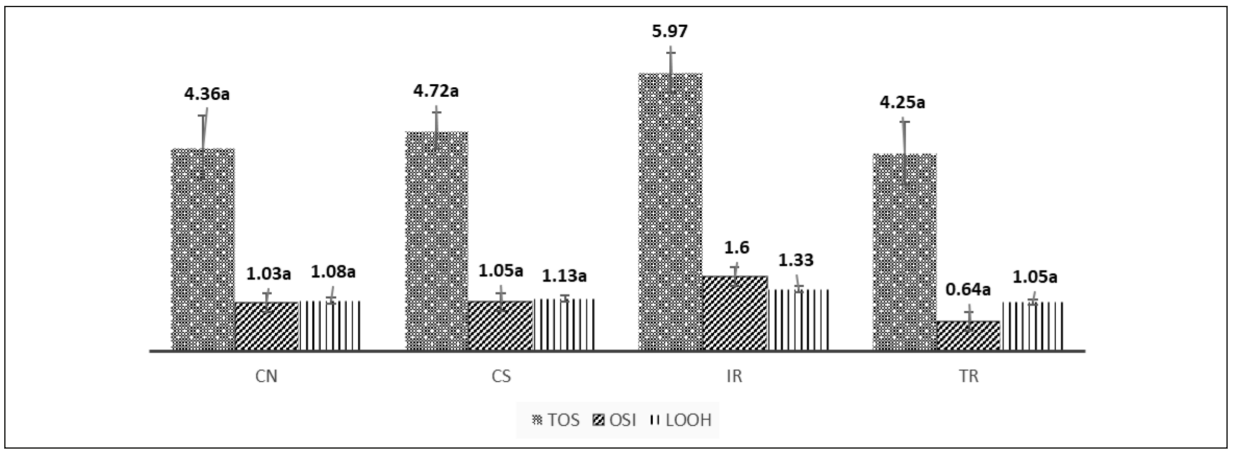

Figure 1. Oxidant parameters measured in the lung tissue of rats. a: $p < 0.001$ *vs*. IR group. TOS, total oxidative status; OSI, oxidative stress index; LOOH, lipid hydroperoxides; CN, normal control group; CS, control shame group; IR, irradiation-alone group; TR, irradiation plus TQ group.

group were statistically higher than other groups ($p < 0.0001$). The levels of TOS, LOOH, and OSI in the group treated with TR were statistically lower compared to the IR group. Compared with the IR group, TOS levels were reduced by 26.9% in the CN group, 20.9% in the CS group, and 28.8% in the TR group, respectively. OSI levels were decreased by 35.6% in the CN group, 34.4% in the CS group, and 51.9% in the TR group, respectively. In addition, LOOH levels were decreased by 18.8% in the CN group, 15% in the CS group, and 21% in the TR group, respectively. This irradiation-induced increase in these oxidative parameters was prevented by TQ ($p < 0.001$).

Antioxidant Parameters

Anti-oxidative parameters of the four groups are also shown in Figures 2-3. The PON activity and TAS level were statistically higher in the TR group compared with other groups ($p < 0.01$ and $p < 0.001$, respectively). Compared with the TR group, TAS levels were reduced by 36% in the CN group, 24% in the CS group, and 37% in the IR group, respectively. In addition, PON activity was decreased by 27% in the CN group, 31% in the CS group, and 35% in the IR group, respectively. No statistical difference was detected among all the groups with respect to the ARE activity, Cp, and -SH levels.

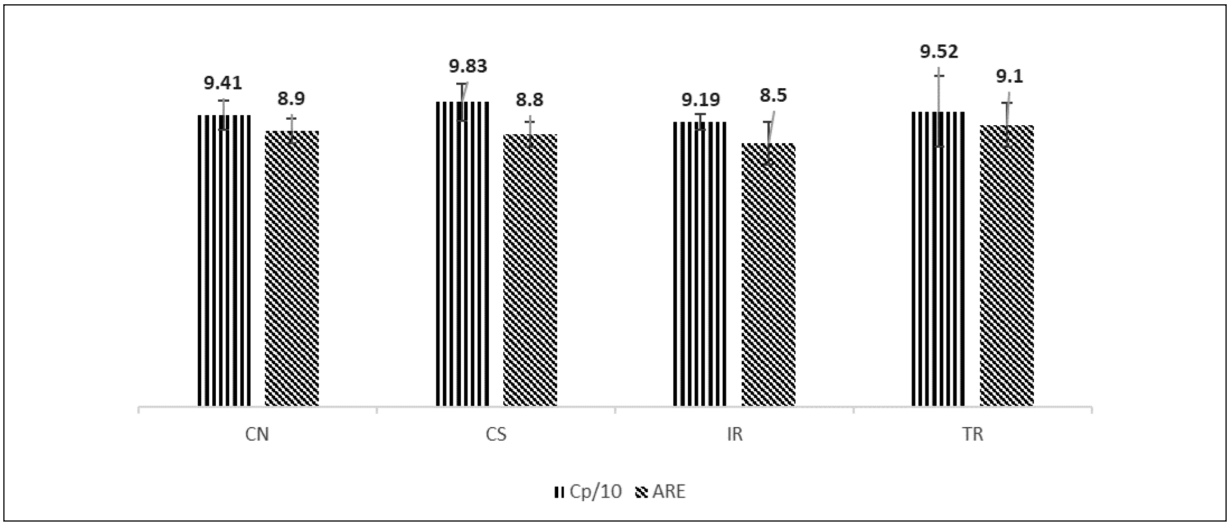

Figure 2. Cp and ARE parameters measured in the lung tissue of rats. Cp, ceruloplasmin; ARE, arylesterase.

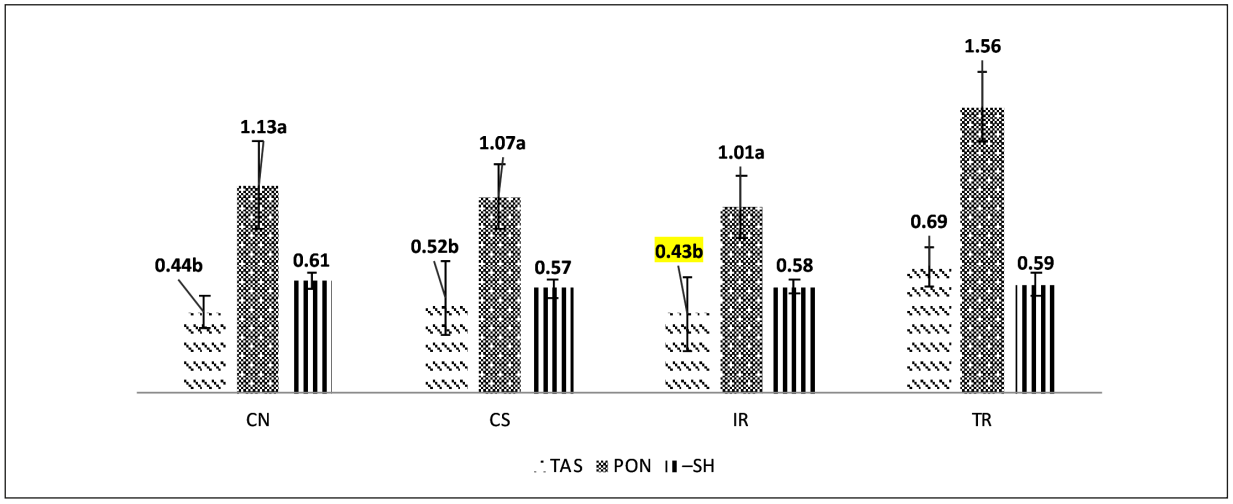

Figure 3. TAS, PON and –SH parameters measured in the lung tissue of rats. a: $p < 0.01$, b: $p < 0.001$ *vs.* TR group. TAS, total antioxidative status; PON, paraoxonase; –SH, sulfhydryl.

Discussion

Although TQ's potent radiation protective effects have been proven in numerous studies, to our knowledge, it is the first study to analyze and compare the effects of TQ on oxidative damage induced by ionizing radiation in rats. The oxidative stress parameters (TOS, LOOH, and OSI) in irradiated rats treated with TQ were found to be significantly lower than those in the control group. However, levels of ARE, SH, and Cp did not exhibit significant differences across the groups. The results of the present study suggest that systemic administration of TQ reduces oxidative damage in irradiated lung tissues of the rat model.

When IR is given to the chest area, it is evident that it may cause harmful side effects depending on the radiation dose applied and that the increased ROS production after exposure to radiation will shift the oxidant/antioxidant balance in the body in favor of oxidants. Oxidative stress caused by oxygen free radicals (OFR) plays an important role in the pathogenesis of many diseases, such as diabetes mellitus, cancer, rheumatoid arthritis, infectious diseases, preeclampsia, lung damage[25-27]. Therefore, we aimed to measure TAS and TOS parameters, as most antioxidant and oxidant parameters can be measured and evaluated together. It should also be noted that measuring only one oxidant or antioxidant parameter will not often provide accurate information about the true oxidative state of the organism. In the present study, we found TOS levels higher in the IR group compared to the control groups. However, we found no significant difference between IR and control groups in terms of TAS levels. OSI may reflect the state of oxidative status more accurately than TAS[28,29]. In this study, the OSI level was significantly higher in the lung tissues of rats in the IR group than were in the control groups. Therefore, this result demonstrated the presence of oxidative stress in radiation-injured lung tissue.

The increase in oxidative stress plays an important role in the pathogenesis of radiation-induced lung tissue, causing lung damage such as radiation pneumonitis (RP) and is a serious side effect of thoracic irradiation. RP due to thoracic RT usually occurs within 6 months and can be potentially fatal, even with appropriate treatment[9]. For these reasons, natural antioxidants, radiation protectors, and antimutagens that have no harmful effects and can protect the cell membranes of living organisms from the side effects of ionizing radiation and OFRs may provide potential benefits[24,25]. The results obtained from the studies[30-32] have shown that TQ detoxifies free radicals, reduces oxidative/nitrosative stress, protects the cell membrane against lipid peroxidation, and provides cell integrity. TQ will further increase the success of the treatment. TQ not only protects cellular membranes but, if used in appropriate doses, also does not have adverse effects on cancer cells[7,13,30,33].

We have not found any research on the protective effect of TQ on radiation-induced lung injury. However, TQ has been reported to prevent lung damage produced by agents other than ionizing radiation. In one study[34], TQ was shown to reduce pulmonary inflammation in a rat allergic asthma model by decreasing Th-2 cytokines and inflammatory cell infiltrates in the lung. It has been reported that TQ exhibited a protective effect against cyclophosphamide-induced pulmonary injury by reducing the restoration of antioxidants and secretions of the proinflammatory cytokine TNF-α in rat serum[35]. In one study[36], TQ treatment was reported to inhibit inflammatory pulmonary responses by significantly reducing peribronchial inflammatory cell infiltrations, alveolar septal infiltrates, alveolar exudates, interstitial fibrosis, and necrosis in rats treated with toluene.

In this study, we found oxidative parameters such as TOS and OSI decreased in irradiated lung tissues of rats treated with TQ compared to the IR group. Significant decreases in OSI and TOS levels in TR groups indicated protective responses against oxidative damage. Our study results are consistent with the above-mentioned studies that have demonstrated the protective effects of TQ against oxidative stress in the lung tissues of rats.

In living organisms, there is a delicate balance between the production and detoxification of OFRs. Overproduction of OFRs and possible cellular damage as a result of this is prevented by enzymatic and non-enzymatic antioxidant defense systems. We evaluated the effects of TQ on enzymatic and non-enzymatic antioxidant defense systems such as ARE, PON, Cp, and total -SH groups. This shows how tissue damage caused by oxidative stress is blocked by the antioxidant defense system. Lipid peroxidation caused by OFRs is one of the most important causes of cell damage[29]. We found that LOOH, one of the lipid peroxidation markers, increased in the IR groups, and the increase in LOOH levels in the TQ-given group was statistically significantly decreased and was close to the values of the control group.

Metabolic changes in cancer are significantly affected by oxidative stress. Oxidative stress and its resulting lipid peroxidation products play a role in oncogenesis. PON-1, one of the three enzyme families called PON-1, PON-2, and PON-3, is one of the main systems that protect the cell from oxidative stress by breaking down lipid peroxides in circulating lipoproteins and in the cytoplasmic and intracellular organelle membranes of cells. PON-1, which is found in the membranes of most cells, is found in circulation and is bound to high-density lipoproteins[37,38]. Several studies[29,39-40] have shown decreased activity of serum PON and ARE in patients with increased oxidative stress and cancer[41]. In this study, we found that PON-1 activity was statistically significantly lower in rats receiving only ionizing radiation compared to the TQ-treated group, and TQ increased PON-1 activity. As stated in previous studies[14,40], lipid peroxides formed as a result of oxidative stress inhibit PON activity. Increased lipid peroxides react covalently with the free sulfhydryl group in cysteine, which is in the active site of the PON1 enzyme, causing its inhibition[38-41]. Therefore, decreased PON1 activity is a net result of increased oxidative stress caused by ionizing radiation. Finally, ARE enzyme activity, Cp, and total SH levels did not differ between groups. IR and TQ treatments have little or no effect on the aforementioned antioxidant defense system parameters.

Limitations

One of the major limitations of this study is the lack of histological evaluation. Although biochemical analyses suggested TQ radio-protective effects against oxidative damage in the lung tissue of irradiated rats, supporting this data with histological evaluations may be reasonable.

Conclusions

The results of our present study support that TQ might be protective in radiation-injured lung tissues. TQ might also be beneficial in the treatment and protection of radiation injury, especially in patients who are treated with thoracic irradiation. However, these results must be interpreted carefully because no histological data are available in this study. Further studies with different study designs are needed to understand more clearly the mechanism involved in modulating oxidative balance through the administration of TQ.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Ethics Approval
This study was conducted at the Department of Medical Biochemistry after obtaining ethical approval from the Animal Ethics Committee of Gaziantep University School of Medicine (Number 2016/15).

Authors' Contribution
SD: designed the study and developed the research questions, SD, ST: performed the experiments and collected the data, SD, ST, AY: analyzed the data and conducted statistical analyses, AY: wrote the paper and prepared the figures.

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Informed Consent
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