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Protective role of alpha lipoic acid against polychlorobiphenyl (Aroclor 1254)-induced testicular toxicity in rats

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Abstract

The present study was aimed to investigate the antioxidant, biochemical, and histological effects of alpha lipoic acid (ALA) on polychlorinated biphenyl (PCB)-induced testicular toxicity in male rats. The rats were divided into five groups: In the control group, the rats were not administered any chemicals for 30 days. In the sham group, the rats were administered corn oil for 30 days. In the PCB group, the rats were administered with Aroclor 1254 for 30 days. In the ALA group, the rats were treated with ALA for 30 days. In the ALA+PCB group, the rats were treated with ALA 24 hours before Aroclor 1254 was administered for 30 days. The total oxidant status (TOS) level in the serum and testis, number of apoptotic cells, vacuolization at the basal membrane, immature spermatids in the tubular lumen, heme oxygenase-1 (HO-1) staining density, and abnormal spermatozoa were significantly increased in the PCB group. Moreover, in the PCB group, the seminiferous tubule diameter (STD) was decreased in stage VII–VIII and XII–XIV tubules. The TOS level in the serum and testis, vacuolization at the basal membrane, immature spermatids in the tubular lumen, and apoptosis were significantly decreased in the ALA+PCB groups. These findings suggested that ALA has a protective role against PCB-induced testicular toxicity.

Keywords: rat, polychlorinated biphenyl, alpha lipoic acid, heme oxygenase-1, testicular histomorphometry, TUNEL.

Introduction

The function and structure of the testicular epithelial tissue in humans and animals is adversely affected by many natural and artificial environmental pollutants such as polychlorinated biphenyls (PCBs) [1], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [2], insecticides [3], xylene, and formaldehyde [4]. PCBs are used as hydraulic oils in vacuum pumps, heat-exchange systems, pesticides, transformers, and capacitors and as additives in adhesives, inks, paints, plastics, copying paper, and sealants. PCBs can spread throughout the ecosystem, including water, air, and soil [5, 6]. Food ingestion is the main line of PCB exposure in the general population. PCBs accumulate more extensively in fatty tissues, because of their great lipophilicity [6].

PCBs have a chemical formula consisting of $C_{12}H_{10-n}Cl_n$. The PCB family forms 209 different congeners [6]. Aroclor 1254 is a commercial mixture of PCBs, in which the mixture contains approximately 54% chlorine by weight [7].

Previous studies suggested that Aroclor 1254 has many deleterious effects on the male reproductive system in humans and animals. Aroclor 1254 exposure reduces body, testis and epididymis weights [7], also degenerates seminiferous tubules [5] and damages spermatogenesis [7]. Moreover, it decreases the diameter of seminiferous tubules, germinal cell layer thickness [5], steroidogenic enzyme activity [8], serum testosterone level [8, 9], epididymal sperm count and sperm motility [5, 10]. On the contrary, it increases abnormal sperm rate [5, 10], proapoptotic Bax gene expression [5] and terminal dUTP nick end labeling (TUNEL)-positive apoptotic cells in the testis [10]. PCB exposure may occur in the production of reactive oxygen species (ROS) [hydroxyl radical (HO•), hydrogen peroxide (H_2O_2)], increase lipid peroxidation, and decrease the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) in epididymal sperm [11] and the mitochondrial fraction of testis [7]. PCBs lead to testis toxicity by induction of oxidative stress in the mitochondrial fraction of the testis [7].

Alpha lipoic acid (ALA) is an antioxidant substance found in mitochondria as a coenzyme. Experimental and clinical studies have shown the potential usefulness of exogenous lipoic acid as a therapeutic agent for the prevention and treatment of free radical-related diseases [12]. Decreasing SOD, CAT, GPx, GR, and testosterone and increasing levels of the malondialdehyde (MAD) in cyclophosphamide (CP)-administered rats were restored toward normal values in the testis of rats that received an injection of ALA before administration of CP [13, 14]. Decreased antioxidant levels, increased lipid peroxidation and the number of abnormal germ cell induced by adriamycin (ADR) were substantially normalized by ALA pretreatment [15].

Heme oxygenase (HO) is an antioxidant substance that plays a role in heme catabolism. Three isoforms of HO (HO-1, -2, -3) have been identified in mammalian cells [16]. HO-1 is expressed in the testis [17] and may have an antiapoptotic effect [16].

Apoptosis, also called programmed cell death, normally occurs in the testis. It has a critical role during the development of spermatogenesis that provides homeostasis of germ cells [18]. Additionally, apoptosis has a major role in the elimination of damaged germ cells because of exposure to environmental pollutants, heat, and chemotherapeutic agents in later life [19].

ALA may prevent adverse effects on the synthesis and use of testosterone, spermatogenesis, apoptosis, and

antioxidant mechanism of PCB in the testis. The aim of the present study was to investigate the antioxidant, histological, and biochemical effects of ALA on PCB-induced testicular toxicity. This study is the first to investigate the protective role of ALA against PCB-induced testicular toxicity.

Materials and Methods

Animals and treatments

All experiments with animals performed in this study were reviewed and approved by the University of Adnan Menderes Institutional Animal Ethics Committee (2010/055). Forty healthy adult male Wistar rats (180–200 g, 12 weeks old) were used in the present study. The animals were obtained from the Experimental Research Centre (Aydın, Turkey) of Adnan Menderes University and were housed in standard plastic cages under standard laboratory conditions (temperature 24±1^{0}C, a 12-hour light/dark cycle). A laboratory rodent diet (Optima Food Company, Izmir, Turkey) and clean drinking water were provided *ad libitum*. The implementation period was 30 days. The animals were randomly divided into five groups consisting of eight rats each. The control group received no application for 30 days. The sham group was administered corn oil at a dose of 2 mL/kg body weight (bw)/day by oral gavage for 30 days. The PCB group was dosed daily with Aroclor 1254 (Sigma 48586) (5 mg/kg bw/day by oral gavage) dissolved in 2 mL/kg bw corn oil for 30 days. The ALA group was administered α-lipoic acid (ALA, Sigma T5625) (25 mg/kg bw/day by oral gavage) dissolved in 2 mL/kg bw corn oil for 30 days. The ALA+PCB group was treated with ALA (dissolved in corn oil, 2 mL/kg bw) at a dose of 25 mg/kg bw and one hour later PCB (dissolved in corn oil, 2 mL/kg bw) at a dose of 5 mg/kg bw for 30 days.

Sample collection and preparation

Rats were weighed at 24 hours after the last treatment. Blood was collected from the heart by cardiac puncture in clean serum separation tubes under Ketamine (20 mg/kg bw, intraperitoneally) and Xylazine (80 mg/kg bw, intraperitoneally) anesthesia, allowed to clot at room temperature and then centrifuged at 3500 rpm for 15 minutes. Serum subsequently was removed and stored at -80^{0}C for biochemical analyses.

Rats were killed by cervical dislocation after the blood samples were taken. The testes and epididymides were removed and cleaned of adhering connective tissue. The testes were weighed. The right testes were fixed in Bouin's fluid for 24 hours for histological examinations. After fixation, tissue samples were transferred to 50% ethanol for 48 hours and then to 70% ethanol for 12 hours, processed through graded alcohols, cleaned in xylene, and embedded in paraffin [20]. The left testes were also fixed in liquid nitrogen. After fixation, the testes were stored at -80^{0}C for biochemical and western blot (HO-1) analyses.

Homogenate preparation for biochemical assay

The testes were removed from the deep freezer and cleaned of adhering connective tissue. Then, the tissue samples were diluted at 1:10 (*v/v*) with phosphate-buffered saline (pH 7.4) in cold glass tubes. For the analysis of total antioxidant status (TAS) and total oxidant status (TOS), tissue samples were homogenized with homogenizer (IKA Overhead Stirrer; Germany) at 2000 rpm for 3 minutes. The homogenates were centrifuged at 11 000 rpm at 4^{0}C for 10 minutes.

Histological techniques

The paraffin tissue blocks were cut serially at intervals of 300 μm and thickness of 6 μm. The histological sections were placed on glass slides. Crossman's triple staining method [21] was used for histomorphometrical analysis and the evaluation of histological changes in the testis.

Histological changes

Two sections from each animal were evaluated under a research microscope for qualitative analysis of histological changes such as vacuolization at the basal membrane, and immature spermatids in the tubular lumen and were scored as follows: 0 – negative, 1 – low, 2 – moderate, and 3 – high.

Histomorphometry

A total of eight serial sections were used for histomorphometrical analysis from each rat. Twenty round or nearly round stage VII–VIII and XII–XIV tubules were randomly selected for each section [22]. The seminiferous tubule diameter (STD) and the seminiferous epithelial height (SE) were measured in eight serial sections (approximately 160 diameter and height measurements per animal). Twenty-five tubules were observed in each section (approximately 200 tubules per animal), and the percentage incidence of the stage XIV tubules was documented [23].

Histochemical staining (TUNEL staining)

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International S7101) was applied to detect the apoptotic cells in two sections from each animal. TUNEL-labeled germ cells were determined in at least 200 seminiferous tubules per testis. The number of TUNEL-positive cells was established per individual seminiferous tubule. Additionally, the apoptosis percentage was calculated by the ratio of the positive seminiferous tubules of apoptosis to the total number of seminiferous tubules in the cross-sections [24].

Immunohistochemistry of HO-1

The expression of HO-1 was investigated with an immunohistochemistry streptavidin–biotin complex (sABC) staining method using a rabbit anti-HO-1 polyclonal antibody (unconjugated, Abcam ab79854) in three sections from each animal. The sections were evaluated under a research microscope for qualitative analysis of HO-1 staining intensity and were scored as follows: 0 – negative, 1 – low, 2 – poor, 3 – moderate, and 4 – high. Twenty round or nearly round tubules were randomly selected on each section (approximately 60 tubules per animal). HO-1 positive cells were stained brown.

SDS-PAGE and western blotting

The levels of HO-1 protein were detected with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and western blotting. Proteins were separated by SDS-PAGE and transferred to polyvinyl difluoride (PVDF, Immobilon-PSQ ISEQ00010) as described by Laemmli [25]. Western blots were performed using an anti-HO-1 polyclonal antibody (unconjugated, Abcam ab79854). Protein bands were visualized by using enhanced chemiluminescence detecting reagent. The intensity of the HO-1 protein bands was quantified using UVP Life Science software (Cambridge, UK).

Biochemical assays

Determination of serum testosterone levels

The serum testosterone level was measured with the enzyme-linked immunosorbent assay (ELISA) method using a commercial ELISA testosterone kit (Cayman Chemical 582701, 96-wells kit). The results were reported as ng/mL.

Determination of total oxidant and antioxidant status levels in serum and testis

The serum and testicular tissue TOS levels were determined with a spectrophotometer (Shimadzu UV-1601, Japan) using a commercial total oxidant status-TOS assay kit (Rel Assay Diagnostics, RL0024). The results were reported as μmol H_2O_2 equivalents (equiv.)/g protein.

The serum and testicular tissue TAS levels were determined with a spectrophotometer (Shimadzu UV-1601, Japan) using a commercial total antioxidant status–TAS assay kit (Rel Assay Diagnostics, RL0017). The results were reported as mmol Trolox equiv./L.

Sperm morphology

The semen samples were isolated from the epididymal tissue of each rat. Smears were prepared on clean glass slides with the Eosin–Nigrosin staining method as described by Hackett & Macpherson [26]. A total of 200 spermatozoa were analyzed on each slide per animal. Total abnormality rates of spermatozoa were expressed as a percentage.

Statistical analysis

SPSS (for Windows, version 15.0) was used for statistical analysis. The results were expressed a mean ± standard deviation (SD). Between-groups differences of the values of the body weight, testis weight, stage VII–VIII and XII–XIV STD and SE, serum testosterone levels, serum and testicular tissue TOS and TAS levels were assessed using one-way analysis of variance (ANOVA) and Duncan *post hoc* tests. Additionally, the histological changes (vacuolization at the basal membrane and immature spermatids in the tubular lumen), total abnormality rates of spermatozoa, number of TUNEL-positive cells, apoptosis percentage, incidence of stage XIV tubules, HO-1 staining, and band intensity were analyzed with Kruskal–Wallis one-way analysis of variance and a *post hoc* multiple comparison test [27]. Moreover, between these parameters differences were assessed using correlation analysis. Differences were considered significant at $p<0.05$.

Results

Body and testis weight

There were no statistically significant differences in body weight between the groups. However, corn oil administration alone (sham) caused a statistically significant increase ($p<0.001$) in the testis weight compared with the other groups (Table 1).

Table 1 – ***Values of body and testis weights***

Groups	*n*	Body weight [g]	Testis weight [g]
		$\bar{x} \pm S_{\bar{x}}$	$\bar{x} \pm S_{\bar{x}}$
Control	8	244.88 ± 6.60	2.54 ± 0.07[b]
Sham	8	271.25 ± 6.95	2.96 ± 0.07[a]
PCB	8	247.00 ± 9.67	2.57 ± 0.10[b]
ALA	8	247.75 ± 9.94	2.43 ± 0.07[b]
ALA+PCB	8	238.25 ± 5.27	2.33 ± 0.09[b]
p		NS	***

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b]Means within each grouping with different letter designations differ significantly. NS: Not significant, *n*: No. of rats, ***$p<0.001$.

Histological appearance

There were no significant differences in the histological appearance between the control, sham control, ALA, and ALA+PCB groups. Stage VII–VIII and XII–XIV seminiferous tubules were discriminated in all groups.

Histological changes

Significant increases in the histological changes such as vacuolization at the basal membrane ($p<0.001$), and immature spermatids in the tubular lumen ($p<0.001$) were observed in only the PCB group compared with the other groups (Figure 1, Table 2).

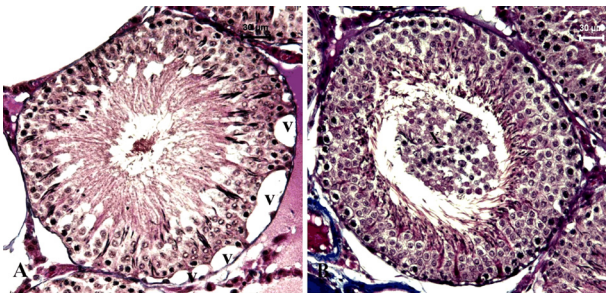

Figure 1 – ***(A and B) The histological changes in PCB group: (A) Vacuolization at the basal membrane (V); (B) Immature spermatids in tubular lumen (X). PCB: Polychlorinated biphenyl. Scale bar: 30 μm.***

Table 2 – ***Values of qualitative analysis of the histological changes in all groups***

Groups	*n*	Vacuolization at the basal membrane	Immature spermatids in the tubular lumen
		$\bar{x}$ *(Md)* $\pm S_{\bar{x}}$	$\bar{x}$ *(Md)* $\pm S_{\bar{x}}$
Control	7	0.95 (1.00) ± 0.13[b]	0.50 (0.00) ± 0.10[b]
Sham	8	0.73 (0.50) ± 0.11[b]	0.47 (0.00) ± 0.11[b]
PCB	8	1.95 (2.00) ± 0.14[a]	1.34 (1.00) ± 0.14[a]
ALA	8	0.78 (1.00) ± 0.08[b]	0.50 (0.00) ± 0.08[b]
ALA+PCB	8	0.59 (0.00) ± 0.09[b]	0.05 (0.00) ± 0.03[c]
p		***	***

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b,c]Means within each grouping with different letter designations differ significantly. Md: Median, *n*: No. of rats, ***$p<0.001$.

Histomorphometry

PCB and ALA+PCB treatment significantly (p<0.001) decreased the STD at stage VII–VIII and stage XII–XIV compared with the control, sham, and ALA groups. There were no statistically significant differences in the SE at stage VII–VIII and stage XII–XIV between groups (Figures 2 and 3).

PCB administration decreased the rate of % stage XIV tubulus compared with the control and sham groups, but these decreases were statistically insignificant (Table 3).

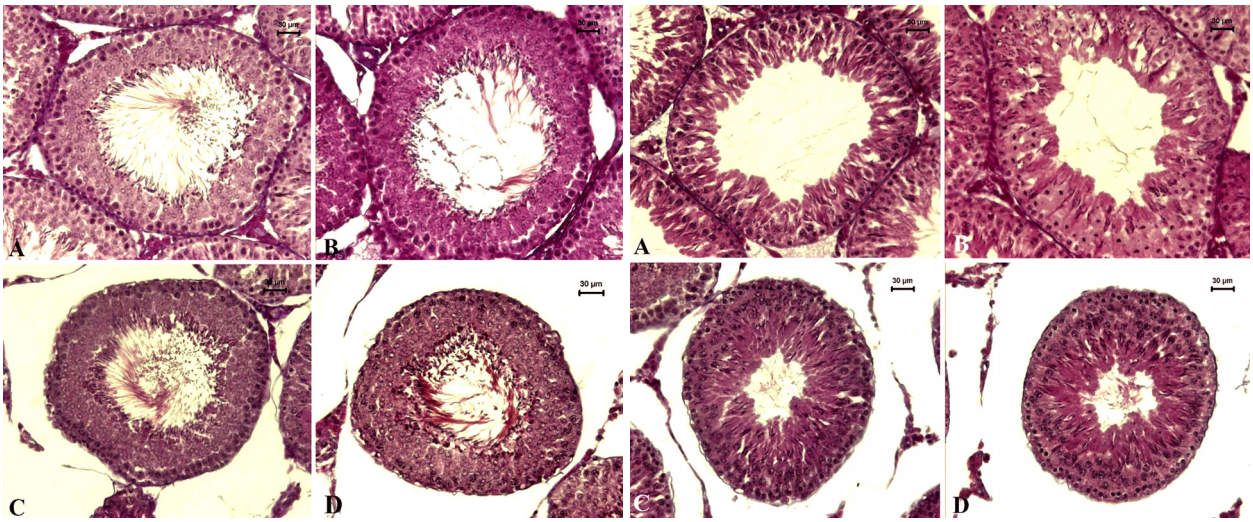

Figure 2 – ***Seminiferous tubules in control (A), sham (B), PCB (C) and ALA+PCB (D) groups at stage VII–VIII. PCB: Polychlorinated biphenyl; ALA: Alpha lipoic acid. Scale bar: 30 µm.***

Figure 3 – ***Seminiferous tubules in control (A), sham (B), PCB (C) and ALA+PCB (D) groups at stage XII–XIV. PCB: Polychlorinated biphenyl; ALA: Alpha lipoic acid. Scale bar: 30 µm.***

Table 3 – ***Histomorphometrical analysis of the testis in all groups***

Groups	*n*	Stage VII–VIII STD [µm]	Stage VII–VIII SE [µm]	Stage XII–XIV STD [µm]	Stage XII–XIV SE [µm]	Stage XIV tubules [%]
		$\bar{x} \pm S_{\bar{x}}$	$\bar{x} \pm S_{\bar{x}}$	$\bar{x} \pm S_{\bar{x}}$	$\bar{x} \pm S_{\bar{x}}$	$\bar{x}$ (Md) $\pm S_{\bar{x}}$
Control	7	274.53 ± 2.57[a]	71.21 ± 0.74	250.85 ± 2.56[a]	79.76 ± 0.90	3.29
Sham	8	277.95 ± 2.50[a]	70.99 ± 0.66	251.24 ± 2.37[a]	77.77 ± 0.77	2.80
PCB	8	262.91 ± 2.53[b]	71.71 ± 0.59	238.34 ± 2.17[b]	77.55 ± 0.71	1.63
ALA	8	274.43 ± 2.22[a]	70.67 ± 0.71	248.59 ± 2.43[a]	79.60 ± 0.85	2.69
ALA+PCB	8	262.35 ± 2.54[b]	70.98 ± 0.62	233.91 ± 2.11[b]	78.07 ± 0.69	2.38
p		***	NS	***	NS	NS

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b]Means within each grouping with different letter designations differ significantly. NS: Not significant, *n*: No. of rats, STD: Seminiferous tubule diameter, SE: Seminiferous epithelial height, ***p<0.001.

Evaluation of germ cell apoptosis

PCB treatment caused significant increases in the number of TUNEL-positive tubules (p<0.001), TUNEL-positive tubules (%) (p<0.001), and TUNEL-positive cells per tubule (p<0.05) compared with the other groups. There were no statistically significant differences in these parameters between the control, sham, ALA, and ALA+PCB groups (Figure 4, Table 4).

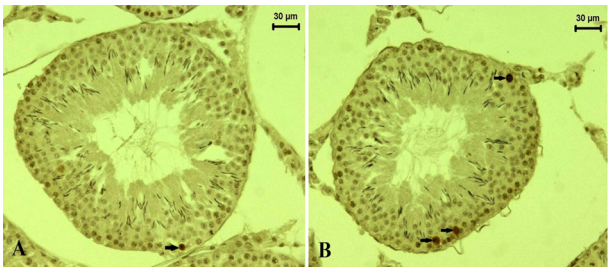

Figure 4 – ***Apoptotic cells in control (A), and PCB groups (B) (black arrows). PCB: Polychlorinated biphenyl. Scale bar: 30 µm.***

Table 4 – ***Evaluation of germ cell apoptosis in the testis from the control and experimental groups***

Groups	*n*	No. of TUNEL-positive cells/tubule	No. of TUNEL-positive tubules	TUNEL-positive tubules [%]
		$\bar{x}$ (Md) $\pm S_{\bar{x}}$	$\bar{x}$ (Md) $\pm S_{\bar{x}}$	$\bar{x}$ (Md) $\pm S_{\bar{x}}$
Control	7	0.28 (0.33) ± 0.05[b]	14.23 (16.00) ± 1.94[b]	7.12[b]
Sham	7	0.27 (0.25) ± 0.04[b]	13.38 (13.00) ± 0.91[b]	6.69[b]
PCB	8	0.46 (0.46) ± 0.04[a]	21.56 (21.00) ± 1.13[a]	10.78[a]
ALA	8	0.26 (0.25) ± 0.05[b]	13.44 (12.50) ± 1.98[b]	6.72[b]
ALA+PCB	8	0.29 (0.31) ± 0.02[b]	13.38 (13.00) ± 1.01[b]	6.69[b]
p		*	***	***

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b]Means within each grouping with different letter designations differ significantly. NS: Not significant, Md: Median, *n*: No. of rats, TUNEL: Terminal dUTP nick end labeling, *p<0.05, ***p<0.001.

Immunohistochemistry

PCB and ALA+PCB treatment increased significantly (p<0.001) the HO-1 staining intensity compared with the control and sham groups. There were no statistically significant differences in the HO-1 staining intensity between the control, sham, and ALA groups (Figure 5, Table 5).

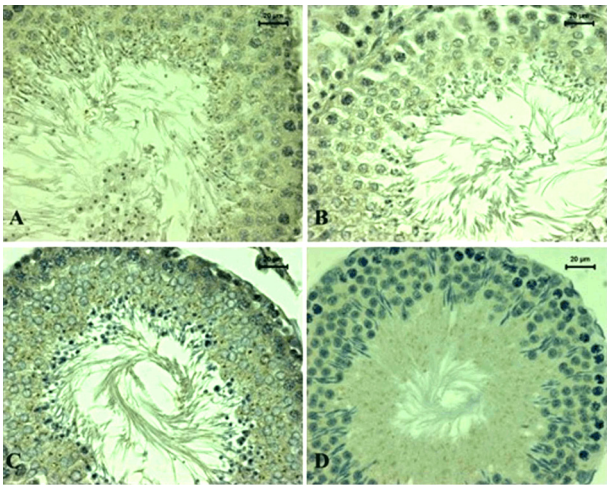

Figure 5 – ***The HO-1 positive cells in control (A), sham (B), PCB (C) and ALA+PCB (D) groups (brown precipitates). PCB: Polychlorinated biphenyl; ALA: Alpha lipoic acid. Scale bar: 20 µm.***

Table 5 – ***HO-1 staining intensity of the testis in all groups***

Groups	*n*	HO-1 staining intensity
		$\bar{x}$ *(Md)* ± $S_{\bar{x}}$
Control	7	1.76 (2.00) ± 0.08[b]
Sham	8	1.65 (2.00) ± 0.07[b]
PCB	8	2.00 (2.00) ± 0.07[a,c]
ALA	7	1.83 (2.00) ± 0.09[b,c]
ALA+PCB	8	2.10 (2.00) ± 0.07[a]
p		***

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b,c]Means within each grouping with different letter designations differ significantly. Md: Median, *n*: No. of rats, HO-1: Heme oxygenase-1, ***p<0.001.

Determination of HO-1 protein with Western blotting

There were no statistically significant differences in the intensity of the HO-1 protein bands between the groups (Figure 6, Table 6).

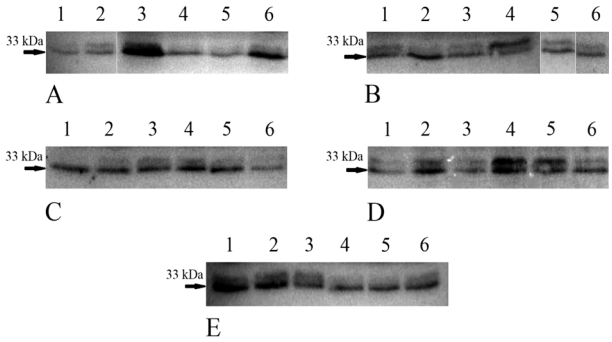

Figure 6 – ***The HO-1 protein bands in control (A), sham (B), PCB (C), ALA (D) and ALA+PCB groups (E) per animal (n=6). PCB: Polychlorinated biphenyl; ALA: Alpha lipoic acid.***

Table 6 – ***Intensity of HO-1 protein bands in all groups***

Groups	*n*	Intensity of HO-1 protein bands [units]
		$\bar{x}$ *(Md)* ± $S_{\bar{x}}$
Control	6	6.88 (6.74) ± 0.63
Sham	6	7.92 (7.69) ± 0.69
PCB	6	6.89 (6.34) ± 0.43
ALA	6	7.37 (7.37) ± 0.06
ALA+PCB	6	7.03(6.92) ± 0.14
p		NS

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. NS: Not significant, Md: Median, *n*: No. of rats, HO-1: Heme oxygenase-1, ***p<0.001.

Biochemistry

Serum levels of testosterone

There were no statistically significant differences in the serum levels of testosterone between the groups (Table 7).

Table 7 – ***Serum levels of testosterone, total oxidant status and total antioxidant status in serum and testis in all groups***

Groups	*n*	Serum levels of testosterone [ng/mL]	Total oxidant status in serum [µmol H_2O_2 equiv./g protein]	Total oxidant status in testis [µmol H_2O_2 equiv./g protein]	Total antioxidant status in serum [mmol Trolox equiv./L]	Total antioxidant status in testis [mmol Trolox equiv./L]
		$\bar{x}$ ± $S_{\bar{x}}$	$\bar{x}$ ± $S_{\bar{x}}$	$\bar{x}$ ± $S_{\bar{x}}$	$\bar{x}$ ± $S_{\bar{x}}$	$\bar{x}$ ± $S_{\bar{x}}$
Control	8	2.98 ± 0.21	4.59 ± 0.44[b]	0.42 ± 0.01[c]	109.10 ± 9.90	25.40 ± 1.64
Sham	8	2.86 ± 0.11	4.51 ± 0.47[b]	0.48 ± 0.01[b,c]	111.73 ± 12.40	29.13 ± 3.41
PCB	8	3.17 ± 0.28	8.03 ± 0.75[a]	0.60 ± 0.03[a]	87.63 ± 2.83	23.78 ± 3.01
ALA	8	2.94 ± 0.20	4.95 ± 0.21[b]	0.51 ± 0.02[b]	98.93 ± 15.46	26.28 ± 3.11
ALA+PCB	8	3.07 ± 0.16	5.97 ± 0.43[b]	0.53 ± 0.03[b]	98.25 ± 6.93	25.94 ± 0.84
p		NS	***	***	NS	NS

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. [a,b,c]Means within each grouping with different letter designations differ significantly. NS: Not significant, Md: Median, *n*: No. of rats, Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ***p<0.001.

Total oxidant and antioxidant status levels in serum and testis

PCB treatment caused significant (p<0.001) increases in the TOS level in the serum and testis compared with the other groups. There were no statistically significant differences in the TOS level in the serum and testis between the sham, ALA, and ALA+PCB groups (Table 7).

There were no statistically significant differences in the TAS level in serum and testis between the groups. Moreover, PCB treatment decreased the TAS level in serum and testis compared with the other groups; this decrease was statistically insignificant (Table 7).

Sperm morphology

PCB and ALA+PCB administration caused significant (p<0.01) increases in the total abnormality rates of spermatozoa (%) compared with the control and sham groups (Figure 7, Table 8).

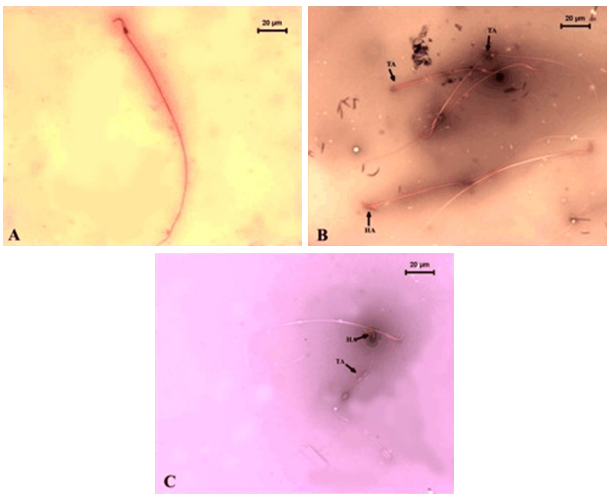

Figure 7 – ***Different types of epididymal spermatozoa. Normal spermatozoa in the control group (A), PCB group (B) and ALA+PCB group (C). HA: Head abnormalities; TA: Tail abnormalities; PCB: Polychlorinated biphenyl; ALA: Alpha lipoic acid. Scale bar: 20 µm.***

Table 8 – ***Abnormality rates of spermatozoa in all groups***

Groups	*n*	Total abnormality rates of spermatozoa $\bar{x}$ *(Md)* ± $S_{\bar{x}}$	Total spermatozoa abnormality [%]
Control	8	6.00 (6.00) ± 1.21^{b,c}	3.00^{b,c}
Sham	8	4.00 (4.50) ± 0.85^{c}	2.00^{c}
PCB	8	13.75 (10.00) ± 2.89^{a}	6.88^{a}
ALA	8	8.13 (9.00) ± 1.36^{b,a}	4.07^{b,a}
ALA+PCB	5	14.40 (16.00) ± 3.19^{a}	7.20^{a}
p		**	**

PCB: Polychlorinated biphenyl, ALA: Alpha lipoic acid. ^{a,b,c}Means within each grouping with different letter designations differ significantly. Md: Median, *n*: No. of rats, **p<0.01.

Correlation analysis

A significant positive correlation was determined between the TOS level in serum and the TOS level in testicular tissue (p<0.05). A positive correlation was statistically significant between the TOS level in testicular tissue and the number of apoptotic cells per tubule and between the total oxidant level in testicular tissue and the number of abnormal spermatozoa (p<0.05). The positive correlation was statistically significant between the HO-1 concentration determined at the microscopic level and the number of abnormal spermatozoa (p<0.05) (Table 9).

Table 9 – ***Correlation analysis between total oxidant and antioxidant status in serum and testis, serum levels of testosterone, number of TUNEL positives cells, HO-1 staining intensity and total abnormality spermatozoa***

	Serum TOS	Testis TOS	Serum TAS	Testis TAS	Serum testosterone	No. of TUNEL-positive cells	HO-1 staining intensity	Total abnormality spermatozoa
Serum TOS	1	0.384*	-0.222	-0.043	-0.183	0.318	-0.124	0.232
Testis TOS	0.384*	1	-0.193	0.057	0.064	0.348*	0.188	0.395*
Serum TAS	-0.222	-0.193	1	-0.090	-0.091	-0.217	-0.022	-0.032
Testis TAS	-0.043	0.057	-0.090	1	0.042	0.019	-0.101	-0.120
Serum testosterone	-0.183	0.064	-0.091	0.042	1	0.310	0.139	0.226
No. of TUNEL-positive cells	0.318	0.348*	-0.217	0.019	0.310	1	0.206	0.225
HO-1 staining intensity	-0.124	0.188	-0.022	-0.101	0.139	0.206	1	0.411*
Total abnormality spermatozoa	0.232	0.395*	-0.032	-0.120	0.226	0.225	0.411*	1

TOS: Total oxidant status, TAS: Total antioxidant status, TUNEL: Terminal dUTP nick end labeling, HO-1: Heme oxygenase-1. Values show the Spearman's correlation coefficient. *p<0.05.

Discussion

Body and testis weight

Ateşşahin *et al.* [5], Aly *et al.* [7], and Murugesan *et al.* [8] reported that PCB administration (intraperitoneally) significantly decreased body and testis weight. In this study, ALA, PCB, and ALA+PCB treatment did not cause significant differences in body weight compared with control and sham groups. The reason for this difference may be the method of PCB administration. Moreover, a portion of the PCB may be eliminated through the digestive tract.

However, Ateşşahin *et al.* [5] and Gray *et al.* [28] observed no significant variation in the testis weight with PCB application (gavage). In the present study, the testis weight was significantly increased only in the sham group. The testicular weight may have increased because pure corn oil increased the level of the energy-giving substance in the diet in the sham group.

Histological appearance

In the present study, the histological appearance of the testicular tissues had a histological appearance in accordance with the literature [29] in the groups (control, sham, ALA, and ALA+PCB) except the PCB group.

França *et al.* [30] reported that the spermatogenic cycle consists of 14 stages in rats. In a study, stages VII–VIII, XII–XIII, and XIV of the spermatogenic cycle were distinguished in all groups as researchers have previously reported.

Histological changes

In a study performed in rats by Ateşşahin *et al.* [5], degeneration of germ cells, epithelial desquamation,

reduction in the number of germ cells, and capillary congestion were observed in the group that had been administered 2 mg/kg bw/day dose Aroclor 1254 for eight weeks.

When the data obtained in the present study were examined, vacuolization at the basal membrane and immature spermatids in the tubular lumen were observed in all groups to be somewhat different from the normal histological appearance. These changes were almost twice as frequent in PCB-administered groups compared to the other groups. These changes were age-related. In a study conducted in mice [31], similar changes were suggested to occur normally from the sixth month.

Gawish [3] examined histological changes occurring in the testis after oral administration of a mixture of Chlorpyrifos (insecticide) and Fenitrothion (pesticide) in rats and detected atrophy, disorganization, and hyperemia in the tubulus. These changes ere significantly reduced with an application of a mixture of Chlorpyrifos+Fenitrothion with ALA. In the present study, a significant reduction was observed vacuolization at the basal membrane ($p<0.001$) and immature spermatids in the tubular lumen ($p<0.001$) in the ALA+PCB group compared to the PCB group.

Histometry

Seminiferous tubule diameter and epithelial height

In studies that investigated the histomorphometrical effect in testes of PCB in mice [32] and TCDD as a dioxin-like PCB compound in rats [2], the diameter of the seminiferous tubule decreased significantly. In the present study, there was no significant difference between the PCB and ALA+PCB groups; the tubulus diameter in the stage VII–VIII and XII–XIV was shorter in both groups compared to the control, sham, and ALA groups ($p<0.001$), which was found similar to previous research results [2, 32].

In studies conducted in rats and mice, intraperitoneal administration of Malathion [33] and Methyl-parathion (MP) [23], in which PCB is used as an additive were determined to cause a statistical reduction in the height of the epithelium of the seminiferous tubule. In the present study, there was no difference statistically in epithelial height between the control and experimental groups. Therefore, in this study, PCB had no effect on the seminiferous epithelium height.

Incidences of stage XIV tubules

Narayana *et al.* [23] determined that the percentage of tubule density in stage XIV was significantly reduced in rats that were administered MP intraperitoneally. In the present study, the percentage of tubule density in stage XIV was significantly decreased in the group that had been administered PCB, but this decrease was not statistically significant. The percentage of tubule density in stage XIV was numerically higher in the ALA+PCB group compared to the PCB group but this increase was not significant.

Germ cell apoptosis

Ateşşahin *et al.* [5] and Cai *et al.* [10] showed that Aroclor 1254 causes apoptosis in the germ cells. In the present study, Aroclor 1254 was confirmed to have an apoptotic effect on the testis.

In a study conducted in rats, the number of TUNEL-positive apoptotic cells was increased by oral administration of a mixture of Chlorpyrifos (insecticide) and Fenitrothion (pesticide), the intensity of apoptosis was significantly reduced by the oral administration of a mixture of Chlorpyrifos+Fenitrothion with ALA [3]. In the present study, the number of apoptotic cells per tubule ($p<0.05$) and the percentage of the number of TUNEL-positive tubules ($p<0.001$) was significantly reduced in the group that had been administered ALA+PCB compared to the group that had been administered PCB. Therefore, ALA was determined to decrease the apoptotic effect of Aroclor 1254.

Intensity of HO-1

In a study conducted in rats, Lee *et al.* [34] determined that the HO-1 level increased in neurons in the brain tissue in the group that had been administered PCB. In this study, the HO-1 protein was determined by the western blot technique, but a statistical difference in the intensity of HO-1 was not detected between the groups. However, the immunohistochemically intensity of HO-1 was higher in the PCB and ALA-PCB groups compared to the control, sham, and ALA groups ($p<0.001$). The increase in the intensity of HO-1 determined with the immunohistochemical method is consistent with the findings of another study [34]. The high intensity of HO-1 in the ALA+PCB group was thought to result from PCB. In this group, the increased intensity of HO-1 together with ALA can decrease apoptosis intensity. A review of the literature showed that no study had investigated the level of HO-1 in PCB toxicity in testis.

Biochemistry

Serum levels of testosterone

In studies performed in rats, PCB has significantly reduced serum testosterone levels [1, 9]. In addition, researchers [10, 28] showed that oral administration of PCB in rats had no significant difference in serum testosterone levels compared to the control group. In this study, there was no statistically significant difference in serum testosterone levels between the groups. In the present study, different from the literature, PCB had no effect on testosterone because PCB was administered orally to the rats and because of the duration of administration.

Total oxidant and antioxidant status levels in serum and testis

Murugesan *et al.* [1] and Aly *et al.* [7] showed that administration of PCB in rats increased the levels of H_2O_2 and hydroxyl radicals and lipid peroxidation by inducing oxidative stress in the testicular tissue and decreased the level of enzymatic antioxidants (SOD, CAT, GPx, GR). The present study showed that the total oxidant level in serum and testicular tissue was significantly increased ($p<0.001$) and the total antioxidant level numerically decreased only in rats that had been administered PCB. The findings are consistent with the literature that oral administration of PCB (Aroclor 1254) has been demonstrated to induce oxidative stress.

Gawish [3] showed in a study performed in rats that the level of plasma malondialdehyde (MDA) increased

by applying oral administration of a mixture of Chlorpyrifos (insecticide) and Fenitrothion (pesticide), ALA + the mixture (Chlorpyrifos+Fenitrothion) reduced the increased MDA level. In the present study, the total oxidant enzyme level in the serum and testicular tissue was significantly reduced ($p<0.001$), and the total antioxidant enzyme level in the serum and testicular tissue also showed a numerical increase in the ALA+PCB group compared to the PCB group. ALA was demonstrated to reduce the effects of oxidative stress in PCB toxicity.

Sperm morphology

In other studies, there was a decrease in sperm count, sperm motility [7, 10], and daily sperm production [7], and an increase in the rate of abnormal sperm [10] after the administration of PCB. In the present study, the proportion of abnormal spermatozoon was significantly higher in the PCB and ALA+PCB groups compared to the other groups ($p<0.01$). An increase in the proportion of abnormal spermatozoon is considered to result from the toxic apoptotic effect of PBC.

It has been demonstrated that the proportion of abnormal spermatozoon increased in rat testes with the administration of adriamycin [15] and the administration of ALA with these toxic substances has corrected abnormal changes. ALA has a protective effect against toxicity in the reproductive system. In this study, the rate of abnormal sperm was statistically higher in the ALA+PCB group compared to the control and sham groups ($p<0.01$). This increase is thought to be prevented by increasing the amount of ALA and the duration of administration.

Correlation analysis

A positive correlation was determined between the total oxidant level in testicular tissue and the number of apoptotic cells per tubule. Apoptosis was demonstrated to be associated with oxidative stress. The data in the ALA+PCB group were indistinguishable from the control group and the group that received only ALA. Therefore, use of ALA reduced the apoptotic effect of PCB.

Conclusions

Administration of PCB in rats increased vacuolization at the basal membrane and immature spermatids in the tubular lumen, decreased the diameters of tubules at stage VII–VIII and XII–XIV, and increased apoptosis, HO-1 staining intensity, the total oxidant level in serum and testicular tissue, and the number of abnormal spermatozoa. The administration of ALA+PCB significantly reduced vacuolization at the basal membrane ($p<0.001$), immature spermatids in the tubular lumen ($p<0.001$), apoptosis rate, and the total oxidant level in serum and testicular tissue observed in the group that had been administered only PCB. Therefore, with the present study, the presence of the protective effect of ALA against the toxic effects of PCB on the male reproductive system was demonstrated. The present study is the first study to investigate the antioxidant effect of ALA against PCB toxicity in the testis. Future studies should change the use rate and the route of administration of ALA to further investigate the antioxidant and anti-apoptotic effects of ALA on the male reproductive system.

Conflict of interests

The authors declare that they have no conflict of interests.

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