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The effects of Coenzyme Q10, Fullerene C60, α–Lipoic Acid on reproductive system of pubertal male rats exposed to Bisphenol A

Los efectos de la coenzima Q10, el fullereno C60 y el ácido α–lipoico en el sistema reproductor de ratas macho púberes expuestas al bisfenol A

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ABSTRACT

Bisphenol A is widely recognized as a significant toxic environmental contaminant globally, primarily due to its extensive industrial and commercial applications. The current study aimed to evaluate the potential of Coenzyme Q10, Carbon–60 fullerene and α–Lipoic Acid, which are known for their significant antioxidant capacity, to reduce the reproductive toxic effects induced by Bisphenol A in male rats. A total of sixty prepubertal male Sprague Dawley rats were assigned to eight experimental groups. The substances were applied by oral gavage at dose–adjusted levels, dissolved in olive oil and/or water, once per day for a duration of seven weeks. The rats were decapitated 24 hours subsequent to the final application thereafter blood and testicular tissues were taken for analysis. The exposure of Bisphenol A resulted in significant elevation in serum and testicular malondialdehyde level and significant reduction in the level of antioxidant enzymes. Epididymal sperm concentration and motility were considerably lower in the Bisphenol A–exposed group against the control group. Marked histopathological changes were evident in the testicular tissue, characterized by degeneration and a notable decrease in germinal cell. It was determined that administration of Coenzyme Q10 and α–Lipoic Acid significantly prevented Bisphenol A–induced oxidative stress and associated complications such as testicular dysfunction and the decreased epididymal sperm quality. In conclusion, Bisphenol A negatively affected the reproductive system biochemically, histologically and reproductively in male rats; Coenzyme Q10, Carbon-60 fullerene, and α–Lipoic Acid alleviated these negative effects.

Key words: α–Lipoic Acid; Bisphenol A; Coenzyme Q10; Fullerene C60; sperm

RESUMEN

El bisfenol A está ampliamente reconocido como un importante contaminante tóxico del medio ambiente a nivel mundial, debido principalmente a sus amplias aplicaciones industriales y comerciales. El presente estudio pretendía evaluar el potencial de la coenzima Q10, el fullereno de carbono–60 y el ácido α–Lipoico, conocidos por su importante capacidad antioxidante, para reducir los efectos tóxicos reproductivos inducidos por el bisfenol A en ratas macho. Se asignó un total de sesenta ratas Sprague Dawley macho prepúberes a ocho grupos experimentales. Las sustancias se aplicaron por sonda oral a dosis ajustadas, disueltas en aceite de oliva y/o agua, una vez al día durante siete semanas. Las ratas fueron decapitadas 24 horas después de la última aplicación, tras lo cual se extrajeron sangre y tejidos testiculares para su análisis. La exposición al bisfenol A produjo una elevación significativa del nivel de malondialdehído sérico y testicular y una reducción significativa del nivel de enzimas antioxidantes. La concentración de espermatozoides en el epidídimo y su motilidad fueron considerablemente menores en el grupo expuesto al bisfenol en comparación al grupo control. Se evidenciaron marcados cambios histopatológicos en el tejido testicular, caracterizados por degeneración y una notable disminución de células germinales. Se determinó que la administración de coenzima Q10 y ácido α–Lipoico previno significativamente el estrés oxidativo inducido por el bisfenol A y las complicaciones asociadas, como la disfunción testicular y la disminución de la calidad del esperma epididimario. En conclusión, el bisfenol afectó negativamente al sistema reproductor desde el punto de vista bioquímico, histológico y reproductivo en ratas macho; la coenzima Q10, el fullereno de carbono–60 y el ácido α–Lipoico aliviaron estos efectos negativos.

Palabras clave: Ácido α–Lipoico; bisfenol A; coenzima Q10; fullereno C60; esperma

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INTRODUCTION

Bisphenol A (BPA), chemically known as 2,2–bis(4–hydroxyphenyl) propane, is a synthetic xenoestrogen and ranks among the most extensively produced industrial chemicals worldwide. It is predominantly employed in the synthesis of polycarbonate plastics, epoxy resins, and thermal papers. Due to its widespread industrial applications, BPA is frequently detected in a broad range of consumer goods, including the inner coatings of food and beverage cans, thermal receipt papers, plastic food containers, electronic devices, medical instruments, and dental sealants. This situation makes the risk of high exposure to BPA inevitable, especially due to contact with food and beverages, environmental contamination or occupational reasons [1]. Although contaminated food and beverages are the primary source of BPA, today BPA is found in water, air, soil and many products that come into contact with [2]. It has been determined that BPA has some estrogenic effects and it may adversely affect endocrine functions by binding to many steroid hormone receptors, especially thyroid and androgen hormone receptors as well as estrogen receptors at a certain level [3, 4]. BPA causes impaired development of reproductive organs, decreased testosterone levels and decreased sperm production in male mice and rats [5]. Moreover, the increase in Reactive Oxygen Species (ROS) production caused by BPA creates an imbalance between ROS and cellular antioxidant defense capacity and consequently causes oxidative stress. [6, 7].

The most important biochemical functions of Coenzyme Q10 (CEQ10), which is mostly found in cell membrane structures and mitochondria at the cellular level, can be expressed as being a strong antioxidant, its connection with mitochondrial energy utilization and its protective effect in maintaining the integrity of the phospholipid membrane structure of the cell [8, 9]. It has been shown that CEQ10 given as an external supplement helps to improve the mitochondrial functions of spermatozoa and gives positive results on infertility [9, 10].

Carbon-60 (C60) fullerene (FUL) is a kind of molecule composed completely of 60 carbon atoms, the third allotrope of carbon and the most foremost member of the nanomaterial class. FUL and its various functionalized derivatives have been reported to exert a broad spectrum of bioactive properties. These nanostructures have also been shown to confer robust protection against oxidative damage in both *in vitro* and *in vivo* [11]. However, there are very few studies on the effects of FUL on reproductive system functions and semen quality [11, 12].

α–Lipoic Acid (ALA), which is found in all cells and plays a fundamental role in mitochondrial dehydrogenase reactions and is soluble in both water and fat, has attracted attention as an important antioxidant substance in recent years. Exogenous ALA supplementation reduces oxidative stress and improves the levels of other antioxidants in many tissues under various physiological and pathological conditions *in vivo* [13].

On the basis of this information, the purpose of the current research was to determine the levels of testicular damage, decrease in epididymal semen quality and decrease in serum testosterone and antioxidant enzyme levels in male rats exposed to BPA from the early pubertal development period and to determine whether CEQ10, FUL and ALA given to the animals as supplements together with BPA have protective or corrective effects in terms of the determined parameters against these negative effects.

MATERIAL AND METHODS

Animals and Experimental Design

The 60 male Sprague–Dawley rats (*Rattus norvegicus*) used in the study. Animals were obtained from Firat University's Experimental Research Center (FÜDAM) in Elazığ, Türkiye. Rats were housed under standard conditions in accordance with the conditions for the care and use of laboratory animals 24 ± 3°C constant temperature, 60–65% humidity and special ventilation system. Throughout the study, animals were fed with standard commercial rat chow and water was provided *ad libitum*.

Ethical committee permission (at the meeting dated 31/10/2018 and numbered 2018/18, decision no 170, protocol no 2018/94) was taken from Firat University Local Ethics Committee for Animal Experiments.

Since the first external sign of pubertal development in male rats is the detachment of the preputial tissue from the glans penis, the onset of puberty was assessed by evaluating preputial separation on a daily basis starting from postnatal day (d) 30 in all groups [14].

After determination of the onset of puberty, the animals were randomly divided into 8 groups and placed in special cages, as follows: Group 1, Control group (n=7) and 0.2 mL olive oil were given. Group 2 was Bisphenol A group (BPA, n=8) and received 25 $mg \cdot kg^{-1} \cdot d^{-1}$ BPA in 0.2 mL olive oil [15]. Group 3 was Coenzyme Q10 group (CEQ10, n=7) and received 40 $mg \cdot kg^{-1} \cdot d^{-1}$ CEQ10 in 0.2 mL olive oil [16]. Group 4 was Fullerene C60 group (FUL, n=7) and received 8 $\mu g \cdot kg^{-1} \cdot d^{-1}$ FUL in aqueous form in 0.2 mL olive oil [12]. Group 5 was α–Lipoic Acid group (ALA, n=7) and received 70 $mg \cdot kg^{-1} \cdot d^{-1}$ ALA in 0.2 mL olive oil [17]. Group 6 was Bisphenol A + Coenzyme Q10 group (BPA + CEQ10, n=8) and received 25 $mg \cdot kg^{-1} \cdot d^{-1}$ BPA and 40 $mg \cdot kg^{-1} \cdot d^{-1}$ CEQ10 in 0.2 mL olive oil. Group 7 was Bisphenol A + Fullerene C60 group (BPA + FUL, n=8) and received 25 $mg \cdot kg^{-1} \cdot d^{-1}$ BPA in 0.2 mL olive oil and 8 $\mu g \cdot kg^{-1} \cdot d^{-1}$ FUL in aqueous form. Group 8 was Bisphenol A + α–Lipoic Acid group (BPA + ALA, n=8) and received 25 $mg \cdot kg^{-1} \cdot d^{-1}$ BPA and 70 $mg \cdot kg^{-1} \cdot d^{-1}$ ALA in 0.2 mL olive oil.

The animals in the groups were given chemicals [Bisphenol A (Aldrich, Taiwan), Coenzyme Q10 (Roth, Germany), α–Lipoic Acid (Merck, Germany)] and C60 Fullerene Hydrated Concentrated Solution (IPAC, Ukraine) prepared at appropriate doses by oral gavage method between 08:00–12:00 daily for 7 weeks from the beginning of the experimental phase.

Spermatological examinations

After the right cauda epididymis was separated and weighed with precision scales (Sartorius, Germany), it was thoroughly sliced and dissected in 1 mL of 0.9% physiological saline in a special petri dish for 2 min using a scalpel and a pair of forceps. After this process, the mixture was incubated at room temperature for 4 h to allow the spermatozoa in the epididymal tissue to pass into the liquid. Following the incubation period, sperm concentration in the cauda

epididymis was calculated with hemocytometric method using a hemacytometer (Marienfeld Superior, Germany) [18].

A blob of Tris buffer solution (Tris (hydroxymethyl) aminomethane 3.63 g, glucose 0.50 g, citric acid 1.99 g and distilled water 100 mL) were prepared on a slide placed on the warming plate of the microscope (Nikon, Japan) and the temperature was adjusted to 37°C, and a small drop of epididymal fluid taken by making a section in the left cauda epididymis was placed on it. This solution was mixed with the help of a coverslip and homogenized. After this, three different fields were examined under 400 magnification of the microscope and the motility rate was subjectively determined as percentage [19].

A blob of Tris buffer solution was placed on a clean slide set at 37°C and a small drop of epididymal fluid obtained by sectioning the left cauda epididymis with a scalpel was placed on it. This solution was mixed and homogenized with the help of a coverslip and a smear was taken with the help of a slide. A special Diff–Quik staining set (Medion Diagnostics / Bioz, Switzerland) was used for staining the smear. The prepared smear was examined under 400 magnifications of a light microscope. In one smear, 200 spermatozoa were examined and the total abnormal sperm ratio was expressed as percentage [20].

Determination of serum testosterone level

At the end of the experiment, testosterone levels in serum samples obtained from the animals were determined by ECLIA method [19]. The blood serum samples obtained from the animals were read on a special device (Cobas 6000, Roche–Hitachi, Germany) with the help of branded testosterone assay kits (Elecsys Testosterone II, Roche, Germany) and the results were recorded as "ng·mL^{-1}".

Oxidative stress analysis

Lipid peroxidation (LPO) levels in the samples were determined using a spectrophotometer (Shimadzu UV-1700, Japan), by measuring thiobarbituric acid reactive substances concentration [21]. Malondialdehyde (MDA), a secondary product generated during LPO, was utilized as a biomarker for oxidative stress. The absorbance of the sample supernatant was read at 532 nm against a blind, and MDA levels were expressed as "nmol·mL^{-1}".

The method established by Sedlak and Lindsay [22], was followed to detect the glutathione (GSH) level in the prepared tissue and serum samples. The prepared mixture was measured for absorbance at 412 nm using a spectrophotometer. GSH levels were expressed in "nmol·mL^{-1}".

The protocol described by Lawrence and Burk [23], was followed to determine glutathione peroxidase (GSH–Px) levels in the prepared tissue and serum samples. After this, the absorbance of the samples was measured at 340 nm for 5 min. GSH–Px activity was reported in "IU·L^{-1}".

Catalase (CAT) activity in prepared tissue and serum samples was determined following the method described by Goth [24]. The colorimetric change resulting from the enzymatic reaction was measured spectrophotometrically at 405 nm against a blank control. CAT activity was quantified based on the reduction of H_2O_2 levels and expressed as "kU·L^{-1}".

Histopathological examination

Bouin's solution was used to fix testicular tissues for 48 h. Then these samples were transferred to 80% ethanol overnight and embedded in paraffin following standard histological procedures. Paraffin–embedded testicular tissues were sectioned at 5 μm thickness using microtome (Leica, Germany), stained with Hematoxylin & Eosin, and evaluated under a light microscope for histopathological alterations. The degree of spermatogenic activity in tubuli seminiferi contorti (TSC) was evaluated according to Johnsen Testicular Scoring Method (TABLE I) [25]. Additionally, the diameters of TSC and the thickness of the germinal cell were measured using an ocular micrometer (Olympus, Japan).

Immunohistochemical examination

The Avidin–Biotin–Peroxidase technique was employed for immunohistochemical evaluation [26]. The sections were then incubated (StainTray slide staining system, Merck, Germany) with Bcl-2 (Bioss, USA, Catalog No: bs-0127R), Bax (Bioss, USA, Catalog No: bs-4563R) and Androgen Receptor (AR) (Bioss, USA, Catalog No: bs-0118R). In the final step, the sections were counterstained with Mayer Hematoxylin (MH), rinsed, coated with immune–mount and examined by light microscopy. Bax and Bcl-2 immunostaining were semi–quantitatively assessed. In AR immunostaining, AR positive germinal cells were counted under 400 magnification of the microscope, 5 TSCs were taken into consideration in 5 different random fields and a total of 25 TSCs were counted, and the data obtained were recorded.

TABLE I
Johnsen Testicular Scoring System

Score	Histological Findings
10	Numerous spermatozoa are present in the TSC lumen. Spermatogenesis is complete, the germinal epithelium is of regular height and the TSC lumen has a normal diameter.
9	Numerous spermatozoa are present in the TSC lumen. There is disorganization of the germinal epithelium (loss of structural integrity) and sequestration of germinal cells (separation of non–viable tissue from viable tissue).
8	Fewer than 5-10 spermatozoa are present in each TSC lumen.
7	Each TSC contains numerous spermatids, spermatocytes and spermatogonium. There are no spermatozoa in the TSC lumen.
6	Each TSC contains numerous spermatocytes, spermatogonium and 5-20 spermatids. There are no spermatozoa in the TSC lumen.
5	Each TSC contains numerous spermatogonium and spermatocytes. However, there are no spermatozoa and spermatids in the TSC lumen.
4	There are numerous spermatogonium in each TSC but fewer than 5 spermatocytes. There are no spermatozoa and spermatids in the TSC lumen.
3	In TSCs, only the spermatogonium is present.
2	Germinal cells are completely obliterated and only Sertoli cells are present.
1	All TSCs lack cells.

TSC: Tubuli seminiferi contorti

Statistical analysis

SPSS (Version 22.0) statistical program was used for statistical analyses. All data obtained were presented as mean ± standard deviation (SD). For the evaluation of the data, normality test was performed first. For data showing normal values, one–way analysis of variance (ANOVA) was used to determine the differences between groups and Tukey test was used for pairwise comparisons. On the other hand, for nonparametric data, Kruskal–Wallis test was used to determine the differences between groups and Mann–Whitney–U test was used for pairwise comparisons. In all analyses, P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Various toxic substances have adverse effects on testes and sperm in rats. [27, 28]. One of these toxic agents is BPA. Today, BPA, a phenolic chemical substance, is one of the leading toxic environmental pollutants worldwide [29].

In the present study, the mean epididymal sperm characteristics and serum testosterone levels of the rats in the groups in the study are presented in TABLE II.

TABLE II
Epididymal sperm characteristics and serum testosterone levels of the study groups (mean ± SD)

Groups	Epididymal Sperm Motility (%)	Epididymal Sperm Concentration ($\times 10^6$)	Abnormal Sperm Rate (%)	Testosterone Level (ng·mL^{-1})
Control	76.81 ± 6.39^{abc}	364.71 ± 49.36^{ab}	7.07 ± 1.84	3.57 ± 2.00
BPA	62.08 ± 8.53^{d}	287.25 ± 35.50^{c}	7.63 ± 1.55	3.10 ± 1.49
CEQ10	86.19 ± 4.05^{a}	361.29 ± 40.72^{ab}	6.29 ± 1.41	4.29 ± 1.66
CEQ10 + BPA	76.66 ± 7.56^{abc}	322.75 ± 44.17^{bc}	6.81 ± 1.03	3.41 ± 1.96
FUL	76.68 ± 7.20^{abc}	333.14 ± 37.95^{abc}	6.15 ± 1.70	3.87 ± 2.04
FUL + BPA	70.00 ± 10.37^{cd}	312.14 ± 45.88^{c}	7.15 ± 1.46	3.29 ± 1.15
ALA	83.80 ± 4.05^{ab}	377.14 ± 47.37^{a}	6.29 ± 1.52	3.96 ± 1.60
ALA + BPA	72.45 ± 8.59^{bc}	326.25 ± 35.56^{bc}	7.44 ± 1.90	3.53 ± 1.68
P	0.001	0.002	not statistically significant	not statistically significant

[a, b, c]: The difference between values with different letters in the same column is statistically significant ($P<0.01$). BPA: Bisphenol A, CEQ10: Coenzyme Q10, ALA: Alpha lipoic acid, FUL: Carbon 60 Fullerene

The present study demonstrated that epididymal sperm motility and concentration were significantly lower in the BPA alone group compared to the control group. In a study conducted was found a significant decrease in epididymal sperm motility in rats exposed to BPA for 60 d [29]. The results of other experimental studies also revealed that BPA exposure caused a decrease in sperm quality, and this was associated with infertility [30, 31]. The results of present study are similar to the results of the studies mentioned above.

It was observed a significant increase in epididymal sperm concentration and motility, and a significant improvement in spermatogenesis ($P<0.01$), and a significant decrease in testicular damage ($P<0.01$) were found in the group administered CEQ10 with BPA and ALA with BPA compared to the group administered BPA alone. However, although a partial increase in epididymal sperm concentration and motility and a slight improvement in terms of testicular damage were observed in the group administered FUL with BPA compared to the BPA group. In a study, an increase in seminal plasma CEQ10 levels was reported to positively affect the motility of spermatozoa by leading to an improvement in mitochondrial oxidative phosphorylation [32]. Likewise, these findings are similar to the results of a research which was reported that FUL administration provided significant protection against damage to seminiferous tubules in the testes of diabetic male rats [12].

In studies conducted on BPA effects, it is possible to find different results especially regarding the relationship between testosterone level and BPA level [33, 34]. This paper presents the testosterone levels of the animals in the BPA group showed a numerical decrease compared to the control group. However, this difference was not statistically significant ($P>0.05$).

MDA and antioxidant enzyme levels (GSH, GSH–Px, and CAT) in blood serum and testicular tissue of the study groups are shown in TABLE III and IV.

TABLE III
Serum MDA and antioxidant enzyme levels of the study groups (mean ± SD)

Groups	MDA (nmol·mL^{-1})	GSH (nmol·mL^{-1})	GSH–Px (IU·L^{-1})	CAT (kU·L^{-1})
Control	2.92 ± 0.39^{a}	0.253 ± 0.029	4.06 ± 0.28^{a}	225.04 ± 88.93^{a}
BPA	4.52 ± 0.60^{c}	0.220 ± 0.016	3.15 ± 0.34^{b}	121.69 ± 23.14^{c}
CEQ10	2.86 ± 0.29^{a}	0.256 ± 0.035	4.06 ± 0.41^{a}	203.03 ± 27.28^{ab}
CEQ10 + BPA	2.96 ± 0.25^{a}	0.246 ± 0.022	3.56 ± 0.38^{ab}	174.52 ± 23.32^{ab}
FUL	3.02 ± 0.36^{a}	0.236 ± 0.018	4.05 ± 0.35^{a}	193.78 ± 28.92^{ab}
FUL + BPA	3.90 ± 0.52^{bc}	0.239 ± 0.030	3.83 ± 0.68^{a}	159.51 ± 37.49^{bc}
ALA	2.93 ± 0.15^{a}	0.273 ± 0.067	4.10 ± 0.32^{a}	191.92 ± 71.97^{ab}
ALA + BPA	3.69 ± 0.29^{b}	0.246 ± 0.038	3.91 ± 0.26^{a}	140.30 ± 32.02^{bc}
P	0.001	not statistically significant	0.001	0.001

[a, b, c]: Different letters in the same column are statistically significant ($P<0.01$). BPA: Bisphenol A, CEQ10: Coenzyme Q10, ALA: Alpha lipoic acid, FUL: Carbon 60 Fullerene, MDA: Malondialdehyde, GSH: Glutathione, GSH–Px: Glutathione peroxidase, CAT: Catalase

In the present study, it was found that serum and testicular tissue MDA levels increased significantly ($P<0.01$) in the BPA–treated group compared to the control group; in addition, antioxidant enzyme levels showed a tendency to decrease in number and especially CAT levels decreased significantly ($P<0.01$). In this study, it was observed that MDA and antioxidant enzyme levels in the group administered CEQ10 alone were at similar levels with the control group, whereas in the group administered CEQ10 together with BPA, MDA levels decreased significantly compared to the BPA group and antioxidant enzyme levels increased and reached the control group levels. These data show that CEQ10 supplementation has a very important effect in preventing oxidative stress caused by BPA in animals. On the other hand, in this study, it was determined that MDA and antioxidant enzyme levels in the FUL

TABLE IV
Testicular tissue MDA and antioxidant enzyme levels of the research groups (mean ± SD)

Groups	MDA (nmol·g^{-1} prot.)	GSH (nmol·g^{-1} prot.)	GSH–Px (IU·g^{-1} prot.)	CAT (k·g^{-1} prot.)
Control	2.68 ± 0.36^{ab}	0.287 ± 0.031	13.70 ± 1.12	30.36 ± 6.07^{a}
BPA	3.73 ± 0.63^{c}	0.223 ± 0.014	13.40 ± 2.38	21.25 ± 1.98^{b}
CEQ10	2.51 ± 0.32^{a}	0.243 ± 0.037	13.55 ± 1.29	28.83 ± 4.46^{a}
CEQ10 + BPA	2.88 ± 0.36^{ab}	0.228 ± 0.026	13.36 ± 1.19	25.77 ± 3.18^{ab}
FUL	2.59 ± 0.32^{a}	0.243 ± 0.046	14.05 ± 1.59	31.07 ± 9.31^{a}
FUL + BPA	3.28 ± 0.42^{bc}	0.231 ± 0.027	13.42 ± 1.62	26.82 ± 1.66^{ab}
ALA	2.50 ± 0.15^{a}	0.237 ± 0.020	13.79 ± 1.16	27.84 ± 3.37^{ab}
ALA + BPA	3.02 ± 0.22^{ab}	0.235 ± 0.035	13.62 ± 2.64	26.58 ± 1.88^{ab}
P	0.001	not statistically significant	not statistically significant	0.004

prot.: Protein, $^{a, b, c}$: Different letters in the same column are statistically significant (P<0.01). BPA: Bisphenol A, CEQ10: Coenzyme Q10, ALA: Alpha lipoic acid, FUL: Carbon 60 Fullerene, MDA: Malondialdehyde, GSH: Glutathione, GSH–Px: Glutathione peroxidase, CAT: Catalase

TABLE V
TSC diameter, germinal cell thickness and Johnsen Testicular Scoring values (mean ± SD)

Groups	TSC Diameter (µm)	Germinal Cell Thickness (µm)	Johnsen Score
Control	245.50 ± 1.87^{bc}	84.80 ± 0.63^{a}	9.45 ± 0.16
BPA	222.20 ± 1.50^{e}	56.10 ± 0.66^{d}	8.88 ± 0.30
CEQ10	250.69 ± 1.41^{a}	80.40 ± 0.53^{ab}	9.43 ± 0.20
CEQ10 + BPA	246.13 ± 1.25^{abc}	75.56 ± 0.44^{bc}	9.33 ± 0.24
FUL	244.85 ± 1.46^{c}	79.71 ± 0.62^{ab}	9.42 ± 0.20
FUL + BPA	238.40 ± 1.58^{d}	69.03 ± 0.70^{c}	9.29 ± 0.29
ALA	249.77 ± 1.56^{abc}	86.55 ± 0.77^{a}	9.50 ± 0.25
ALA + BPA	234.86 ± 1.80^{d}	80.85 ± 0.70^{ab}	9.00 ± 0.19
P	0.001	0.001	not statistically significant

$^{a, b, c, d, e}$: Different letters in the same column are statistically significant (P<0.01). BPA: Bisphenol A, CEQ10: Coenzyme Q10, ALA: Alpha lipoic acid, FUL: Carbon 60 Fullerene, TSC: Tubuli seminiferi contorti

alone treated group showed similar levels with the control group (P>0.05), while in the group treated with FUL together with BPA, MDA levels decreased numerically and antioxidant enzyme levels increased compared to the BPA group, but the difference was found to be statistically insignificant (P>0.05). This result shows that the effect of FUL in preventing the oxidative stress caused by BPA is limited. As presented in the study, serum and testicular tissue MDA levels in the group administered ALA alone were similar to the control group, while in the group administered ALA with BPA, MDA levels were significantly decreased (P<0.01) compared to the BPA group. Exposure to environmental toxic substances such as BPA increases ROS production, leading to inadequate testicular antioxidant system and testicular oxidative stress [29].

In other study, it has been reported that CEQ10 supplementation significantly decreases free radical levels and significantly increases the total antioxidant capacity in the body, especially CAT and SOD [35]. On the other hand, the effect of CEQ10 on MDA has been investigated by many scientists and it has been found to significantly decrease MDA levels [36]. In another research, ALA was found to significantly reduce MDA levels [37]. Similarly, other researchers have found that plasma MDA levels were higher in diabetic rats compared to normal rats of the same age and observed that supplementation of these rats with ALA decreased MDA to basal levels along with other oxidative stress parameters [38]. This finding is similar to the results of the above studies.

Histopathologic evaluations of TSC diameter, germinal cell thickness and Johnsen Scores determined with ocular micrometer are shown in TABLE V.

In terms of TSC diameter averages, the highest diameter value was observed in the group given CEQ10 alone, while the best value among the treatment groups was observed in the group given CEQ10 together with BPA. On the other hand, when a general evaluation was made in terms of germinal cell thickness, it was determined that the closest germinal cell thickness to the control group was found in the group given ALA alone, while the most significant increase in germinal cell thickness among the treatment groups was found in the BPA+ALA group.

AR, Bax and Bcl–2 immunostaining mean score values of all groups are given in TABLE VI.

When the groups in the study were evaluated in terms of AR positive cells, it was determined that the count of germinal cells positive for AR was highest in ALA and CEQ10 groups, while AR positivity was significantly lower (P<0.01) in the group given BPA alone (FIG. 1). Analysis of Bax immunostaining intensity and distribution revealed that apoptosis was highest in the BPA group and lowest in the control, CEQ10, and ALA groups (FIG. 2). When the antiapoptotic protein Bcl-2 positivity in TSC was evaluated in general, no statistically significant difference was observed between the study groups (P>0.05).

TABLE VI
AR, Bax and Bcl-2 immunostaining mean score values in the testes of animals in the research groups (mean ± SD)

Groups	AR	Bax	Bcl-2
Control	46.53 ± 0.63^{bc}	0.72 ± 0.04^{a}	0.92 ± 0.03
BPA	30.55 ± 0.71^{d}	0.97 ± 0.04^{c}	0.85 ± 0.04
CEQ10	48.06 ± 0.74^{a}	0.69 ± 0.05^{a}	0.91 ± 0.04
CEQ10 + BPA	42.11 ± 0.66^{b}	0.83 ± 0.04^{b}	0.86 ± 0.03
FUL	45.03 ± 0.49^{b}	0.76 ± 0.04^{ab}	0.92 ± 0.03
FUL + BPA	38.28 ± 0.87^{c}	0.81 ± 0.05^{b}	0.86 ± 0.04
ALA	49.73 ± 0.58^{a}	0.71 ± 0.05^{a}	0.89 ± 0.04
ALA + BPA	44.14 ± 0.66^{b}	0,85 ± 0.05^{b}	0.79 ± 0.04
P	0.001	0.001	not statistically significant

$^{a, b, c, d}$: Different letters in the same column are statistically significant (P<0.01). BPA: Bisphenol A, CEQ10: Coenzyme Q10, ALA: Alpha lipoic acid, FUL: Carbon 60 Fullerene, AR: Androgen receptor

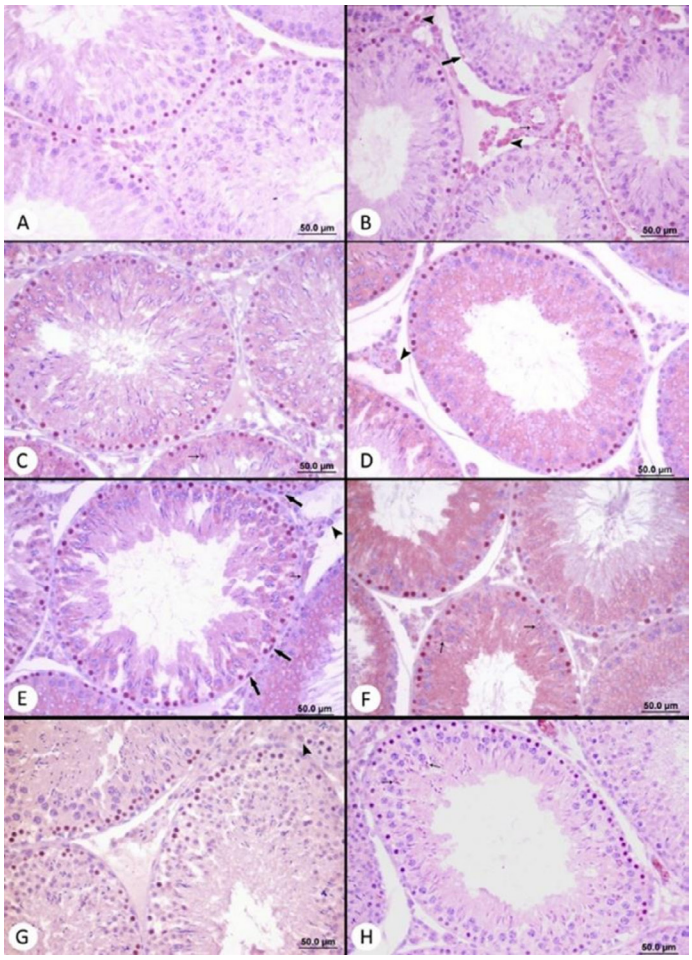

FIGURE 1. Androgen receptor (AR) immunostaining results. (A) AR positivity localized in spermatogonium of the control group, (B) AR expression observed in Leydig cells (arrowheads), myoepithelial cells (large arrow), vascular smooth muscle cells, and sparse spermatogonium in the BPA group, (C) predominant AR positivity in spermatogonium and few spermatids (small arrow) in the ALA group, (D) AR immunoreactivity confined to Leydig cells (arrowhead) in the BPA+ALA group, (E) AR expression detected in spermatogonium of the FUL group, as well as Sertoli cells (large arrows) and Leydig cells (arrowhead) (F) AR positivity noted in spermatids (small arrows) alongside spermatogonium in the BPA+FUL group, (G) mild AR staining in Sertoli cells (arrowhead) with spermatogonium in the CEQ10 group, (H) AR positivity present in spermatids (small arrows) and spermatogonium in the BPA+CEQ10 group, MH ×200 [Original]

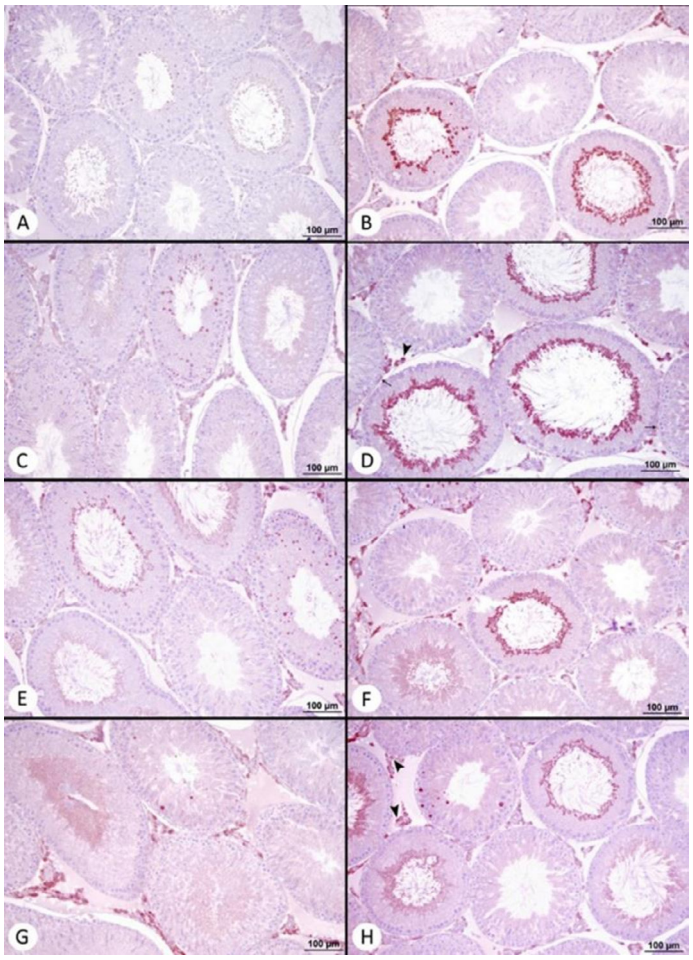

FIGURE 2. Bax immunostaining results. (A) Bax positivity observed in the germinal epithelium of TSC in the control group, (B) Bax expression detected in spermatocytes, Leydig cells, and germinal cells within TSCs in the BPA group, (C) mild Bax positivity present in Leydig cells and germinal epithelium in the ALA group, (D) Bax immunoreactivity noted in spermatocytes, Leydig cells (arrowhead), and myoepithelium (small arrow) in the BPA+ALA group, (E) Bax positivity identified in Leydig cells along with germinal epithelium and spermatocytes in the FUL group, (F) Bax expression evident in germinal epithelium, spermatocytes, and Leydig cells in the BPA+FUL group, (G) pronounced Bax positivity localized in Leydig cells in the CEQ10 group, (H) Bax positivity observed in Leydig cells (arrowheads) in the BPA+CEQ10 group, MH ×100 [Original]

Testicular germ cell apoptosis occurs naturally and continually throughout life. Proapoptotic (Bax) and antiapoptotic (Bcl–2) proteins are common during the peak of apoptosis following cellular stress [39]. The apoptotic pathway involves the proteins Bax and Bcl–2. Increased Bcl–2 protein expression demonstrates lower apoptosis, but increased Bax protein expression indicates apoptosis [40]. Previous studies have demonstrated that BPA leads to apoptosis in tissues such as brain and testis [41, 42] by increasing the Bax ratio and decreasing the Bcl–2 ratio. This increase in apoptosis may be due to BPA–induced increased oxidative stress. AR has important roles in spermatogenesis and fertility and moderates the effects of androgens [43]. Previous studies have reported that BPA adversely affects AR through multiple mechanisms [44, 45].

CONCLUSIONS

Serum and testicular MDA levels were significantly elevated ($P<0.05$), some antioxidant enzyme levels decreased ($P<0.05$), TSCs in the testes decreased in diameter and germinative cell layer, and epididymal sperm concentration and motility decreased ($P<0.05$). It can be said that supplementation of CEQ10 or ALA to humans or animals with infertility problems as a result of BPA exposure may be effective in preventing the negative effects of BPA on reproduction and/or mitigating the damage caused. However, although FUL seems to be less effective in preventing these effects, the level of efficacy should be examined with new studies involving different doses and application times.

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable requests.

Author Contributions

Tutku Can ACISU and Mustafa SÖNMEZ: Conceptualization, supervision, methodology, writing–original draft preparation, review & editing. Serkan Ali AKARSU, Gaffari TÜRK, Seyfettin GÜR, Şeyma ÖZER KAYA, and Serap DAYAN CİNKARA; Methodology, data curation. Songül ÇERİBAŞI, Özgür BULMUŞ and Abdurrauf YÜCE; Methodology. conceptualization, validation.

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