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In Vivo Radioprotective Effects of *Nigella sativa* L Oil and Reduced Glutathione Against Irradiation-Induced Oxidative Injury and Number of Peripheral Blood Lymphocytes in Rats

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ABSTRACT

Radiotherapy is one of the most common therapies for treating human cancers. Several studies have indicated that irradiation induces reactive oxygen species (ROS), which play an important role in radiation damage of the cell. It has been shown that *Nigella sativa* L. (NS) and reduced glutathione (GSH) have both an antiperoxidative effect on different tissues and a scavenger effect on ROS. The purpose of this study was to determine the antioxidant and radioprotective roles of NS and GSH against irradiation-induced oxidative injury in an experimental model. The NS group was administrated NS (1 mL/kg body weight), the GSH group was injected GSH (150 mg/kg body weight) and the control group was given physiologic saline solution (1 mL/kg body weight) for 30 consecutive days before exposure to a single dose of 6 Gy of radiation. Animals were sacrificed after irradiation. Malondialdehyde, nitrate, nitrite (oxidative stress markers) and ascorbic acid, retinol, β-carotene, GSH and ceruloplasmin (nonenzymatic antioxidant markers) levels and peripheral blood lymphocytes were measured in all groups. There were statistically significant differences between the groups for all parameters ($P < 0.05$). Whole-body irradiation caused a significant increase in blood malondialdehyde, nitrate and nitrite levels. The blood oxidative stress marker levels in irradiated rats that were pretreated with NS and GSH were significantly decreased; however, nonenzymatic antioxidant levels were significantly increased. Also, our results suggest that NS and GSH administration prior to irradiation prevent the number of alpha-naphthyl acetate esterase peripheral blood T lymphocytes from declining. These results clearly show that NS and GSH treatment significantly antagonize the effects of radiation. Therefore, NS and GSH may be a beneficial agent in protection against ionizing radiation-related tissue injury.

INTRODUCTION

The potential application of radioprotective antioxidant nutrients and phytochemicals in the event of planned exposures or radiation accidents has been studied for more than 50 years. Scientific and technological advancements have further increased the radiation burden in humans, because exposure to low levels of radiation has become common during medical diagnostic procedures, space or air travel, cosmic radiation and through the use of certain electronic gadgets. Other sources of radiation exposure include radon in houses, contamination from weapons testing sites, nuclear accidents and radiotherapy. Ionizing radiation may cause cancer, death, and loss of neural function in humans and animals. It also induces mutation, chromosomal aberrations and apoptosis in cells (1,2).

Among the various physical/chemical agents, radiation is an important source in the generation of oxygen-derived free radicals and excited states. In actively metabolizing cells, there is considerable water apart from the target macromolecules of DNA, proteins, lipids and so on. The exposure of biological systems to radiation results in a radiolytic cleavage of water, giving rise to e^-_{aq}, $OH^\bullet$ and H. However, ionizing radiation can break chemical bonds and cause ionization of biologically important macromolecules such as nucleic acids, membrane lipids and proteins (3–5). Due to the presence of polyunsaturated fatty acids, membranes are highly susceptible to oxidative damage induced by reactive oxygen species (ROS) generated during radiation (6). Hence, compounds that are capable of protecting cellular membranes against ionizing radiation in particular, and free radicals in general, will have potential benefits as radioprotectors, antioxidants and antimutagens (7). Antioxidant systems have a fundamental role in defending organisms against irradiation-induced oxidative stress. Antioxidants are molecules that can prevent or reduce the extent of oxidative destruction of biomolecules when present in small concentrations compared with the biomolecules they are supposed to protect (8).

A larger number of plants such as *Nigella sativa* L. (NS) and their purified constituents have been shown to exhibit potentially beneficial therapeutic effects. NS, commonly known as black seed, is a seed of capsulated plants, and belongs to the Ranunculaceae family. The seed has been used in many countries in the Middle East and East Asia as a natural remedy. NS contains >30 w/w of

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fixed oil and 0.40–0.45 w/w of a volatile oil. The volatile oil has been shown to contain 18.4–24% thymoquinone and 46% monoterpenes such as *p*-cymene and α-pinene (9,10). Recently, clinical and animal studies have shown that an extract of black seed has many therapeutic effects, including anti-inflammatory (11), antiviral (12), antihelminthic (13), antibacterial (14), antitumor (15), antihistaminic (10), immunomodulative (16) and hepatoprotective (17). Although the plant has many different effects, to our knowledge there are no reports on the radioprotective effect of NS.

Aerobic organisms are protected against free radicals by antioxidant defense systems such as reduced glutathione (GSH). GSH is an important cellular antioxidant and plays a major role in protecting cells against oxidative stress. Functions of GSH in reductive processes are essential for synthesis and degradation of proteins, formation of the deoxyribonucleotid precursors of DNA, regulation of enzymes and protection of the cells against ROS and free radicals produced even in normal metabolism (18–20).

Nonspecific esterase is widely distributed in various types of cells. Cytochemical esterase activity is commonly used to differentiate types of leukocytes and leukemia cells (21). Alpha-naphthyl acetate esterase (ANAE) is a nonspecific esterase. The pattern of esterase activity revealed by this method provides a discriminating marker for mature T lymphocytes showing dense, localized, dot-like positive responses. Low-dose, whole-body gamma-irradiation decreases the lymphocyte pool, causing homeostasis-driven proliferation of lymphocytes. This proliferation restores the T cell memory compartment, producing more clones that are reactive to self-antigens; naïve T cells are reconstituted by new arrivals from the thymus (22). Vijayalaxmi *et al.* (23) reported that human peripheral blood lymphocytes that were pretreated with melatonin *in vitro* and then exposed to gamma radiation *in vitro* exhibited a significantly reduced incidence of chromosomal aberrations and micronuclei as compared with similarly irradiated lymphocytes that were not pretreated with melatonin.

In the present study, we investigated the radioprotective effects of NS and GSH on the activities of oxidant and antioxidant substances such as malondialdehyde (MDA), nitrate, nitrite, GSH, ascorbic acid, retinol, β-carotene and ceruloplasmin levels in rats exposed to radiation.

MATERIALS AND METHODS

Chemicals. GSH, thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, ethylenediamene tetraacetic acid (EDTA), (5,5-dithiobis-[2-nitrobenzoic acid]), phenylendiamine, sodium azide, 2,4-dinitrophenylhydrazine, ethanol, hexane, sodium nitrite, sodium nitrate, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride and vanadium (III) chloride were purchased from Sigma. All other chemicals and reagents used in this study were analytical grade.

Plant materials and extraction procedure. The NS seeds were purchased from a local herb store in Afyon, Turkey. The seeds of NS were powdered in a mixer, placed in a distillation flask and the volatile oil with 0.2% yield was collected by steam distillation.

Animals and experimental design. Before commencing the work we obtained permission from the Institutional Animal Ethics Committee of Ege University. Thirty male Wistar albino rats weighing 200–230 g (average age, 16 weeks) were used in this study. The animals were housed in macrolon cages under standard laboratory conditions. They were fed standard laboratory chow and water before the experiment. The animal room was windowless with automatic temperature (22 ± 1°C) and lighting control (14 h light/10 h dark). All procedures were performed in sterile conditions. All animals received human care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health. The experiment lasted for 30 days.

The animals were randomly divided into three groups: Group I (NS), Group II (GSH) and Group III (control), each containing 10 animals. Groups I, II and III received a daily intraperitoneal injection of 1 mL/kg NS, 150 mg/kg GSH or 1 mL/kg physiologic saline solution, respectively for 30 days. After 30 days, the three groups received 6.0 Gy of whole-body radiation (1.55 Gy/min). Irradiation was performed using a ^{60}Co teletherapy unit (EMS, Canada). Twenty-four hours after the radiation exposure, all groups were sacrificed under ether anesthesia. Blood samples were collected by cardiac puncture using heparinized syringes. Whole blood was collected into heparinized tubes and whole blood MDA and GSH levels were assessed on the same day. Blood was also collected into a polystyrene microtube and after clotting, centrifuged at 4000 *g* for 7 min and the serum removed using EDTA-washed Pasteur pipettes. The serum was stored in polystyrene plastic tubes at −70°C until analysis.

Biochemical analysis. Whole-blood MDA (as an important indicator of lipid peroxidation) levels were measured using the method described by Jain *et al.* (24). The principle of the method is the spectrophotometric measurement of the color that occurs during the reaction of thiobarbituric acid with MDA. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of malondialdehyde–thiobarbituric acid complex and expressed in nmol/mL. Whole blood GSH concentration also was measured via a spectrophotometric method (25).

The concentrations of nitric oxide (nitrate and nitrite) were detected using the methods described by Miranda *et al.* (26). Nitrite and nitrate calibration standards were prepared by diluting sodium nitrite and sodium nitrate in pure water. After loading the plate with samples (100 μL), addition of vanadium (III) chloride (100 μL) to each well was rapidly followed by addition of the Griess reagents sulfanilamide (50 μL) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 μL). (The Griess solutions may also be premixed immediately prior to application to the plate.) Nitrite mixed with Griess reagents forms a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as N-1-(naphthyl) ethylenediamine. Blank sample values were obtained by substituting diluting medium for a Griess reagent. Nitrite was measured in a similar manner except that samples and nitrite standards were exposed only to Griess reagents. The absorbance at 540 nm was read to assess the total level of nitrite and nitrate in all samples (26).

Serum vitamin C (ascorbic acid) level was determined after derivatization with 2,4-dinitrophenylhydrazine (27). The levels of β-carotene at 425 nm and vitamin A (retinol) at 325 nm were detected after the reaction of serum:ethanol:hexane at the ratio of 1:1:3, respectively (28). Ceruloplasmin (CLP) level was studied using the spectrophotometric method (29).

Histological analyses. Heparinized blood samples were taken from rats for ANAE staining for the purpose of this study. For ANAE demonstration (21), air-dried blood smears were fixed in glutaraldehyde-acetone mixture (glutaraldehyde [Merck 4239], 9 mL; distilled water, 21 mL and acetone [Merck 2500], 45 mL) for 3 min at −10°C. Following fixation, the smears were rinsed in distilled water and then allowed to dry at room temperature for 30 min. Blood smears were incubated (pH 7.2) for 4 h, and after incubation the preparations were washed in distilled water and counterstained with 1% methyl green for 30 min. Following dehydration in increasing concentrations of ethanol, the preparations were cleaned in xylene and mounted in DPX. After application of ANAE enzyme stain, they were examined with a light microscope. The proportions of ANAE(+) and ANAE(−) lymphocytes were determined for each blood smear by counting 500 lymphocytes in a total of five areas (100 in each area).

All values were expressed as mean ± SD. Statistical analysis of data was performed using a one-way analysis of variance and Tukey's posttest. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Levels of whole-blood MDA and GSH, and serum nitrate, nitrite, ascorbic acid, retinal, β-carotene and CLP in all subjects are shown in Figs. 1–4. The figures show a statistically significant difference between the groups for all parameters ($P < 0.05$). Lipid peroxidation levels were determined by measuring the TBARS concentration. Treatment of rats with NS and GSH did not alter lipid peroxidation in whole blood compared with the normal physiologic saline solution–treated control group after irradiation

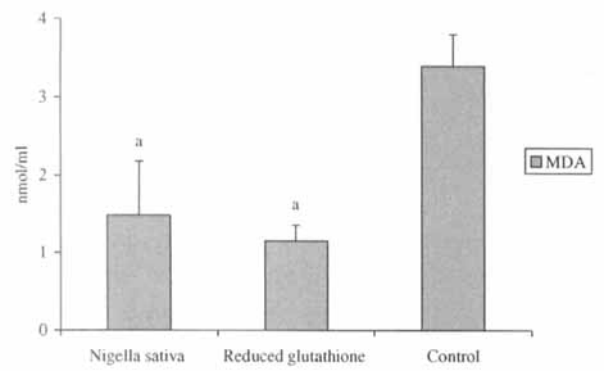

Figure 1. Effects of *Nigella sativa* and reduced glutathione treatment on lipid peroxidation levels of irradiated rats. Malondialdehyde (MDA, nmol/mL) is the end product of lipid peroxidation and serves as an index of oxidative damage. The data are expressed as mean ± SD. a, $P < 0.001$ with respect to control.

(Fig. 1). The indicator of lipid peroxidation MDA was markedly decreased in both the NS and GSH treated groups compared with that of the control group ($P < 0.001$), which was pretreated with physiologic saline solution. The levels of nitrate and nitrite in the NS and GSH groups were less than those of the control group (Fig. 2). Serum nitrate levels were significantly lower in the NS and GSH groups than in the control group ($P < 0.05$ and $P < 0.01$, respectively). Serum nitrite levels were also lower in the study groups compared with those in the control group ($P < 0.05$).

The nonenzymatic antioxidant defense system was increased in the NS and GSH treated groups. Antioxidant vitamins such as ascorbic acid (vitamin C), retinal (vitamin A) and β-carotene concentrations were found to be increased in NS and GSH treated groups compared with those of the control (Fig. 3). The serum ascorbic acid level in the GSH-treated group and the β-carotene level in the NS-treated group were significantly higher than levels in the control group ($P < 0.05$). Serum retinol levels were also increased in the NS and GSH treated groups compared with levels in the control group ($P < 0.01$ and $P < 0.001$, respectively). Increased GSH and CLP levels were observed in the NS and GSH treated groups, and these values were significant (Fig. 4; $P < 0.01$, $P < 0.01$ and $P < 0.001$, $P < 0.05$ for the NS-treated and GSH-treated groups compared with the control group, respectively).

The results of ANAE staining are shown in Table 1. In a light microscope examination, ANAE(+) lymphocytes had one to four

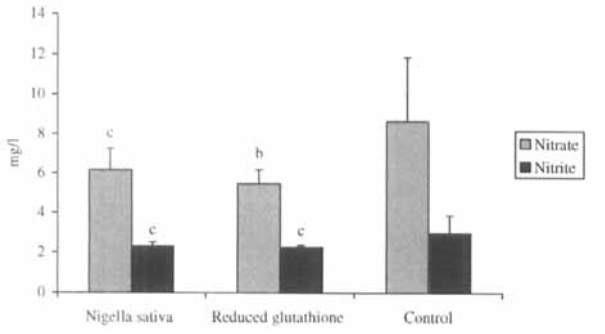

Figure 2. Effects of *Nigella sativa* and reduced glutathione treatment on nitric oxide (nitrate, mg/L and nitrite, mg/L) levels of irradiated rats. It is well known that nitric oxide possesses both antioxidant and pro-oxidant properties. The data are expressed as mean ± SD. b, $P < 0.01$; c, $P < 0.05$ with respect to control.

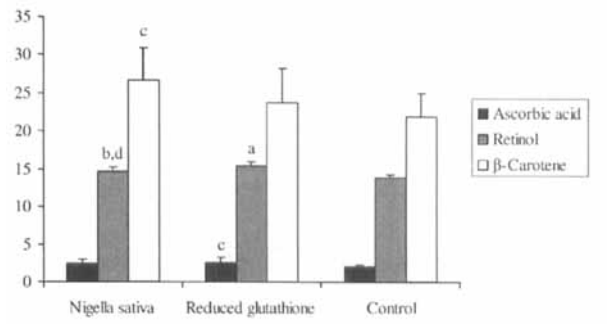

Figure 3. Effects of *Nigella sativa* and reduced glutathione treatment on levels of ascorbic acid (vitamin C, mg/dL), retinol (vitamin A/10, μg/dL) and β-carotene (μg/dL) of irradiated rats. The data are expressed as mean ± SD. a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$ with respect to control. d, $P < 0.01$ with respect to GSH.

distinct, red-brownish granules representing the reaction product by which they recognized T lymphocytes (Figs. 5 and 6). The numbers of ANAE(+) lymphocytes in the control was lower than those in NS and GSH treated groups ($P < 0.05$).

DISCUSSION

Radiotherapy is one of the most common therapies for treating human cancers. Eighty percent of patients with cancer need radiotherapy at some time or other, either for a curative or a palliative purpose. To obtain optimum results, a judicious balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required. To obtain better tumor control with a higher dose, the normal tissues should be protected against radiation injury. Thus, the role of radioprotective compounds is very important in clinical radiotherapy (2).

Radiation is a known to be a producer of ROS. Ionizing radiation–induced injury to living cells is mediated through the generation of oxygen-derived free radicals and hydrogen peroxide (30). ROS are overproduced and result in lipid peroxidation and oxidative damage. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules. Aerobic organisms are protected against ROS by enzymatic antioxidant (superoxide dismutase, glutathione peroxidase and catalase) and nonenzymatic antioxidant (such as β-carotene, retinol, vitamin C, GSH and CLP) defense systems (17,31).

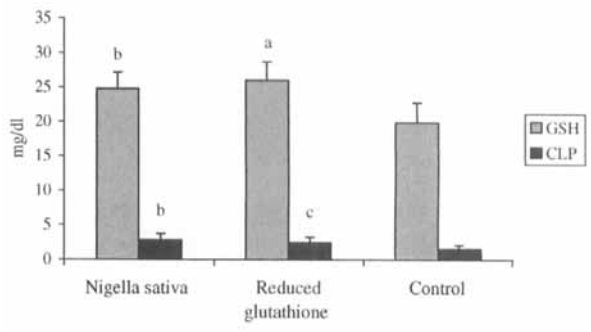

Figure 4. Effects of *Nigella sativa* and reduced glutathione treatment on reduced levels of glutathione (GSH, mg/dL) and ceruloplasmin (CLP, mg/dL) of irradiated rats. The data are expressed as mean ± SD. a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$ with respect to control.

Table 1. ANAE staining in peripheral blood lymphocytes in irradiated rats

Groups	% of ANAE(+) lymphocytes	% of ANAE(−) lymphocytes
Control	73.7 ± 7.4*	26.3 ± 3.5
Nigella sativa	81.1 ± 9.7	18.9 ± 2.8
Reduced glutathione	79.2 ± 13.8	20.8 ± 6.2

*$P < 0.05$ with respect to *Nigella sativa* and reduced glutathione groups.

One of the major forms of cellular damage induced by ionizing radiation is lipid peroxidation. The lipid peroxidation of cell membranes results in MDA formation (32), then an increase in the MDA concentration in blood and tissues possibly leading to tissue damage (33) and damage to the other enzyme systems and DNA (34). Many investigators have reported that irradiation increased the formation of MDA as the main product of lipid peroxidation (1,35–39). According to the results of the present study, when rats were exposed to a single dose (6.0 Gy) of whole-body irradiation, lipid peroxidation was found to be significantly increased in the blood (Fig. 1). Our results are consistent with the published literature. The MDA levels in the groups receiving irradiation plus NS or GSH treatment were significantly decreased compared with the group that received irradiation only. These results revealed that NS and GSH clearly decreased the lipid peroxidation in blood induced by whole-body irradiation.

Nitric oxide (nitrate and nitrite) is an inorganic free radical gas produced from L-arginine by a family of isoenzymes called nitric oxide synthases. Nitric oxide is known, together with ROS, to induce cytotoxicity and cytostasis. Several studies have shown that nitric oxide and H_2O_2-induced oxidative damage exhibit similar cytotoxicity (40). Under conditions of oxidative stress induced by irradiation, nitric oxide is often produced; its cytotoxicity is caused by production of $ONOO^-$, a toxic oxidant, generated when the nitric oxide couples with O_2^- (41). The processes triggered by $ONOO^-$ include initiation of lipid peroxidation, inhibition of mitochondrial respiration, inhibition of membrane pumps, depletion of glutathione, and damage to DNA (42). Taysi *et al.* (39) and Agrawal *et al.* (43) reported that nitric oxide levels in blood exposed to whole-body irradiation were significantly increased in the liver. Also in this study, nitric oxide levels in rats exposed to radiation were found to be increased before the treatment of rats with NS and GSH (Fig. 2). The result showing low MDA and nitric oxide levels in the NS and GSH treatment groups suggests that NS and GSH have a protective effect against radiation.

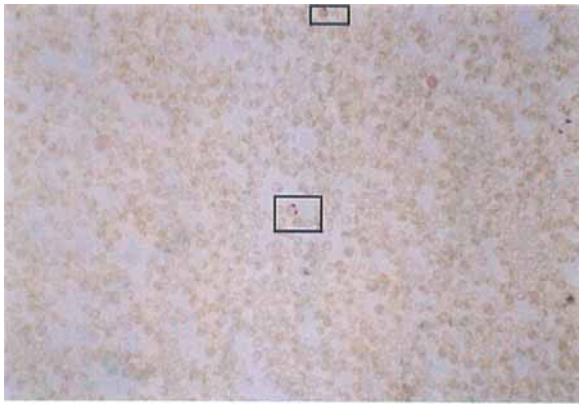

Figure 5. Alpha-napththyl acetate esterase (ANAE) staining in the control group. ANAE-positive granules–T lymphocyte (windows). ANAE, 360×.

Figure 6. Alpha-napththyl acetate esterase (ANAE) staining in the *Nigella sativa* group. ANAE-positive granules–T lymphocyte (arrow) and A: ANAE-negative–T lymphocyte. ANAE, 360×.

One of the potential properties of NS is the ability of one or more of its constituents to reduce toxicity due to its antioxidant activities. The majority of studies that have been performed to evaluate the different effects of NS have been confined to address its antitoxic properties (9–17). In addition to this, many investigators have determined the antitumor activities of NS *in vivo* (44,45). These studies have shown the potential immunomodulatory and immunotherapeutic effects of NS. However, to our knowledge, there has been no study to date on the radioprotective effect of NS, and this is the first study on the radioprotective effect of NS.

GSH is a versatile protector. Several distinct mechanisms of radioprotection by GSH can be identified, including radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state. Of these mechanisms, the most important one is probably hydrogen donation to DNA radicals. Because competing reactions are very rapid, this mechanism requires a high concentration of GSH (46). It is postulated that ionizing radiation would rapidly oxidize the thiol groups of cells. In accordance with this hypothesis, radiation decreases the cellular concentration of GSH and leads to formation of glutathione disulfide. Radiosensitivity of cells depends on the intracellular thiol level. Administration of various thiols can protect the cells and animals against the effects of radiation (18,19) In this study, the GSH supplement was very potent in the antioxidative defense system. Although the oxidant levels such as MDA and nitric oxide were decreased, ascorbic acid, retinol, β-carotene, GSH and CLP antioxidant levels were increased in the GSH group. Some investigators measured GSH levels in animals exposed to radiation (1,37,38) and noted that the GSH levels were significantly lower in the groups exposed to radiation compared with the control groups. Our GSH results are consistent with these studies (Fig. 4).

Antioxidant vitamins such as ascorbic acid, retinol and β-carotene play an important role in reducing or eliminating the oxidant damage produced by ROS in the short and long terms. In the present study, we also measured serum antioxidant vitamin capacity and, all of the antioxidant vitamins levels were increased in both the NS and GSH treatment groups (Fig. 4).

In a report by Ragan *et al.* (47) of hematological and immunological effects of microwaves in mice, bone marrow

cellularity was significantly reduced in one of six groups exposed at 10 mW/cm^2, in contrast with an increase in bone marrow cellularity in two studies exposed at 5 mW/cm^2. Trosi *et al.* (48) reported that the number of lymphoblasts in irradiated animals was lower throughout the experiment compared with the matched sham-exposed rats, but a significant decrease was obtained at 15 and 30 days into the experiment. In the same study, the absolute number of lymphocytes in bone marrow did not significantly differ between animals in the treated and untreated groups.

ANAE(+) lymphocyte counts were significantly protected with a 1 mL/kg/day dose of NS oil and a 150 mg/kg GSH against irradiation. The number of ANAE(−) lymphocytes in control animals was higher than in other experimental groups ($P < 0.05$). This study suggests that NS oil and GSH administration prevents a decrease in peripheral T lymphocytes by radiation when given before exposure to irradiation. This result is parallel with the findings by Koc *et al.* (49).

In conclusion, the original feature of the present study is the measurement of the levels of oxidant and antioxidant in rats exposed to radiation to determine the radioprotective effect of NS. Additionally, we have shown that antioxidant vitamin levels were increased in the GSH treatment groups. Moreover, oxidative stress markers were decreased, whereas antioxidant levels were increased in the NS and GSH treatment groups compared with the irradiation-only group. Finally, NS and GSH have clear antioxidant properties and are protective against irradiation; furthermore, they may be used as an antioxidant against oxidative injury.

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