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Investigation of the effect of Coenzyme–Q10 on Cyclophosphamide induced testicular damage in male rats

Investigación del efecto de la coenzima Q10 sobre el daño testicular inducido por ciclofosfamida en ratas macho

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ABSTRACT

Cyclophosphamide (CP) is one of the frequently preferred chemotherapeutic agents Worldwide. CP has negative effects on the testes, spermatogenesis, and reproductive hormones. The aim of this study was to determine the protective effect of Coenzyme Q10 (CoQ10) on the damage caused by CP. CoQ10 is use in the treatment of infertility problems and is naturally found in the testes and seminal fluid. Thirty-six Albino Wistar male rats were divided into six groups (Control, Sham, Cyclophosphamide (CP), Coenzyme Q10 (CoQ10), CP + CoQ10 I, CP + CoQ10 II), with six animals in each group. Semen analysis included investigations of sperm DNA damage, motility, abnormal sperm ratio, and density. Histopathological examination and assessment of oxidative stress parameters in the testes were conducted. Additionally, serum levels of FSH, LH, and Testosterone were measured. CoQ10 administration increased the motility rate, density, and Testosterone levels in testicular damage caused by CP (P<0.05). Furthermore, it was observed that the abnormal sperm ratio, sperm DNA damage, and oxidative stress were reduced (P<0.05). Based on the results of this study, the use of CoQ10 in conjunction with CP has the potential to alleviate male infertility problems that may arise from CP administration.

Key words: Coenzym Q10; cyclophosphamide; sperm; testis

RESUMEN

La ciclofosfamida (CP) es uno de los agentes quimioterapéuticos preferidos en todo el mundo. La CP tiene efectos negativos sobre los testículos, la espermatogénesis y las hormonas reproductivas. El objetivo de este estudio era determinar el efecto protector de la coenzima Q10 (CoQ10) sobre los daños causados por la CP. La CoQ10 se utiliza en el tratamiento de problemas de infertilidad y se encuentra de forma natural en los testículos y el líquido seminal. Se dividieron 36 ratas macho Albino Wistar en seis grupos (Control, Sham, Ciclofosfamida (CP), Coenzima Q10 (CoQ10), CP + CoQ10 I, CP + CoQ10 II), con seis animales en cada grupo. El análisis del semen incluyó investigaciones del daño del ADN espermático, la motilidad, la proporción de espermatozoides anormales y la densidad. Se realizó un examen histopatológico y una evaluación de los parámetros de estrés oxidativo en los testículos. Además, se midieron los niveles séricos de FSH, LH y testosterona. La administración de CoQ10 aumentó la tasa de motilidad, la densidad y los niveles de testosterona en el daño testicular causado por CP (P<0,05). Además, se observó que se redujo la proporción anormal de espermatozoides, el daño del DNA espermático y el estrés oxidativo (P<0,05). En base a los resultados de este estudio, el uso de CoQ10 junto con CP tiene el potencial de aliviar los problemas de infertilidad masculina que pueden surgir de la administración de CP.

Palabras clave: Ciclofosfamida; coenzima Q10; esperma; testículo

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INTRODUCTION

Cyclophosphamide (CP) is one of the most commonly used chemotherapeutic drugs in both Veterinary and Human Health [1]. Apart from its use in cancer treatment, it is also employed as an immunosuppressive drug in autoimmune diseases and organ transplants [2]. CP has a cytotoxic alkaline nature, and its metabolites, aldophosphamide mustard and acrolein, inhibit deoxyribonucleic acid (DNA) synthesis [3, 4]. Due to its ability to penetrate the blood–testicular barrier, CP and its metabolites cause various dysfunctions in the testes, including decreased testicular weight, loss of germ cells, degeneration of Tubulus Seminiferus Contortus, oligospermia, azoospermia, increased oxidative stress, and decreased Testosterone levels [5, 6, 7].

Coenzyme Q10 (CoQ10) is often preferred in the treatment of idiopathic male infertility problems. It is widely distributed in tissues and organs and exhibits prolonged action [8]. CoQ10 plays a role in energy mobilization and protection against lipid peroxidation in spermatozoa [9]. Acting on mitochondria, CoQ10 serves as an electron carrier and reduces oxidative stress [10]. Mitochondria, located in the middle part of spermatozoa, are responsible for various metabolic activities such as steroid synthesis, energy production, calcium metabolism, and cell apoptosis [11]. CoQ10, distributed in these mitochondria, acts as an energy promoter and antioxidant [12]. It is involved in gene expression, membrane stability, and cell signaling, protecting cells from damage and abnormal growth [13]. CoQ10 protects cells against apoptosis, increases energy levels, and possesses anti-inflammatory properties. Furthermore, its nanoparticle structure enables it to penetrate the blood–testis barrier [14, 15].

The aim of this study was to investigate the protective effect of CoQ10 on CP-induced testicular damage at different time points, focusing on the testes, semen, sperm DNA damage, and reproductive hormones.

MATERIAL AND METHODS

Animals

Adult, pathogen-free male Albino Wistar rats (*Rattus norvegicus*) were obtained from Van Yuzuncu Yil University. The animals used in the study were approximately 3–4 months old and weighed between 200–250 g. They were provided with *ad libitum* access to food and water and were kept under a 12-h light-dark cycle. The housing conditions maintained an average temperature of 22–24°C and relative humidity of 55–60%. Each cage contained 6 animals, and the experiment was conducted with these groups.

Groups

Control (n=6): 0.5 mL of physiological saline daily administered by oral gavage for 4 weeks

Sham (Olive Oil) (n=6): 0.5 mL of olive oil (*Olea europaea*) daily administered by oral gavage for 4 weeks (Coenzyme Q10 (CoQ10) dissolves in olive oil).

Cyclophosphamide (CP) (n=6): CP (Endoxan, Eczacıbaşı, Turkey) at a dose of 6 mg·kg^{-1} was dissolved in 0.5 mL of physiological saline and administered by oral gavage for 4 weeks.

Coenzyme Q10 (CoQ10) (n=6): CoQ10 (Ocean, Germany) at a dose of 2.8 mg·kg^{-1} was dissolved in 0.5 mL of olive oil and administered by oral gavage for 4 weeks.

CP + CoQ10 I (n=6): 6 mg·kg^{-1} CP + 2.8 mg·kg^{-1} CoQ10 administered by oral gavage for 4 weeks.

CP + CoQ10 II (n=6): 6 mg·kg^{-1} CP administered by oral gavage for 4 weeks + 2.8 mg·kg^{-1} CoQ10 administered by oral gavage at 3 and 4 weeks.

Sperm examination

Motility examination (Progressive motility)

The sperm sample was obtained by epididymis puncture immediately after sacrifice and was placed on a glass slide on the heating table (Mshot, TP-R282-M, China) set to 38°C. The coverglass was closed at an angle of 45° and motility (in %) detected by microscopy (Nikon, Eclipse E200, Japan), at 40x magnification. Uniform, linear, forward-moving spermatozoa were compared to immobile, swirling and quivering spermatozoa [16].

Density analysis

After epididymal puncture, 0.1 mL of sperm sample was added to Eppendorf tubes with 0.5 mL Hayem solution (Norateks, Germany). Sperm count per mL was calculated on a Thoma cell counting chamber [16].

Abnormal sperm ratio

The sperm obtained by epididymis puncture was transferred to Eppendorf tubes with 0.5 mL Hancock solution (Norateks, Germany). At least 400 sperm samples were examined at 40x magnification to determine the ratio. The proportion of spermatozoa with anomalies in the head, tail and neck part was determined [16].

Sperm DNA damage

The DNA Fragmentation Index was assessed using the Halomax® kit (Spain). Sperm DNA damage was calculated following the protocol of the Halotech Halomax HT-RN40 kit. A total of 600 sperm were counted for each group, and the damage ratio was calculated using the following formula:

$$Sperm\ DNA\ fragmentation\ (SDF) = \frac{Fragmented + Degraded}{Total\ Sperm\ Counted}$$

Oxidative stress

Collection of tissue samples for RNA isolation and preparation for analysis

Testicular tissues were collected under sterile conditions and stored at -80 °C (ILDAM, DF-210, Turkey) until the study day. On the study day, the tissues were allowed to thaw at room temperature, and approximately 30 mg of tissue was taken into sterile tubes. The tissues were then homogenized by adding 0.2 mL of sterile phosphate buffer. The homogenized tissues were centrifuged (Hettich, Rotofix 32, Germany), and the liquid portion of the tube was discarded. The pellet was used for total Ribonucleic acid (RNA) isolation.

RNA extraction and analysis – cDNA extraction

Total mRNA was extracted from the obtained pellets using the Trizol Reagent-chloroform method [7]. The amount and purity of the extracted mRNA were measured using a spectrometer (Biochrom,

Anthos Zenyth 200RT, UK). A nanodrop spectrophotometer device (BioDrop, UK) was used for the quantitative evaluation of total RNA.

To obtain complementary DNA (cDNA), reverse transcription was performed using the Wizscript kit (Wizbio WizScript cDNA Synthesis Kit, Korea) according to the protocol, with the Rotor-Gene Q Software-Run device. The expression levels of oxidative/antioxidant genes (GPX1, NCF1, NOS2, SOD1) were analyzed.

Real Time-qPCR

Using the obtained cDNAs, the mRNA transcription levels of the target genes (GPX1, NCF1, SOD1, NOS2) were determined in TABLE I.

TABLE I
Primary sequence sequence of target genes

Gene	Primary sequence sequencing	
	F: 5′→3′	R: 5′→3′
Actin Beta (ACTB)	CTCCTCAAGGATGGCACC	GCTCATTGTAGAAAGTGTGGT
GPX-1	TCCACCGTGTATGCCTTCTC	TCTCTTCATTCTTGCCATTCTCC
NCF1	GTCGGAGAAGGTGGTCTACAG	CGATAGGTCTGAAGGATGATGG
SOD1	GCTTCTGTCGTCTCCTTGCT	CATGCTCGCCTTCAGTTAATCC
NOS2	TCTTCAGAGTCAAATCCTACCA	TCTATTTCCTTTACGGCTTCC

Optimized primer conditions were determined for each gene. An example of RT-qPCR reaction conditions is provided in TABLE II. The RT-qPCR reactions were performed using the ROTOR-GENE Q system (Qiagen, Germany). To determine gene expression patterns related to oxidative stress, the transcription levels of GPX1, NCF1, SOD1, and NOS2 were measured. ACTB (Actin Beta) was used as a control gene in the expression analysis. SYBR Green master mix (ENZO Life Science, cat: ENZ-NUC104-0200) was used for amplification detection. The Ct (cycle threshold) values were determined at the beginning of the logarithmic phase of the amplifications for each sample. The gene expression was analyzed using the 2-ΔΔCt method, and the fold changes in expression were compared to the control group.

Histopathological examination

At the end of the experiment, necropsies were performed on the rats, and samples of testis tissue were collected. The tissue pieces were fixed in Bouin's solution and underwent routine tissue processing. They were embedded in paraffin blocks, and 4 µm sections were obtained using a microtome (Leica, RM2235, Germany). The sections were stained with hematoxylin and eosin (H-E) and examined under a light microscope. Morphological findings were photographed and evaluated.

TABLE II
RT-qPCR Reaction conditions

Reaction content	For one sample	Reaction Cycle	
Tampon (2X)	10 µL	95°C | 2 min	denaturation
Primer and control Primer (Actin Beta)	Forward : 0.5 µl Reverse : 0.5 µl	95°C | 5 s *58°C – 60°C	40 cycle
dH2O	8.4 µL		
cDNA	0.6 µL		
Total	20 µL		

*: The binding temperature varied according to the primers. Melting Curve: Ramp 50–99°C (1°C increment) 90°C | 5 s

ELISA (FSH, LH, Testosterone)

Blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 1,118 G for 15 min to separate the serum. The serum samples were then analyzed using the FSH (AD3200 Ra), LH (AD1683 Ra), and Testosterone (AD1386 Ra) ELISA kits following the protocols provided by Andy Gene, USA.

Statistical analysis

Statistical analysis was performed using the SPSS v.20 software package (Chicago, IL, USA). All data were expressed as mean ± standard deviation. One-way ANOVA followed by post hoc multiple comparisons (Tukey's test) was used for comparative analysis between the groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Sperm examination

The motility and density parameters in the CP, CP+CoQ10 I, and CP+CoQ10 II groups were significantly lower compared to the other groups ($P<0.001$). Additionally, the rates of abnormal sperm and sperm DNA damage (FIG 1A-1F), were significantly higher in the CP, CP+CoQ10 I, and CP+CoQ10 II groups compared to the other groups ($P<0.001$)(TABLE III).

ELISA (Hormone levels)

In the CoQ10 group, serum levels of Testosterone, FSH, and LH showed a statistically significant increase compared to the other groups ($P<0.001$). On the other hand, in the CP group, the Testosterone level was significantly decreased compared to the other groups ($P<0.001$)(TABLE IV).

Oxidative stress

The GPX1 was statistically increased in the CoQ10, CP+CoQ10 I, and CP+CoQ10 II groups ($P<0.001$). NCF1 gene expression was significantly increased in the CoQ10 group ($P<0.001$) and decreased in the CP, CP+CoQ10 I, and CP+CoQ10 II groups ($P<0.001$). SOD1 was statistically increased in the CP+CoQ10 I and CP+CoQ10 II groups ($P<0.001$)(TABLE V).

In paired comparisons between the CP group and the CP+CoQ10 I and CP+CoQ10 II groups, it was found that sperm motility, density, and NCF1 gene expression statistically increased, while sperm DNA damage and abnormal sperm rates statistically decreased ($P<0.05$).

Histopathological findings

Microscopically, the testicular sections from the Control group (FIG.2A), Sham group (FIG.2B), and CoQ10 group (FIG.2C) exhibited normal histological appearances. Spermatogenesis was found to be higher in the testicles of rats in the CoQ10 group compared to the Control and Sham groups. In contrast, the CP group showed noticeable changes in the testicular tissue. There was diffuse loss of spermatozoa in the tubular lumens, and germ cells appeared dissociated from the

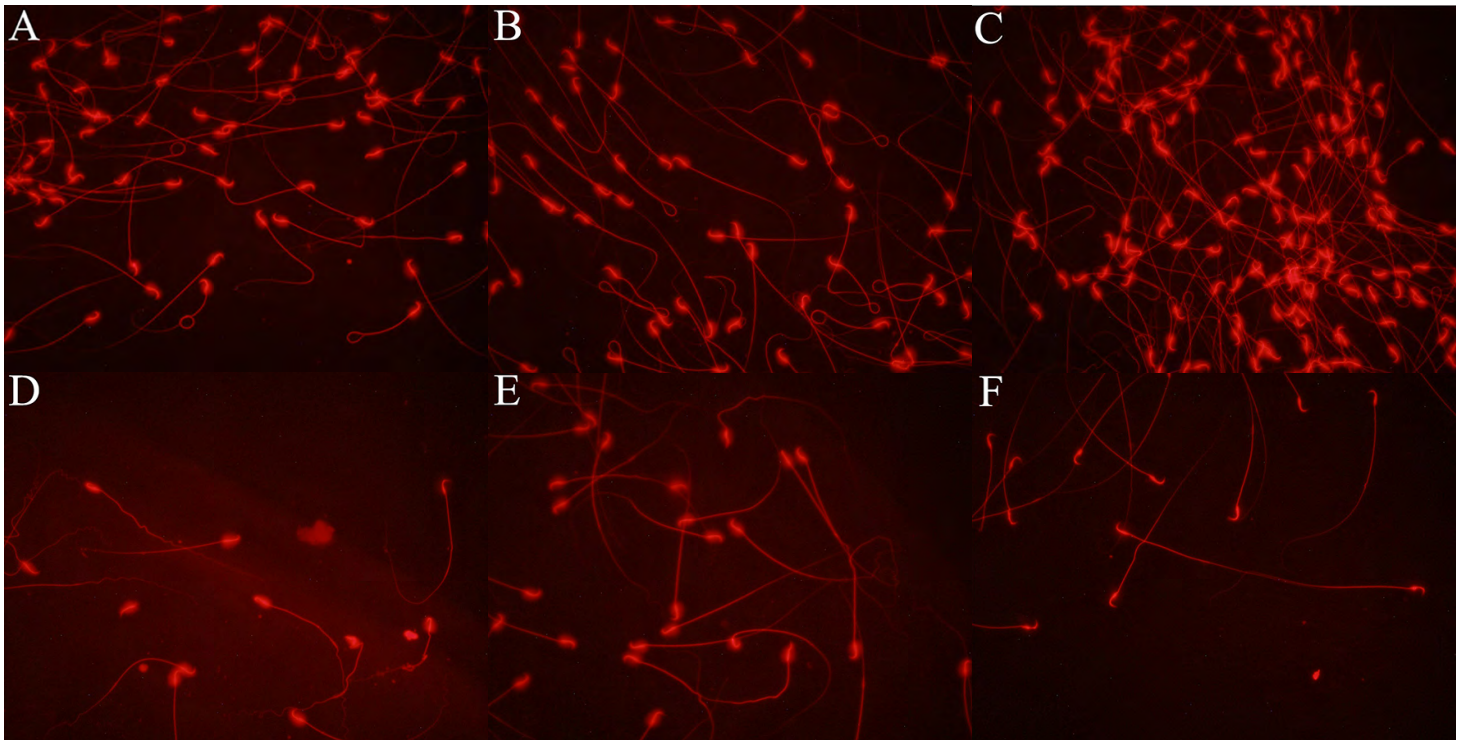

FIGURE 1. Sperm DNA damage. Control (A), Sham (B), CoQ10 (C), CP (D), CP+CoQ10 I (E) and CP+CoQ10 II (F) groups

TABLE III
Sperm parameters (Motility, Density, Abnormal sperm) and Sperm DNA Damage results

	Control	Sham	CP	CoQ10	CP+CoQ10 I	CP+CoQ10 II
Motility (%)	83.33±5.16	85.00±5.47	55.00±5.47*[a]	86.66±5.16	68.33±7.52*[b]	60.00±6.32*[b]
Density (x10^9)	2.19±0.15	2.20±0.13	1.69±0.06*[a]	2.34±0.05	1.96±0.09*[b]	1.8±0.19*[b]
Abnormal sperm (%)	17.00±1.26	16.33±1.21	44.66±3.72*[a]	16.50±1.37	30.16±4.21*[b]	37.83±3.43*[b]
Sperm DNA Damage (%)	15.3	15.5	42.6*[a]	11.6	30.1*[b]	33.6*[b]

*means: statistically significant difference between the groups in the same row (P<0.001), [a,b]means: statistically significant difference in pairwise comparisons (CP–CP+CoQ10 I and CP–CP+CoQ10 II) (P<0.05)

TABLE IV
Hormone levels (FSH, LH, Testosteron)

	Control	Sham	CP	CoQ10	CP+CoQ10 I	CP+CoQ10 II
FSH (pg·mL^{-1})	367.48±23.31	384.66±12.17	379.36±21.32	415.73±13.91*	370.40±35.69	382.39±44.08
LH (pg·mL^{-1})	220.01±26.35	232.11±64.56	251.83±19.20	308.53±19.50*	253.37±40.22	220.81±53.22
Testosterone (pg·mL^{-1})	167.80±14.75	172.77±7.28	136.38±21.17*	185.95±7.03*	168.90±14.85	166.72±7.84

*means; statistically significant difference between the groups in the same row (P<0.001)

TABLE V
Oxidative stress (GPX1, NCF1, SOD1, NOS2) results

	Control	Sham	CP	CoQ10	CP+CoQ10 I	CP+CoQ10 II
GPX1	1.11±0.17	0.90±0.16	1.20±0.20	2.73±0.39*	5.07±1.72*	2.50±0.49*
NCF1	1.10±0.09	0.81±0.05	0.19±0.18*[a]	5.34±1.68*	0.48±0.09*[b]	0.56±0.18*[b]
NOS2	0.95±0.15	0.85±0.63	0.87±0.41	1.20±0.07	1.02±0.28	1.06±0.28
SOD1	0.95±0.06	1.21±0.48	0.97±0.32	1.03±0.45	3.51±0.65*	3.99±0.59*

*means: statistically significant difference between the groups in the same row (P<0.001), [a,b]means: statistically significant difference in pairwise comparisons (CP–CP+CoQ10 I and CP–CP+CoQ10 II) (P<0.05)

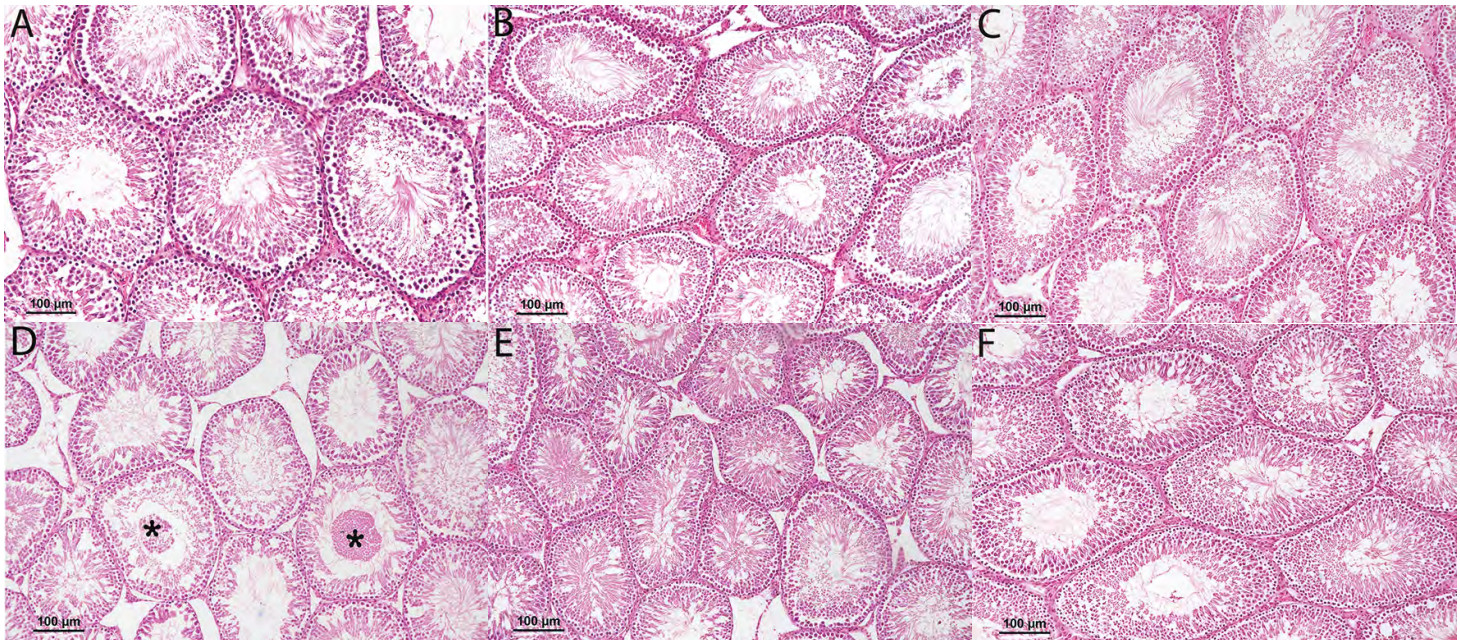

FIGURE 2. Histopatology of Testes. Histopathological appearance of cross–sections of the rat testes (H&E staining, Bar; 100 µm). Control (A), Sham (B) and CoQ10 (C) groups showed normal seminiferous tubule morphology and spermatogenic cells in advanced stages. CP group showed arrested spermatogenesis (*). The seminiferous tubules were irregular and shrunken. In the lumen of the seminiferous tubules desquamated degenerated spermatogonia were obserrved. In CP+CoQ10 I (E) and CP+CoQ10 II (F) groups the morphology of the seminiferous tubules were almost normal

basal membrane. Additionally, several accumulations of immature germinal cells were observed in the lumen of seminiferous tubules (FIG.2D). However, in the CP+CoQ10 I and CP+CoQ10 II groups, these histological abnormalities were significantly reduced, and the testis appeared closer to its normal histological appearance (FIG.2E–2F).

The testis is known to be highly susceptible to damage from chemotherapeutic agents, metals, and clastogenic substances [17]. The metabolites of chemotherapeutic drugs can disrupt DNA synthesis in cells undergoing meiosis and mitosis, leading to cell apoptosis and the production of reactive oxygen species (ROS) [18]. Cyclophosphamide (CP), a commonly used chemotherapeutic agent, can cause damage to the testicles. This damage is primarily attributed to the alkaline metabolites of CP, such as aldophosphamide mustard and acrolein [19].

In this study and others studies carried out by applying CP, it has been observed, that the sperm motility rate and density decrease, the ratio of abnormal sperm increases, and sperm DNA damage is enhanced. Additionally, histopathological damage to the testes, decreased testosterone levels, and increased oxidative stress have been detected [1, 3, 7, 19, 20, 21, 22, 23, 24, 25].

CoQ10 has been investigated in several studies for its effects on testicular damage caused by various agents, including cadmium [26], lead [27], radiation [17], and smoking [10] were investigated. However, in the literature review, no study has been found regarding the protective effect of CoQ10, administered at different time points, on the damage induced by CP application.

When comparing Testosterone levels, it was observed that the CP group had low levels, while the CoQ10 group had higher levels (P<0.001). The CP+CoQ10 I and CP+CoQ10 II groups exhibited intermediate levels. CoQ10 is believed to have a positive effect on Testosterone levels. Testosterone is produced through enzymatic processes involving StAR, P450, CYP11A1, CYP17A1, 3β–HSD, and 17β–HSD. CoQ10 enhances Testosterone levels by facilitating the transport of cholesterol to the outer mitochondrial membrane of StAR [28, 29, 30]. Conversely, CP decreases Testosterone levels by inducing atrophy and toxicity in Leydig cells [1, 21].

Sperm DNA damage was significantly higher in the CP, CP+CoQ10 I, and CP+CoQ10 II groups (P<0.001). However, it was observed that the CP+CoQ10 I and CP+CoQ10 II groups had lower DNA damage sizes compared to the CP group (P<0.05). Sperm DNA damage is closely associated with reactive oxygen species (ROS), lipid peroxidation, and antioxidants. This study, along with several others [3, 4, 5] has determined that CP leads to an increase in ROS and lipid peroxidation in the testicles. CoQ10, being a natural antioxidant and ROS scavenger, is naturally present in seminal fluid [31, 32]. These inherent properties of CoQ10 contribute to the reduction of sperm DNA damage and oxidative stress (P<0.05).

The motility rate was found to be lower in the CP, CP+CoQ10 I, and CP+CoQ10 II groups (P<0.001). However, it was observed that the motility rate was improved in the CP+CoQ10 I and CP+CoQ10 II groups (P<0.05). CoQ10 plays a role in maintaining the energy mobilization of spermatozoa through its interaction with mitochondria, thus enhancing the motility rate of sperm [9]. Mitochondria have various functions, including steroid synthesis, energy production, and apoptosis regulation [11]. CoQ10 helps minimize flagellum and axonemal damage in the spermatozoa structure, thereby maintaining the motility rate [21].

It was determined that the density decreased and the rate of abnormal sperm increased in the CP group (P<0.001). In the pairwise comparison between CP – CP+CoQ10 I and CP – CP+CoQ10 II, the protective effect of CoQ10 becomes evident (P<0.05). In this study and several studies on CP, it has been observed that CP leads to

cytoplasmic vacuolization in tubules, nuclear membrane disorders, necrotization [24], germ cell vacuolization [1], damage to the integrity of the germinal epithelium [7], and loss of germ cells [6]These histopathological changes in the testis have a negative impact on spermatogenesis, density, and the rate of abnormal sperm.

CONCLUSIONS

Based on the results of this study, it was concluded that CoQ10 can reduce the negative effects of Cyclophosphamide on testis, testicular oxidative stress, sperm DNA damage and reproductive hormones, and may have a protective effect on male reproductive fertility.

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Ethical statement

This study was approved by the Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (YUHAD-YEK, Date: 28/11/2019; Decision number: 2019/11).

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