

Original Paper

Thymoquinone Rescues T Lymphocytes from Gamma Irradiation-Induced Apoptosis and Exhaustion by Modulating Pro-Inflammatory Cytokine Levels and PD-1, Bax, and Bcl-2 Signaling

Mona Samy Guida^a Ali Abd El-Aal^b Yehya Kafafy^c Safwat Farid Salama^c
Badr Mohamed Badr^c Gamal Badr^d

^aDepartment of Pediatrics, Faculty of Medicine, Mansoura University, Mansoura, ^bDepartment of Zoology, Faculty of Science, Ain Shams University, Ain Shams, ^cDepartment of Radiation Biology, National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, ^dLaboratory of Immunology and Molecular Physiology, Zoology Department, Faculty of Science, Assiut University, Assiut, Egypt

Key Words

Apoptosis • Bcl-2 signaling • Bax • Gamma radiation • Thymoquinone • T cells

Abstract

Background/Aims: Recent studies have shown that thymoquinone (TQ) exerts protective effects against ionizing radiation-induced cataracts in lens after total cranium irradiation of rats. Nevertheless, there is no published work investigated the effects of TQ on T cell development and biology in animal models exposed to gamma radiation. Therefore, in the present study we focused on determining the effects of TQ on radiation damage in the thymus, radiation-induced T cell imbalance, and on immune dysfunction induced by gamma-rays. **Methods:** Three groups of rats were used: a control group, a gamma-irradiated group, and a gamma-irradiated group that was orally supplemented with TQ. Serum lipid profiles, malondialdehyde (MDA) levels, and pro-inflammatory cytokine levels were measured to assess gamma irradiation-induced oxidative stress and inflammatory capacity. T cell apoptosis was evaluated by annexin V/propidium iodide staining followed by flow cytometry analysis. The expression of pro-apoptotic proteins such as Bax and caspase-3, the anti-apoptotic protein Bcl-2, and an exhaustion marker of T cells (PD-1) in CD4⁺ and CD8⁺ T cell populations was evaluated using flow cytometry analysis. The T cell architecture of the thymus gland was evaluated by histological analysis. **Results:** Exposure to gamma radiation increased triglyceride, cholesterol, LDL-C, MDA, TNF- α and IL-6 levels and decreased HDL-C levels. The altered lipid profile and MDA and pro-inflammatory cytokine (TNF- α and IL-6) levels induced by exposure to gamma radiation were significantly restored in TQ-treated gamma-irradiated rats. Rats exposed to gamma radiation exhibited increased exhaustion of T lymphocytes via down-regulation of Bcl-

Dr. Gamal Badr,

Associate Professor of Immunology, Zoology Department, Faculty of Science, Assiut University, 71516 Assiut, (Egypt); Tel. +201110900710, Fax +20882080642, E-Mail badr73@yahoo.com, E-Mail gamal.badr@aun.edu.eg

2 expression and upregulation of PD-1, Bax, and caspase-3 expression, which sensitized these cells to apoptosis. Interestingly, treatment of gamma-irradiated rats with TQ decreased T cell exhaustion and apoptosis by modulating the expression of Bcl-2, PD-1, Bax, and caspase-3. **Conclusions:** Our results provide evidence for the beneficial effects of TQ as an effective radioprotective candidate that enhances cellular immunity.

© 2016 The Author(s)
Published by S. Karger AG, Basel

Introduction

Radiation is an important environmental factor with hazardous effects on health, including oxidative stress [1, 2], hematopoietic system dysfunction [3], genetic mutations [4] and immune dysfunction [5]. Ionizing radiation (IR) administered during radiotherapy generates free radicals when it passes through living tissues. IR generates reactive oxygen species (ROS) as a result of water radiolysis. These ROS can induce oxidative damage to vital cellular molecules and structures, including DNA, lipids, proteins and membranes [6, 7]. DNA damage can result in chromosomal abnormalities, gene mutations and cell death [8]. Products of lipid peroxidation such as malondialdehyde (MDA) can interact with and alter macromolecules, potentially causing disease [9, 10]. Thus, the modification of lipids and proteins by ROS has been implicated in the etiology of radiation-induced physiological disorders and diseases.

The immune system is one of the most important defense mechanisms against environmental agents. IR induces impairment of the immune response as well as a persistent inflammatory status through the deregulation of cytokine production [11, 12]. Furthermore, the dysregulation of immune homeostasis, particularly CD4⁺ T cell function, may influence health status and impede tissue repair after exposure to IR [13]. Dainiak et al. [14] proposed that IR induces a reduction in lymphocytes and halts the proliferation of hematopoietic progenitors, leading to the suppression of immune function and increasing the risk of infection. IR also induces pro-inflammatory processes in which tumor necrosis factor (TNF- α), interferon (IFN- γ) and interleukin levels are altered, eventually leading to inflammatory disorders [15].

Naturally occurring antioxidants may provide protection against irradiation and may have therapeutic potential when administered after irradiation. Several plant constituents possess considerable free radical-scavenging or antioxidant activity [16, 17]. Thymoquinone (TQ) is an abundant *Nigella sativa* essential oil compound that is responsible for many of the seed's antioxidant and anti-inflammatory effects [18]. TQ also has promising antitumor effects [19, 20]. Woo et al. [21] observed that TQ is a free radical and superoxide radical scavenger and preserves the activity of various antioxidant enzymes. The potential immunomodulatory effects of TQ include significantly enhanced splenocyte proliferation, macrophage function, and natural killer (NK) antitumor activity [22]. *N. sativa* and its component, TQ, are promising natural radioprotective agents against the immunosuppressive and oxidative effects of IR [23]. Oral administration of TQ significantly reduces levels of the pro-inflammatory mediators IL-1 β , IL-6, TNF- α , and IFN- γ and increases IL-10 levels [24].

In this study, we aimed to investigate the radioprotective activity of TQ in rats irradiated by gamma -rays. We focused on determining the effects of TQ on radiation damage in the thymus, radiation-induced T cell imbalance, and on immune dysfunction induced by gamma-rays.

Materials and Methods

Chemicals

TQ was purchased from Sigma Chemical Company (St Louis, MO, USA). TQ was > 99% pure and was reconstituted in saline at a concentration of 5 mg/ml. This stock solution was stored at 4°C in 15-ml centrifuge tubes wrapped in aluminum foil to prevent dimer formation.

Irradiation

Whole-body gamma-irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt, using a (¹³⁷cesium) Gamma Cell-40 biological irradiator. Animals were irradiated at a fractionated dose level of 4 Gy delivered at a dose rate of 0.012 Gy/s.

Animals and experimental design

Forty adult male rats (*Rattus norvegicus*) weighing 150 - 170 g were obtained from Theodor Bilharz Research Institute, Cairo, Egypt. All animal procedures were performed in accordance with the guidelines for the care and use of experimental animals established by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the protocols of the National Institutes of Health. The Animal Ethics Committee of the Department of Radiation Biology, NCRRT, Atomic Energy Authority, Egypt, approved the protocol used in this study in accordance with the Declaration of Helsinki. Animals were allowed to acclimate for 2 weeks before the experiment and were housed in metal cages in a well-ventilated room. The animals were maintained under standard laboratory conditions (25°C, 60-70% relative humidity and a 12-hour light/dark cycle) and fed a standard commercial pellet diet and water. Animals were divided into three groups (10 animals per group). In the control group, rats were administered vehicle (saline) by gavage for 28 consecutive days. The irradiated group was exposed to γ -rays (4 Gy/14 days for 28 days). The TQ + irradiated group was exposed to gamma-rays (4 Gy/14 days for 28 days) and received TQ (5 mg/kg, gavage) 1 hour after irradiation for 28 consecutive days.

Lipid profile analysis

Lipid profiles were determined using BioMerieux kits and a standard assay method. Cholesterol levels were evaluated using the cholesterol esterase method. Triglycerides were measured using the lipase method. HDL, LDL, and chylomicrons were precipitated with phosphotungstic acid. The amount of cholesterol bound to HDL was determined using the cholesterol oxidase method and the phosphotungstate-magnesium salt method using a Cholesterol E-Test Kit (Wako, Osaka, Japan) according to the manufacturer's instructions.

Lipid peroxidation analysis

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form complex reactive compounds, such as MDA. Thiobarbituric acid-reactive substances (TBARS), the final products of lipid peroxidation, were assayed spectrophotometrically.

Cytokine measurement

The levels of the plasma cytokines IL-6 and TNF- α were determined by ELISA using commercially available kits (R&D Systems, USA) according to the manufacturer's instructions. The cytokine concentrations were then calculated from a standard cytokine curve included on the same plate as the samples.

Flow cytometry analysis

Cell surface antigen expression was determined by single-parameter fluorescence-activated cell sorter (FACS) analysis as previously described [25-27] using the following monoclonal antibodies (mAbs): FITC-conjugated anti-CD4, FITC-conjugated anti-CD8, and FITC-conjugated mouse isotype-matched control mAbs, all purchased from BD Biosciences. After incubation in culture for 4 hours, PBMCs were washed 3 times in PBS, washed 2 times with FACS buffer (1% BSA in PBS) and then double stained with FITC-conjugated anti-CD4 and FITC-conjugated anti-CD8 or -IgG2a isotype control at 10 μ l of antibody/10⁶ cells at 4°C for 30 minutes. The cells were then washed with FACS buffer, resuspended and stored in 500 μ l of 2% paraformaldehyde solution. A FACSCalibur flow cytometry instrument (Becton Dickinson) was used for data acquisition and analysis. After viable cell gating, 15000 events per sample were analyzed. For each marker, the threshold of positivity was defined beyond the nonspecific binding observed in the presence of a relevant isotype control mAb.

Apoptosis assays

The percentage of lymphocytes undergoing apoptosis was determined by flow cytometry. Dead cells were identified using the Trypan blue exclusion test. To distinguish viable, early apoptotic, late apoptotic and necrotic cells, the cells were washed and incubated in PBS containing 30% human AB serum (4°C for

30 minutes) prior to staining with annexin V-FITC and propidium iodide (PI) (15 minutes at 25°C) using a commercial kit according to the manufacturer's instructions (Abcam, Canada). The cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) within 1 hour of staining, and the percentage of cells undergoing apoptosis was determined. The expression of caspase-3, Bax and Bcl-2 was evaluated by staining with FITC-conjugated anti-caspase-3, PE-conjugated anti-Bax and PE-conjugated anti-Bcl-2 or -IgG2a isotype control at 10 μ l of antibody/10⁶ cells.

Programmed cell death 1 (PD-1) protein expression

PE-labeled anti-mouse PD-1 and isotype control IgG mAbs were purchased from BD Biosciences (San Jose, CA, USA). After washing with cold PBS, 1 \times 10⁶ PBMCs were pelleted by centrifugation at 300 \times g for 10 minutes at room temperature. The cells were subsequently incubated with PE-labeled anti-mouse mAbs for 30 minutes at 4°C, followed by a final washing in cold 1X PBS. The cells were then washed twice and fixed in 1X PBS containing 2% paraformaldehyde. PD-1 expression on viable cells was quantified by flow cytometry analysis.

Histopathological examination

The thymus tissue was isolated from control and treated animal groups and was transferred immediately to 10% formalin for 24 hr and dehydrated in ascending grades of ethanol (50-100%). Clearing was done in xylene and embedded in paraffin wax. Sections (4 μ m thick) were prepared and then stained with hematoxylin and eosin (H & E). The preparations obtained were visualized using a light microscopy at a magnification of 100 X.

Statistical analysis

Results are expressed as the mean \pm SEM. Variation between groups was measured by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Statistical significance was considered at P < 0.05.

Results

Effects of thymoquinone on lipid profiles and pro-inflammatory cytokines elevated by exposure to gamma irradiation

Exposure to a high dose of gamma -irradiation induces dyslipidemia due to elevated serum triglyceride and cholesterol levels, increased levels of low-density lipoprotein (LDL) particles and decreased levels of high-density lipoprotein (HDL). This dyslipidemia induces immune cell exhaustion. We therefore monitored the lipid profiles of the 3 groups of animals. Serum levels of MDA, a marker of oxidative lipid damage and a major oxidative product of peroxidized polyunsaturated fatty acids, were significantly increased in irradiated rats compared with the control group (Fig. 1A). HDL-C levels were significantly lower in the irradiated group than the control group (Fig. 1B). By contrast, serum levels of LDL-C, triglycerides and cholesterol were significantly higher in irradiated rats than in the control group. Interestingly, a normal lipid profile was restored in irradiated rats following TQ treatment. We also monitored the plasma levels of various pro-inflammatory cytokines that can alter showed that immune cell function can be altered (Fig. 1C). The data acquired for eight individual rats per group are shown. In the irradiated group, we observed aberrant and significantly elevated levels of TNF- α and IL-6 compared with the control group, indicating prolonged pro-inflammatory conditions after exposure to gamma irradiation. By contrast, pro-inflammatory cytokine levels were significantly decreased in irradiated rats treated with TQ compared with untreated irradiated rats.

Thymoquinone treatment reduces gamma irradiation-mediated PBMCs exhaustion

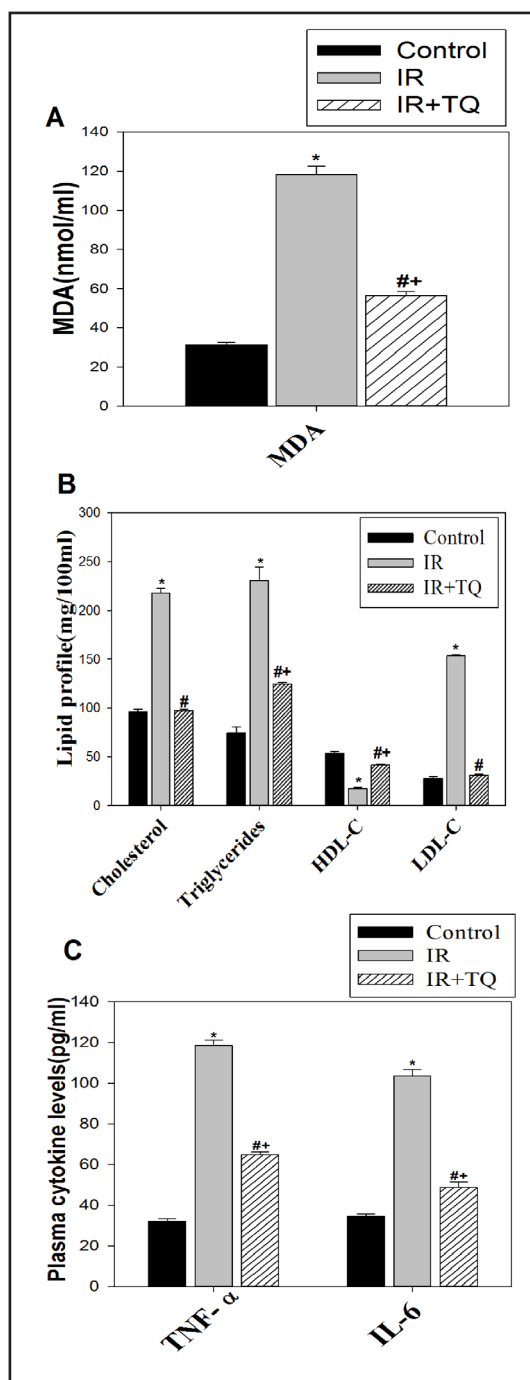
To further confirm that gamma-rays induce dyslipidemia and a sustained inflammatory condition, resulting in lymphocyte exhaustion, PD-1 expression was analyzed by flow cytometry (Fig. 2A). The results of one representative experiment out of eight revealed that,

Fig. 1. Effect of gamma-rays on serum lipid profiles and pro-inflammatory cytokines. The levels of MDA (A), cholesterol, triglycerides, HDL-C and LDL-C (B) were determined in control rats (black bars), gamma ray-irradiated rats (gray bars) and gamma ray-irradiated rats treated with thymoquinone (TQ) (hatched bars), as described in the Materials and Methods. The pooled data for eight individual rats from each group are expressed as the mean level of each lipid \pm SEM (n = 8). The levels of plasma pro-inflammatory cytokines (TNF- α and IL-6) were measured in each group of rats using ELISA and are presented as the cytokine levels (pg) per ml of plasma, expressed as the mean \pm SEM (n = 8) (C). *P < 0.05, gamma-irradiated rats vs. control rats; #P < 0.05, gamma-irradiated rats + TQ vs. gamma-irradiated rats; and *P < 0.05, gamma-irradiated rats + TQ vs. control rats (ANOVA with Tukey's post-test).

the percentage of PD-1 expression in stained PBMCs was low (14.54%) in cells isolated from control rats. By contrast, PBMCs isolated from the irradiated group were characterized by aberrant upregulation of PD-1 expression (41.89%). Interestingly, cells isolated from the irradiated group treated with TQ exhibited an obvious decrease in PD-1 expression (22.48%). The upregulation of PD-1 expression is an indicator of continuous activation and exhaustion. Data acquired from eight individual rats per group are presented as the mean MFI \pm SEM for the expression of PD-1 (Fig. 2B) and revealed that PBMCs in the irradiated group were likely exhausted and that TQ treatment restored the functional state of the PBMCs.

Thymoquinone supplementation decreases gamma irradiation-induced perturbations in T cells

To investigate the effects of TQ on irradiation-induced immune dysfunction, the numbers of CD4⁺ and CD8⁺ cells were measured using flow cytometry. The results of one representative experiment out of eight are shown in Fig. 3A. The mean percentage of CD4⁺ cells was significantly lower in the irradiated group (10.82%) than the control group (17.90%). By contrast, the irradiated group treated with TQ exhibited a significant increase in CD4⁺ cells (20%). Furthermore, as shown in Fig. 3B, the mean percentage of CD8⁺ cells was significantly lower in the irradiated group (6.04%) than the control group (14.60%). By contrast, the irradiated group treated with TQ exhibited a significant increase in CD8⁺ cells (16.50%). Data acquired from eight individual rats per group (Fig. 3C) revealed that the percentages of CD4⁺ and CD8⁺ cells were both significantly decreased in the irradiated group compared with the control group, indicating prolonged pro-inflammatory conditions after exposure to gamma irradiation. By contrast, TQ supplementation significantly increased the percentage



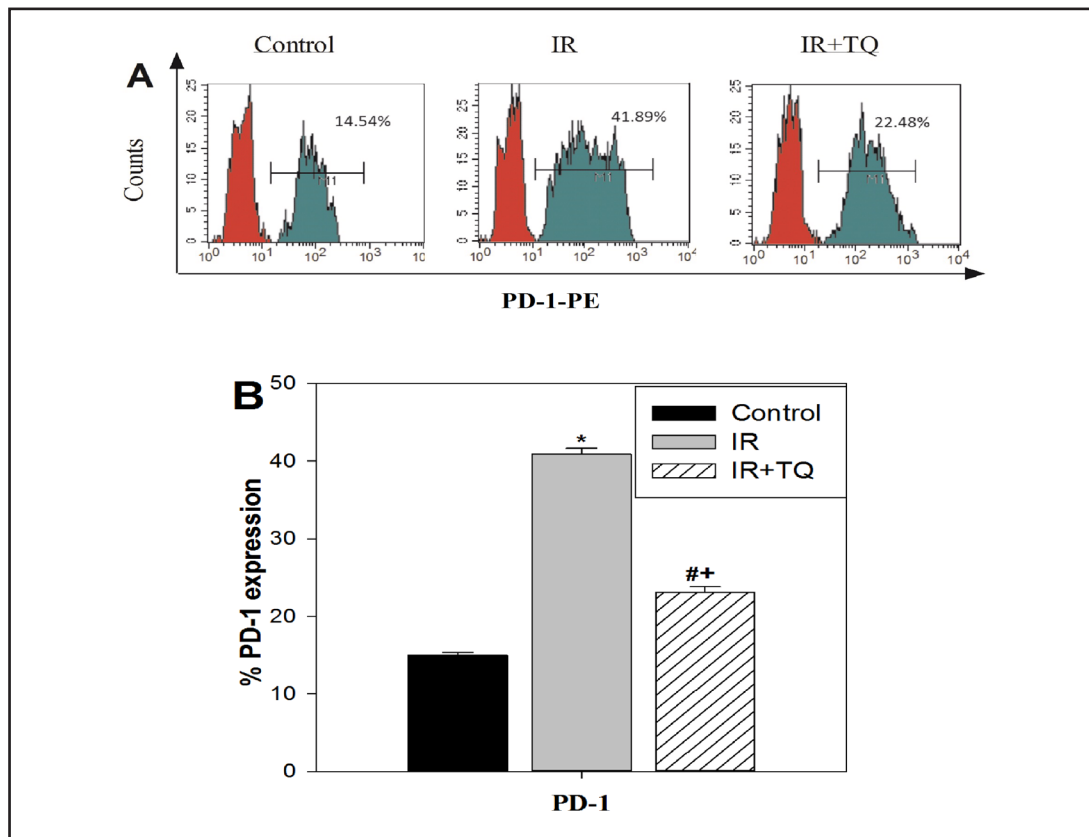


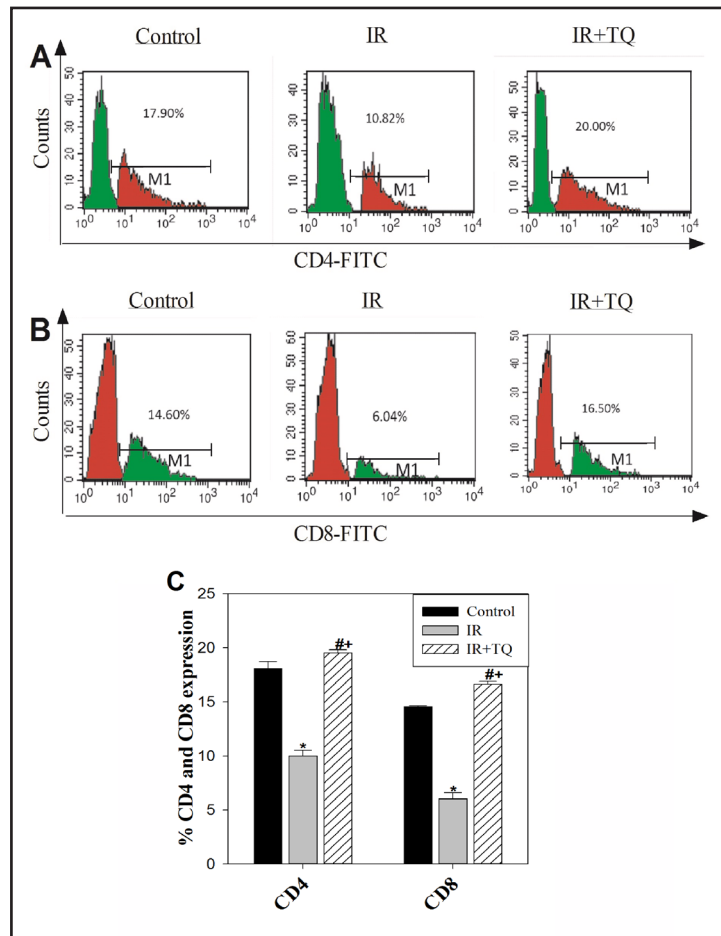
Fig. 2. Gamma-rays increase PD-1 expression and lymphocyte exhaustion. PBMCs were isolated from each group and stained for 30 minutes at 4°C with PE-conjugated anti-PD-1 or isotype control IgG mAbs. The cells were then washed twice and fixed in PBS containing 2% paraformaldehyde, followed by flow cytometry analysis of PD-1 expression on viable cells. The numbers shown correspond to the mean fluorescence intensity of PD-1 expression on labeled cells (A). The pooled data for eight individual rats from each group results are expressed as the mean PD-1 expression \pm SEM (n = 8) (B). *P < 0.05, gamma-irradiated rats vs. control rats; #P < 0.05, gamma-irradiated rats + TQ vs. gamma-irradiated rats; and †P < 0.05, gamma-irradiated rats + TQ vs. control rats (ANOVA with Tukey's post-test).

of CD4⁺ and CD8⁺ cells compared with untreated irradiated rats, which indicates that TQ can ameliorate the radiation-induced decreases in T cell subsets and play important roles in immune system.

Thymoquinone treatment partially reduces apoptosis induced by exposure to gamma irradiation

To investigate the protective effects of TQ on the survival of PBMCs after exposure to gamma irradiation, PBMCs isolated from control, irradiated, and irradiated + TQ-treated rats were stained with annexin V/PI and then analyzed by flow cytometry to determine the percentages of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant), late apoptotic cells (upper right quadrant), and necrotic cells (upper left quadrant). The data of one representative experiment out of eight are shown in the dot plot (Fig. 4A), which reveals that 6.11% and 8.66% of cells underwent late apoptosis and early apoptosis, respectively, in the control group, compared with a significant increase 33.33% and 22.19%, respectively, in the irradiated group. By contrast, treatment with TQ significantly reduced the percentage of late apoptotic cells and early apoptotic cells to 21.27% and 16.46%, respectively, compared with the untreated irradiated group. Pooled data for eight individual rats in each group (Fig. 4B) indicated that treatment with TQ significantly (P < 0.01) decreased apoptosis and necrosis in PBMCs induced by exposure to gamma irradiation.

Fig. 3. Gamma-irradiation induces perturbations in T cell populations. PBMCs were isolated from each group and stained for 30 minutes at 4°C with FITC-conjugated anti-CD4, FITC-conjugated anti-CD8 and/or isotype control IgG mAbs. The cells were then washed twice and fixed in PBS containing 2% paraformaldehyde, followed by flow cytometry analysis of CD4⁺ expression (A) and CD8⁺ expression (B). The accumulated data for CD4⁺ and CD8⁺ expression in all groups in eight experiments are expressed as the means of CD4⁺ and CD8⁺ expression ± SEM (n = 8) (C). *P < 0.05, gamma-irradiated rats vs. control rats; #P < 0.05, gamma-irradiated rats + TQ vs. gamma-irradiated rats; and *P < 0.05, gamma-irradiated rats + TQ vs. control rats (ANOVA with Tukey's post-test).



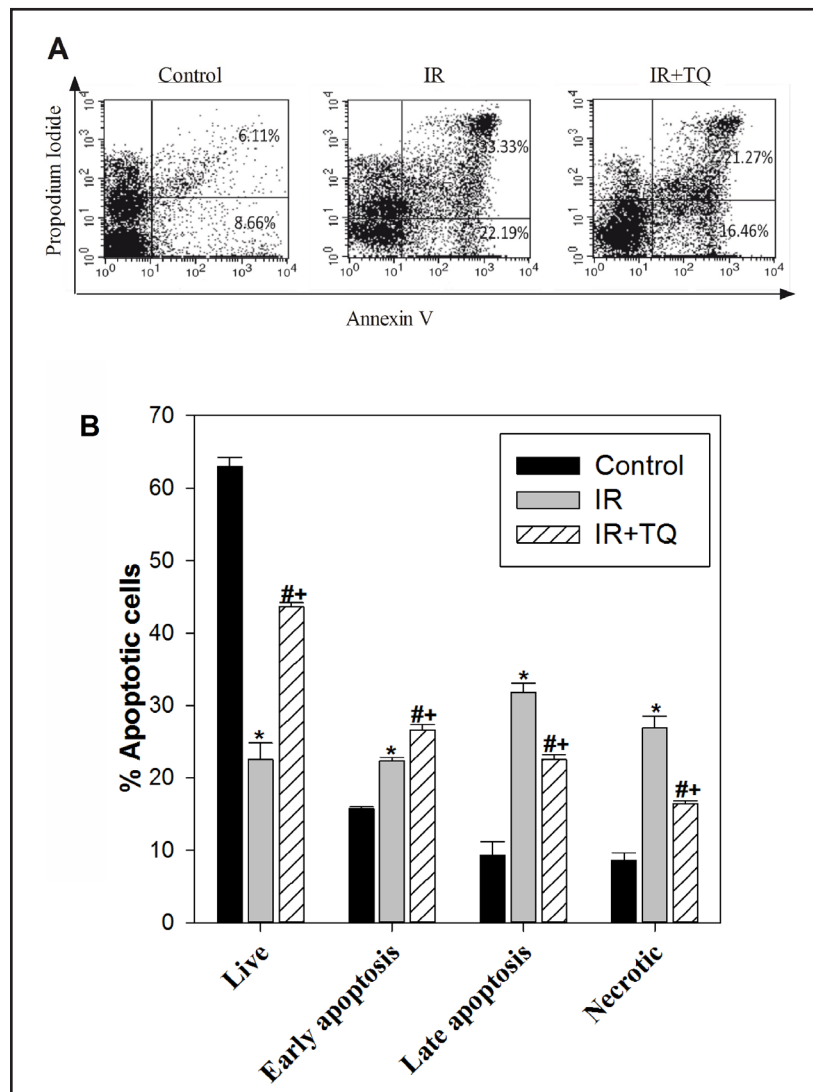
Influence of thymoquinone treatment on apoptotic and anti-apoptotic proteins altered by exposure to gamma irradiation

To investigate the mechanism by which TQ attenuates radiation-induced apoptosis, Bcl-2, Bax and caspase-3 expression on PBMCs isolated from control, irradiated, and irradiated + TQ-treated rats were examined by flow cytometry. The results of one representative experiment out of eight are shown in Fig. 5A. These results demonstrated that the mean percentage of cells expressing Bcl-2 decreased significantly in the irradiated group (18.22%) compared with the control group (30.85%). Interestingly, the irradiated group treated with TQ exhibited a significant increase in Bcl-2 expression (33.88%). Moreover, as shown in Fig. 5B, the mean percentage of cells expressing Bax increased significantly in the irradiated group (22.76%) compared with the control group (10.62%). The irradiated group treated with TQ also exhibited a significant increase in Bax expression (13.48%). Furthermore, as shown in Fig. 5C, the mean percentage of cells expressing caspase-3 increased significantly in the irradiated group (20.60%) compared with the control group (9.97%). The irradiated group treated with TQ also exhibited a significant increase in caspase-3 expression (14.14%). The cumulative results for eight individual rats from each group (Fig. 5D) indicated that the percentages of cells expressing caspase-3 and Bax were markedly decreased in the TQ-treated irradiated group, whereas Bcl-2 expression was increased by TQ supplementation.

Thymoquinone treatment reverses the histological changes in the thymus induced by gamma irradiation

Because the thymus is the primary lymphoid organ in which T cells undergo differentiation and maturation, we next investigated whether TQ supplementation could reverse the histological changes in the thymus induced by exposure to gamma-rays. The

Fig. 4. Gamma-rays induce apoptosis of T lymphocytes. The potential of gamma-rays to induce apoptosis of T cells was determined by flow cytometry based on the patterns of annexin V and propidium iodide (PI) staining. (A) One representative experiment in which the late apoptotic (upper right quadrant) and necrotic (lower right quadrant) PBMCs are separated into distinct groups is shown. (B) Accumulated data from annexin V/PI staining from eight experiments across all groups, expressed as the mean percentage of T cell population undergoing apoptosis \pm SEM (n = 8). *P < 0.05, gamma-irradiated rats vs. control rats; #P < 0.05, gamma-irradiated rats + TQ vs. gamma-irradiated rats; and *P < 0.05, gamma-irradiated rats + TQ vs. control rats (ANOVA with Tukey's post-test).

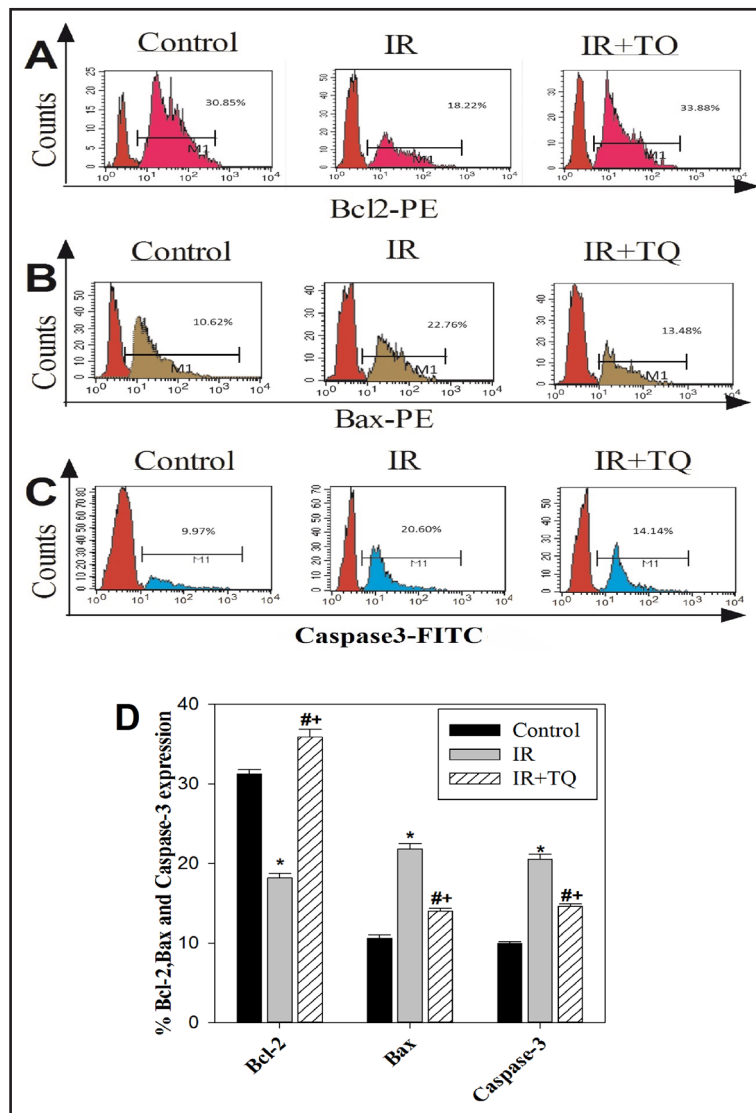


normal thymus in the control group displayed incompletely separated lobules with a large, densely stained cortex and a narrow, well organized medulla (Fig. 6A). In the gamma ray-irradiated group, the thymus exhibited marked degenerative changes in the thickness of the cortex, with visible bodies of macrophages and medullary epithelial cells scattered throughout the cortex. The degenerated medulla displayed lymphocytic depletion, invading fibroblasts, and prominent hemorrhages scattered throughout the medulla (Fig. 6B). Treatment with TQ nearly restored the normal organization of the thymus in the irradiated group (Fig. 6C).

Discussion

According to previous published works, it has been shown that radiation exposure is divided into low-dose (< 1Gy) and high-dose (> 1Gy) [28]. While, varying degrees of clinical toxicity from ionizing radiation may develop after a mild (1 – 2Gy), moderate (2 – 4Gy), severe (4 – 6Gy), very severe (6 – 8Gy), or lethal (>8Gy) exposure (International Atomic Energy Agency, 1998). In this context threshold dose of ionizing radiation exists for a biological effect remains debatable and classified the sub-lethal dose to be (2 - 4Gy) or (2 - 6Gy). It was provided a correlation between absolute lymphocyte count and ionizing radiation dose

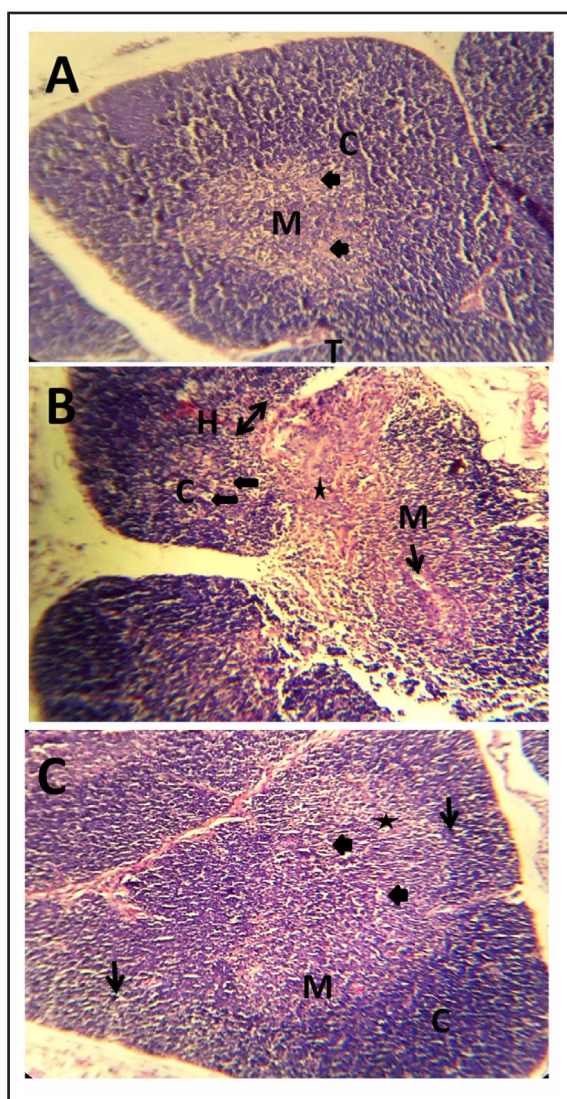
Fig. 5. Thymoquinone attenuates gamma-ray-induced apoptosis. The mechanism by which gamma-rays induce apoptosis of T cells was determined by flow cytometry analysis of anti-apoptotic and pro-apoptotic protein expression. PBMCs were stained for 30 minutes at 4°C with PE-conjugated anti-Bcl2, PE-conjugated anti-Bax, FITC-conjugated anti-caspase-3 or isotype control IgG mAbs. The cells were then washed twice and fixed in PBS containing 2% paraformaldehyde, followed by flow cytometry analysis of Bcl2 expression (A), Bax expression (B) and caspase-3 expression (C) on viable cells. (D) Accumulated data for Bcl-2, Bax and caspase-3 expression from eight experiments across all groups, expressed as the mean percentage of PBMCs \pm SEM (n = 8). *P < 0.05, gamma-irradiated rats vs. control rats; #P < 0.05, gamma-irradiated rats + TQ vs. gamma-irradiated rats; and *P < 0.05, gamma-irradiated rats + TQ vs. control rats (ANOVA with Tukey's post-test).



investigated that acute radiation syndrome on circulating lymphocytes varying from mild to lethal and (4Gy) is the critical dose to give a severe reduction in the lymphocyte [28]. Therefore, the researchers have focused to study the protective effects of natural antioxidants against (4Gy) gamma radiation-induced cellular damage to normal tissues [29, 30].

Accordingly, in the present study we choose (4Gy) as sub-lethal dose induced immune suppression and defect in T-cells. We also used fractionated dose of (4Gy) for several reasons: 1) to study the acute effect of sub-lethal gamma radiation dose induced immune imbalance which is in consistent with previous study showed that fractions of (4Gy) cause total peripheral T cell decline and immunosuppressive effect [31]; 2) according to Boston university committee about irradiation of rodents demonstrated that fractionation of irradiation dose (the total irradiation dose of lethal dose split into two equal parts of sub-lethal dose (4Gy) separated by 14 days time interval) is the optimum method in order to minimize morbidity and mortality of rats; 3) our preliminary results showed that gamma irradiated rats for 14 days is mainly the optimal period to induce hematopoietic syndrome by gamma radiation; and 4) we used fractionated sub-lethal dose as a simulative for what applied during radiotherapy where human patients take more fraction doses. Moreover, TQ was orally supplemented for 28 days according to previous studies revealed the immune-modulator effects of TQ for 28 consecutive days [32, 33].

Fig. 6. Gamma-irradiation induces histological changes in the thymus. One representative experiment out of five is shown. Thymus section from a control, non-irradiated rat (A) showing incompletely separated thymic lobules with thin trabeculae (T), a cortex (C) formed of densely packed thymocytes, a lightly stained medulla (M), and Hassall's corpuscles (arrowheads) (H&E, $\times 100$). (B) Thymus section from a gamma ray-irradiated rat showing a disorganized thymus with visible macrophage bodies (arrowheads), hemorrhagic areas (H) and medullary epithelial cells (double headed arrow) scattered in the cortex (C). The degenerated medulla (M) shows lymphocytic depletion, invasion of fibroblasts (star) and focal areas of macrophage activity (arrow) (H&E, $\times 100$). (C) Thymus section of a thymoquinone (TQ)-treated, gamma ray irradiated rat showing a large, densely stained cortex (C), packed thymocytes and the aggregation of macrophages (arrows). The narrow, well organized medulla (M) contains blood capillaries, epithelial reticular cells (star) and Hassall's corpuscles (arrowheads) (H&E, $\times 100$).



Exposure to IR results in DNA damage, which induces apoptosis in radiation-sensitive tissues, including lymphocytes [28]. Immune suppression contributes to alterations in peripheral lymphoid populations and the impairment of immune responses [34, 35]. Natural antioxidants play central roles in enhancing the immune system through mechanisms dependent on oxidative stress, which seems to play significant roles in many human diseases. In this context, we previously demonstrated the beneficial effects of TQ in the induction of growth arrest of multiple myeloma cancer cells and its protective anti-diabetic effects [36-39]. Moreover, we provided clear evidence for the effects of other natural antioxidants (camel whey protein and bee propolis) as immune-modulators in accelerating the healing process of diabetic wounds in experimental animal models [40-46]. Most interesting, we demonstrated that natural antioxidants isolated from snake and ant venoms enhanced normal lymphocyte functions and exerted antitumor effects in different human and animal cancer cells [47-53].

The present results show that fractionated, sub-lethal doses of gamma-irradiation produce significant oxidative damage. Whole-body exposure of rats to high-energy radiation causes tissue damage in several organs, as assessed by increased lipid peroxidation [54, 55]. Thus, the modification of lipids by ROS has been implicated in the etiology of radiation-induced physiological disorders and diseases [56]. In the present study, a significant increase in the levels of serum cholesterol, triglycerides, and LDL and diminished levels of serum HDL were observed post-irradiation, suggesting that IR-induced oxidative stress alters lipid

metabolism and the levels of serum lipoproteins and supporting an association between IR-induced oxidative stress and elevated levels of lipid fractions [56]. Therefore, we suggest that oxidative stress might be an important contributor to altered lipid metabolism following radiation exposure. In the present study, administration of TQ effectively decreased MDA levels and attenuated the alterations in the lipid profiles induced by gamma-irradiation, clearly indicating that TQ effectively reduces IR-induced oxidative stress. TQ was previously suggested to preserve the activity of various antioxidant enzymes and act as a free radical scavenger [24].

In addition to increasing oxidative stress by increasing lipid peroxidation and altering lipid levels, exposure to gamma-irradiation also stimulates the production of pro-inflammatory cytokines (TNF- α and IL-6). Treatment with TQ reduced this inflammatory condition and decreased TNF- α and IL-6 levels. Radiation induces inflammatory responses [57, 58]. The release of pro-inflammatory cytokines such as TNF- α and IL-6 aggravates the IR-induced inflammatory cascade and plays a role in the development of late radiation effects [59]. Serum TNF- α and inflammatory changes are markedly reduced in rats administered TQ [60]. Tekeoglu et al. reported that TQ has anti-inflammatory effects and decreases the levels of inflammatory cytokines in circulation [61]. Similarly, antioxidant and anti-inflammatory properties of TQ have been reported in several diseases [62]. The present study demonstrates that TQ treatment prevents inflammatory changes induced by gamma-irradiation in rats.

The remarkable dynamic changes in inflammatory cytokine levels and increases in oxidative stress suggest that these changes may contribute to the impairment of immune responses. PD-1 is a member of the immunoglobulin superfamily, and its expression is inducible in T cells, B cells, and activated monocytes [63]. PD-1 signaling was recently identified as an important mechanism of antigen-specific T-cell dysfunction in chronic infections in humans [64-65]. In the present study, PBMCs isolated from irradiated rats were functionally exhausted and were characterized by high PD-1 expression. Local upregulation of the PD-L1/PD-1 axis following exposure to IR suppresses immune responses and limits the full expression of antitumor immunity [66]. Fascinatingly, TQ supplementation regulated PD-1 expression, suggesting that TQ may provide a protective effect against irradiation-induced immunosuppression.

We observed a significant decrease in CD4⁺ and CD8⁺ T cells that was positively correlated with lymphocyte exhaustion in irradiated rats. Significant decreases in naïve T cells have been observed in atomic bomb survivors [67]. The decreased proportion of CD4⁺ naïve T cells were attributed to reduced T cell responses to mitogens and proliferation [68]. By contrast, TQ supplementation ameliorated the decrease in the proportions of CD4⁺ and CD8⁺ T cells following exposure to gamma-irradiation. Consistent with these results, treatment with *N. sativa* oil induces an increase in the CD4⁺ to CD8⁺ T cell ratio and an increase in NK cell function [69]. *N. sativa* seeds and their component, TQ, have a stimulatory effect on the proliferation of T cells and responses to different mitogens [70].

In this context, to determine the mechanism by which IR induces apoptosis, an Annexin V/PI assay was performed to quantify the apoptotic cell populations. Significant increases in both the early and late stages of apoptosis and necrosis were observed. Moreover, treatment with TQ decreased apoptosis induced by gamma-irradiation in PBMCs. To further analyze the possible mechanism underlying IR-induced apoptosis, we examined the expression of Bcl-2, Bax and caspase-3 in PBMCs after exposure to gamma-irradiation. In the irradiated group, the expression of Bcl-2 and caspase-3 increased significantly, whereas Bax expression decreased markedly. Consistent with these findings, it has been reported that cell apoptosis induced by radiation is regulated by a complex balance between pro-apoptotic factors, including caspase-3, c-caspase-3 and Bax, and anti-apoptotic factors, including the Bcl-2 family [71, 72]. Recently, it has been shown that induction of apoptosis in T cells was associated with imbalance activation of caspase and Bcl-2 [73-75]. In the present study, we observed that TQ reduced radiation-induced caspase-3 and Bax activation and enhanced Bcl-2 expression. Indeed, *N. sativa* and its component TQ enhance innate immunity functions and decrease apoptosis in lymphocytes in peripheral blood [76]. Our results indicate that TQ can prevent

radiosensitive cells from entering apoptosis. The structure of the thymus was also examined in the present study. In the irradiated group the cortex and medulla were seriously damaged and lymphocytes were reduced. These tissue structures were effectively protected by TQ supplementation. Our results indicate that TQ rescues T cells from IR-induced apoptosis and impairment of T cell maturation within the thymus.

In summary, this study demonstrates that the radio-protective effect of TQ on immune system is attributable to its antioxidant and anti-inflammatory capacities. TQ relieved the damage to the immune system induced by IR via reducing immune dysfunction by reducing the apoptosis of T cells either directly or by attenuating the destruction of the thymus as the site of T cell maturation.

Acknowledgments

The authors are grateful to the Department of Radiation Biology, National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt, for providing support.

Disclosure Statement

The authors declare they have no competing interest, state that the manuscript has not been published or submitted elsewhere, state that the work complies with the Ethical Policies of the Journal and state that the work has been conducted under internationally accepted ethical standards after relevant ethical review.

References

- 1 Verma AR, Vijayakumar M, Rao CV, Mathela CS: *In vitro* and *in vivo* antioxidant properties and DNA damage protective activity of green fruit of *Ficus glomerata*. *Food Chem Toxicol* 2010;48:704–709.
- 2 Yao L, Wang Z, Zhao H, Cheng C, Fu X, Liu J, Yang X: Protective effects of polysaccharides from soybean meal against X-ray radiation induced damage in mouse spleen lymphocytes. *Int J Mol Sci* 2011;12:8096–8104.
- 3 Li YR, Cao W, Guo J, Miao S, Ding GR, Li KC, Wang J, Guo GZ: Comparative investigations on the protective effects of rhodioside, ciwujianoside-B and astragaloside IV on radiation injuries of the hematopoietic system in mice. *Phytother Res* 2011;25:644–653.
- 4 Sangsliwan T, Haghdoost S: The nucleotide pool, a target for low-dose gamma-ray-induced oxidative stress. *Radiat Res* 2008;170:776–783.
- 5 Yang RY, Pei XR, Wang JB, Zhang Z, Zhao H, Li Q, Zhao M, Li Y: Protective effect of a marine oligopeptide preparation from Chum Salmon (*Oncorhynchus keta*) on radiation-induced immune suppression in mice. *J Sci Food Agric* 2010;90:2241–2248.
- 6 Cadet J, Bellon S, Douki T, Frelon S, Gasparutto D, Muller E, Pouget JP, Ravanat JL, Romieu A, Sauvaigo S: Radiation-induced DNA damage: formation, measurement, and biochemical features. *J Environ Pathol Toxicol Oncol* 2004;23:33–43.
- 7 Esterbauer H: Estimation of peroxidative damage: a critical review. *J Environ Pathol Toxicol Oncol* 1996;23:33–43.
- 8 Jalal SH: Flavonoids and genomic instability induced by ionizing radiation. *Drug Discov Today* 2010;15:907–918.
- 9 Box HC, Maccubbin AE: Lipid peroxidation and DNA damage. *Nutrition* 1997;13(10):920–921.
- 10 Petersen DR, Doorn JA: Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med* 2004;37:937–945.
- 11 Oradovskaia IV, Pashchenkova IuG, Feoktistov VV, Nikonova MF, Vikulov GK, Bozheskaia NV, Smirnova NN: The epidemiological analysis of monitoring of the immune status in liquidators of consequences of the Chernobyl accident for early identification of risk groups and diagnostics of oncological diseases. Report 2. Dependence of frequency and changes in the immune status on risk factors of radiation accident. *Radiats Biol Radioecol* 2011;51:117–123.

- 12 Borja-Aburto VH, Bustamante-Montes P, Garcia-Sancho MC, Villa-Romero AR: Ionizing radiation at low doses and cancer: epidemiological controversy. *Rev Invest Clin* 1990;42:312–316.
- 13 Makidono R, Ouchida T, Makidono A, Jingu K: Severe damage of CD4-2H4+ T subpopulation cells (naive T cells and suppressor/inducer) by radiation therapy, their recovery being promoted by a plant alkaloid. *Nihon Igaku Hoshasen Gakkai Zasshi* 1992;52:223–228.
- 14 Dainiak N, Waselenko JK, Armitage JO, MacVittie TJ, Farese AM: The hematologist and radiation casualties. *Hematology Am Soc Hematol Educ Program* 2003;1:473–496.
- 15 Hayashi T, Morishita Y, Kubo Y, Kusunoki Y, Hayashi I, Kasagi F, Hakoda M, Kyoizumi S, Nakachi K: Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors. *Am J Med* 2005;118:83–86.
- 16 Majdalawieh AF, Hmaidan R, Carr RI: Nigella sativa modulates splenocyte proliferation, Th1/Th2 cytokine profile, macrophage function and NK anti-tumor activity. *J Ethnopharmacol* 2010;131:268–275.
- 17 Assayed ME: Radioprotective effects of black seed (Nigella sativa) oil against hemopoietic damage and immunosuppression in gamma-irradiated rats. *Immunopharmacol Immunotoxicol* 2010;32:284–296.
- 18 Gali-Muhtasib H, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, Evert M, El-Najjar N, Peters B, Jurjus A, Roessner A, Schneider-Stock R: Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med* 2008;12:330–342.
- 19 Badary OA, Gamal El-Din AM: Inhibitory effects of thymoquinone against 20-methylcholanthrene-induced fibrosarcoma tumorigenesis. *Cancer Detect Prev* 2001;25:362–368.
- 20 Badary OA, Al-Shabanah OA, Nagi MN, Al-Rikabi AC, Elmazar MM: Inhibition of benzo(a)pyrene-induced forestomach carcinogenesis in mice by thymoquinone. *Eur J Cancer Prev* 1999;8:435–440.
- 21 Woo CC, Kumar AP, Sethi G, Tan KH: Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol* 2012;83:443–451.
- 22 Srinivasan M, Sudheer AR, Rajasekaran KN: Effect of curcumin analog on gamma-radiation-induced cellular changes in primary culture of isolated rat hepatocytes *in vitro*. *Chem Biol Interact* 2008;176:1–8.
- 23 Verma AR, Vijayakumar M, Rao CV, Mathela CS: *In vitro* and *in vivo* antioxidant properties and DNA damage protective activity of green fruit of Ficus glomerata. *Food Chem Toxicol* 2010;48:704–709.
- 24 Umar S, Zargan J, Umar K, Ahmad S, Katiyar CK, Khan HA: Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats. *Chem Biol Interact* 2012;197:40–46.
- 25 Sayed D, Badr G, Maximous D, Mikhail NN, Abu-Tarboush F, Alhazza IM: HLA-G and its relation to proliferation index in detection and monitoring breast cancer patients. *Tissue Antigens* 2010;75:40–47.
- 26 Badr G, Mohany M: Maternal perinatal undernutrition attenuates T-cell function in adult male rat offspring. *Cell Physiol Biochem* 2011;27:381–390.
- 27 Badr BM, Moustafa NA, Eldien HM, Mohamed AO, Ibrahim HM, El-Elaimy IA, Mahmoud MH, Badr G: Increased levels of type 1 interferon in a type 1 diabetic mouse model induce the elimination of B cells from the periphery by apoptosis and increase their retention in the spleen. *Cell Physiol Biochem* 2015;35:137–147.
- 28 Dainiak N: Hematologic consequences of exposure to ionizing radiation. *Exp Hematol* 2002;30:513–528.
- 29 Velho-Pereira R, Kumar A, Pandey PN, Mishra KP, Jagtap AG: Radioprotection by macerated extract of nigella sativa in normal tissues of fibrosarcoma bearing mice. *Indian J Pharm Sci* 2012;74:403–414.
- 30 Xiao S, Zhang W, Manley N: The acute effects of sub-lethal gamma irradiation on the thymus and T cells were directly affected by age, sex and radiation exposure dose. *J Immunol* 2012;188:115.8.
- 31 Olivier G, Benderitter M, Linard C: Acute and persisting Th2-like immune response after fractionated colorectal gamma-irradiation. *World J Gastroenterol* 2008;14:7075–7085.
- 32 Hadjzadeh MA, Mohammadian N, Rahmani Z, Rassouli FB: Effect of thymoquinone on ethylene glycol-induced kidney calculi in rats. *Urol J* 2008;5:149–155.
- 33 Mohany M, El-Feki M, Refaat I, Garraod O, Badr G: Thymoquinone ameliorates the immunological and histological changes induced by exposure to imidacloprid insecticide. *J Toxicol Sci* 2012;37:1–11.
- 34 Kusunoki Y, Hayashi T: Long-lasting alterations of the immune system by ionizing radiation exposure: Implications for disease development among atomic bomb survivors. *Int J Radiat Biol* 2008;84:1–14.
- 35 Chernyshov VP, Vykhoanets EV, Slukvin II, Antipkin YG, Vasyuk AN, Strauss KW: Analysis of blood lymphocyte subsets in children living on territory that received high amounts of fallout from Chernobyl accident. *Clin Immunol Immunopathol* 1997;84:122–128.

- 36 Badr G, Lefevre EA, Mohany M: Thymoquinone inhibits the CXCL12-induced chemotaxis of multiple myeloma cells and increases their susceptibility to Fas-mediated apoptosis. *PLoS One* 2011;6:e23741.
- 37 Badr G, Mohany M, Abou-Tarboush F: Thymoquinone decreases F-actin polymerization and proliferation of human multiple myeloma cells through suppression of STAT3 phosphorylation and Bcl2/Bcl-XL expression. *Lipids Health Dis* 2011;10:236.
- 38 Badr G, Mahmoud MH, Farhat K, Waly H, Al-Abdin OZ, Rabah DM: Maternal supplementation of diabetic mice with thymoquinone protects their offspring from abnormal obesity and diabetes by modulating their lipid profile and free radical production and restoring lymphocyte proliferation via PI3K/AKT signaling. *Lipids Health Dis* 2013;12:37.
- 39 Badr G, Alwasel S, Ebaid H, Mohany M, Alhazza I: Perinatal supplementation with thymoquinone improves diabetic complications and T cell immune responses in rat offspring. *Cell Immunol* 2011;267:133-140.
- 40 Badr G, Badr BM, Mahmoud MH, Mohany M, Rabah DM, Garraud O: Treatment of diabetic mice with undenatured whey protein accelerates the wound healing process by enhancing the expression of MIP-1 α , MIP-2, KC, CX3CL1 and TGF- β in wounded tissue. *BMC Immunol* 2012;13:32.
- 41 Badr G: Camel whey protein enhances diabetic wound healing in a streptozotocin-induced diabetic mouse model: The critical role of β -Defensin-1, -2 and -3. *Lipids Health Dis* 2013;12:46.
- 42 Badr G, Mohany M, Metwalli A: Effects of undenatured whey protein supplementation on CXCL12- and CCL21-mediated B and T cell chemotaxis in diabetic mice. *Lipids Health Dis* 2011;10:203.
- 43 Badr G, Ebaid H, Mohany M, Abuelsaad A: Modulation of immune cell proliferation and chemotaxis towards CC chemokine ligand (CCL)-21 and CXC chemokine ligand (CXCL)-12 in un-denatured whey protein-treated mice. *J Nutr Biochem* 2012;23:1640-1646.
- 44 Badr G: Supplementation with undenatured whey protein during diabetes mellitus improves the healing and closure of diabetic wounds through the rescue of functional, long-lived wound macrophages. *Cell Physiol Biochem* 2012;3:571-582.
- 45 Al Ghamdi AA, Badr G, Hozzein WN, Allam A, Al-Waili NS, Al-Wadaan MA, Garraud O: Oral supplementation of diabetic mice with propolis restores the proliferation capacity and chemotaxis of B and T lymphocytes towards CCL21 and CXCL12 by modulating the lipid profile, the pro-inflammatory cytokine levels and oxidative stress. *BMC Immunol* 2015;16:54.
- 46 Hozzein WN, Badr G, Al Ghamdi AA, Sayed A, Al-Waili NS, Garraud O: Topical application of propolis enhances cutaneous wound healing by promoting TGF-beta/Smad-mediated collagen production in a streptozotocin-induced type I diabetic mouse model. *Cell Physiol Biochem* 2015;37:940-954.
- 47 Badr G, Al-Sadoon MK, El-Toni AM, Daghestani M: *Walterinnesia aegyptia* venom combined with silica nanoparticles enhances the functions of normal lymphocytes through PI3K/AKT, NF κ B and ERK signalling. *Lipids Health Dis* 2012;11:27.
- 48 Badr G, Al-Sadoon MK, Rabah DM: Therapeutic efficacy and molecular mechanisms of snake (*Walterinnesia aegyptia*) venom-loaded silica nanoparticles in the treatment of breast cancer- and prostate cancer-bearing experimental mouse models. *Free Radic Biol Med* 2013;65:175-189.
- 49 Al-Sadoon MK, Rabah DM, Badr G: Enhanced anticancer efficacy of snake venom combined with silica nanoparticles in a murine model of human multiple myeloma: molecular targets for cell cycle arrest and apoptosis induction. *Cell Immunol* 2013;284:129-138.
- 50 Al-Sadoon MK, Abdel-Maksoud MA, Rabah DM, Badr G: Induction of apoptosis and growth arrest in human breast carcinoma cells by a snake (*Walterinnesia aegyptia*) venom combined with silica nanoparticles: crosstalk between Bcl2 and caspase 3. *Cell Physiol Biochem* 2012;30:653-665.
- 51 Badr G, Al-Sadoon MK, Rabah DM, Sayed D: Snake (*Walterinnesia aegyptia*) venom-loaded silica nanoparticles induce apoptosis and growth arrest in human prostate cancer cells. *Apoptosis* 2013;18:300-314.
- 52 Sayed D, Al-Sadoon MK, Badr G: Silica nanoparticles sensitize human multiple myeloma cells to snake (*Walterinnesia aegyptia*) venom-induced apoptosis and growth arrest. *Oxid Med Cell Longev* 2012;2012:386286.
- 53 Badr G, Garraud O, Daghestani M, Al-Khalifa MS, Richard Y: Human breast carcinoma cells are induced to apoptosis by samsun ant venom through an IGF-1-dependant pathway, PI3K/AKT and ERK signaling. *Cell Immunol* 2012;273:10-16.
- 54 Koc M, Taysi S, Buyukokuroglu ME, Bakan N: The effect of melatonin against oxidative damage during total-body irradiation in rats. *Radiat Res* 2003;160:251-255.

- 55 Sener G, Jahovic N, Tosun O, Atasoy BM, Yeğen BC: Melatonin meliorates ionizing radiation-induced oxidative organ damage in rats. *Life Sci* 2003;74:563–572.
- 56 Onody A, Csonka C, Giricz Z, Ferdinandy P: Hyperlipidemia induced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts. *Cardiovasc Res* 2003;58:663–670.
- 57 Yuan H, Goetz DJ, Gaber MW, Issekutz AC, Merchant TE, Kiani MF: Radiation-induced up regulation of adhesion molecules in brain microvasculature and their modulation by dexamethasone. *Radiat Res* 2005;163:544–551.
- 58 Minafra L, Bravatà V: Cell and molecular response to IORT treatment. *Transl Cancer Res* 2014;3:32-47.
- 59 Boerma M, Hauer-Jensen M: Preclinical research into basic mechanisms of radiation-induced heart disease. *Cardiol Res Pract* 2011;2011:858-262.
- 60 Helal GK: Thymoquinone supplementation ameliorates acute endotoxemia-induced liver dysfunction in rats. *Pak J Pharm Sci* 2010;23:131-137.
- 61 Tekeoglu I, Dogan A, Ediz L, Budancamanak M, Demirel A: Effects of thymoquinone (volatile oil of black cummin) on rheumatoid arthritis in rat models. *Phytother Res* 2007;21:895–897.
- 62 Salem ML: Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. *Int Immunopharmacol* 2005;5:1749–1770.
- 63 Greenwald RJ, Freeman GJ, Sharpe AH: The B7 family revisited. *Annu Rev Immunol* 2005;23:515-548.
- 64 Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R: Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;439:682-687.
- 65 Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD: PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006;443:350-354.
- 66 Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C: PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006;80:11398-11403.
- 67 Deng L, Liang H, Burnette B, Beckett M, Darga T, Weichselbaum RR, Fu YX: Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. *J Clin Invest* 2014;124:687-695.
- 68 Kusunoki Y, Kyoizumi S., Hirai Y, Suzuki T, Nakashima E, Kodama K, Seyama T: Flow cytometry measurements of subsets of T, B and NK cells in peripheral blood lymphocytes of atomic bomb survivors. *Radiat Res* 1998;150:227–236.
- 69 Yamaoka M, Kusunoki Y, Kasagi F, Hayashi T, Nakachi K, Kyoizumi S: Decreases in percentages of naïve CD4 and CD8 T cells and increases in percentages of memory CD8 T-cell subsets in the peripheral blood lymphocyte populations of Abomb survivors. *Radiat Res* 2004;161:290–298.
- 70 Haq A, Lobo PI, Al-Tufail M, Rama NR, Al-Sedairy ST: Immunomodulatory effect of *Nigella sativa* proteins fractionated by ion exchange chromatography. *Int J Immunopharmacol* 1999;21:283–295.
- 71 Haq A, Abdullatif M, Lobo PI, Khabar KS, Sheth KV, al-Sedairy ST: *Nigella sativa*: effect on human lymphocytes and polymorphonuclear leukocyte phagocytic activity. *Immunopharmacology* 1995;30:147–155.
- 72 Stahnke K, Mohr A, Liu J, Meyer LH, Karawajew L, Debatin KM: Identification of deficient mitochondrial signaling in apoptosis resistant leukemia cells by flow cytometric analysis of intracellular cytochrome c, caspase-3 and apoptosis. *Apoptosis* 2004;9:457-465.
- 73 Wu ZS, Yao YM, Hong GL, Xu XP, Liu Y, Dong N, Zheng JY, Lu ZQ, Zhao GJ, Zhu XM, Zhang QH, Sheng ZY: Role of mitofusin-2 in high mobility group box-1 protein-mediated apoptosis of T cells *in vitro*. *Cell Physiol Biochem* 2014;33:769-783.
- 74 Szabo I, Trentin L, Trimarco V, Semenzato G, Leanza L: Biophysical characterization and expression analysis of Kv1.3 potassium channel in primary human leukemic B cells. *Cell Physiol Biochem* 2015;37:965-978.
- 75 Li Q, Wang Y, Li H, Shen G, Hu S: Ox-LDL influences peripheral Th17/Treg balance by modulating Treg apoptosis and Th17 proliferation in atherosclerotic cerebral infarction. *Cell Physiol Biochem* 2014;33:1849-1862.
- 76 Wilkins RC, Kutzner BC, Truong M, McLean JR: The effect of the ratio of CD4+ to CD8+ T-cells on radiation-induced apoptosis in human lymphocyte subpopulations. *Int J Radiat Biol* 2002;78:681-688.