Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2021 Nov 12; 9(A):993-1005.
https://doi.org/10.3889/oamjms.2021.7063
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pathophysiology*

Protective Potential of Ginseng and/or Coenzyme Q10 on Doxorubicin-induced Testicular and Hepatic Toxicity in Rats

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Edited by: Sinisa Stojanoski
Citation: Khodir S, Alafify A, Omar E, Al-Gholam M. Protective Potential of Ginseng and/or Coenzyme Q10 on Doxorubicin-induced Testicular and Hepatic Toxicity in Rats. Open-Access Maced J Med Sci. 2021 Nov 12; 9(A):993-1005. https://doi.org/10.3889/oamjms.2021.7063
Keywords: Doxorubicin; Nuclear factor E2-related factor 2; Caspase-3; Ginseng; Coenzyme Q10
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Received: 14-Aug-2021
Revised: 05-Sep-2021
Accepted: 02-Nov-2021

Funding: This research did not receive any financial support
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Although doxorubicin (DOX) is a successful cancer chemotherapeutic, side effects limit the clinical utility of DOX-based therapy, including male infertility and hepatotoxicity.

AIM: The objective of the study was to evaluate the testicular and hepatoprotective effect of ginseng and/or coenzyme Q10 (CoQ10) in rats exposed to DOX and the possible underlying mechanisms.

MATERIALS AND METHODS: Fifty adult male albino rats were divided into (10/group) control, DOX group, DOX/Gin group, DOX/CoQ10 group, and DOX/Gin+CoQ10 group. Serum testosterone, serum liver enzymes, fasting serum cholesterol and triglyceride (TG), tissue malondialdehyde (MDA), tissue superoxide dismutase (SOD), serum tumor necrosis factor-alpha (TNF-α), serum interleukin-6 (IL-6), serum interleukin 10 (IL-10), nuclear factor E2-related factor 2 (Nrf2) gene expression in liver and testis, and organ indices were measured. Histopathological and immunohistochemical assessments of apoptotic marker caspase 3 in testis and liver were also performed.

RESULTS: DOX-induced toxicity is associated with a significant decrease in serum testosterone, testis and liver index values, testicular and hepatic SOD, testicular and hepatic Nrf2 gene expression, and serum IL-10. However, there was a significant increase in serum liver enzymes, serum cholesterol and TG, testicular and hepatic MDA, serum TNF-α, and serum IL-6 when compared with the control group. The combination of ginseng and CoQ_{10} resulted in significant improvement of DOX-induced changes when compared with other treated groups.

CONCLUSION: Ginseng and CoQ_{10} have valuable therapeutic effects on DOX-induced testicular and hepatic toxicity through upregulation of Nrf2 gene expression, inhibition of apoptosis, antioxidant, anti-inflammatory, and hypolipidemic effects.

Introduction

According to the global statistics, the neoplasm is one of the main reasons for morbidity and mortality worldwide. Frequently encountered issues are seeking medical care in late stages of the disease and inaccessibility of diagnosis. Despite the active development of various approaches to cancer treatment, systemic chemotherapy remains the mainstay cancer treatment. The pharmacological development in the second half of the 20th century led to a stable annual increase in the life expectancy of cancer patients [1]. The anthracycline antibiotic doxorubicin (DOX) is one of the applied antitumor agents against human malignancies [2]. However, many toxic adverse effects of DOX on organs, such as the liver and testis, have also been established [3]. DOX could impede spermatogenesis [4]. The liver is the chief detoxifying tissue; therefore, it is the target of excessive amounts of genotoxic composites and anticancer drugs including DOX. Approximately 40% of patients on DOX suffer from liver injury and can result in liver failure and death [3]. Thus, it is critically important to understand how DOX impairs organ function to generate targeted interventions to improve health outcomes [5].

The main mechanism of DOX-induced cytotoxicity is due to the generation of reactive oxygen species (ROS) [6]. Maintaining the balance between the production of ROS and the availability of antioxidant enzymes, such as superoxide dismutase (SOD), is consequently critical. This could be an important mechanism for preventing oxidative stress-induced tissue damage. Another mechanism is cellular apoptosis in the liver and testes following DOX treatment [7].

Many genes and signaling pathways play a mechanistic role in the pathogenesis of Dox-induced cytotoxicity. Nuclear factor E2-related factor 2 (Nrf2) is referred to as the master regulator of the antioxidant response. It modulates the expression of a series of genes encoding antioxidant proteins expression through the antioxidant response element (ARE) in the liver [8]. Under normal conditions, Nrf2 binds to the kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm [9]. In response to oxidative or electrophilic stress, Keap1 is oxidized resulting in decreased affinity for Nrf2, which allows it to accumulate in the nucleus

to induce the detoxifying and antioxidant defense genes expression [10]. DOX exposure also activates the expression of nuclear factor kappa B cell (NF-κB) which in turn releases pro-inflammatory mediator, including tumor necrosis factor-alpha (TNF-α) [11]. These inflammation factors have been thought to be the focus of investigating inflammatory injury. Thus, activating the Nrf2 signaling pathway and inhibiting inflammatory response might be a beneficial molecular target to prevent DOX-induced organ injury.

Controlling the oxidative injury by a variety of antioxidant or anti-apoptotic agents has been employed to counteract Dox-induced damage [12]. Nevertheless, so far, there is still no single agent proven effective enough to prevent or reverse this adverse effect. Ginseng is a medicinal herbal plant that has been used for thousands of years as an adaptogen to increase physical energy and enhance fertility [13] and hepatoprotective activities [14]. The pharmacological properties of ginseng are mainly attributed to ginsenosides [15]. Ginsenosides are bioactive compounds that have antioxidant, anti-inflammatory [16], hypocholesterolemic, and hypolipidemic effects [17].

Coenzyme Q10 (CoQ10) is an important cellular antioxidant. [18], with free radical scavenging activities [19] and anti-inflammatory effects [20]. CoQ10 can modulate the expression of Nrf2 after exercise training, supporting the role of CoQ10 in antioxidant defense and inflammation [21].

Therefore, both ginseng and CoQ10 have the potential to protect against tissue injury induced by DOX. This encouraged us to conduct the present study to evaluate the testicular and hepatoprotective effect of ginseng and/or CoQ10 in rats exposed to DOX and the possible underlying mechanisms. In addition, we investigated the impact of DOX-induced cytotoxicity on Nrf2 expression in testis and liver, and whether it has been improved by ginseng and/or CoQ10 treatment or not as a suggested mechanism of action.

Materials and Methods

Animals

This study was carried out following the regulations of the Animal Experimentation Ethics Committee of the Faculty of Medicine Menoufia University. Fifty adult male Wister rats weighing 150–180 g were used. The animals were housed at 20–24°C with a 12 h light and 12 h dark cycle and they were provided with standard rat chow and tap water freely available**.** Rats were allowed to acclimatize for 10 days before the start of experiments.

Experimental design

Rats were randomly divided into five groups (10 rats each):

1. Control group: Rats received a single intraperitoneal (i.p.) injection of 1 ml normal saline for each rat
2. DOX group: DOX-induced testicular and hepatic toxicity was achieved by a single intraperitoneal injection of each rat by DOX in a dose of 15 mg/kg [22], [23]. DOX, a product of HIKMA specialized pharmaceuticals Badr city, Cairo, Egypt, was available in the form of a vial with the trade name “Adricin.” Each vial contains DOX HCL 50 mg/25 ml
3. DOX/ginseng treated (DOX/Gin) group: Rats received the combination of a single dose of DOX (15 mg/kg, i.p.) and ginseng a product of EIPICO, 10th of Ramadan city, Sharkia, Egypt (under license of Pharmaton SA, Lugano, Switzerland), was available in the form of capsules with the trade name “Ginsana.” Each capsule contained 100 mg of ginseng. The contents of the capsule were withdrawn by a syringe and dissolved in distilled water and given to the rats in a dose of 400 mg/kg once daily for 4 weeks by oral gavage [24]
4. DOX/CoQ10 treated (DOX/CoQ10) group: Rats received the combination of a single dose of DOX (15 mg/kg, i.p.) and CoQ10 (MEPACO, Egypt). CoQ10 was dissolved in distilled water and given orally by gavage in a dose of (10 mg/kg/day) for 4 weeks [22], [25]
5. DOX/ginseng+CoQ10treated(DOX/Gin+CoQ10) group: Rats received the combination of a single dose of DOX (15 mg/kg, i.p.) and both of ginseng (400 mg/kg/day) and CoQ10 (10 mg/kg/day) for 4 weeks.

All treatments were started on the next day of DOX injection. At the end of the study, all rats were weighed and blood samples were withdrawn for subsequent biochemical analysis. Thereafter, all rats were sacrificed, and liver and testis were quickly dissected after being weighed.

The organ indices were calculated by the following formula:

$$\text{Organ index} = \frac{\text{Organ weight}}{\text{Body weight}} \times 100$$ [26].

The right lobe of the liver and part of the right testis were stored at –80°C for real-time PCR analysis of the nrf2 gene. The remaining part of the right testis and lobe of the liver was homogenized for biochemical analysis while the left testis and remaining part of the liver were prepared for histological and immunohistochemical analyses.

Blood sampling and biochemical analysis

At the end of the study period, animals have fasted overnight, and then, retro-orbital blood samples were collected, allowed to coagulate for 30 min at room temperature, and then centrifuged at 2000 rpm for 15 min. Serum was collected and frozen at –80°C until analyzed.

Serum interleukin 10 (IL-10), serum interleukin 6 (IL-6), serum TNF-α, and serum testosterone levels were measured using the corresponding rat enzyme-linked immunosorbent assay kits (IL-10: ERI3010-1, Assaypro LLC, Saint Charles, Missouri, USA), (TNF−α: ERT2010-1, Assaypro LLC, Saint Charles, Missouri, USA), (IL-6: ab100772, Abcam, Cambridge, UK**),** (Testosterone: ab108666, Abcam, Cambridge, UK**)** according to the manufacturer's instructions. Tissue malondialdehyde (MDA), tissue SOD, serum liver enzymes (alanine transaminase [ALT], aspartate transaminase [AST], alkaline phosphatase [ALP], gamma-glutamyl transferase [GGT]), fasting serum cholesterol, and fasting serum triglyceride (TG) were determined using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt).

Tissue homogenate preparation

Specimens from the liver and testis were weighted and homogenized separately with a tissue homogenizer (MPW120, MPW Medical Instruments, China). For estimation of tissue MDA level, tissues were homogenized in phosphate buffer saline 50 mM pH 7.4. For the estimation of SOD, tissues were homogenized in potassium phosphate buffer 10 mM pH 7.4. The crude tissue homogenate was centrifuged at 10,000 rpm, for 15 min in an ice-cold centrifuge, and the resultant supernatant was collected and stored at −80°C for assay.

Quantitative assay of gene expression using reverse transcriptase polymerase chain reaction technique (RT-PCR)

Relative mRNA levels of the Nrf2 gene in the liver and testis were analyzed by RT-PCR. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted RNA was stored at –80°C till the time of use. The first step, PCR was complementary DNA (cDNA) synthesis (reverse transcription step) using the ThermoScript™ RT reagent kits (Invitrogen). Then, cDNAs were amplified by PCR assays with SYBR Green Mix kits (Stratagene, USA). A cycle threshold (Ct) value was obtained from each amplification curve. Glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene. Data analysis was done using 7500 ABI PRISM (Applied Biosystems, USA) v.2.0.1. The relative quantification of Nrf2 gene expression was calculated using the comparative ΔΔCt method [27]. The primers employed for the Nrf2 gene were as follows:

- Forward primer: 5'-AGCAAGACTTGGGCCACTT-3'
- Reverse primer: 5'-GATGGAGGTTTCTGTCGTTTTC-3'

Histopathological analysis

Fresh specimens of the right lobe of the liver and the left testis were collected and fixed immediately in 10% neutral buffered formalin. Paraffin sections (5 μm in thickness) were prepared and stained with H&E to verify histological details [28].

The primary monoclonal antibody was the mouse monoclonal primary antibody to caspase-3 (Ab-7, Mouse Mab. MS.). The cellular reaction appeared as brownish cytoplasm of the cells.

Immunostaining for caspase-3 was performed according to the manufacturer's instructions. Briefly, 5 μm sections of liver and testis were deparaffinized, rehydrated, rinsed in tap water, and embedded in 3% H2O2 for 10 min to block endogenous peroxidase. Sections were immersed in an antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6) and subjected to heat-induced antigen retrieval for 20 min in a microwave. Non-specific protein binding was blocked by a blocking solution (phosphate-buffered saline [PBS] and 10% normal goat serum). The slides were incubated with the diluted primary antibody (caspase-3, Abcam, working dilution 1:500). Sections were incubated with secondary biotinylated antibodies (goat anti-mouse IgG; Sigma Aldrich, St. Louis, USA) for 20 min. The streptavidin-peroxidase complex was then applied to sections for 10 min. The secondary antibody binding was visualized by incubating sections with 3, 3-diaminobenzidinetetrahydrochloride (DAB; Sigma Aldrich, St. Louis, USA). Finally, slices were rinsed with PBS. Finally, the sections were counterstained with hematoxylin (H), dehydrated, and coverslipped.

Statistical analysis

The data were tabulated and analyzed by Statistical Package for the Social Sciences (SPSS) software using statistical package version 16 (SPSS, Inc., USA). Quantitative data were expressed as mean ± standard deviation (X±SD). The significance of differences between groups was determined by one-way analysis of variance followed by a *post hoc* Tukey test. $p < 0.05$ was considered statistically significant ($p < 0.05$).

Results

Serum testosterone

The mean value of serum testosterone was significantly lower in the DOX group when compared to the control group (0.57 ± 0.1 vs. 4.63 ± 0.38 ng/ mL, p < 0.001). Serum testosterone levels were significantly higher in DOX/Gin (1.53 ± 0.32 ng/ mL) DOX/CoQ10 (1.71 ± 0.15 ng/mL), and DOX/ Gin+CoQ10 (3.15 ± 0.26 ng/mL) groups when compared to the DOX group (p < 0.001) and significantly lower when compared to the control group (p < 0.001). Serum testosterone level of DOX/Gin+CoQ10 was significantly higher when compared to DOX/Gin and DOX/CoQ10 (p < 0.001). There was no statistically significant difference in the serum testosterone level of DOX/Gin and DOX/CoQ10 groups (p > 0.05) (Figure 1).

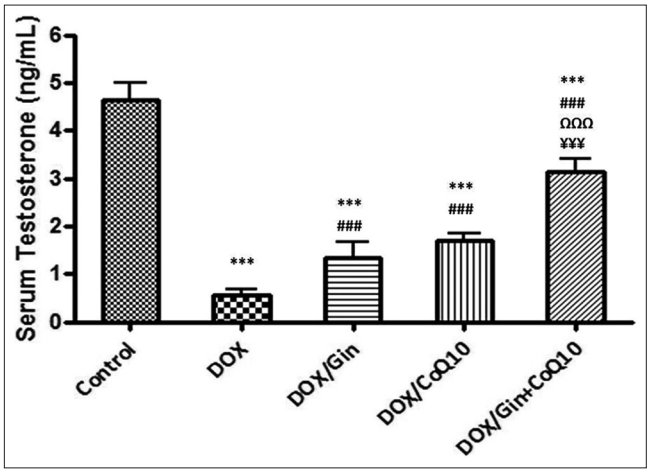

*Figure 1: Serum testosterone (ng/mL) in all studied groups (***: p < 0.001 vs. control group, ###: p < 0.001 vs. DOX group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

Serum liver enzymes

Regarding serum liver enzymes results, DOX group showed significantly higher ALT (168.93 ± 11.26 vs. 38.85 ± 4.11 U/L), AST (204.8 ± 10.24 vs. 53.5 ± 4.39 U/L), ALP (301.53 ± 7.51 vs. 108.87 ± 10.78 U/L), and GGT (17.35 ± 2.13 vs. 5.03 ± 0.65 U/L) levels when compared to the control group (p < 0.001). Serum ALT, AST, ALP, and GGT were significantly lower in DOX/ Gin (118.5 ± 8.11, 144.27 ± 4.88, 247 ± 7.66, and 12.8 ± 1.26 U/L, respectively), DOX/CoQ10 (120.6 ± 13.18, 140.03 ± 8.21, 242.67 ± 10.39, and 12.3 ± 1.56 U/L, respectively), and DOX/ Gin+CoQ10 (83.68 ± 7.2, 103.75 ± 5.72, 200.37 ± 10.72, and 8.37 ± 0.497 U/L, respectively) groups when compared to the DOX group (p < 0.001) and significantly higher than that of the control group (p < 0.001). There were significantly lower values of DOX/Gin+CoQ10 when compared to DOX/Gin and DOX/CoQ10 (p < 0.001). However, there was insignificant difference in serum liver enzymes of DOX/ Gin and DOX/CoQ10 groups (p > 0.05 (Figure 2).

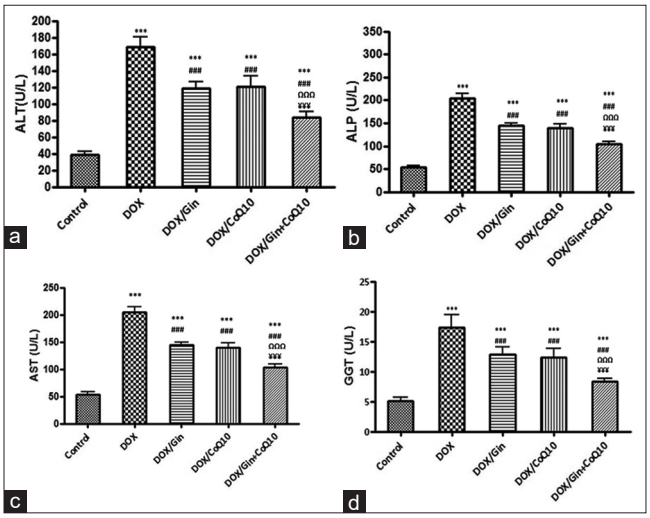

*Figure 2: (a) Serum alanine transaminase (ALT) (U/L). (b) Serum alkaline phosphatase (ALP) (U/L). (c) Serum aspartate transaminase (AST) (U/L). (d) Serum gamma-glutamyl transferase (GGT) (U/L), in all studied groups (***: p < 0.001 vs. control group, ###: p < 0.001 vs. DOX group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/ CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

Testis and liver indices and serum lipids

The mean values of testis and liver index were significantly lower in the DOX group when compared to the control group (0.44 ± 0.03 vs. 0.75 ± 0.04, 2.01 ± 0.13 vs. 2.91 ± 0.16, respectively, p < 0.001). Testis and liver index values were significantly higher in DOX/Gin (0.56 ± 0.03, p < 0.001 and 2.27 ± 0.11, p < 0.01, respectively), DOX/CoQ10 (0.58 ± 0.01, p < 0.001 and 2.25 ± 0.11, p < 0.05, respectively), and DOX/Gin+CoQ10 groups (0.63 ± 0.04 and 2.62 ± 0.06 [p < 0.001], respectively) when compared to the DOX group and significantly lower when compared to the control group (p < 0.001). Testis index value of DOX/Gin+CoQ10 was significantly higher when compared to DOX/Gin (p < 0.05) and insignificant difference with DOX/CoQ10 (p > 0.05). The liver index value of DOX/Gin+CoQ10 was significantly higher when compared to DOX/Gin and DOX/CoQ10 (p < 0.001). There was no statistically significant difference in testis and liver index values of DOX/Gin and DOX/CoQ10 groups (p > 0.05) (Figure 3a).

Regarding lipid profile results, DOX group showed significantly higher serum cholesterol (158.67 ± 7.31 vs. 90.16 ± 6.11 mg/dl) and TG (115.15 ± 5.67 vs. 40.92±4.78 mg/dl) levels when compared to control group (p < 0.001). Serum cholesterol and TG were significantly lower in DOX/Gin

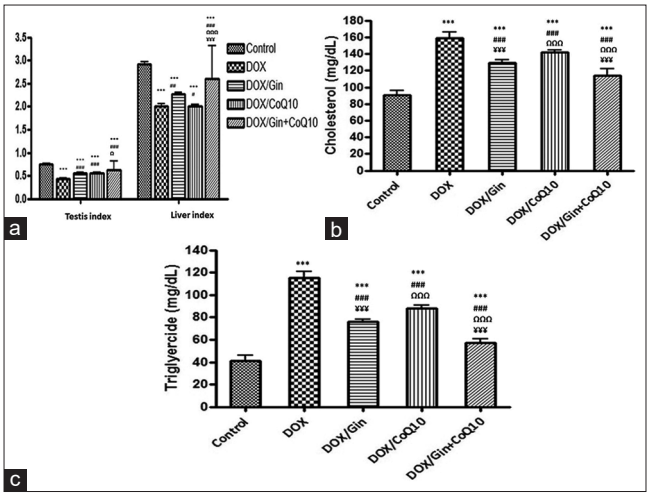

*Figure 3: (a) Testis and liver indices. (b) Serum cholesterol (mg/dl). (c) Serum triglyceride (mg/dl), in all studied groups (***: p < 0.001 vs. control group, #: p < 0.05 vs. DOX group, ##: p < 0.01 vs. DOX group, ###: p < 0.001 vs. DOX group, Ω: p < 0.05 vs. DOX/Gin group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/ CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

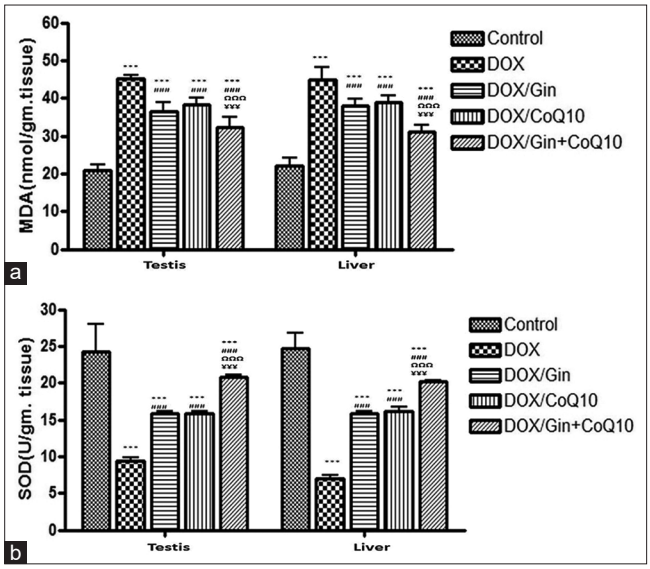

*Figure 4: (a) Testicular and hepatic malondialdehyde (nmol/ gm.tissue). (b) Testicular and hepatic superoxide dismutase (U/ gm.tissue), in all studied groups (***: p < 0.001 vs. control group, ###: p < 0.001 vs. DOX group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/ CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

(129.67 ± 3.07 and 75.85 ± 2.08 mg/dl, respectively), DOX/CoQ10 (141.98 ± 2.54 and 88.3 ± 2.47 mg/dl, respectively), and DOX/Gin+CoQ10 (114.17 ± 7.78 and 57.5 ± 3.08 mg/dl, respectively) groups when compared to the DOX group ($p < 0.001$) and significantly higher when compared to the control group ($p < 0.001$). There were significantly lower values of DOX/Gin+CoQ10 when compared to DOX/Gin and DOX/CoQ10 ($p < 0.05$). There were significantly lower values of DOX/Gin group when compared with DOX/CoQ10 group ($p < 0.001$) (Figure 3b and c).

Testicular and hepatic MDA and SOD

The mean values of testicular and hepatic MDA were significantly higher in the DOX group when compared to the control group (45.31 ± 0.93 vs. 20.83 ± 1.65 and 44.98 ± 3.19 vs. 22.1 ± 2.05 nmol/gm.tissue, respectively, $p < 0.001$). However, testicular and hepatic MDA mean values were significantly lower in DOX/Gin (36.6 ± 2.22 and 37.93 ± 1.98 nmol/gm.tissue, respectively), DOX/CoQ10 (38.33 ± 1.83 and 38.99 ± 1.74 nmol/gm.tissue, respectively), and DOX/Gin+CoQ10 groups (32.41 ± 2.47 and 31.02 ± 1.81 nmol/gm.tissue, respectively) when compared to the DOX group ($p < 0.001$) and significantly higher when compared to the control group ($p < 0.001$). Testicular and hepatic MDA mean values of DOX/Gin+CoQ10 were significantly lower when compared to DOX/Gin and DOX/CoQ10 ($p < 0.001$). There was no statistically significant difference in testicular and hepatic MDA levels of DOX/Gin and DOX/CoQ10 groups ($p > 0.05$) (Figure 4a).

The mean values of testicular and hepatic SOD were significantly lower in the DOX group when compared to the control group (9.36 ± 1.59 vs. 24.25 ± 3.81 and 6.99 ± 1.46 vs. 24.7 ± 2.14 U/gm tissue, respectively, $p < 0.001$). Testicular and hepatic SOD levels were significantly higher in DOX/Gin (15.82 ± 0.99 and 15.83 ± 0.95 respectively), DOX/CoQ10 (15.82 ± 0.92 and 16.21 ± 1.69, respectively), and DOX/Gin+CoQ10 groups (20.75 ± 1.22 and 20.17 ± 0.74 U/gm tissue, respectively) when compared to the DOX group ($p < 0.001$) and significantly lower when compared to the control group ($p < 0.001$). Testicular and hepatic SOD levels of DOX/Gin+CoQ10 were significantly higher than those of DOX/Gin and DOX/CoQ10 ($p < 0.001$). There was no statistically significant difference in testicular and hepatic SOD mean values of DOX/Gin and DOX/CoQ10 groups ($p > 0.05$) (Figure 4b).

Serum TNF-α, IL-6, and IL-10

The mean values of serum TNF-α and IL-6 levels were significantly higher in the DOX group when compared to the control group (56.58 ± 3.11 vs. 15.92 ± 3.12 ng/mL and 141.67 ± 3.98 vs. 74.58 ± 4.57 Pg/mL, respectively, $p < 0.001$). Serum TNF-α and IL-6 levels were significantly lower in DOX/Gin (43.08±3.35ng/mL and 111.98±4.83Pg/mL, respectively), DOX/CoQ10 (43 ± 3.35 ng/mL and 110.85 ± 8.28 Pg/mL, respectively), and DOX/Gin+CoQ10 groups (33.83 ± 4.12 ng/mL and 94.33 ± 6.89 Pg/mL, respectively) when compared to the DOX group ($p < 0.001$) and significantly higher when compared to the control group ($p < 0.001$). Serum TNF-α and IL-6 levels of DOX/Gin+CoQ10 were significantly

lower when compared to DOX/Gin and DOX/CoQ10 ($p < 0.001$). There was no statistically significant difference in serum TNF-α and IL-6 levels of DOX/Gin and DOX/CoQ10 groups ($p > 0.05$). However, the mean value of serum IL-10 was significantly lower in the DOX group when compared to the control group (5.42 ± 0.95 vs. 17.67 ± 1.78 ng/mL, $p < 0.001$). Serum IL-10 was significantly higher in DOX/Gin (8.78 ± 0.73), DOX/CoQ10 (8.67 ± 0.87 ng mL), and DOX/ Gin+CoQ10 (12.8 ± 0.96 ng/mL) groups when compared to the DOX group ($p < 0.001$) and significantly lower when compared to the control group ($p < 0.001$). Serum IL-10 level of DOX/Gin+CoQ10 was significantly higher when compared to DOX/Gin and DOX/CoQ10 ($p < 0.001$). There was no statistically significant difference in the mean values of serum IL-10 level of DOX/Gin and DOX/CoQ10 groups ($p > 0.05$) (Figure 5a-c).

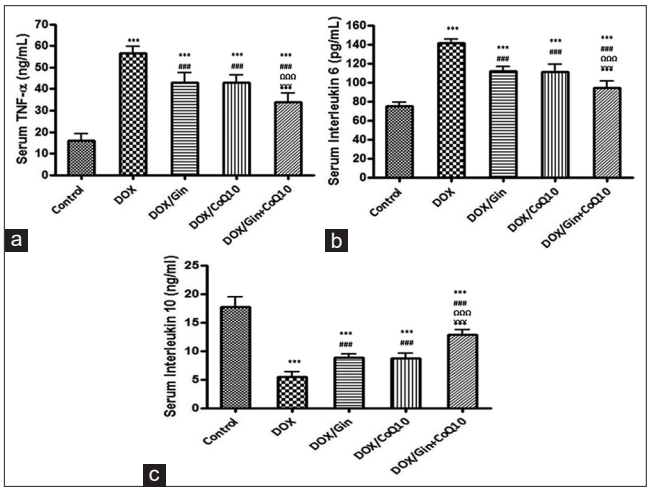

*Figure 5: (a) Serum tumor necrosis factor-alpha (ng/mL). (b) Serum interleukin-6 (pg/mL). (c) Serum interleukin 10 (ng/mL), in all studied groups (***: p < 0.001 vs. control, ###: p < 0.001 vs. DOX group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/ CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

Testicular and hepatic Nrf2 gene expression

Expression of testicular and hepatic Nrf2 gene in DOX group was significantly downregulated when compared to the control group (0.107±0.013 vs. 1±0, 0.205±0.019 vs. 1±0, respectively, $p < 0.001$). However, expression of testicular and hepatic Nrf2 genes in DOX/Gin (0.49±0.035 and 0.598±0.011, respectively), DOX/CoQ10 (0.413 ± 0.055 and 0.515 ± 0.086, respectively), and DOX/Gin+CoQ10 (0.732 ± 0.046 and 0.878 ± 0.044, respectively) groups was significantly upregulated when compared to DOX group ($p < 0.001$) but significantly lower than control group. Expression of testicular and hepatic Nrf2 gene in DOX/Gin+CoQ10 group was significantly upregulated when compared to DOX/Gin and DOX/CoQ10 ($p < 0.05$). Expression of testicular and hepatic Nrf2 gene in DOX/Gin was significantly upregulated when compared to DOX/CoQ10 ($p < 0.05$) (Figure 6).

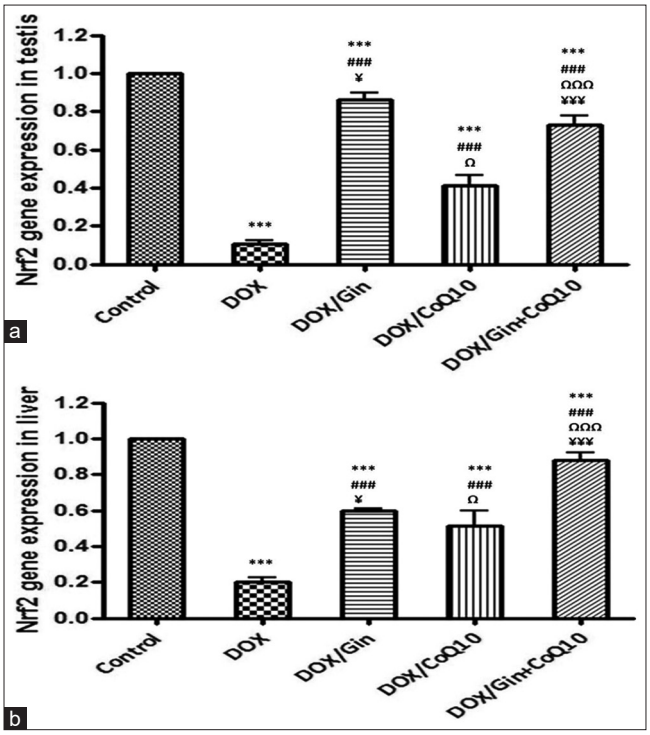

*Figure 6: (a) Nuclear factor E2-related factor 2 gene expression in testis. (b) Nuclear factor E2-related factor 2 gene expression in liver, in all studied groups (***: p < 0.001 vs. control group, ###: p < 0.001 vs. DOX group, Ω: p < 0.05 vs. DOX/Gin group, Ω Ω Ω: p < 0.001 vs. DOX/Gin group, ¥: p < 0.05 vs. DOX/CoQ10 group, ¥¥¥: p < 0.001 vs. DOX/CoQ10 group). DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated. Data are shown as means ± SD (n = 10). ANOVA was used to make group comparisons*

Histopathological results

Histological examination of testis tissue of the control group stained with H&E showed rounded to oval seminiferous tubules, separated by a minimal amount of interstitial tissue containing Leydig cells, and lined by multiple layers of germinal epithelium. This germinal epithelium revealed pyramidal Sertoli cells and oval spermatogonia with darkly stained nuclei close to the basement membrane. Primary spermatocytes were the largest one seen within the seminiferous tubules. Spermatids were small cells arranged in 2–4 rows lying close to the lumen. Moreover, eosinophilic threads representing tails of the sperms were observed in the lumen of most of the tubules. Spindle-shaped myoid cells resting on the outer surface of the basement membrane were also detected (Figure 7).

Seminiferous tubules of DOX group showed loss of architecture, significant disorganization in the basal membrane and few disorganized germ cell layers associated with vacuolization, and degeneration of the

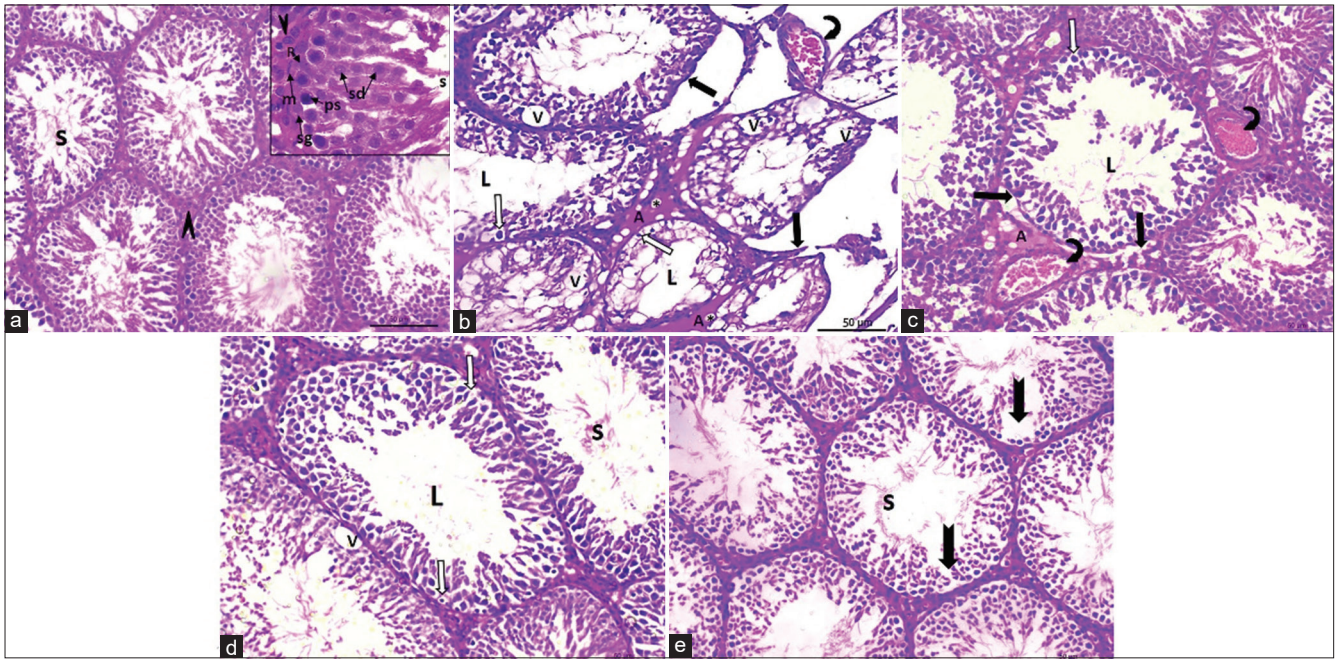

Figure 7: Representative micrographs of H&E testicular section of all experimental groups showing (a) seminiferous tubules of control group containing different stages of spermatogenic cells; spermatogonia (sg), primary spermatocytes (ps), spermatids (sd), and the Sertoli cells (R) in between. Numerous mature spermatozoa (S) appear in the lumen. The myoid cells (m) are arranged outside the basal lamina. A rounded Leydig cells with an acidophilic cytoplasm (head arrow) are scattered between the seminiferous tubules H&E, ×20 and ×1000. Seminiferous tubules of DOX group (b) showed distortion of the seminiferous tubules with few germ cells some are disorganized with deeply stained pyknotic nuclei (white arrow), others showed cytoplasmic vacuolation (V). The germ cells detached from basement membrane (black arrow). The lumen of tubules contains few spermatozoa (L). Wide intertubular space appeared containing homogenous acidophilic material (A), many vacuoles (star) and congested dilated blood vessels (curved arrow). Administration of Gin (c) revealed apparent a smaller number of pyknotic germ cells (white arrow), mild detachment from basement membrane (black arrow). The lumen of tubules contains moderate number of spermatozoa (L). Intertubular space appeared containing homogenous acidophilic material (A), less vacuoles (star) and congested dilated blood vessels (curved arrow). Administration of CoQ10 (d) resulted in amelioration of the architecture with less vacuolated germ cells (V), apparent a smaller number of pyknotic germ cells (white arrow) and few sperms (S) in the lumen (L). Combination of Gin and CoQ10 (e) induced significant improvement in the histopathological picture with loss of germ cells from some seminiferous tubules (notched arrow) (H&E, ×20, 50 μm scale)

lining epithelial cells. The lumen of tubules revealed few spermatozoa. The intertubular space appeared wide, contained homogenous acidophilic material, and congested dilated blood vessels. These changes were ameliorated in the groups treated with Gin or CoQ10. Moreover, the combination of Gin- and CoQ10-induced significant improvement in the histopathological results compared to the use of each of these drugs alone (Figure 7).

The control hepatic tissue showed normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm, and few spaced hepatic sinusoids arranged in-between the hepatic cords. The sinusoids were lined by flat endothelial cells. DOX group hepatic sections showed dissolution of hepatic cords, which appeared as empty vacuoles with shrunken, irregular, and darkly stained nuclei. Furthermore, there was a higher tendency for liver fibrosis manifested by the presence of many spots of focal cellular aggregations (Figure 8).

The DOX/Gin treated group showed mild cloudy swelling and inflammation. Hepatic lesions were improved in animals, which received Dox and CoQ10 treatment; whereas the hepatic tissues in the DOX/Gin+CoQ10 treated group were apparently normal (Figure 8).

Immunohistochemical and morphometric results

Administration of DOX was associated with a significant increase in area % of the caspases-3 immunohistochemical staining (59.6 ± 8.53 vs. 0.80 ± 0.86, $p < 0.001$) compared to the control group. The treatment with Gin, CoQ10, or Gin+CoQ10 showed significantly lower caspases-3 area % of immunohistochemical staining (34.00 ± 7.62, 24.00 ± 3.67, and 13.40 ± 3.85, respectively, $p < 0.001$) compared with the DOX group. Moreover, the Gin+CoQ10 combination induced a significant reduction in the caspase-3 immunoreactivity compared to the use of each of these drugs alone (Figure 9).

The liver sections of the Dox group showed a significant increase in area % of positive immune reaction for caspase-3-positive cells as compared to the control group (69.00 ± 5.43 vs. 0.60 ± 0.55, $p < 0.001$). DOX/Gin, DOX/CoQ10 treated as well as DOX/Gin+CoQ10 treated groups showed a significant decrease in area % of positive immune reaction for caspase-3-positive cells as compared to the DOX group (33.80 ± 8.93, 21.80 ± 4.32, and 14.80 ± 3.70, $p < 0.001$, respectively) but Gin+CoQ10 combination induced a significant reduction in the caspase-3

immunoreactivity compared to the use of each of these drugs alone (Figure 9).

Discussion

Although DOX is a successful cancer chemotherapeutic, side effects limit the clinical utility of DOX-based therapy, including male infertility [4] and hepatotoxicity [3]. The results of the DOX group revealed a significant decrease in serum testosterone and testis index value when compared with the control group. These results were in accordance with previous reported results [4], [29]. The antineoplastic agents can disturb Leydig cells directly [30]. Thus, the reduction in circulating testosterone is supposed to be resulting from a direct poisonous effect of DOX on the Leydig cells. A marked decrease in testis index value by DOX can be result of reduced number of germ cells, atrophy of Leydig cells, and lower rate of spermatogenesis, as

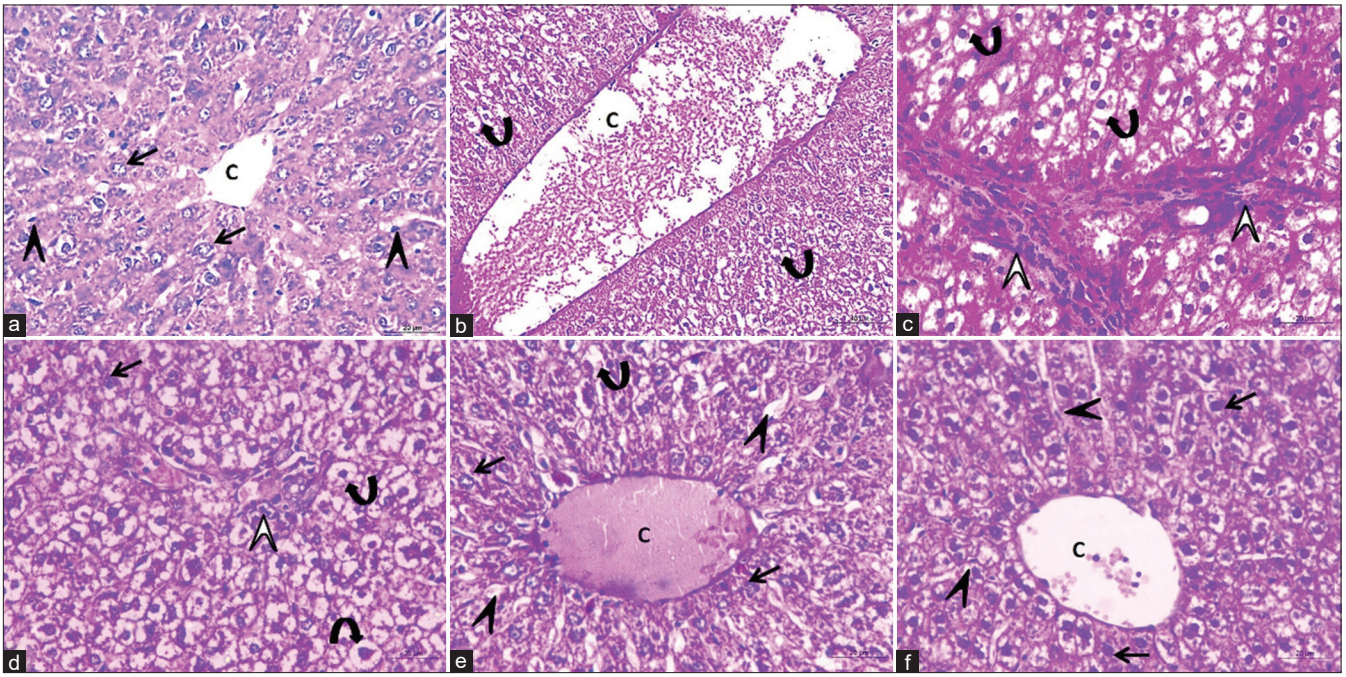

Figure 8: Representative micrographs of H&E liver sections of different experimental groups showing normal hepatic lobular architecture showing the central vein (C) at the center of classic hepatic lobule and cords of hepatocytes with large rounded vesicular nuclei (arrows) radiating from it and separated by the blood sinusoids (arrowheads). The sinusoids were lined by flat endothelial cells. DOX group (b and c), dilated congested central vein (C) could be detected. The hepatic sections showing dissolution of hepatic cords, which appeared as empty vacuoles with shrunken, irregular, and darkly stained nuclei (curved arrows). Many spots of focal cellular aggregations (white arrowheads) could be detected. The DOX/Gin treated group (D) showing hepatocytes appeared as empty vacuoles with shrunken, irregular, and darkly stained nuclei (curved arrows), spots of focal cellular aggregations (white arrowheads), and normal hepatocytes (arrow). Animals, which received Dox and CoQ10 treatment showing normal hepatocytes (arrows) and blood sinusoids (black arrowheads), degenerated hepatocytes (curved arrow) and congested dilated central vein (C); whereas the hepatic tissues in the DOX/Gin+CoQ10 treated group were apparently as control group except for dilated central vein (C). (a, c, d, e and f; Scale bar 20 µm, B; scale bar 40 µm)

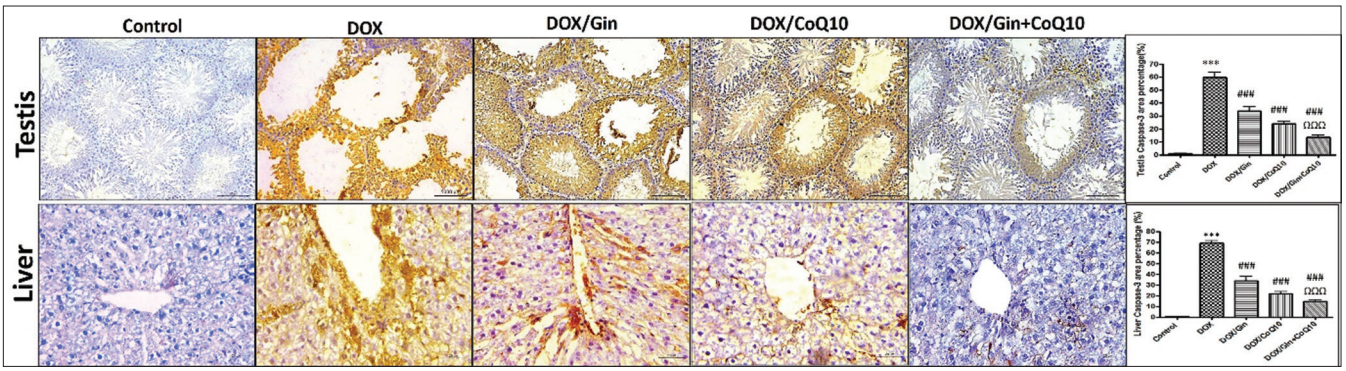

*Figure 9: Representative caspase-3 staining of rat testis and liver of different groups: DOX administration upregulated expression of caspase-3 immunoexpressing in both testis and liver tissues, ***p < 0.001 versus control group. This increase was significantly decreased in DOX/Gin, DOX/CoQ10, and DOX/Gin+CoQ10 groups; ## p<0.001 versus DOX group. Scale bar 20 µM, ×400. DOX: Doxorubicin, DOX/Gin: Doxorubicin/ginseng treated, DOX/CoQ10: Doxorubicin/CoQ10 treated, DOX/Gin+CoQ10: Doxorubicin/ginseng+CoQ10 treated*

confirmed by our histological findings which showed that the seminiferous tubules of DOX group showed loss of architecture, significant disorganization in the basal membrane, and few disorganized germ cell layers associated with vacuolization, and degeneration of the lining epithelial cells. This is in agreement with previous reported studies [26].

The leakage of hepatocellular enzymes is used as a hepatotoxicity marker. The results of the DOX group revealed a significant increase in serum liver enzymes (ALT, AST, ALP, and GGT) and a decrease in liver index value when compared with the control group, following our results previous reported studies [3], [31]. The DOX prompted hepatotoxicity can be attributed to free radical-induced oxidative stress mechanism during hepatic drug metabolism [32]. This evokes ROS initiated lipid peroxidation favoring hepatocyte damage and creating ALT and AST spillage into the serum. This was confirmed by our histological results of liver, in agreement with previous results [31].

However, rats treated with ginseng revealed a significant increase in serum testosterone and testis index value when compared with the DOX group, and this is in accordance with previous results [33]. Ginseng has potent effects on sexual function and could relieve senile testicular dysfunction [34]. In addition, ginseng rescued testicular impairment in aged rats through regulation of the oxidative defense systems [35]. Ginseng improved spermatogenesis in experimental models of testicular dysfunction [36]. Furthermore, our histological results revealed amelioration in changes induced by DOX in rats treated by ginseng.

Treatment with ginseng revealed, also, a significant decrease in serum liver enzymes and increase in liver index value when compared with the DOX group in accordance with our results previous reported study [24]. Similar previous studies demonstrated that ginseng extract had antioxidant activity and acted as a free radical scavenger [37], [38]. It has been reported that ginsenosides are responsible for its hepatoprotective effect by destroying lipid peroxyl radicals and ROS [37] and this was confirmed by our histological results.

Treatment of rats with CoQ10 revealed a significant increase in serum testosterone and testis index value when compared with the DOX group. Oda *et al*. stated that supplementation of CoQ10 to oxytetracycline-treated rats showed elevated serum levels of testosterone and restored relative testis weight [25]. Furthermore, CoQ10 as an antioxidant suppressed lipid peroxidation in testis. Similarly, oral CoQ10 induced an increase in SOD activity in infertile men [39].

Treatment with CoQ10 revealed also, significant decrease in serum liver enzymes and increase in liver index value when compared with the DOX group. These results are in accordance with previous reported results [40], and this was confirmed by our histological results.

The results of the Dox group revealed significant elevation of serum cholesterol and TG when compared with the control group. These results have been in accordance with previous reported results [41]. However, rats treated with ginseng revealed a significant decrease in serum cholesterol and TG levels when compared with DOX group. Hafez *et al*. revealed that the increased levels of TGs and total cholesterol were restored to their normal values with carbon tetrachloride (CCl4) + ginseng [24]. Ginseng products are responsible for its hypolipidemic effect [42]. Treatment with CoQ10 revealed a significant decrease in serum cholesterol and TG when compared with the DOX group and a significant increase when compared with DOX/Gin group. The improvement in the hypolipidemic effect of atorvastatin by CoQ10 has already been reported in Guinea pigs [43].

Oxidative stress plays an essential role in DOX-induced toxicity through the formation of ROS [7]. The results of the Dox group revealed significant elevation of testicular and hepatic MDA levels and a significant decrease in testicular and hepatic SOD when compared with the control group. These results are in accordance with previous reported studies [29], [31]. It has been demonstrated that oxygen radical-induced damage of lipids in the membrane is the key factor for DOX-induced toxicity [44]. The adverse effects of DOX result mainly from its essential tendency to produce free radicals and block antioxidant enzymes in various tissues [45]. However, rats treated with ginseng revealed a significant decrease in testicular and hepatic MDA levels and a significant increase in testicular and hepatic SOD when compared with the DOX group. Ginseng has potent antioxidant effects and increases the protein expression level of antioxidants in rats [46]. Ginseng prevented severe testicular toxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats through its antioxidant activity [47]. It has been reported that ginsenosides are responsible for its hepatoprotective effect by destroying lipid peroxyl radicals and ROS [48].

Treatment with CoQ10 revealed a significant decrease in testicular and hepatic MDA levels and a significant increase in testicular and hepatic SOD when compared with the DOX group. These results are accordance with the previous study [49]. Saleh *et al*. concluded that the supplementation of CoQ10 significantly ameliorated liver function and hepatic antioxidant defense capacity and attenuated ROS levels [49]. Studies have shown that the CoQ10 has free radical scavenging activities and liver injury reducing properties [50]. CoQ10 is a powerful antioxidant. CoQ10 inhibits the generation of ROS [51] and scavenges lipid peroxidation products during free radical reactions [52]. There was no significant difference between DOX/Gin and DOX/CoQ10 groups.

The results of the Dox group revealed significant elevation of pro-inflammatory markers serum TNF-α and IL-6 levels and a significant decrease of

anti-inflammatory marker serum IL-10 when compared with the control group. These results are in accordance with previous reported studies [53]. DOX exposure activates the expression of NF-κB and nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome which, in turn, releases pro-inflammatory mediator including TNF-α [11]. However, treatment with ginseng revealed a significant decrease in serum TNF-α and interleukin-6 levels and an increase of serum IL-10 levels when compared with the DOX group. Hafez *et al*. revealed that ginseng extract increased the expression of IL-10, in CCl4-induced liver injury [24].

Treatment with CoQ10 revealed a significant decrease in serum TNF-α and IL-6 levels and a significant increase of serum IL-10 levels of DOX/CoQ10 when compared with the DOX group. Following our results, Saleh *et al*. reported that supplementation of CoQ10 significantly ameliorated liver function and the production of pro-inflammatory cytokines [49]. CoQ10 exhibits anti-inflammatory properties by reducing the release of pro-inflammatory cytokines during inflammatory injury [54], [55]. There was no statistically significant difference in serum TNF-α and IL-6 levels of DOX/Gin and DOX/CoQ10 groups.

In our results, the DOX/Gin+CoQ10 group showed significant improvement in all measured biochemical parameters and testis and liver indices when compared with DOX, DOX/Gin, and DOX/CoQ10 groups. This attributed to the potent effects of both ginseng and CoQ10 through synergistic properties.

The real-time PCR results for the Nrf2 gene demonstrated a significant downregulation of the expression of testicular and hepatic Nrf2 in DOX group when compared with the control group. These results have been in accordance with previous reported studies [31]. DOX hepatic damage may likewise lay on lessened hepatic Nrf2 protein expression and elevated MDA level favoring oxidative stress with hepatocyte apoptosis. These mechanisms were initially proposed to be part of the downregulation of hepatic Nrf2 expression. First, reduced glutathione can react with cysteine residues in proteins to form disulfides and this chemical process is known as S-glutathionylation [56]. Curiously, S-glutathionylation can modulate Nrf2 gene expression [57]. Nrf2 has been expressed in the control group which can be attributed to the fact that even in normal cells, ROS are produced but in a controlled fashion to help in different physiological processes within the cell [58].

Treatment of the DOX group by ginseng revealed significant upregulation of testicular and hepatic Nrf2 gene activity when compared with the DOX group. Ning *et al*. revealed that ginseng upregulated the protein expression of Nrf2 which was decreased in the CCl_4 group, suggesting that ginseng enhanced the antioxidant ability through activation of the Nrf2 signaling pathway [59].

Treatment of the DOX group by CoQ10 revealed significant upregulation of testicular and hepatic Nrf2 gene expression of DOX/CoQ10 when compared with the DOX group. However, it was significantly downregulated when compared to DOX/Gin group. Tarry-Adkins *et al*. revealed that CoQ_{10} may exert antifibrotic effects through the activation of the Nrf2/Nrf2/ARE pathway [60]. The antioxidant genes involved in the Nrf2/ARE pathway were increased by CoQ_{10} supplementation [61]. The combined group shows significant upregulation of Nrf2 gene expression when compared with other treated groups.

Our results revealed that administration of DOX was associated with a significant increase in the caspases-3 immunohistochemical staining of testis and liver compared to the control group. This finding was consistent with previous reported studies [62], [63]. The treatment with ginseng showed a significantly decrease of caspases-3 immunohistochemical staining of testis and liver compared with the DOX group. There results in accordance with previous reported results [63]. The treatment with CoQ10 also showed significantly lower values of caspases-3 immunohistochemical staining of testis and liver compared with the DOX group. The previous studies showed the therapeutic effects of CoQ10 on metabolic stress by inhibition of apoptosis in hepatocytes [64]. Gin+CoQ10 group showed significantly lower expression of caspases-3 immunohistochemical staining compared with the DOX group.

Conclusion

DOX has many toxic adverse effects in organs, such as liver and testis**.** The mechanisms behind DOX-induced cytotoxicity are complex and continue to be not fully understood. To the best of our knowledge, this is first study elucidating the potential effects of ginseng combined with CoQ_{10} on DOX-induced toxicity. Based on our biochemical, molecular, and immunohistochemical findings, we believe that ginseng and CoQ_{10} have valuable therapeutic effects on DOX-induced toxicity through upregulation of Nrf2 gene expression, inhibition of apoptosis, antioxidant, anti-inflammatory, and hypolipidemic effects.

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