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REVIEW ARTICLE

Attenuating effect of lycopene and ellagic acid on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced spermiotoxicity and testicular apoptosis

Mustafa Sönmez[1], Gaffari Türk[1], Ali Osman Çeribaşı[2], Fatih Sakin[3], and Ahmet Ateşşahin[4]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, Elazığ, Turkey, [2]Department of Pathology, Faculty of Veterinary Medicine, Fırat University, Elazığ, Turkey, [3]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay, Turkey, and [4]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Fırat University, Elazığ, Turkey

Abstract

This study was conducted to investigate the prophylactic effects of lycopene (LC) and ellagic acid (EA) on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced testicular and spermatozoal toxicity. These toxicological changes are associated with the oxidative stress and apoptosis in male rats. Forty-eight male rats were allocated to one of six groups of 8 rats each: control, LC, EA, TCDD, TCDD+LC, and TCDD+EA. The control group was treated with 0.5 mL/rat slightly alkaline solution+0.5 mL/rat corn oil every other day. The LC group was treated with 0.5 mL/rat slightly alkaline solution+0.5 mL/rat corn oil containing 10 mg/kg of LC every other day. The EA group received 0.5 mL/rat corn oil+0.5 mL/rat slightly alkaline solution containing 2 mg/kg of EA every other day. The TCDD group received 0.5 mL/rat corn oil containing 100 ng/kg/day of TCDD+0.5 mL/rat slightly alkaline solution. The TCDD+LC group was treated with 0.5 mL/rat TCDD+0.5 mL/rat LC. The TCDD+EA group was treated with 0.5 mL/rat TCDD+0.5 mL/rat EA. All treatments were made by gavage, and the experimental period was maintained during 8 weeks. Sperm motility, concentration, and abnormal sperm rate in epididymal tissue, testicular tissue lipid peroxidation (LPO), antioxidant enzyme activity, histopathological changes, and apoptosis (i.e., Bax and Bcl-2 proteins) were determined. TCDD exposure resulted in significant decreases in sperm motility, concentration, testicular superoxide dismutase activity, germinal cell-layer thickness, Johnsen's testicular score, and significant increases in abnormal sperm rate, testicular malondialdehyde, glutathione levels, Bax-positive staining, and Bax-positive apoptotic cell score, along with some testicular histopathological lesions. TCDD treatment did not affect significantly catalase activity. However, combined treatment with LC or EA, in addition to TCDD, prevented the development of TCDD-induced damages in sperm quality, testicular histology, and LPO. Improvements in testicular apoptosis after the administration of LC and EA to TCDD-treated rats were minimal, but not statistically significant. TCDD-induced lipid peroxidation leads to functional and structural damages, as well as apoptosis, in spermatogenic cells of rats. Both LC and EA protected against the development of these effects.

Keywords: Environmental pollutant, carotenoid, polyphenol, male reproduction, oxidative stress

Introduction

With the development of industry and agriculture, environmental pollutants have drawn concerns because of their potential health impacts on humans and animals (Ateşşahin et al., 2010). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic environmental pollutants that disrupts normal endocrine functions in humans (Mocarelli et al., 2008) and animals (Hwang et al., 2004; Fisher et al., 2005; Choi et al., 2007; Wintermyer and Cooper, 2007; Arima et al., 2009). It is formed as a by-product in the manufacture of chlorinated hydrocarbons, in the incineration process of municipal waste, paper and pulp bleaching, and in emission from steel foundries and motor vehicles (Skene et al., 1989). The male reproductive system is a sensitive target of TCDD or other dioxin congeners, with effects of TCDD exposure that include increased gonadotropins (e.g.,

Address for Correspondence: Gaffari Türk, Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, 23119 Elazığ,Turkey; Fax:+90 424 238 81 73; E-mail: gturk@firat.edu.tr

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follicle-stimulating hormone and leutinizing hormone) and decreased testosterone concentrations (Choi et al., 2007), reduced testis, epididymis and accessory gland weights (Latchoumycandane and Mathur, 2002; El-Tawil and Elsaieed, 2005), reduced sperm count (Choi et al., 2007), sperm motility (El-Sabeawy et al., 1998; Yamano et al., 2009), increased abnormal sperm rate (Bell et al., 2007), and damage to the normal testicular structure (Rune et al., 1991; El-Sabeawy et al., 1998; Choi et al., 2007).

The possible explanations for TCDD-induced male reproductive toxicity are that TCDD exposure activates the aryl hydrocarbon receptor (Fisher et al., 2005) and leads to oxidative stress (Latchoumycandane and Mathur, 2002; Latchoumycandane et al., 2002; 2003; El-Tawil and Elsaieed, 2005). Oxidative stress is an imbalance between the production of free radicals and synthesis of antioxidant defenses against them. Free radicals are normally generated in subcellular compartments, particularly mitochondria, which are subsequently scavenged by antioxidant defense systems of the corresponding cellular compartments. However, this balance can easily be broken by chemicals that disrupt the prooxidant-antioxidant balance, leading to cellular dysfunction. Additionally, the mitochondrial membrane of spermatozoa is susceptible to lipid peroxidation (LPO), as this compartment is rich in polyunsaturated fatty acids and has been shown to contain low amounts of antioxidants (Agarwal et al., 2008a, 2008b). Apoptosis normally ensures an optimal number of testicular germ cells that can be supported by Sertoli cells, and it is triggered in specific areas of the seminiferous epithelium, controlling the spermatogonial population (Blanco-Rodriguez, 1998). However, an increase in the frequency of testicular apoptosis is seen as the side effects of some environmental pollutants, such as Aroclor 1254 (Ateşşahin et al., 2010) and TCDD (Schultz et al., 2003).

Lycopene (LC), a nonprovitamin A carotenoid, has potent antioxidant properties and quencher of singlet oxygen ($^1O_2$) and other free radicals, resulting in protection against LPO and oxidative DNA damage both *in vivo* and *in vitro* (Seren et al., 2008). Ellagic acid (EA) is an important component of fruits and vegetables and is partly responsible for their beneficial health effects against oxidation-linked chronic diseases, such as cancer and cardiovascular diseases. It is believed that EA functions either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating/inducing cellular antioxidant enzyme systems (Vattem and Shetty, 2005). The present study was designed to investigate whether LC or EA would have a protective effect against TCDD-induced changes in epididymal sperm characteristics and testicular tissue.

Materials and methods

Chemicals

TCDD was purchased from AccuStandard® (New Haven, Connecticut, USA). LC 10% FS (Redivivo TM, Code 7803) was obtained from DSM Nutritional Products (İstanbul, Turkey). EA was supplied from Fluka (Steinheim, Germany), and the other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA).

Animals and experimental design

Forty-eight healthy adult male Sprague-Dawley rats (8 weeks old) were used in this study. The animals were obtained from Fırat University Experimental Research Center (Elazığ, Turkey) and were housed under standard laboratory conditions (temperature, 24 ± 3°C; humidity, 40–60%; 12-hour light-and-dark cycle). A commercial pellet diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water were given *ad libitum*. The protocol for animal use was approved by the Institutional Review Board of the National Institute of Health and Local Committee on Animal Research.

TCDD was given to the animals by gavage at the dose of 100 ng/kg/day. LC was suspended in corn oil and administered to the animals by gavage at the dose of 10 mg/kg/every other day. EA is poorly dissolved under neutral conditions. Therefore, it was dissolved in alkaline solution (0.01 N NaOH; approximately pH 12). The final solution (pH ≈8) after the addition of EA was administered to the animals by gavage at the dose of 2 mg/kg/every other day. The doses of TCDD (Latchoumycandane and Mathur, 2002; El-Tawil and Elsaieed, 2005), LC, and EA (Ateşşahin et al., 2010; Türk et al., 2010) used in this study were selected on the basis of the previous studies. Body weights were measured weekly during the experimental period. Dosing solutions were adjusted for changes in body weights during the administration period. All treatments continued for 8 weeks. Because rats need a period of 48–52 days to complete a spermatogenic cycle, including spermatocytogenesis, meiosis, and spermiogenesis, the administration period was set at 8 weeks (Ateşşahin et al., 2010; Türk et al., 2010).

The animals were randomly divided into six experimental groups of 8 rats each. These groups were arranged as follows: group 1: control: treated with placebo, received 0.5 mL/rat slightly alkaline solution+0.5 mL/rat corn oil every other day; group 2: LC: treated with 0.5 mL/rat slightly alkaline solution+0.5 mL/rat corn oil containing 10 mg/kg LC every other day; group 3: EA: received 0.5 mL/rat corn oil+0.5 mL/rat slightly alkaline solution containing 2 mg/kg EA every other day; group 4: TCDD: received 0.5 mL/rat corn oil containing 100 ng/kg/day TCDD+0.5 mL/rat slightly alkaline solution; group 5: TCDD+LC: treated with 0.5 mL/rat TCDD+0.5 mL/rat LC; and group 6: TCDD+EA: treated with 0.5 mL/rat TCDD+0.5 ml/rat EA.

Sample collection and homogenate preparation

The rats were killed by cervical dislocation under slight ether anaesthesia at the end of 8 weeks. Testes and epididymides were removed and cleared of adhering

connective tissue. Blood samples were collected from vena cava via sterile injector containing heparin and centrifuged at 3,000 × *g* for 10 minutes to obtain plasma. One of the testes was fixed in 10% neutral formalin solution for histopathological and immunohistochemical examinations. The other testis and plasma samples were also stored at–20°C for biochemical analyses. Testes were taken from a–20°C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a 9-fold volume of phosphate buffer (pH 7.4). For the enzymatic analyses, testes were minced in a glass and homogenized by a Teflon-glass homogenizator for 3 minutes in cold physiological saline on ice (Ateşşahin et al., 2010; Türk et al., 2010).

Sperm analyses

The epididymal sperm concentration in the right cauda epidymal tissue was determined with a hemocytometer (Türk et al., 2007). Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. The percentage of sperm motility was evaluated using a light microscope with a heated stage. To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin-nigrosin (1.67% eosin, 10% nigrosin, and 0.1 M of sodium citrate) were prepared. The slides were then viewed under a light microscope at 400X magnification. A total of 300 spermatozoa were examined on each slide (2,400 cells in each group), and the head, tail, and total abnormality rates of spermatozoa were expressed as a percentage (Türk et al., 2007, 2008).

Testicular tissue LPO level and antioxidant enzyme activity

LPO levels were spectrophotometrically measured according to the concentration of thiobarbituric acid reactive substances (TBARs), and the amount of malondialdehyde (MDA) produced was used as an index of LPO. The MDA level was expressed as nmol/mL (Placer et al., 1966). Reduced glutathione (GSH) levels were spectrophotometrically determined at 412 nm using the method described by Sedlak and Lindsay (1968)and expressed as nmol/ml. Glutathione peroxidase (GSH-Px) activity was spectrophotometrically determined according to the method of Lawrence and Burk (1976). Protein concentrations were determined using the method of Lowry et al. (1951). GSH-Px activity was expressed as IU/g protein. Catalase (CAT) activity was spectrophotometrically determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as kU/g protein, where k is the first-order rate constant (Aebi, 1983). Superoxide dismutase (SOD) activity was spectrophotometrically measured using xanthine and xanthine oxidases to generate superoxide radicals, which react with nitroblue tetrazolium (NBT), and are expressed as U/mL (Flohe and Otting, 1984).

Testosterone

Plasma testosterone level was measured by the enzyme-linked immunosorbent assay (ELISA) method using DRG® ELISA testosterone (ELISA EIA-1559, 96 wells; DRG Instruments, GmbH, Marburg, Germany), according to the manufacturer's instructions, and are expressed as ng/dL.

Histopathology and apoptosis determined by immunohistochemistry

The histopathological and immunohistochemical evaluations were conducted in a "blinded" mode. The testicular tissues were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 μm, and were stained with hematoxylin and eosin. Light microscopy was used to measure diameters of seminiferous tubules (DSTs) and germinal cell-layer thicknesses (GCLTs) and to evaluate testicular damage. Johnsen's testicular score (Johnsen, 1970) was performed for control and treatment groups. All cross-sectioned tubules were evaluated systematically, and a score between 1 (*very poor*) and 10 (*excellent*) was given to each tubule, according to Johnsen's criteria. Twenty-five tubules were evaluated for each animal.

The avidin-biotin-peroxidase method was used for immunohistochemical analyses (Jahnukainen et al., 2004). Testis tissues, embedded in paraffin and sectioned at 4 μm, were deparaffinized with xylene and dehydrated with an alcohol series. Testicular sections were incubated in 0.01 M of Na-citrate for 20 minutes to bring into the open the antigenic receptors. These were washed with phosphate-buffer solution (PBS) and incubated in 3% H_2O_2, which was prepared with PBS, for 10 minutes to inactivate endogenous peroxidase activity. Nonspecific binding was blocked by incubation with 1% untreated goat serum for 1 hour. Testicular tissues were then incubated with primary rabbit polyclonal antibodies directed against Bax (proapototic protein) and Bcl-2 (antiapoptotic protein) at dilutions of 1:200 and 1:400, respectively, in PBS containing 0.1% goat serum at 37°C for 1 hour. Testicular sections were washed again in PBS and incubated with biotinylated secondary antibodies, which were diluted at the rate of 1:1000 in PBS containing 0.1% goat serum, (secondary biotinylated goat antirabbit IgG) for 30 minutes, and thereafter tissues were washed with PBS and incubated with avidin-conjugated horseradish peroxidase for 1 hour. 3-amino-9-etilcarbazole (AEC) was used as a color-determining substrate. The reaction was stopped at the moment that color change occurred after the addition of this solution to the testicular tissues. At the last stage, testicular tissues were washed with tap water for 2 minutes after they were stained with Mayer's hematoxylin for 15 seconds. Stained tissues were covered with immune-mount, and then Bax- and Bcl-2-positive spermatogenic cells (from spermatogonia to elongated spermatid) were evaluated under a light microscope and scored as follows (Kandi Coşkun and Çobanoğlu, 2005): 0: negative stained cells; 1: <25% positive stained cells; 2:

26–50% positive stained cells; 3: 51–75% positive stained cells; and 4: >75% positive stained cells.

Statistical analysis

All values are presented as mean ± SEM. Differences were considered to be significant at $P<0.05$. One-way analysis of variance (ANOVA) and post-hoc Tukey-HSD test were used to determine differences between groups (Akgül, 2003). The SPSS/PC program (version 10.0; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

Results

Epididymal sperm characteristics

Table 1 lists changes in epididymal sperm characteristics in response to the various treatments. Although treatment with LC and EA alone had no effect on any of the parameters evaluated, TCDD treatment alone significantly decreased sperm motility ($P<0.05$) and concentration ($P<0.01$) and significantly increased the head ($P<0.01$), tail ($P<0.05$), and total ($P<0.05$) abnormality rate of spermatozoa, compared with values in the control group. A significant increase in sperm motility ($P<0.05$) and concentration ($P<0.01$) and significant decrease in head ($P<0.01$), tail ($P<0.05$), and total ($P<0.05$) abnormality rate of spermatozoa were observed in the TCDD+LC and TCDD+EA groups, compared with values for the TCDD-treated group.

Biochemical parameters

Testicular tissue LPO levels, antioxidant enzyme activities, and plasma testosterone levels are presented in Table 2. Although treatment with TCDD alone significantly ($P<0.05$) increased MDA levels, compared with the control group, LC or EA administrations to TCDD-treated rats significantly ($P<0.05$) reduced these increased MDA levels, compared with levels in the TCDD group. Treatment with LC alone and TCDD significantly ($P<0.05$) increased the GSH level, compared with the control group. In addition, concomitant administration of LC or EA with TCDD provided a more significant ($P<0.05$) increase in the TCDD-induced increased GSH level, compared with the values in control and TCDD-alone groups. Although TCDD-alone treatment did not affect GSH-Px activity, coadministration of LC and TCDD caused a significant ($P<0.05$) increase in GSH-Px activity, compared with the values in the TCDD-alone group. There was no significant difference in CAT activity between the control and TCDD-alone, TCDD-alone and TCDD+LC, TCDD-alone, and TCDD+EA groups. TCDD treatment alone significantly ($P<0.05$) decreased SOD activity, compared with values in the control group. However, LC or EA administration to TCDD-treated rats tended to increase SOD activity, but the differences did not reach statistical significance. No statistically significant differences were observed among any of the groups with respect to plasma testosterone levels.

Testicular histopathology and immunohistochemistry

The histological appearance of the testis in control (Figure 1A), LC (Figure 1B), and EA (Figure 1C) groups were normal. The histopathological changes, such as degeneration, desquamation, disorganization, and reduction in germinal cells, interstitial edema, and congestion, were observed in the TCDD-alone group (Figure 2A), compared with the control group. However, LC (Figure 2B) and EA (Figure 2C) administrations to TCDD-treated rats markedly improved these histological changes induced by TCDD. Significant ($P<0.05$) decreases in GCLT and Johnsen's testicular score were observed in the TCDD group. However, both LC and EA administration to TCDD-treated animals significantly ($P<0.05$) prevented the TCDD-induced decreases in these parameters (Table 3).

There were no immunohistochemically significant differences among control (Figure 3A), LC (Figure 3B), and EA (Figure 3C) groups in terms of Bax-positive staining. However, Bax-positive cells were observed more frequently in the TCDD-treated (Figure 4A) rat-testis sections than in the control group rat-testis sections. The intense staining was observed in almost all the spermatogenic cell types (from spermatogonia to elongated spermatid) in the TCDD-treated rat testis sections. A moderate decrease in intense staining was observed in both TCDD+LC (Figure 4B) and TCDD+EA (Figure 4C) groups, compared with the TCDD-alone group. In addition, TCDD administration significantly ($P<0.05$) increased the scores of Bax-positive cells, compared with the control group. However, LC or EA administration to TCDD-treated rats tended to decrease

Table 1. Mean ± SEM values of sperm parameters.

Parameters					
		Epididymal sperm concentration	Abnormal sperm rate (%)		
Groups	Sperm motility (%)	(million/g tissue)	Head	Tail	Total
Control	$77.77 \pm 2.94^{a,b}$	347.5 ± 11.5^{A}	2.28 ± 0.31^{A}	$3.78 \pm 0.78^{a,c}$	$6.06 \pm 2.01^{a-bc}$
LC	85.53 ± 1.86^{b}	344.6 ± 9.7^{A}	2.16 ± 0.39^{A}	1.83 ± 0.37^{a}	3.99 ± 1.67^{a}
EA	82.76 ± 3.15^{b}	351.3 ± 11.5^{A}	1.89 ± 0.41^{A}	$2.55 \pm 0.31^{a,c}$	$4.44 \pm 0.72^{a,b}$
TCDD	62.77 ± 6.05^{c}	245.1 ± 10.1^{B}	6.22 ± 0.88^{B}	7.22 ± 0.50^{b}	13.44 ± 0.78^{d}
TCDD+LC	83.32 ± 4.21^{b}	319.3 ± 17.1^{A}	3.33 ± 0.15^{A}	$4.26 \pm 0.93^{a,c}$	$7.59 \pm 0.87^{b,c}$
TCDD+EA	81.10 ± 4.61^{b}	304.9 ± 14.8^{A}	3.43 ± 0.71^{A}	4.43 ± 0.27^{c}	7.86 ± 0.95^{c}

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a, b, c, and d: $P<0.05$; A and B: $P<0.01$).

Table 2. Mean ± SEM values of testicular tissue MDA, GSH levels and GSH-Px, CAT, SOD activities, and plasma testosterone levels.

	Biochemical pParameters					
Groups	MDA (nmol/mL)	GSH (nmol/mL)	GSH-Px (IU/g protein)	CAT (kU/g protein)	SOD (U/mL)	Testosterone (ng/dL)
Control	93.4 ± 12.0[a]	6.5 ± 1.2[a]	10.2 ± 1.0[a,b]	6.2 ± 0.4[a]	1.72 ± 0.28[a]	392 ± 65[a]
LC	106.6 ± 8.9[a]	10.4 ± 0.6[b]	8.5 ± 1.1[a]	13.4 ± 0.9[b]	1.79 ± 0.26[a]	289 ± 65[a]
EA	105.8 ± 15.4[a]	8.7 ± 0.7[ab]	10.2 ± 2.4[a,b]	6.5 ± 0.3[a]	1.60 ± 0.29[a]	354 ± 73[a]
TCDD	170.3 ± 11.8[b]	16.6 ± 2.6[b]	11.9 ± 3.1[a,b]	6.8 ± 0.6[a,c]	0.46 ± 0.08[b]	283 ± 68[a]
TCDD+LC	48.4 ± 5.4[c]	27.5 ± 3.7[c]	27.3 ± 5.8[c]	8.0 ± 0.3[c]	0.79 ± 0.11[b]	284 ± 44[a]
TCDD+EA	56.3 ± 2.9[c]	27.0 ± 4.2[c]	18.9 ± 2.5[b]	7.6 ± 0.4[a,c]	0.62 ± 0.04[b]	301 ± 90[a]

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a, b, and c: $P < 0.05$).

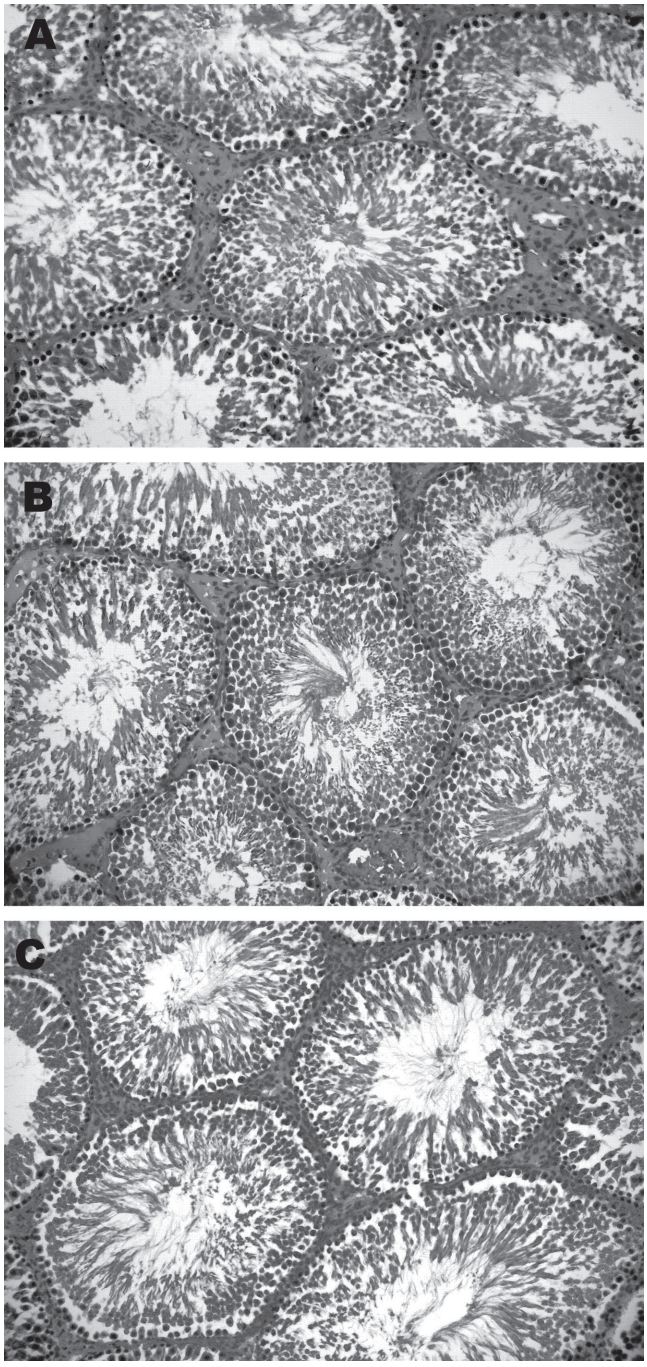

Figure 1. Normal histological appearance of seminiferous tubules in control (**A**), LC (**B**), or EA (**C**) groups (H&E, 100X). (See colour version of this figure online at www.informahealthcare.com/dct)

the scores of Bax-positive cells induced by TCDD, but the differences did not reach statistical significance (Table 3). In other words, improvements in Bax-positive apoptotic cell scores provided by LC or EA administration to TCDD-treated rats were minimal. With respect to Bcl-2-positive staining (Figure 3D–F and Figure 4D–F) and Bcl-2 antiapoptotic cell scores (Table 3), there were no immunohistochemically significant differences between any of the groups.

Discussion

TCDD has led to partial or total reproductive dysfunction in humans (Mocarelli et al., 2008) and different species of animals, such as the rat (Latchoumycandane and Mathur, 2002; El-Tawil and Elsaieed, 2005; Choi et al., 2007), mouse (Fisher et al., 2005), guinea pig (Hwang et al., 2004), rhesus monkey (Arima et al., 2009), and oyster (Wintermyer and Cooper, 2007).

The structure of mature sperm plasma membrane is consistent throughout, in that it is composed of three layers or zones: lipid bilayer, phospholipid-water interface, and glycocalyx. A major part of plasma membrane consists of lipid bilayer and phospholipid-water interface layers. Because sperm plasma membranes contain large quantities of lipids (i.e., polyunsaturated fatty acids) and their cytoplasm contains low concentrations of scavenging enzymes, they are particularly susceptible to the damage induced by excessive reactive oxygen species (ROS; Agarwal et al., 2008a, 2008b). ROS can attack the unsaturated bonds of the membrane lipids in an autocatalytic process, with the genesis of peroxides, alcohol, and lipidic aldehydes as a by-product of the reaction. Thus, the increase of free radicals in cells can induce LPO by the oxidative breakdown of polyunsaturated fatty acids in the membranes of cells. Obviously, peroxidation of sperm lipids destroys the structure of the lipid matrix in membranes of spermatozoa, and it is associated with a rapid loss of intracellular adenosine triphosphate (ATP), leading to axonemal damage, decreased sperm viability, and increased midpiece morphological defects, and it even completely inhibits spermatogenesis, in extreme cases (Türk et al., 2007, 2008). It has been reported that TCDD exposure results in decreased sperm count (Choi et al., 2007), sperm motility (El-Sabeawy et al., 1998; Yamano et al., 2009), and increased abnormal sperm

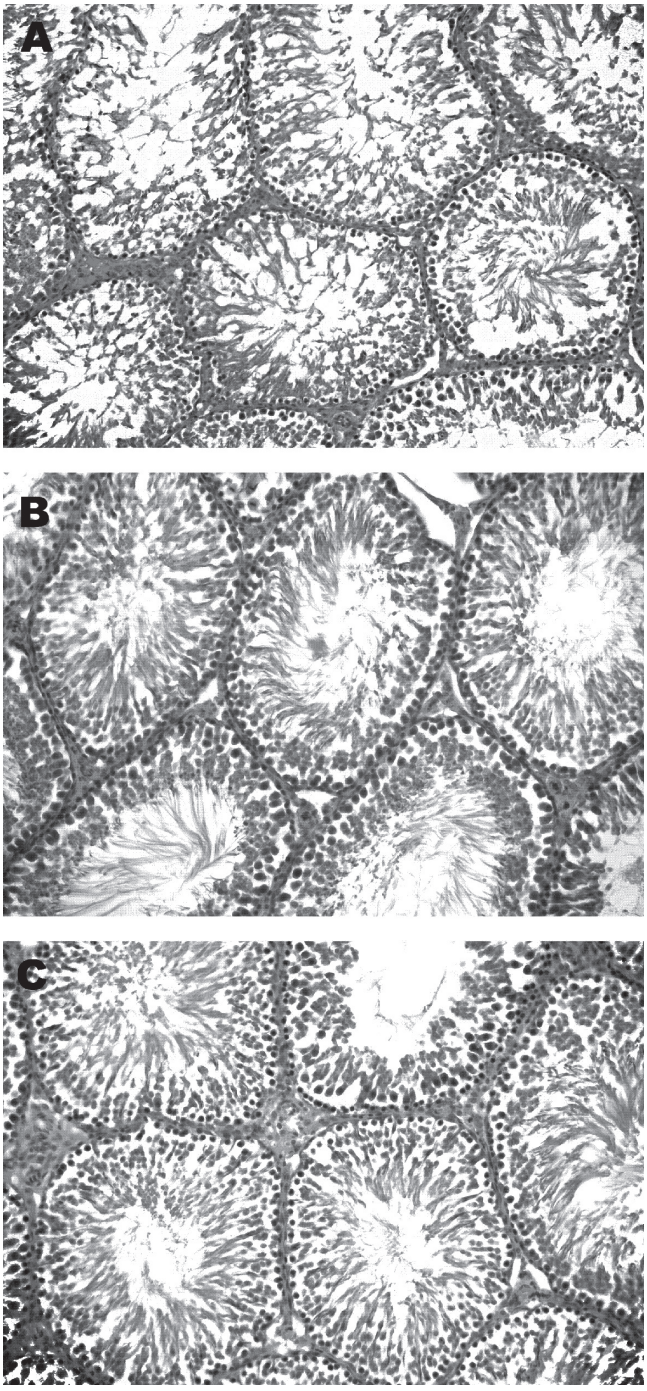

Figure 2. Disorganization, degeneration, and reduction in germinal cells along with interstitial edema and capillary congestion in TCDD alone (**A**), with significant improvement in testicular view and normal spermatogenesis in the TCDD+LC (**B**) and TCDD+EA (**C**) groups (H&E, 100X). (See colour version of this figure online at www.informahealthcare.com/dct)

rate (Bell et al., 2007) in male rats. In our previous study (Ateşşahin et al., 2010), we found that Aroclor 1254 exposure for 8 weeks caused reduced sperm motility, concentration, and increased abnormal sperm rate. In this study, TCDD-exposed rats had lower sperm concentration, motility, and higher abnormal sperm rate than the corresponding control group. Our findings are in agreement with the above-cited reports. It has been reported that TCDD decreases testosterone concentration (Choi et al., 2007) and increases the production of ROS that leads to LPO (Latchoumycandane and Mathur, 2002; El-Tawil and Elsaieed, 2005). The damage observed in sperm quality after TCDD exposure in the present study may be attributed to the peroxidation of polyunsaturated fatty acids in plasma membranes of spermatozoa, loss of ATP, and damaged flagellum, which are important machinery for the sperm motility, decreased daily sperm production due to the reduced testosterone concentration, impaired spermatogenesis, and DNA.

It has been reported that TCDD causes significant histopathological gonadal lesions, such as decreased intercellular contact between Sertoli cells and neighboring germ cells, increased necrotic germ cells, increased Sertoli cells with lipid droplets and phagolysosomes (Rune et al., 1991), atrophied seminiferous tubules, reduced germ cells, and decreased Johnsen's testicular score (Choi et al., 2007). In the present study, administration of TCDD resulted in significant decrease in GCLT and Johnsen's testicular score, along with degeneration, desquamation, disorganization, and reduction in germinal cells, interstitial edema, and congestion. The damage observed in the histological structure of the testis in this work may be elucidated with either decreased testosterone, which stimulates spermatogenesis, in particular, spermiogenesis or increased oxidative stress and that is a chemical mechanism capable of disrupting the structure and function of the testis.

Bax and Bcl-2 are members of a growing family of genes that are involved in promoting either cell survival or death via apoptosis. Proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins exist in the culmination of apoptosis after the onset of cellular stress. The ratio of these molecules has been implicated as a critical determinant of cell fate, such that elevated Bcl-2 favors extended survival of cells and increasing levels of Bax expression accelerate cell death (Sinha Hikim and Swerdloff, 1999). Schultz et al. (2003) have reported that TCDD exposure causes an increase in the frequency of testicular apoptosis. Similarly, in a study by Ateşşahin et al., (2010), it has been reported that an increase in apoptotic germ cells associated with oxidative stress is seen in Aroclor 1254–treated rats. In this study, a significant increase in Bax-positive apoptotic staining (from spermatogonia to elongated spermatids) and the scores of Bax-positive cells were observed in TCDD-treated rats, compared with the control group. Our findings are in agreement with the above reports. H_2O_2, one of the ROS, induces testicular germ-cell apoptosis by extrinsic and intrinsic mechanisms as well other regulatory pathways (Maheshwari et al., 2009). Elevated apoptotic cell rates after exposure to TCDD observed in this study may be explained by increased ROS and LPO levels in testicular tissue or direct DNA and chromatin damage to germ cells.

ROS, including superoxide anion ($O_2^{-\bullet}$), hydroxyl ($^{\bullet}OH$) radical, peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, as well as nonradical species, such as $^1O_2$, ozone

Table 3. Mean ± SEM values of DST, GCLT Johnsen's testicular, and immunohistochemical scores.

	Parameters				
Groups	DST (μm)	GCLT (μm)	Johnsen's testicular score	Bax- positive cell score	Bcl-2- positive cell score
Control	223.6 ± 2.2	76.4 ± 0.1[a]	9.6 ± 0.2[a]	0.33 ± 0.21[a]	0.33 ± 0.21
LC	225.1 ± 2.0	75.7 ± 1.2[a]	10.0 ± 0.0[a]	0.17 ± 0.17[a]	0.50 ± 0.22
EA	224.5 ± 1.9	74.7 ± 0.1[a]	10.0 ± 0.0[a]	0.67 ± 0.21[a]	0.50 ± 0.22
TCDD	215.5 ± 2.2	49.2 ± 0.5[b]	7.2 ± 0.2[b]	1.83 ± 0.40[b]	0.67 ± 0.21
TCDD+LC	219.8 ± 2.1	65.5 ± 0.7[c]	9.5 ± 0.2[a]	1.17 ± 0.17[b]	0.50 ± 0.22
TCDD+EA	219.3 ± 11.3	66.9 ± 0.7[c]	9.3 ± 0.2[a]	1.33 ± 0.33[b]	0.67 ± 0.22

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a, b, c, and d: $P < 0.05$).

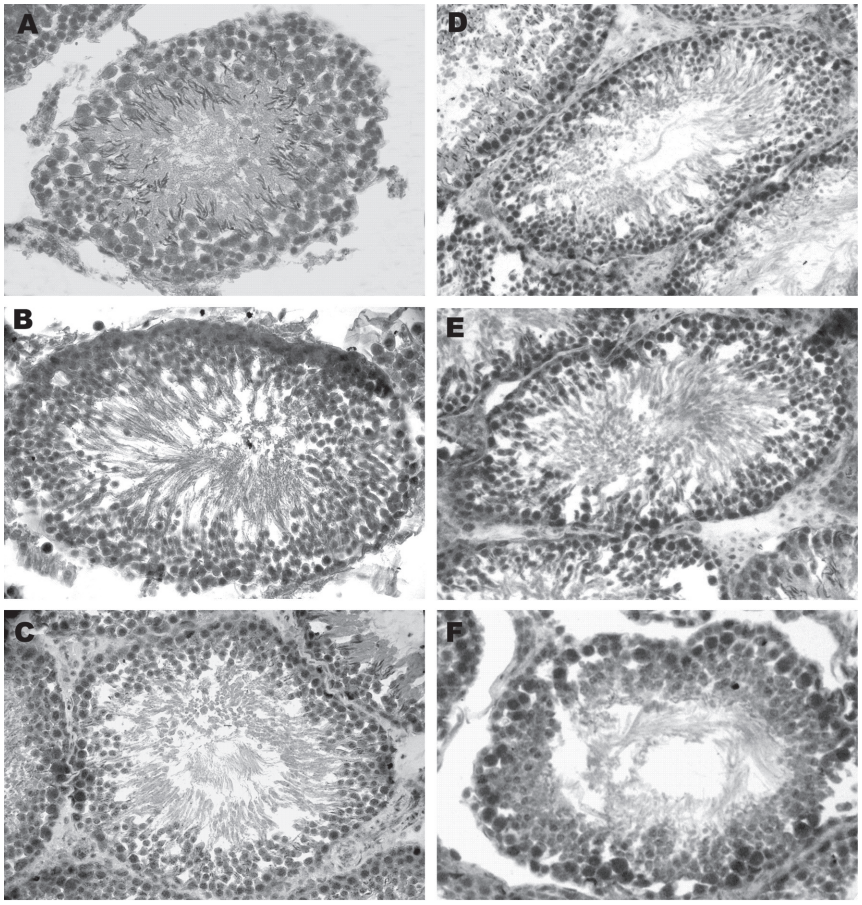

Figure 3. Bax positive in seminiferous tubules in control (**A**), LC (**B**), and EA (**C**) groups and Bcl-2 positive in seminiferous tubules in control (**D**), LC (**E**), and EA (F) groups (200X). (See colour version of this figure online at www.informahealthcare.com/dct)

(O_3), and H_2O_2, are produced during the use of oxygen in normal metabolism and are required for some physiological evidences in the male reproductive system. However, overproduction of ROS leads to LPO, resulting in oxidative stress. Cells have antioxidant mechanisms to decrease, partially or totally, ROS production. Antioxidant enzymes, such as SOD and CAT, react with radicals $O_2^{-\bullet}$ and H_2O_2, respectively. GSH-Px scavenges alkyl ($R^\bullet$), $RO^\bullet$, and $ROO^\bullet$ radicals that may be formed from oxidized membrane components, and it uses GSH as a substrate (Agarwal et al., 2008a, 2008b). In addition to the activation of the aryl hydrocarbon receptor (Fisher et al., 2005), increased LPO is also reported to be responsible for the toxic mechanism of TCDD on the male reproductive system (Latchoumycandane and Mathur, 2002; Latchoumycandane et al., 2002, 2003; El-Tawil and Elsaieed, 2005). It has been reported that TCDD exposure results in increased H_2O_2 and LPO levels and decreased enzymatic (e.g., SOD, CAT, and GSH-Px) and nonenzymatic (e.g., GSH) antioxidants (Latchoumycandane and Mathur, 2002; Latchoumycandane et al., 2002, 2003; El-Tawil and Elsaieed, 2005). It was observed that TCDD given alone significantly increased MDA and GSH levels and significantly decreased SOD activity in this study. The reason for increased MDA levels is the TCDD-induced excessive production of free radicals and, consequently, elevated LPO. The decrease in SOD activity may be attributed to the excessive utilization of this enzyme in order

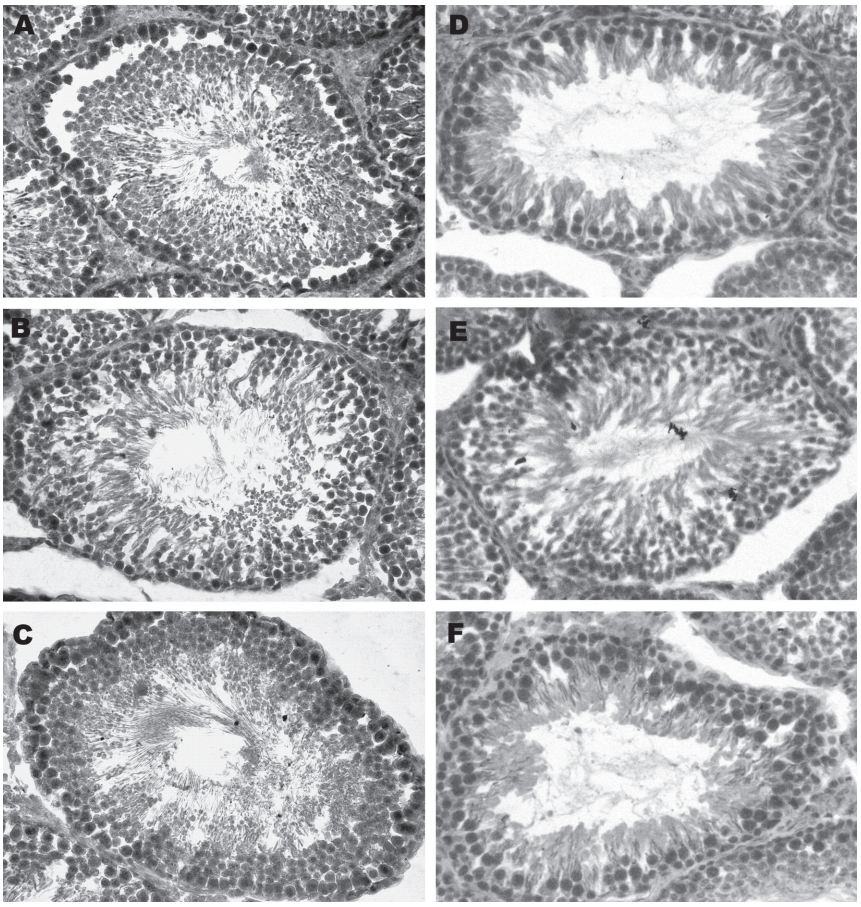

Figure 4. Bax positive in seminiferous tubules in TCDD (**A**), TCDD+LC (**B**), and TCDD+EA (**C**) groups and Bcl-2 positive in seminiferous tubules in TCDD (**D**), TCDD+LC (**E**), and TCDD+EA (**F**) groups (200X). (See colour version of this figure online at www.informahealthcare.com/dct)

to scavenge the free radicals. Salvemini et al. (1999) have reported that GSH synthesis may be induced in cells exposed to oxidative stress as an adaptive process. In the same way, Yılmaz et al. (2006) have suggested that, under conditions of oxidative stress, there may be positive regulation of GSH biosynthesis, resulting in increased levels of GSH. In this study, the increase that was seen in GSH levels after TCDD exposure may have been due to the adaptive process of GSH.

LC, the most effective antioxidant among the carotenoids, is known as a highly efficient scavenger of $^1O_2$ and other excited species (Seren et al., 2008). Being a strong antioxidant, EA attenuates the damaging effect of H_2O_2 and scavenges $O_2^{-\bullet}$ and $^\bullet OH$ by its metal-chelating property, thus providing protection against LPO (Vattem and Shetty, 2005). It has been reported that there is a protective effect of vitamin E (Latchoumycandane and Mathur, 2002) against TCDD-induced oxidative stress and of LC and EA against Aroclor 1254–induced LPO, impaired sperm quality, damaged histological structure, and apoptosis (Ateşşahin et al., 2010) in rat testis. In addition, in our earlier studies, we found that LC and EA protected LPO-induced testicular histological lesions and spermatozoal toxicity (Türk et al., 2007, 2008) and apoptosis (Türk et al., 2010). In the present study, concomitant administration of LC and EA with TCDD provided significant decrease in MDA levels, and significant increase in GSH contents, GCLT, and Johnsen's testicular score, and significant improvements in all sperm parameters evaluated after the 8 weeks of the administration period. However, there was no significant increase in SOD and CAT activity, compared with the TCDD-alone group. Simultaneous administration of LC with TCDD, but not EA, provided a significant increase in GSH-Px activity compared, with the TCDD-alone group. Improvements in Bax- and Bcl-2--positive staining and pro- and antiapoptotic cell scores provided by LC or EA administration to TCDD-treated rats were minimal and not statistically significant. These improvements in testicular structure, sperm quality, oxidant/antioxidant balance, and apoptosis after LC or EA administrations may be explained by their potent free radical–scavenging and antioxidant effects.

Conclusions

In conclusion, this study supports that LC and EA have attenuating effects against testicular and spermatozoal toxicity induced by TCDD. These effects of LC and EA seem to be closely involved with the suppressing of

LPO and enhancing of antioxidant enzyme activities. Therefore, antioxidants from food consumed by human and animals, such as LC and EA, may attenuate the negative effects of certain environmental pollutants.

Declaration of interest

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