Coenzyme Q10 Alleviates Cyclophosphamide Testicular Toxicity in Rats Via Activating PPAR-Γ and Nrf2 Signaling, and Down-Regulating NF-Kb And Bax/Bcl-2 Ratio

Rania M. Tawfik[1*], Mahmoud A. Nomier[1], Samah M. Ahmed[2] and Omaima I. Abdel Hamid[1]

[1] Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

[2] Department of Medical Histology and Cell Biology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Corresponding author: Rania M. Tawfik, Associate lecturer of Forensic Medicine and Clinical Toxicology, Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

E-mail: nonamagdiano@gmail.com

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Abstract

Cancer and autoimmune diseases are both treated with the chemotherapy drug cyclophosphamide (CP). However, because of its harmful side effects, particularly testicular toxicity, its use is restricted. The aim of this experiment was to evaluate the effect of coenzyme q10 (CoQ10) on CP induced testicular toxicity in adult albino rats. Sixty adult male albino rats (180-200 g) were divided into 6 groups, 3 control groups and 3 experimental groups {CoQ10-treated group, CP-treated group and CP+CoQ10-treated group}. After 4 weeks of treatment, blood samples were collected for biochemical studies, while tissues were taken for oxidative stress markers, qRT-PCR, histopathological and immunohistochemical studies. Our outcomes clarified that CP provoked a highly significant decrease in reproductive hormones (Testosterone, FSH and LH), anti-oxidant enzymes (SOD, CAT, GSH) and conversely (MDA) levels were markedly elevated. It activated the inflammatory pathway manifested by elevated serum inflammatory cytokines (TNF-α, IL-1β) and up-regulated testicular NF-kB p65 gene. CP affected PPAR-γ and Nrf2 pathway by down-regulating their gene expression in testicular tissues. It also activated apoptotic pathway by up-regulation of Bax and down regulation of Bcl-2 leading to elevated apoptotic index (Bax/Bcl-2) ratio in testicular tissues. However, treatment with CoQ10 ameliorated reproductive hormones, increased anti-oxidant enzymes, up-regulated PPAR-γ and Nrf2 gene expression besides regulated inflammation and apoptosis. Histological improvement of testes also strengthened the defensive effects of CoQ10. These outcomes advocated CoQ10 as a potent natural remedy that reduces the damaging effects of CP on the testes.

Keywords: Cyclophosphamide, Coenzyme Q10, testes, PPAR-γ, Nrf2, Bax/Bcl-2.

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Introduction

Cyclophosphamide (CP) is an anticancer drug and immunosuppressive agent extensively applied for the treatment of both neoplastic and non-neoplastic diseases [1]. Regardless of CP efficacy, its use is often restricted by related dangerous lethal outcomes, including reproductive toxicity [2], hepatotoxicity [3], nephrotoxicity [3] and genotoxicity [4].

Because cells in the reproductive system divide quickly, CP administration can cause serious side effects [5]. Reproductive toxicity provoked by testicular weight loss, seminiferous tubules atrophy, low blood testosterone level, oligospermia and azoospermia [6].

The exact mechanism of CP toxicity resulting from acrolein metabolite as CP is converted into acrolein and phosphoramide mustard in the liver by hepatic microsomal cytochrome p-450 [7]. Acrolein metabolite triggers reactive oxygen (ROS) species generation [8]. In turn, ROS provoke lipid peroxidation, oxidative DNA damage and inflammation that trigger the apoptotic signaling pathways and therefore implicated in CP organ toxicity [9].

Enhancing the body's antioxidant defenses with natural antioxidants is crucial to reduce the harmful effects of CP and its reactive metabolites. Because of this, effective drugs are required to protect healthy organs from the damaging adverse effects of chemotherapy [3].

Coenzyme Q10 (CoQ10), also known as ubiquinone, is the only naturally occurring, lipid-soluble vitamin-like substance that is produced endogenously [10]. CoQ10 is abundant in meat, fish, nuts, broccoli, cauliflower, and certain oils so it can be taken exogenously with foods [11].

Coenzyme Q10 is a strong lipophilic antioxidant for reducing the level of free radicals in the body [12] as it is essential for avoiding lipid, protein, and DNA oxidation [13].

Coenzyme Q10 has anti-inflammatory and anti-apoptotic effects as many findings verified the preventive effect of CoQ10 in various forms of inflammatory and apoptotic tissue injury [14, 15].

It is used as a dietary supplement and as a co-therapy for a wide range of illnesses, such as diabetes, cancer, cardio vascular diseases, and neurological disorders [16]. Therefore, the goal of the current investigation was to evaluate coenzyme q10's protective outcomes against cyclophosphamide-induced testicular toxicity in adult albino rats.

Materials and methods

Chemicals

Cyclophosphamide (C7H15Cl2N2O2P) is available as pale yellow crystalline odorless powder with ≥ 97% HPLC and CAS number 50-18-0 and coenzyme q10 (C59H90O4) is available as a yellow to dark orange crystalline odorless powder with ≥ 98% HPLC and CAS number 303-98-0 obtained from Sigma Aldrich Pharmaceutical Co., Steinheim, Germany. Distilled water as a solvent agent for cyclophosphamide and corn oil as a solvent agent for coenzyme q10 (in form of oily solution) were obtained from El Gomhoria Pharmaceutical Co., Zagazig, Egypt.

Experimental Design

Ethical approval

The experiment design was permitted by the Institutional Animal Care and Use Committee (IACUC) of Zagazig University in Egypt (ZU-IACUC/3/F/3/202) and accompanied in agreement with ARRIVE guidelines.

Sixty adult male albino rats (12 ± 1 week) weighing 180–200 g were employed. The rat species were gained from the animal house of the faculty of medicine, Zagazig University. The rats were retained in isolated cages (2 rats per cage) and fed normal rat food under regular laboratory and ambient conditions. The animals were treated in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals [17].

This study design included six equal experimental groups, each containing ten rats. Group I was used as control (given regular diet and tap water). Group II: each rat was given distilled water (CP solvent) in a dose of 1ml once daily by oral gavage for 4 weeks. Group III: each rat was given corn oil (CoQ10 solvent) in a dose of 1 ml once daily by oral gavage for 4 weeks. Group IV: each rat was given CoQ10 in a dose of 10 mg/kg body weight in 1ml corn oil once daily by oral gavage for 4 weeks [18] (El-Sheikh et al., 2014). Group V: each rat was given CP in a dose of 8.2 mg/kg body weight {1/20 of LD50} in 1ml distilled water once daily by oral gavage for 4 weeks [19]. Group VI: each rat was given both CP and CoQ10 with the same doses for the same duration. Rats were given CoQ10 two hours before CP administration. The bodyweight was recorded before starting the experiment.

Blood and testicular tissue collection

Venous blood samples were taken from the retro-orbital plexus of animals while they were under intra-peritoneal injection of 50 mg/kg of thiopental anaesthesia. Blood samples were drawn into clean test tubes, and the sera were separated by centrifugation following the method clarified by van Herck et al. [20] for the biochemical examination of reproductive hormones {testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH)}, pro-inflammatory markers {tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β)}. After blood collection, all rats were scarified, testes were removed from each rat, and one portion of the tissue was immediately transported on dry ice, and stored at −80 °C to obtain homogenates for the analysis of oxidative stress markers {malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH)} and qRT-PCR gene expression {Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-ɣ), Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear Factor kappa-Beta (NF-kB p65)}. The remaining components were quickly preserved in 10 % formal saline for histopathological studies and immunohistochemical examination of B-cell lymphoma -2 (Bcl-2) and Bcl-2 associated x (Bax) proteins.

Reproductive hormones assay (Testosterone, FSH and LH)

Serum testosterone (ng/mL) was measured colorimetrically using a competitive ELISA kit obtained from Biodiagnostic Co., Egypt, according to the technique described by Demetrious [21]. Testosterone hormone in the sample competes with a testosterone horseradish peroxidase conjugate for binding to the coated antibodies. Serum FSH and LH (mU/L) were measured colorimetrically using two distinct sandwich-ELISA kits acquired from Biodiagnostic Co., Egypt, according to Beastall et al. [22] approach. Avidin coupled to horseradish peroxidase and biotinylated detection antibodies specific for various hormones are added.

Pro-inflammatory markers assay (TNF- α and IL-1β)

Serum TNF- α and IL-1β was determined using rat TNF- α ELISA Kit with Cat. No. (MBS2507393) and rat IL-1β ELISA Kit with Cat. No. (ab255730) respectively achieved from MyBiosource (San Diego, California, United States) based on Sandwich-ELISA principle. OD was calculated spectrophotometrically at a wavelength of 450 nm, where the values were correlated to the rat TNF-α and IL-1β concentration.

Oxidative stress markers assay (MDA, SOD, CAT and GSH)

Lipid peroxidation products MDA (umol/g protein) activity was assessed at a wave length of 534 nm using MDA colorimetric kits achieved from Biodiagnostic Co., Giza, Egypt with a Cat. No. (MD 2529), according to the method suggested by Ohkawa et al. [23] (1979). Superoxide dismutase (SOD) (U/mg protein) activity was assessed using SOD Kit achieved from Cus-abio Biotech Co., Ltd with a Cat No. (CSB-E08555r) following the method proposed by Nishikimiet al. [24] based on inhibition of nitroblue tetrazolium reduction. Catalase (CAT) (U/g protein) activity was evaluated according to Aebi [25] approach based on peroxide removal method. Reduced glutathione (GSH) (umol/mg protein) activity was estimated spectrophotometrically according to the method recommended by Mannervik [26] based on reduction of glutathione disulfide.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assessment of PPAR-γ, Nrf2, NF-kB p65 in testicular tissues

Total RNA was extracted from 50 mg of testicular tissue using Trizol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer´s recommendation, and for assessing the RNA quality, the A260/A280 ratio was analyzed using the NanoDropVR ND-1000 Spectrophotometer. Then, cDNA was created using the HiSenScriptTM RH (-) cDNA Synthesis Kit (iNtRON Biotechnology Co., Seoul, South Korea), after which the primers were made in accordance with the manufacturer's instructions (Sangon Biotech., Beijing, China) in table -1[27]. Utilizing TOPrealTM qPCR 2X PreMIX, real-time RT-PCR was carried out using the Mx3005P real-time PCR equipment (Agilent) by Stratagene (Santa Clara, CA, USA) according to

manufacturer's instructions [28]. Denaturation at 95 degrees Celsius for 12 minutes was followed by 40 cycles of denaturation for 20 seconds, annealing for 30 seconds at 60 degrees Celsius, and extension for 30 seconds at 72 degrees Celsius. After PCR amplification, a melting curve analysis was done. Using the mRNA expression of a well-known housekeeping gene, Gapdh (Table -1), the expression level of the target genes were standardized. Using the 2 -ΔΔCT approach, outcomes are presented as fold-changes from the control group [29].

Table 1: Forward and reverse of the primers for Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-ɣ), Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear Factor kappa-Beta (NF-kB p65), and the housekeeping g Gapdh gene according to khamis et al. [26].

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size	GenBank Accession number
Gapdh	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	143	NM_017008.4
PPAR-ɣ	CCTGAAGCTCCAAGAATACC	GATGCTTTATCCCCACAGAC	153	NM_013124.3
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC	121	NM_031789.2
NF-kB p65	TCTCAGCTGCGACCCCG	TGGGCTGCTCAATGATCTCC	150	AF _079314

II- Histopathological study

Testes were sectioned into 5 um thick successive sections using a Leica RM 2135 Bio Cut Rotary Microtome, mounted on glass slides, stained with hematoxylin and eosin (H&E), and then inspected under a light microscope. Testes were fixed in 10% formal saline for 48 hours prior to use [30].

III- Immunohistochemical examination of B-cell lymphoma -2 (Bcl-2) and Bcl-2 associated x (Bax) proteins

For the immunohistochemical analysis, testicular sections were microwave-irradiated in 0.1 mol/l sodium citrate buffer (pH 6.0) for 20 minutes after deparaffinization and rehydration. Endogenous peroxidases were bleached with 3% H2O2, followed by a 10-minute rinse in Trisbuffer (pH 7.4). Rabbit polyclonal anti-Bcl-2 antibody (1:50; ab59348; Abcam, Cambridge, UK) and rabbit monoclonal anti-Bax antibody (1:250; ab32503; Abcam, Cambridge, UK) were incubated overnight on testicular sections before being washed in Tris buffer. By exclusion of the primary antibodies, the specificity of the antibodies was examined. Mayer's hematoxylin was

utilized as a counterstain after tissue visualization with 3.30-diaminobenzidine (DAB). Testicular sections were then mounted with DPX and cover-slipped after being dried in xylene [31]. Positive results for Bcl-2 and Bax immunoreaction were indicated by brown cytoplasmic staining of testicular cells.

IV- Morphometric study

The interactive measure menu on the image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Pathology Department, Faculty of Dentistry, Cairo University, Egypt, was used to evaluate the area percent of Bax and Bcl-2 proteins in immunohistochemically stained testicular sections. The measuring frame was chosen to have a standard area of 118476.6 m2 so that the brown positive immune reaction could be seen and measured. Ten readings from five non-overlapping testicular sections were taken from each rat in all groups.

V-Statistical analysis

Data were presented as a mean and standard deviation (X±SD) for all groups. The Chicago, USA-based SPSS application, version 21, was utilized. One-way analysis of variance (ANOVA), followed by the LSD test for multiple comparisons between various groups, was used to statistically evaluate whether there was a significant difference. When the probability values (P) were less than 0.05, they were deemed significant, and when they were less than 0.001, they were deemed extremely significant [32].

Results

There was no observed significant difference between the groups receiving distilled water, corn oil, and CoQ10 and the control group as regard all the measures parameters.

Reproductive hormones (Testosterone, FSH and LH)

There was a highly significant reduction in serum testosterone, FSH and LH hormone levels in CP treated group in comparison with the control group ($P<0.001$). While, there was a highly significant elevation in serum testosterone, FSH and LH hormone levels in CP+CoQ10 treated group towards the control when compared to CP treated group ($P<0.001$) as shown in Table-2.

Pro-inflammatory markers (TNF- α and IL-1β)

There was a highly significant elevation in serum TNF- α and IL-1β levels in CP treated group in comparison with the control group ($P<0.001$). While, there was a highly significant reduction in serum TNF- α and IL-1β levels in CP+CoQ10 treated group towards the control when compared to CP treated group ($P<0.001$) as shown in Table -2.

Oxidative stress markers (MDA, SOD, CAT and GSH)

There was a highly significant elevation in MDA level and a highly significant reduction in SOD, CAT and GSH levels of testicular tissue in CP treated group in comparison with the control group (P<0.001). While, there was a highly significant reduction in MDA level and a highly significant elevation in SOD, CAT and GSH levels of testicular tissue in CP+CoQ10 treated group towards the control when compared to CP treated group (P<0.001) as shown in Table-2.

RT-PCR (PPAR-γ, Nrf2 and NF-kB p65) gene expression

There was a highly significant reduction in PPAR-γ and Nrf2 expression levels and a highly significant elevation in NF-kB p65 expression level of testicular tissue in CP treated group in comparison with the control group (P<0.001). While, there was a highly significant elevation in PPAR-γ and Nrf2 expression levels and a highly significant reduction in NF-kB p65 expression level of testicular tissue in CP+CoQ10 treated group toward the control when compared to CP treated group (P<0.001) as shown in Table-3.

Correlation between reproductive hormones and oxidative stress markers

There was significant positive correlation between reproductive hormone levels (testosterone & LH & FSH) and oxidative stress markers (GSH & CAT & SOD) While, there was significant negative correlation between reproductive hormone levels and MDA level as shown in Tale-4.

Correlation between reproductive hormones and pro-inflammatory markers

There was significant negative correlation between reproductive hormone levels (testosterone & LH & FSH) and pro-inflammatory markers (TNF- α & IL-1β) (Table-4).

Correlation between reproductive hormones and gene expression

There was significant positive correlation between reproductive hormones (testosterone & LH & FSH) with (PPAR-γ & Nrf2) gene expression. While, there was significant negative correlation between hormone levels and NF-kB p65 gene expression (Table-4).

Correlation between oxidative stress markers and gene expression

There was significant positive correlation between MDA level and NF-kB p65 gene expression. While, there was significant negative correlation between MDA level and (PPAR-γ & Nrf2) gene expression. There was significant positive correlation between oxidative stress markers (GSH & CAT & SOD) and (PPAR-γ & Nrf2) gene expression. While, there was significant negative correlation between oxidative stress markers (GSH & CAT & SOD) and NF-kB p65 gene expression (Table-5).

Correlation between pro-inflammatory markers and gene expression

There was significant positive correlation between pro-inflammatory markers (TNF- α & IL-1β) and NF-kB p65 gene expression. While, there was significant negative correlation between pro-inflammatory markers (TNF- α & IL-1β) and (PPAR-γ & Nrf2) gene expression (Table-5).

Table (2): Statistical comparison between mean values of serum testosterone (ng/mL), FSH (mU/L), LH (mU/L) hormones, serum TNF- α (pg/ml), IL-1β (pg/ml), testicular MDA (umol/gm), SOD (U/mg), CAT (U/gm) and GSH (umol/mg) in group I (–ve control), group II (+ve control distilled water), group III (+ve control corn oil), group IV (CoQ10), group V (CP) and group VI (CP+CoQ10) after 4 weeks of administration using ANOVA (analysis of variance) and LSD post-hoc test.

Group (n=10) / Parameters	Group I (-v) control	Group II (+ve) control (Distilledwater)	Group III (+ve) control (Corn oil)	Group IV (CoQ10)	Group V (CP)	Group V (CP+CoQ10)	F	p-value
			Mean ± SD					
Testosterone (ng/mL)	150.76±2.88	150.64±2.43	151.38±0.96	150.93±3.0#	87.96±5.28a	146.21±3.33*b	778.816	<0.001**
FSH (mU/L)	29.21±2.10	28.22±1.46	28.29±1.46	28.48±1.94#	11.35±0.78a	27.72±0.95*b	509.522	<0.001**
LH (mU/L)	19.13±0.68	18.94±0.36	18.65±0.38	18.94±0.46#	8.28±0.85a	18.44±0.49*b	777.310	<0.001**
TNF-α (pg/ml)	41.35±1.74	41.03±1.73	40.27±1.32	41.17±1.62#	113.35±2.26a	43.35±1.167*b	5305.89	<0.001**
IL-1β (pg/ml)	21.05±2.11	20.99±1.37	22.38±1.26	21.23±2.14#	71.42±2.35a	23.74±2.76*b	1368.77	<0.001**
MDA (umol/gm)	1.88±0.67	1.64±0.45	1.44±0.46	1.62±0.39#	4.53±0.64a	2.52±0.54*b	49.319	<0.001**
SOD (U/mg)	8.86±0.72	9.11±0.89	8.44±0.97	9.21±0.71#	5.18±0.42a	8.21±0.60*b	109.017	<0.001**
CAT (U/gm)	140.82±3.96	141.7±2.58	140.50±2.64	140.99±3.58#	77.61±6.72a	136.37±2.68*b	548.925	<0.001**
GSH (umol/mg)	9.12±0.61	9.45±1.03	9.16±0.86	9.54±0.84#	5.04±0.58a	8.49±0.57*b	141.690	<0.001**

SD: Standard Deviation. **: highly significant (P<0.001) n: Number of rats in each group F: ANOVA test. P: level of significance CP: Cyclophosphamide CoQ10: Coenzymeq10 -ve: negative $^{\#}p>0.05$, $^{*}p<0.05$, $^{a}p<0.001$ when values are compared to control group, $^{b}p<0.001$ when values are compared to CP group.

Table (3): Statistical comparison between mean values of PPAR-γ, Nrf2 and NF-kB p65 expression in testicular tissues in group I (–ve control), group II (+ve control distilled water), group III (+ve control corn oil), group IV (CoQ10), group V (CP) and group VI (CP+CoQ10) after 4 weeks of administration using ANOVA (analysis of variance) and LSD post-hoc test.

Group(n=10) / Genes	Group I (-ve) control	Group II (+ve) control (Distilled water)	Group III (+ve) control (Corn oil)	Group IV CoQ10	Group V CP	Group VI (CP+CoQ10)	F	p-value
Mean ± SD								
PPAR-γ	1.15±0.28	1.33±0.08	1.32±0.12	1.21±0.13$^{\#}$	0.49±0.06^{a}	0.99±0.04^{*b}	42.767	<0.001**
Nrf2	0.96±0.06	0.93±0.06	0.94±0.04	0.98±0.02$^{\#}$	0.44±0.11^{a}	0.90±0.08^{*b}	114.05	<0.001**
NF-kB p65	1.04±0.08	1.037±0.03	1.05±0.05	1.03±0.08$^{\#}$	2.43±0.26^{a}	1.213±0.13^{*b}	182.859	<0.001**

SD: Standard Deviation **: highly significant (P<0.001) n: Number of rats in each group F: ANOVA test. P: level of significance CP: Cyclophosphamide COQ10: Coenzyme q10 -ve: negative $^{\#}$p>0.05, *p<0.05, ^{a}p<0.001 when values are compared to control group, ^{b}p<0.001 when values are compared to CP group.

Table (4): Pearson correlation between reproductive hormones (Testosterone, LH, FSH) & oxidative stress markers (MDA, GSH, CAT, SOD) & pro-inflammatory markers (TNF-α, IL-1β) and gene expression (PPAR-γ, Nrf2, NF-kB p65)

Reproductive hormones	Oxidative stress markers				Pro-inflammatory markers		Gene expression			P-value
	MDA	GSH	CAT	SOD	TNF- α	IL-1β	PPAR-γ	Nrf2	NF-kB p65	
	(r)									
Testosterone	-0.898	0.876	0.976	0.839	-0.982	-0.981	0.854	0.925	-0.953	<0.001**
LH	-0.882	0.885	0.947	0.860	-0.956	-0.979	0.879	0.884	-0.967	<0.001**
FSH	-0.876	0.894	0.944	0.833	-0.944	-0.966	0.826	0.907	-0.945	<0.001**

r: correlation coefficient.
**: statistically highly significant (P<0.001)

Table (5): Pearson correlation between gene expression (PPAR-γ, Nrf2, NF-kB p65) & oxidative stress markers (MDA, GSH, CAT, SOD) and pro-inflammatory markers (TNF-α, IL-1β)

Gene expression	Oxidative stress markers				Pro-inflammatory markers		P-value
	MDA	GSH	CAT	SOD	TNF	IL-1β	
	(r)						
PPAR-γ	-0.816	0.793	0.854	0.771	-0.849	-0.876	<0.001**
Nrf2	-0.821	0.813	0.935	0.785	-0.933	-0.917	<0.001**
NF-kB p65	0.851	-0.860	-0.948	-0.832	0.955	0.967	<0.001**

r: correlation coefficient.
**: statistically highly significant (P<0.001)

Table (6): Comparison between group I (–ve control), group II (+ve control distilled water), group III (+ve control corn oil), group IV (CoQ10), group V (CP) and group VI (CP+CoQ10) in mean of area % Bcl-2 and Bax immunoreaction in testicular sections after 4 weeks of administration using ANOVA test and LSD post-hoc test.

Group (n=10) / Parameters	Group I (-ve) control	Group II (+ve) control (Distilled water)	Group III (+ve) control (Corn oil)	Group IV CoQ10	Group V (CP)	Group VI (CP+CoQ10)	F	P-value
	Mean ± SD							
Area% of Bcl-2 Immunoreaction	60.33±17.03	61.02±00.03	60.13±16.03	61.30±00.07$^{\#}$	10.38±0.07^{a}	42.32±30.22^{*b}	1.012	<0.05*
Area% of Bax immunoreaction	12.01±10.15	11.25±13.15	12.45±10.13	11.03± 9.7$^{\#}$	73.67±34.01^{a}	27.05± 45.01^{*b}	12.24	<0.001**

SD: Standard deviation n: Number of rats in each group *: Significant (P<0.05) **: Highly significant (P<0.01), $^{\#}$p>0.05 *p<0.05, ^{a}p<0.001 when values are compared to control group, ^{b}p<0.001 when values are compared to CP group.

Histopathological result

Histological examination of the control groups and CoQ10 group showed the same histological results. Slides showed preserved architecture with numerous packed seminiferous tubules that were covered by connective tissue capsule. These tubules were lined by stratified germinal epithelium, which contained a variety of cell types (spermatogonia, primary

spermatocytes, secondary spermatocytes and spermatids). The lumen of seminiferous tubules contained spermatozoa. Between the seminiferous tubules was a narrow interstitium with interstitial cells and blood vessels (Figure 1.a & 1.b &1.c & 1.d & 1.e). Sections of the CP-treated testicular slides stained with H&E revealed disorganised seminiferous tubules; their germinal epithelium separated from their basal lamina and had dark pyknotic nuclei. There was also shrinkage of the seminiferous tubules with irregular basement membranes and wide separation between the tubules. Other tubules had thickened capsule with congested dilated sub-capsular blood vessels, and loss their lining germinal epithelium. Decrease in the height and disorganized germinal epithelial lining was seen. Some spermatozoa were found in the tubule lumen. Acidophilic hyaline material and vacuolations were noticed in the interstitium in between the seminiferous tubules (Figure 1.f & 1.g & 1.h). H & E-stained sections of CP+CoQ10-treated testicular slides revealed improved histological structure. Normal germinal epithelium lined the seminiferous tubules. Spermatozoa were found in the tubule lumen. There was also Leydig cells and slightly congested vessels in the interstitium (Figure 1.i).

Immunohistochemical result:

Immunohistochemical analysis of the control groups, CoQ10 group revealed the same findings. Immunohistochemical reaction for Bcl-2 stained testicular sections of the control groups and CoQ10 group revealed positive Bcl-2 immunoreaction in the cytoplasm of germinal epithelial cells (Figure 2.a & 2.b). In CP treated group, negative Bcl-2 immunoreaction in the cytoplasm of germinal epithelial cells was noticed (Figure 2.c). In CP+CoQ10 treated group, marked improvement with strong positive Bcl-2 immunoreaction was detected in the cytoplasm of most of the germinal epithelial cells (Figure 2.d). Immunohistochemically stained sections for Bax in testicular tissue of the control groups and CoQ10 group revealed negative Bax immunoreaction in the cytoplasm of germinal epithelial cells (Figure 3.a & 3.b). In CP treated group, strong positive Bax immunoreaction was seen in the cytoplasm of germinal epithelial cells (Figure 3.c). In CP+CoQ10 treated group, marked improvement with negative Bax immunoreaction was found in the cytoplasm of the majority of germinal epithelial cells (Figure 3.d).

Morphometric result

There was a highly significant increase in Bax and a highly significant decrease in Bcl-2 expression levels with significant elevation in apoptotic index (Bax/Bcl-2 ratio) in CP treated group in comparison with the control groups ($P<0.001$). While, there was a highly significant decrease in Bax and a highly significant increase in Bcl-2 expression levels with significant reduction in apoptotic index (Bax/Bcl-2 ratio) in CP+CoQ10 treated group towards the control when compared to CP treated group ($P<0.001$) as shown in Table-6.

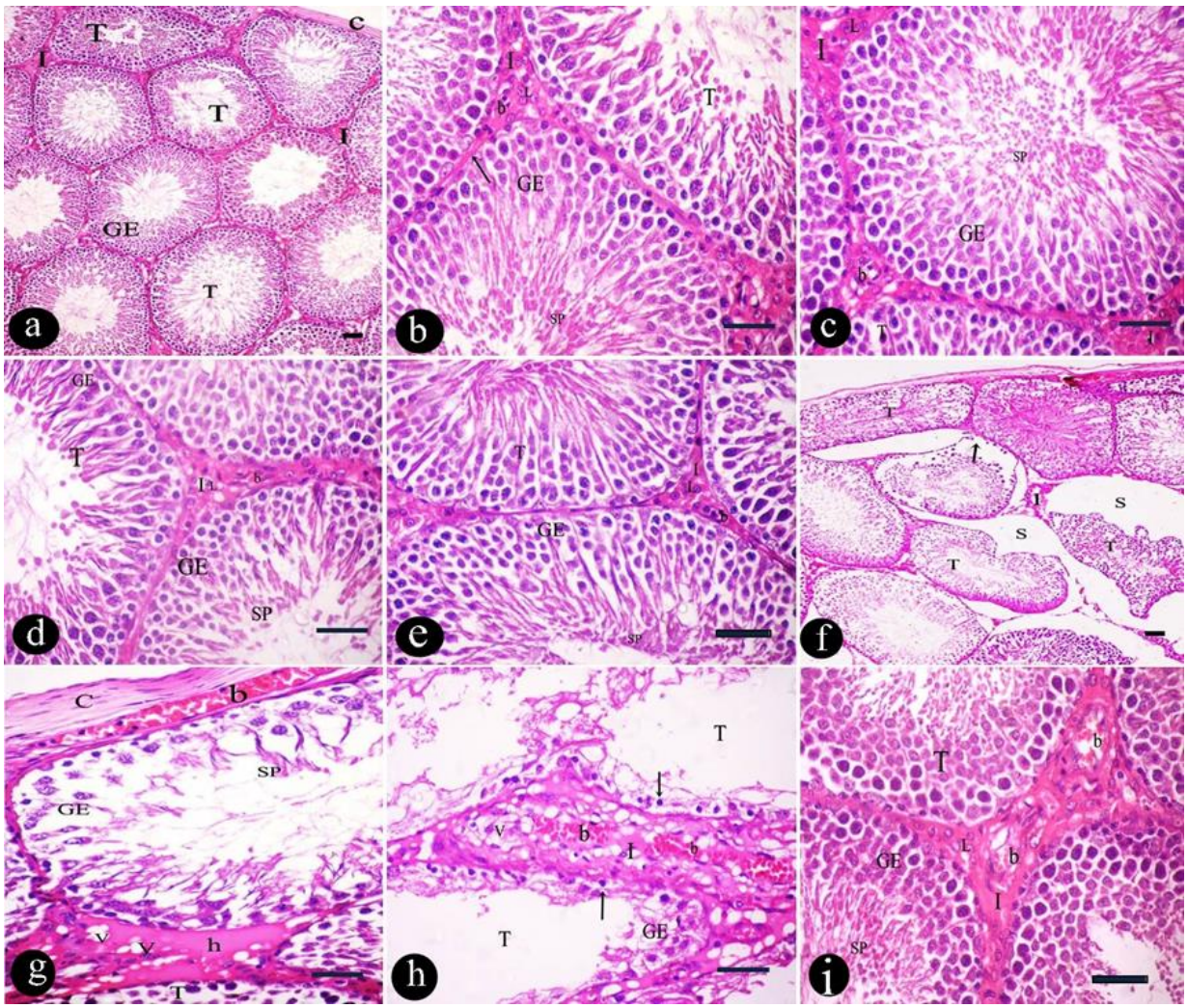

Figure 1. H&E-stained sections of **a:** -ve control group showing normal testicular histology with numerous packed seminiferous tubules (T) that are covered by connective tissue capsule (C) and lined by stratified germinal epithelium (GE) with narrow interstitium (I) is present inbetween them. **b:** -ve control group reveals also normal histological structure with normal seminiferous tubules (T) that are lined by stratified germinal epithelium (GE) and resting on regular basal lamina (arrow). Spermatozoa (SP) are present in the lumen of seminiferous tubules with narrow interstitium (I) contains Lydig cells (L) and blood vessels (b) in between tubules. **c:** +ve control (distilled water) group showing the same normal seminiferous tubules (T) that are lined by germinal epithelium (GE) with spermatozoa (SP) in the lumen and narrow interstitium (I) contains Lydig cells (L) cells and blood vessels (b) in between tubules. **d:** +ve control (corn oil) group showing the same normal seminiferous tubules (T) that are lined by germinal epithelium (GE) with spermatozoa (SP) in the lumen and narrow interstitium (I) containing Lydig cells (L) cells and blood vessels (b) in between tubules. **e:** CoQ10 group showing the same normal histological structure with packed seminiferous tubules (T) that are lined by germinal epithelium (GE) with spermatozoa (SP) in the lumen and narrow interstitium (I) contains Lydig cells (L) cells and blood vessels (b) in between tubules. **f:** CP group showing disorganized seminiferous tubules

(T); their germinal epithelium separated from their basal laminae (arrow). Shrinkage of the seminiferous tubules with irregular basement membranes and wide separation (S) between the tubules are also seen. Distorted interstitium (I) can also be noticed inbetween tubules. **g:** CP group showing seminiferous tubules (T) have thickened capsule (C) with congested dilated subcapsular blood vessel (b) with loss of their lining germinal epithelium (GE). Decrease in the height and disorganized germinal epithelial lining is seen (GE). Few spermatozoa (SP) are present in the lumen of the tubules. Acidophilic hyaline material (h) and vacuolations (V) are detected in the interstitium. **h:** CP group showing marked affection of seminiferous tubules (T) having marked decrease in the height of the germinal epithelium (GE). Pyknotic cells have dark stained nuclei (arrow). Distorted interstitium (I) with dilated congested blood vessels (b) and marked vacuolations (V) are noticed. **i:** CP+CoQ10 group showing improvement of the histological structure of the testicular tissue. Seminiferous tubules (T) are lined by normal germinal epithelium (GE). Spermatozoa (SP) are present in the lumen of the tubules. Interstitium (I) containing Leydig cells (L) and slightly congested blood vessels (b) is also detected. All groups: H&Ex400 (scale bar 30 µm) only **a&f**: H&E×100 (scale bar 50 µm).

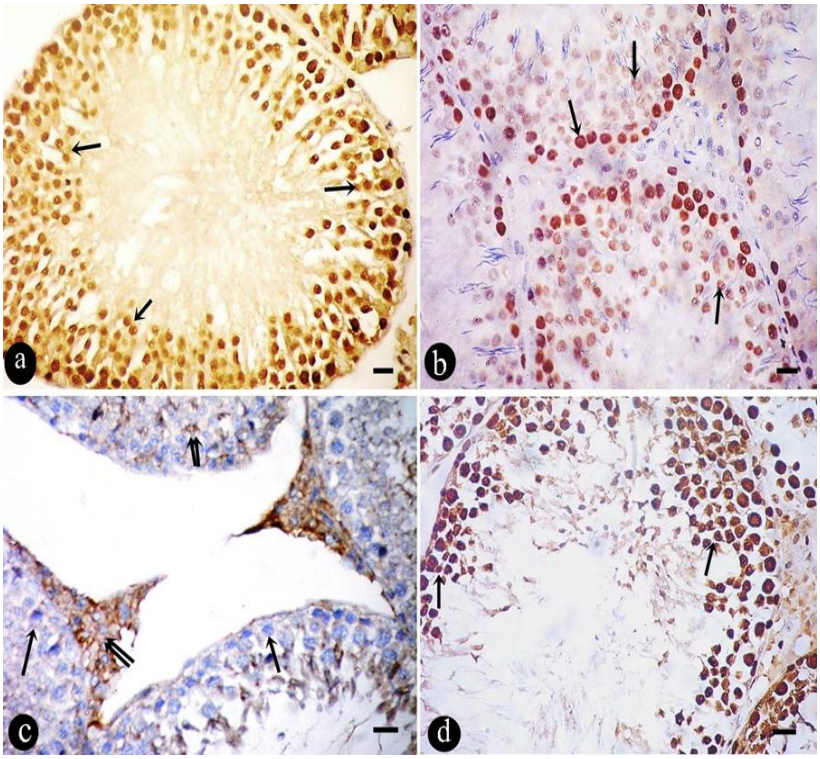

Figure 2. **a**: Immunohistochemical reaction for Bcl-2 stained sections in the testes of the control groups showing strong positive immunoreaction for Bcl-2 in the nuclei and cytoplasm of germinal epithelial cells (arrow). **b**: In CoQ10 treated group, strong positive immunoreaction for Bcl-2 in the nuclei and cytoplasm of germinal epithelial cells (arrow). **c**: CP treated group showing negative immunoreaction for Bcl-2 in the cytoplasm of germinal epithelial cells (arrow) with positive immunoreaction is seen in some interstitial cells and few spermatocytes (double arrow). **d**:

CP+CoQ10 treated group reveals strong positive immunoreaction for Bcl-2 in the nuclei and cytoplasm of most of the germinal epithelial cells (arrow), Bcl-2 ×400 (scale bar 30 mm).

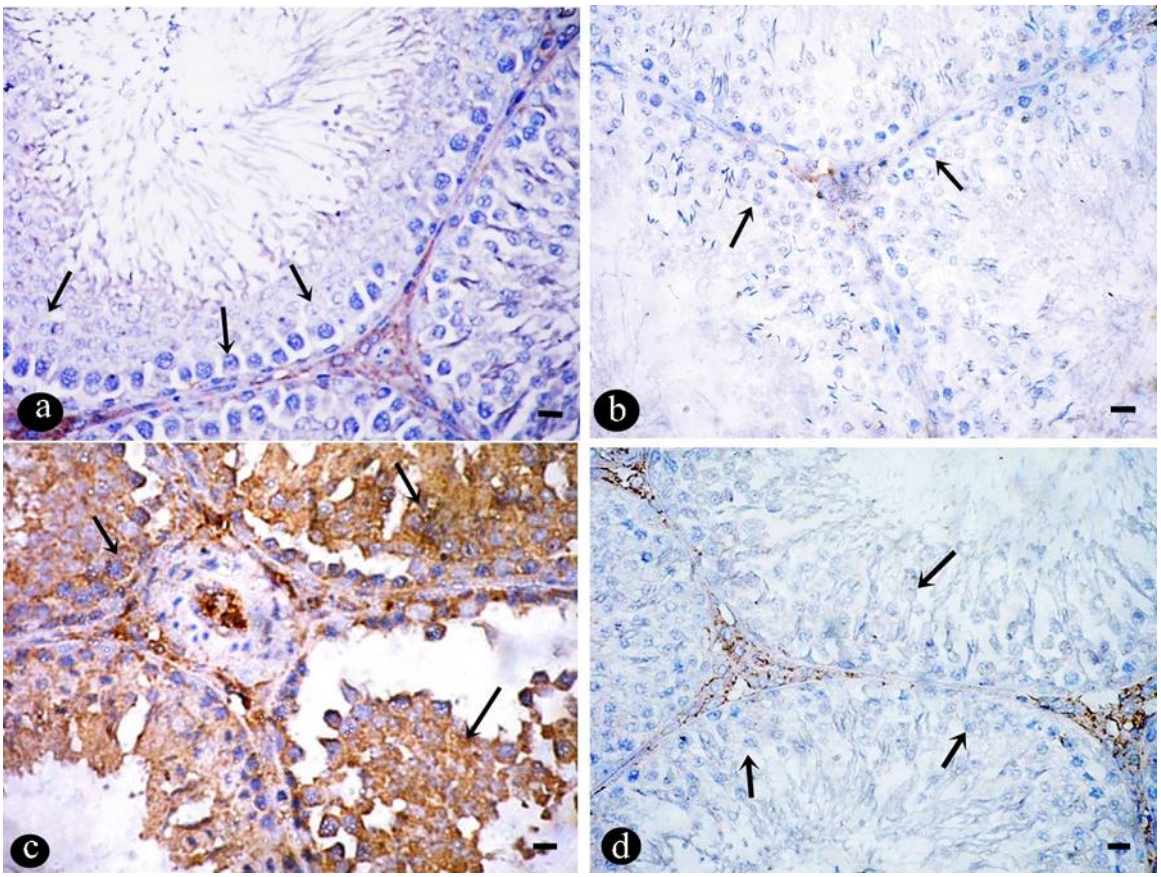

Figure 3. **a:** Immunohistochemical reaction for Bax stained sections in the testes of the control groups showing negative immunoreaction for Bax in the cytoplasm of germinal epithelial cells (arrow). **b:** In CoQ10 treated group, showing negative immunoreaction for Bax in the cytoplasm of germinal epithelial cells (arrow). **c:** CP treated group showing strong positive immunoreaction for Bax in the cytoplasm of germinal epithelium (arrow). **d:** CP+CoQ10 treated group reveals negative immunoreaction for Bax in the cytoplasm of germinal epithelial cells (arrow), Bax ×400 (scale bar 30 mm).

Discussion

The present findings were designed to evaluate the influence of CoQ10 on cyclophosphamide induced testicular toxicity in adult albino rats. According to the present study's findings, testosterone, LH, and FSH serum levels in the CP-treated group were significantly lower than those in the control group. These findings agreed with the results of Wang et al. [33]. Cyclophosphamide toxicity induced marked reduction of serum testosterone level due to induction of oxidative stress that caused damage to DNA, protein and key enzymes implicated in testicular steroidogenesis and spermatogenesis as 3b-hydroxysteroid dehydrogenase and 17b-hydroxysteroid dehydrogenase [34]. This also leads to Leydig cell degeneration which is responsible for

testosterone secretion [35]. Our outcomes also revealed that CP caused a significant rise in testicular MDA, confirming CP-induced lipid peroxidation. Caglayan et al. [3] explained that lipid peroxidation is caused by CP attacks on the membrane phospholipids, which are extremely sensitive to ROS, resulting in MDA elevation. There was a highly significant decrease in SOD, CAT and GSH activities in testicular tissues of CP-treated group compared to control in accordance with the findings of Ebokaiwe et al. [36]. Ayoka et al. [37] described that low serum levels of gonadotropins (FSH & LH) is due to a direct toxic and apoptotic effect of CP on the anterior pituitary gland that is essential for the secretion of these hormones. In contrast to our study's findings, Kaya et al. [38] found no change in serum levels of testosterone, LH, or FSH after CP application in Sprague-Dawley rats at a single dose of (200 mg/kg). This could be because of differing CP doses and durations.

The results of the current investigation revealed a highly significant increase in serum levels of TNF-α and IL-1β in CP-treated group in comparison with the control groups. These findings are in line with the study of Abd-ElRaouf et al. [39]. Inflammation is controlled and induced in part by oxidative stress [40]. Stress signaling and pro-inflammatory pathways can be activated by the sustained ROS and RNS production caused by CP application [41]. CP is converted into active metabolites (acrolein and phosphoramide mustard) by hepatic microsomal enzymes [42]. Acrolein is cytotoxic and promotes intracellular ROS and NO production, which results in the generation of peroxide and the creation of oxidative stress [43]. Rezaei et al. [44] recognized the role of oxidative stress-mediated inflammation in the pathophysiology of CP-induced testicular toxicity.

The results of the current investigation showed that there was a highly significant drop in PPAR-γ expression level in the testicular tissues of the CP-treated group compared to the control group. The current findings are along with the study directed by Abu-Risha et al. [45]. Zarei and Shivanandappa [46] reported that CP administration also induced down-regulation of PPAR-γ gene expression that activated NF-κB signaling pathways which mediated triggering of pro-inflammatory markers (TNF- α and IL-1β). While according to Abo-Salem [47], cyclophosphamide was shown to suppress PPAR-γ expression in testes due to up-regulation of TNF-α, IL-1β and TGF-β expression which in turn suppress PPAR-γ expression. PPAR-γ deficiency in testicular tissues leads to disturbance in spermatogenesis and testicular homeostasis [48].

According to the present findings, there was a highly significant elevation in NF-kB p65 expression in testicular tissues of CP-treated group compared to control. These findings are matched with Rezaei et al. [44]. It is widely known that CP activates NF-kB p65, which is transported into the nucleus and responsible for the transcription of inflammatory markers such as TNF-α, IL-1β, IL-6, COX-2, and iNOS [49]. Additionally, NF-kB p65 not only targets inflammation directly but also indirectly controls cell division, proliferation, and death [50]. The main mechanism of CP-induced NF-κB signaling activation is CP-induced oxidative stress [51]. Oxidative stress activates NF-kB, which then produces pro-inflammatory cytokines that increase

tissue damage [52]. Therefore, antioxidants may be able to guard against the harmful effects brought on by CP [53]. In addition, present investigation revealed a highly significant decrease in Nrf2 expression level in testicular tissues of CP-treated group compared to control. This finding is in line with the study conducted by Fusco et al. [54]. Iqubal et al. [55] mentioned that Nrf2 inhibition may occur from the active CP metabolites' induction of ROS and suppression of the endogenous antioxidant defense system. Also, CP was discovered to disrupt the Nrf2/ARE/HO-1 antioxidant signaling pathway in rats by the same mechanism [56].

The histological findings detected in the CP-treated group runs in accordance with Adana et al. [57] where they showed that CP exposure caused significant morphological alterations in the testicular parenchyma, disorganization and degeneration of germ cells. The thickness of the germinal epithelium lining of the tubules also significantly decreased. In line with Ahmed et al. [58], the mechanism of CP-induced inhibition of B-spermatogonia mitosis may be caused by oxidative stress induction, which caused polyunsaturated fatty acids to be peroxidized by free radicals in spermatozoa plasma membranes, increasing membrane permeability and harming germ cells and sperms. Furthermore, DNA damage may be to blame for the increased testicular morphological abnormalities and abnormal sperm forms [59].

The highly significant increase in Bax with a highly significant decrease in Bcl-2 expression in cytoplasm of germinal epithelial cells in CP-treated rats together with significant elevation of the apoptotic index (Bax/Bcl-2 ratio) runs in consistent with Wang et al. [33] and Abu-Risha et al. [45] who observed that CP administration promotes testicular apoptosis. Exposure to CP causes germ cell apoptosis to increase [60]. The higher ratio of Bax to Bcl-2 caused by CP was connected to the higher apoptotic rate of germ cells [61]. This imbalance caused the discharge of cytochrome c from the mitochondria, which started the basic apoptotic cascade and led to cell death [62]. According to Mansour et al. [63], CP-induced apoptosis is believed to be caused by increased levels of ROS/RNS in CP-treated rats, which then cause DNA damage in testicles and accelerate the rate of apoptosis.

Co-administration of coenzyme q10 with cyclophosphamide revealed a highly significant rise in testosterone, FSH and LH serum levels compared to CP-treated group. There are no previous reports described the outcome of coenzyme q10 on CP-induced testicular toxicity, however, Mohammed Ali et al. [64] described the defensive role of CoQ10 on sodium fluoride-induced reproductive toxicity by elevation of serum level of testosterone and LH hormones. According to Asl et al. [65], CoQ10 supplementation greatly increases GnRH gene expression, which in turn raises the levels of FSH and LH hormones. Serum testosterone level and sperm count increased due to the raised FSH and LH levels. Because CoQ10 supplementation motivates the production of FSH and LH hormones from the hypothalamus and pituitary gland, it has a significant regulatory effect on the hypothalamo-pituitary gonadal axis. CoQ10 has been confirmed to have positive effects on the metabolism and production of reproductive hormones [66].

Co-administration of CoQ10 with CP revealed a highly significant increase in SOD & CAT & GSH activities and a highly significant decrease in MDA activity in testicular tissues compared to CP-treated group. These findings are in line with a study by El-Khadragy et al. [15]. Hussein et al. [12] reported that CoQ10 exhibits a prominent antioxidant effect and decreases the level of oxidative stress parameters. As it can scavenge free radicals and protected membrane phospholipids, mitochondrial membrane protein from oxidative stress and lipid peroxidation-induced damage [67]. Indeed, CoQ10 suppressed NADPH-oxidase expression, a great source of O2 • – [68], inhibits excess NO production [69] so can protect against lipid, protein and DNA oxidation [70].

Also, Co-administration of coenzyme q10 with cyclophosphamide revealed a highly significant reduction in serum levels of TNF-α and IL-1β compared to CP-treated group. Our findings are in accordance with the study directed by Khodir et al. [71] who observed the protective effect of CoQ10 administration on doxorubicin-induced testicular toxicity in rats by marked decrease in serum levels of TNF-α and IL-1β. Coenzyme Q10 is convoluted in the prevention of inflammation in liver [72], kidney [14] and testis [15]. By hindering the release of pro-inflammatory cytokines like TNF-α and IL-1β, which are linked to organ damage, coenzyme q10 demonstrates anti-inflammatory properties [73]. Li et al. [74] confirmed that CoQ10 decreases inflammatory cytokines levels resulting in inhibition of the matrix metalloproteinase which ultimately led to less inflammation and fibrosis.

Coenzyme Q10 significantly increased the PPAR-γ gene expression in testicular tissues compared to CP-treated group. The present findings are in line with the report directed by Rahmani et al. [75] who observed the protective effect of CoQ10 supplementation on PPAR-γ gene expression in patients with polycystic ovary syndrome. Coenzyme Q10 may stimulate PPAR-γ gene expression via the calcium-mediated adenosine monophosphate activated protein kinase signal pathway. In addition, CoQ10 partially attenuated the influence of TNF-α on PPAR-γ suggesting the protective role of CoQ10 [76]. Because PPAR-γ is convoluted in the regulation of a wide range of critical lipid metabolic genes, motivation of PPAR-γ has been shown to reduce testicular oxidative stress in rats [77]. PPAR-γ signaling up-regulation may therefore offer a defense against testicular damage brought on by CP [78]. CoQ10 administration with CP revealed a highly significant increase in Nrf2 gene expression in testicular tissues compared to CP-treated group. The current investigation supported by El-Khadragy et al. [15] who reported the protective effect of CoQ10 administration on lead acetate-induced testicular damage in rats by increasing the expression of Nrf2 in rats. CoQ10 also significantly reduced NF-κB gene expression in testicular tissues in accordance with Mohamed and Said [79] who found that CoQ10 co-treatment reduces intestinal NF-κB gene expression level following radiation exposure in rat models, which indicates the anti-inflammatory impact of CoQ10. According to Tarry-Adkins et al. [72], CoQ10 activates the Nrf2/ARE pathway, which sequentially suppresses the production of the NF-kB p65 gene resulting in reduction of pro-inflammatory cytokines.

Co-administration of CoQ10 with CP showed marked improvement of the testicular histological structure. The present investigation is along with the finding directed by El-Khadragy et al. [15] who stated that CoQ10 supplementation enhanced testicular histological changes following lead acetate exposure, which is consistent with the findings of our investigation and suggests that CoQ10 can operate as a natural beneficial agent to prevent testicular damage. Coenzyme Q10 significantly accelerated the restoration of spermatogenesis, combated changes to sperm characteristics, and improved pathological testicular ultra-structural changes and testicular DNA damage, which mitigated the harmful effect on spermatogenesis caused by toxins [80]. CoQ10 administration with CP revealed marked improvement with a significant reduction in Bax and rise in Bcl-2 expression. In addition, Apoptotic index (Bax/Bcl-2 ratio) revealed a significant reduction indicating the anti-apoptotic effect of CoQ10. The present study matched with the study demonstrated by Said et al. [81] who stated that CoQ10 motivated regulation of apoptotic index (Bax/Bcl-2 ratio). Numerous studies have described CoQ10's anti-apoptotic properties [14]. According to Papucci et al. [82], CoQ10's anti-apoptotic properties are due to its ability to prevent DNA breakage, prevent mitochondrial depolarization, and raise ATP level. Also, CoQ10 reduces the activity of the mitochondrial complex I, which inhibits the nuclear translocation of proteins that cause apoptosis and thus prevents cell death [83]. Moreover, Mahmoud and Al Dera [84] reported that co-activation of PPAR-γ and Nrf2 (1) promoted expression of antioxidant proteins protecting cells from oxidative stress (2) down-regulated NF-kB and iNOS, and prevented the production of pro-inflammatory markers protecting cells from inflammation (3) protected against apoptosis by induction of Bcl-2 expression.

Conclusion

Results of biochemical, histological, immunohistochemical, and morphometric examinations on adult albino rats exposed to CP showed that this exposure caused apparent testicular damage. Administration of CoQ10 may offer protection against these harmful consequences. It is recommended that clinicians should administrate CP with the proper dose and duration and need to consider possible side effects, interactions, and associated toxicities for patients before administering CP. Advise male patients to the potential risks for infertility as fertility may be impaired in patients being treated with CP. For those patients, CoQ10 can be administered as a nutritional supplement to lessen the harmful effects of CP. It will take more research using various CoQ10 levels to evaluate the abilities of the antioxidant.

Disclosure statement

The author (s) did not disclose any potential conflicts of interest.

Data availability

The data arrangements used and evaluated in this present study were available from the corresponding author upon reasonable request.

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