

Antiperoxidative and anti-apoptotic effects of lycopene and ellagic acid on cyclophosphamide-induced testicular lipid peroxidation and apoptosis

Gaffari Türk^{A,E}, Ali Osman Çeribaşı^B, Fatih Sakin^C, Mustafa Sönmez^A and Ahmet Ateşşahin^D

^ADepartment of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ, Turkey.

^BDepartment of Pathology, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ, Turkey.

^CDepartment of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Mustafa Kemal University, 31040, Hatay, Turkey.

^DDepartment of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ, Turkey.

^ECorresponding author. Email: gturk@firat.edu.tr

Abstract. The present study was conducted to investigate the possible protective effects of lycopene (LC) and ellagic acid (EA) on cyclophosphamide (CP)-induced testicular and spermatozoal toxicity associated with the oxidative stress and apoptosis in male rats. Forty-eight healthy adult male Sprague-Dawley rats were divided into six groups of eight rats each. The control group was treated with placebo; the LC, EA and CP groups were given LC (10 mg kg⁻¹), EA (2 mg kg⁻¹) and CP (15 mg kg⁻¹), respectively, alone; the CP+LC group was treated with a combination of CP (15 mg kg⁻¹) and LC (10 mg kg⁻¹); and the CP+EA group was treated with a combination of CP (15 mg kg⁻¹) and EA (2 mg kg⁻¹). All treatments were maintained for 8 weeks. At the end of the treatment period, bodyweight and the weight of the reproductive organs, sperm concentration and motility, testicular tissue lipid peroxidation, anti-oxidant enzyme activity and apoptosis (i.e. Bax and Bcl-2 proteins) were determined. Administration of CP resulted in significant decreases in epididymal sperm concentration and motility and significant increases in malondialdehyde levels. Although CP significantly increased the number of Bax-positive (apoptotic) cells, it had no effect on the number of Bcl-2-positive (anti-apoptotic) cells compared with the control group. However, combined treatment of rats with LC or EA in addition to CP prevented the development of CP-induced lipid peroxidation and sperm and testicular damage. In conclusion, CP-induced lipid peroxidation leads to structural and functional damage, as well as apoptosis, in spermatogenic cells of rats. Both LC and EA protect against the development of these detrimental effects.

Additional keyword: sperm characteristics.

Introduction

Cyclophosphamide (CP), a cytotoxic alkylating agent, has anti-cancer and immunosuppressive effects for organ transplantation and autoimmune diseases (Dollery 1999). Despite its therapeutic importance, a wide range of adverse effects, including reproductive toxicity, have been reported for the drug. Spermatogenic cells are a particular target for the damaging effects of CP because it targets fast-dividing cells, changing the composition of the sperm nuclear matrix (Codrington *et al.* 2007) and inducing DNA cross-links, DNA double-strand breaks (Aguilar-Mahecha *et al.* 2005) and germ cell apoptosis (Cai *et al.* 1997; He *et al.* 2006). Decreased reproductive organ weights, oligo-, azoo- and teratozoospermia, low levels of testosterone and LH, atrophied seminiferous tubules, degenerated spermatogenic cells and apoptosis are some of the detrimental effects induced by

CP (Elangovan *et al.* 2006; Selvakumar *et al.* 2006; Chamorro-Cevallos *et al.* 2008; Tripathi and Jena 2008; İlbey *et al.* 2009). The precise mechanism by which CP causes testicular and other organ toxicity is not fully known; however, numerous studies have shown that exposure to CP can disrupt the redox balance of tissues, suggesting that biochemical and physiological disturbances may result from oxidative stress (Selvakumar *et al.* 2005a, 2005b; Ahmadi *et al.* 2008). Free radicals are normally generated in subcellular compartments of the testis, particularly mitochondria, which are subsequently scavenged by anti-oxidant defence systems of the corresponding cellular compartments (Agarwal *et al.* 2008a). However, this balance can easily be broken by chemicals such as CP, which disrupt the pro-oxidant–anti-oxidant balance, leading to cellular dysfunction (Howell and Shalet 2005). In addition, the mitochondrial membrane of

spermatozoa is more susceptible to lipid peroxidation, because this compartment is rich in polyunsaturated fatty acids and has been shown to contain low amounts of anti-oxidants (Aitken and McLaughlin 2007; Agarwal *et al.* 2008c).

Recently, there has been increased interest in understanding the roles and mechanisms of the carotenoids and phytochemicals as inhibitors of oxidative stress. Lycopene (LC), a carotenoid occurring naturally in tomatoes, has attracted considerable attention as an anti-oxidant. Of the various defence strategies, LC is most likely involved in the scavenging of two of the reactive oxygen species (ROS), singlet molecular oxygen ($^1\text{O}_2$) and peroxy radicals. It has been reported that LC efficiently scavenges peroxy radicals, especially at low oxygen tension, and contributes to the defence against lipid peroxidation (Stahl and Sies 2003). Of all the phytochemicals, ellagic acid (EA) has received the most attention because of its wide array of biological properties, including radical scavenging, chemoprevention (Ateşşahin *et al.* 2007; Yüce *et al.* 2007; Türk *et al.* 2008), anti-atherogenic and anti-apoptotic (Yu *et al.* 2005) actions and oestrogen receptor modulation (Papoutsis *et al.* 2005). EA contains four hydroxyl groups and two lactone groups, with the hydroxyl groups known to increase anti-oxidant activity in lipid peroxidation and to protect cells from oxidative damage (Pari and Sivasankari 2008). In the light of this information, the present study was designed to investigate whether LC or EA have a possible protective effect in rats against CP-induced changes in the characteristics of epididymal spermatozoa and testicular tissue associated with oxidative stress and apoptosis.

Materials and methods

Chemicals

CP (500 mg; Endoxan) was purchased from Eczacıbaşı-Baxter (İstanbul, Turkey); LC 10% FS (Redivivo; code 7803) was obtained from DSM Nutritional Products (İstanbul, Turkey); and EA was obtained from Fluka (Steinheim, Germany). All other chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).

Animals and experimental design

Forty-eight healthy adult male Sprague-Dawley rats (8 weeks old) were used. The rats were obtained from Fırat University Experimental Research Centre (Elazığ, Turkey) and were housed under standard laboratory conditions ($24 \pm 3^\circ\text{C}$, 40–60% humidity, 12-h light–dark cycle). A commercial pellet diet (Elazığ Food Co., Elazığ, Turkey) and fresh drinking water were available *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.

Rats were randomly divided into six experimental groups of eight rats as follows: (1) a control group, given 0.5 mL slightly alkaline solution + 0.5 mL corn oil every other day; (2) the LC group, given 0.5 mL slightly alkaline solution + 0.5 mL of a 10 mg kg^{-1} LC solution every other day; (3) the EA group, given 0.5 mL corn oil + 0.5 mL of a 2 mg kg^{-1} EA solution every other day; (4) the CP group, given 0.5 mL of 15 mg kg^{-1} CP + a mixture of slightly alkaline solution and corn oil (0.5 mL) once a week; (5) the CP+LC group, given 0.5 mL of 15 mg kg^{-1}

CP once a week + 0.5 mL of a 10 mg kg^{-1} LC solution every other day; and (6) the CP+EA group, given 0.5 mL of 15 mg kg^{-1} CP once a week + 0.5 mL of a 2 mg kg^{-1} EA solution every other day. The LC was suspended in corn oil, whereas EA, which is difficult to dissolve, was suspended in alkaline solution (0.01 M NaOH, $\sim\text{pH } 12$). The pH of the final solution administered to rats was ≈ 8 . The doses of LC and EA used in the present study were based on those used in earlier studies (Türk *et al.* 2007, 2008). However, because the duration of the treatment period in the present study was longer than that in the previous studies, the doses of the anti-oxidants used were decreased. All treatments were applied by gavage. Because the spermatogenic cycle, including spermatocytogenesis, meiosis and spermiogenesis, in rats is 48–52 days (Bennett and Vickery 1970), the treatment period in the present study was set at 8 weeks.

Sample collection and homogenate preparation

Rats were killed under light ether anaesthesia at the end of 8 weeks. The testes, epididymides, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. Blood samples were collected and centrifuged at 3000g for 10 min to obtain plasma. One of the testicles was fixed in 10% neutral formalin solution for immunohistochemical examination. The other testicle and plasma samples were stored at -20°C until used for biochemical analyses. Testis tissues were taken from the deep-freezer and weighed. They were then transferred to cold glass tubes and diluted with a ninefold volume of phosphate buffer (pH 7.4). For the enzymatic analyses, testicular tissues were minced and then homogenised using a Teflon–glass homogeniser at 16 000g for 3 min in cold physiological saline on ice.

Sperm analyses

Epididymal sperm concentration

The epididymal sperm concentration was determined using a haemocytometer according to the method of Türk *et al.* (2007, 2008) with some modifications. The cauda of the right epididymis was finely minced using anatomical scissors in 1 mL isotonic saline in a Petri dish. The tissue was then squashed using tweezers for 2 min and allowed to set at room temperature for 4 h to enable the migration of all spermatozoa from the epididymal tissue into the fluid. After incubation, the epididymal tissue–fluid mixture was filtered through a $3\text{-}\mu$ mesh to separate the supernatant from the tissue. The supernatant, containing all the epididymal spermatozoa, was drawn into a pipette designed for counting red blood cells up to the 0.5 mark. A solution containing 0.595 M sodium bicarbonate, 1% formalin and 0.025% eosin was then drawn up into the pipette up to 101 mark. This resulted in a dilution rate of 1 : 200. The contents of the pipette were mixed by holding the ends of the pipette between the thumb and index finger and shaking the pipette vigorously for 100 back-and-forth (30 cm) movements. The bulb of the pipette contained a small glass bead that made thorough mixing possible. After mixing, sufficient solution was expelled from the pipette to ensure that diluents containing no spermatozoa were flushed from the capillary. Approximately $10 \mu\text{L}$ diluted sperm suspension was

transferred to both counting chambers of an improved Neubauer (Deep 1/10 mm; LAB ART, Darmstadt, Germany) and allowed to stand for 5 min. The spermatozoa in both chambers were counted under a light microscope at $\times 200$ magnification.

Sperm motility

The percentage of motile spermatozoa was determined using a light microscope with a heated stage as described by Sönmez *et al.* (2005). To this end, a slide was placed on the heated stage (37°C) of a light microscope, 100 μL of Tris buffer solution (0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid) were placed on the slide, followed by the addition of 10 μL of fluid obtained from the left cauda epididymis and the solutions mixed using a coverslip. The percentage of motile spermatozoa was determined visually under the microscope at $\times 400$. Motility estimates were obtained from three different fields in each sample. The mean of these three estimates was used as the final motility score.

Biochemical analyses

Lipid peroxidation

Testicular tissue lipid peroxidation levels were determined as thiobarbituric acid-reactive substances (TBARS; Placer *et al.* 1966). The amount of malondialdehyde (MDA) produced was used as an index of lipid peroxidation. Briefly, one volume of test sample and two volumes of stock reagent (15% w/v trichloroacetic acid (TCA) in 0.25 M HCl and 0.375% w/v thiobarbituric acid in 0.25 M HCl) were mixed in a centrifuge tube. The solution was heated in boiling water for 15 min. After cooling, the precipitate was removed by centrifugation at 1500g for 10 min and the absorbance of the supernatant was read on a spectrophotometer at 532 nm against a blank containing all reagents except the test sample (2R/UV-visible; Shimadzu, Tokyo, Japan). The concentration of MDA is expressed as μM .

Glutathione

Reduced glutathione (GSH) levels in testicular tissue were determined at 412 nm using the method described by Sedlak and Lindsay (1968). Briefly, samples were precipitated with 50% TCA and then centrifuged at 1000g for 5 min. The reaction mixture contained 0.5 mL supernatant, 2.0 mL Tris-EDTA buffer (0.2 M, pH 8.9) and 0.1 mL of 0.01 M 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min before absorbance was read at 412 nm in a spectrophotometer. Concentrations of GSH are given in μM .

Glutathione peroxidase activity and protein concentration

Glutathione peroxidase (GPx) activity in testicular tissue was determined according to the method of Lawrence and Burk (1976). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH, 1 IU mL^{-1} oxidised glutathione (GSSG) reductase, 1 mM GSH and 0.25 mM H_2O_2 . An enzyme source (0.1 mL) was added to 0.8 mL of this solution and the mixture was incubated at 25°C for 5 min before initiation of the reaction with the addition of 0.1 mL peroxide solution. Absorbance was recorded

at 340 nm for 5 min on a spectrophotometer. GPx activity was calculated from the slope of the lines as μmol NADPH oxidised per min. Activity in the blank, in which the enzyme had been replaced with distilled water, was subtracted from each sample value. Protein concentrations were determined using the method of Lowry *et al.* (1951). GPx activity is expressed as IU g^{-1} protein.

Catalase

Testicular catalase activity was determined by measuring the decomposition of H_2O_2 at 240 nm, according to the method described by Aebi (1983). Catalase activity is expressed as $k\text{U g}^{-1}$ protein, where k is the first-order rate constant.

Superoxide dismutase

Testicular superoxide dismutase (SOD) activity was determined using xanthine and xanthine oxidase to generate superoxide radicals that react with nitroblue tetrazolium (NBT; Flohe and Otting 1984). Briefly, each sample was diluted 1 : 10 with phosphate buffer (50 mM, pH 7.5). The assay solution, containing sodium carbonate buffer (50 mM, pH 10), 0.1 mM xanthine, 0.025 mM NBT, 0.1 mM EDTA, xanthine oxidase (0.1 U mL^{-1} in 2 M ammonium sulfate) and sample, was mixed in a cuvette. One unit of SOD activity was defined as the amount of enzyme required to inhibit NBT by 50%. SOD activity was determined spectrophotometrically at 560 nm as the degree of inhibition and is expressed as U mL^{-1} .

Testosterone

Plasma testosterone concentrations were measured by ELISA (ELISA EIA-1559, 96 Wells kit; DRG Instruments, Marburg, Germany) according to the manufacturer's instruction. Concentrations are reported as ng dL^{-1} .

Apoptotic and anti-apoptotic cell rates determined by immunohistochemistry

The avidin-biotin-peroxidase method was used for immunohistochemical analyses (Jahnukainen *et al.* 2004). Testes tissues, which were embedded in paraffin and sectioned at 4 μm , were deparaffinised with xylene and dehydrated with an alcohol series. Testicular sections were incubated in 0.01 M Na-citrate for 20 min to bring out the antigenic receptors. Sections were washed with phosphate-buffered saline (PBS) and were then incubated in 3% H_2O_2 , prepared in PBS, for 10 min to inactivate endogenous peroxidase activity. Non-specific binding was blocked by incubation of samples with 1% untreated goat serum for 1 h. Testicular tissues were then incubated with primary rabbit polyclonal antibodies directed against Bax (a pro-apoptotic protein) and Bcl-2 (an anti-apoptotic protein) at dilutions of 1 : 200 and 1 : 400, respectively, in PBS containing 0.1% goat serum at 37°C for 1 h. Testicular sections were washed again in PBS and were incubated with biotinylated secondary antibodies, which were diluted 1 : 1000 in PBS containing 0.1% goat serum (secondary biotinylated goat anti-rabbit IgG) for 30 min. Thereafter, tissues were washed with PBS and were incubated with avidin-conjugated horseradish peroxidase for 1 h. 3-Amino-9-ethylcarbazole (AEC) was used as colour-determining substrate. The reaction was stopped the moment the colour

Table 1. Absolute and relative reproductive organ weight

Data are the mean \pm s.e.m. Values with different superscript letters within the same column differ significantly ($P < 0.05$). LC, lycopene; EA, ellagic acid; CP, cyclophosphamide

Group	Absolute weight (mg)				Relative weight (mg g ⁻¹ bodyweight)			
	Testis	Epididymis	Seminal vesicles	Ventral prostate	Testis	Epididymis	Seminal vesicles	Ventral prostate
Control	1345.0 \pm 25.7	454.2 \pm 9.5	901.7 \pm 32.2	455.0 \pm 26.1	4.99 \pm 0.33	1.66 \pm 0.12	3.42 \pm 0.27	1.72 \pm 0.14 ^{ab}
LC	1353.3 \pm 119.8	473.7 \pm 7.2	918.3 \pm 64.7	463.3 \pm 11.2	5.10 \pm 0.11	1.80 \pm 0.07	3.75 \pm 0.42	1.74 \pm 0.06 ^{ab}
EA	1348.3 \pm 93.3	456.0 \pm 6.1	985.0 \pm 47.5	483.3 \pm 10.2	5.12 \pm 0.08	1.73 \pm 0.02	3.76 \pm 0.23	1.84 \pm 0.03 ^a
CP	1290.0 \pm 32.3	430.0 \pm 16.1	793.3 \pm 34.9	420.0 \pm 12.1	4.53 \pm 0.19	1.52 \pm 0.10	2.80 \pm 0.18	1.48 \pm 0.08 ^b
CP+LC	1323.3 \pm 87.4	465.8 \pm 6.1	905.0 \pm 48.5	429.2 \pm 13.1	4.88 \pm 0.29	1.73 \pm 0.08	3.34 \pm 0.35	1.64 \pm 0.06 ^{ab}
CP+EA	1334.2 \pm 99.0	432.5 \pm 16.8	838.3 \pm 55.0	426.7 \pm 25.9	4.84 \pm 0.20	1.57 \pm 0.06	3.01 \pm 0.09	1.53 \pm 0.05 ^{ab}

Table 2. Sperm parameters and immunohistochemical scores

Data are the mean \pm s.e.m. Values with different superscript letters within the same column differ significantly ($P < 0.05$). LC, lycopene; EA, ellagic acid; CP, cyclophosphamide

Group	Sperm motility (%)	Epididymal sperm concentration (million/g tissue)	Bax-positive cell score	Bcl-2-positive cell score
Control	77.77 \pm 2.94 ^{ab}	347.5 \pm 11.5 ^a	0.33 \pm 0.21 ^a	0.33 \pm 0.21 ^a
LC	85.53 \pm 1.86 ^b	344.6 \pm 9.7 ^{ab}	0.17 \pm 0.17 ^a	0.50 \pm 0.22 ^a
EA	82.76 \pm 3.15 ^b	351.3 \pm 11.5 ^a	0.67 \pm 0.21 ^a	0.50 \pm 0.22 ^a
CP	51.07 \pm 3.72 ^c	288.8 \pm 3.1 ^b	2.17 \pm 0.41 ^b	0.57 \pm 0.17 ^a
CP+LC	79.42 \pm 2.35 ^{ab}	329.9 \pm 16.4 ^{ab}	1.17 \pm 0.17 ^c	1.00 \pm 0.00 ^b
CP+EA	68.87 \pm 4.01 ^a	313.0 \pm 21.3 ^{ab}	1.33 \pm 0.21 ^c	1.00 \pm 0.00 ^b

change occurred after the addition of AEC to the samples. Finally, samples were washed with tap water for 2 min after they had been stained with Mayer's haematoxylin for 15 s. Stained tissues were covered with immune-mount (Lab Vision, UK, cat. Number: TA-060-UG) and then Bax- and Bcl-2-positive spermatogenic cells (from spermatogonia to elongated spermatids) were evaluated under a light microscope and scored according to Kandi Coşkun and Çobanoğlu (2005) as follows: 0, no 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 data are presented as the mean \pm s.e.m. Data were analysed using SPSS Version 10.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test were used to evaluate differences between groups. $P < 0.05$ was considered significant.

Results

Bodyweight and reproductive organ weight

There were no significant differences in mean bodyweight at the end of the 8-week treatment period between the control, LC, EA, CP, CP+LC and CP+EA groups (275.6 \pm 17.2, 263.3 \pm 8.4, 263.5 \pm 4.8, 287.3 \pm 14.0, 273.8 \pm 9.6 and 279.0 \pm 17.9 g, respectively). Absolute and relative reproductive organ weights are given in Table 1. Treatment with LC and EA alone had

no effect on the absolute and relative organ weights compared with those in the control group. Although there was a tendency for decreased absolute and relative organ weights following CP administration, the difference did not reach statistical significance compared with the control group. Similarly, although there was a tendency for LC or EA administration to CP-treated animals to increase absolute and relative organ weights compared with CP treatment alone, the differences did not reach statistical significance.

Sperm concentration and motility

Table 2 lists changes in epididymal sperm characteristics in response to the various treatments over the 8 weeks. Although treatment with LC and EA alone had no effect on any of the parameters evaluated, CP treatment significantly ($P < 0.05$) decreased sperm motility and concentration compared with values in the control group. A significant increase in sperm motility ($P < 0.05$) was observed in the CP+LC and CP+EA groups compared with values for the CP-treated group. Although there was a tendency for LC or EA administration to CP-treated rats to bring sperm concentration back towards control values, the difference did not reach statistical significance compared with CP treatment alone.

Biochemical parameters

Testicular tissue lipid peroxidation levels, anti-oxidant enzyme activities and plasma testosterone concentrations are given

Table 3. Malondialdehyde and reduced glutathione concentrations and glutathione peroxidase, catalase and superoxide dismutase activity in testicular tissue and plasma testosterone concentrations

Data are the mean \pm s.e.m. Values with different superscript letters within the same column differ significantly ($P < 0.05$). LC, lycopene; EA, ellagic acid; CP, cyclophosphamide; MDA, malondialdehyde; GSH, reduced glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; k , first-order rate constant

Group	MDA (μM)	GSH (μM)	GPx (IU g^{-1} protein)	Catalase ($k\text{U g}^{-1}$ protein)	SOD (U mL^{-1})	Testosterone (ng dL^{-1})
Control	93.4 \pm 12.0 ^{ab}	6.54 \pm 1.15 ^a	10.24 \pm 1.04	6.20 \pm 0.35 ^a	1.72 \pm 0.28	392 \pm 65
LC	106.6 \pm 8.9 ^{bc}	10.4 \pm 0.6 ^b	8.45 \pm 1.10	13.35 \pm 0.89 ^c	1.79 \pm 0.26	289 \pm 66
EA	105.9 \pm 15.4 ^{bc}	8.74 \pm 0.67 ^{ab}	10.24 \pm 2.44	6.50 \pm 0.32 ^a	1.60 \pm 0.29	354 \pm 74
CP	131.3 \pm 9.9 ^c	7.50 \pm 0.76 ^a	5.12 \pm 1.15	11.47 \pm 0.73 ^{bc}	1.38 \pm 0.17	206 \pm 43
CP+LC	83.2 \pm 10.6 ^{ab}	8.53 \pm 0.67 ^{ab}	7.60 \pm 1.29	10.9 \pm 1.2 ^b	2.24 \pm 0.52	146 \pm 36
CP+EA	66.4 \pm 10.9 ^a	6.80 \pm 1.10 ^a	10.01 \pm 1.95	10.54 \pm 0.68 ^b	1.82 \pm 0.23	183 \pm 31

in Table 3. Although treatment with CP alone significantly ($P < 0.05$) increased MDA levels compared with the control group, LC and in particular EA administration to CP-treated rats significantly ($P < 0.05$) reduced these increased MDA levels compared with levels in the CP-treated group. Only LC caused a significant increase in GSH levels compared with the control group. Although CP treatment alone decreased GPx activity by 50% compared with that in the control group, LC and EA administration to CP-treated rats increased the CP-induced decrease in GPx activity (by 48.4% and 95.5%, respectively). Despite this, there were no significant differences in GPx activity between the treatment groups. Catalase activity in the LC, CP, CP+LC and CP+EA groups was significantly ($P < 0.05$) higher than that in the control and EA groups. Although CP administration tended to decrease SOD activity and plasma testosterone concentrations compared with the control group, the differences did not reach statistical significance.

Apoptotic and anti-apoptotic cell rates

Immunohistochemical analysis of Bax staining failed to demonstrate any significant difference between the control (Fig. 1a), LC (Fig. 1b) and EA (Fig. 1c) groups. However, Bax-positive cells were observed more frequently in testis sections from CP-treated rats (Fig. 2a) than in the control group. Intense staining was observed in almost all the spermatogenic cell types (from spermatogonia to elongated spermatids) in testis sections from CP-treated rats. A decrease in this intense staining was observed in both the CP+LC (Fig. 2b) and CP+EA (Fig. 2c) groups compared with CP treatment alone. Administration of CP significantly ($P < 0.05$) increased the number of Bax-positive cells compared with the control group (Table 2), which was reduced by LC or EA treatment ($P < 0.05$).

Immunohistochemical analysis of Bcl-2 staining failed to demonstrate any significant difference between the control (Fig. 1d), LC (Fig. 1e), EA (Fig. 1f) and CP (Fig. 2d) groups. However, significant intense staining was seen in almost all spermatogenic cells in both the CP+LC (Fig. 2e) and CP+EA (Fig. 2f) groups compared with the other four groups. Thus, although CP administration itself did not affect the number of Bcl-2-positive cells compared with the control group (Table 2), concomitant administration of LC or EA resulted in a

significant ($P < 0.05$) increase in the number of Bcl-2-positive anti-apoptotic cells.

Discussion

Treatment with cytotoxic chemotherapy is associated with significant gonadal damage. Alkylating agents, such as CP, are the most common agents implicated in causing this damage. Reproductive cells and tissues remain stable when the balance between free radical production and scavenging anti-oxidants is maintained. Free radicals can attack and inactivate or alter the biological activity of molecules such as lipids and proteins, which are essential for cell function (Agarwal *et al.* 2008a, 2008b). CP causes excessive production of oxygen-derived free radicals in the testis (İlbey *et al.* 2009). Although some authors (Elangovan *et al.* 2006; Vaisheva *et al.* 2007; Tripathi and Jena 2008; İlbey *et al.* 2009) have reported that CP administration causes significant decreases in bodyweight and reproductive organ weight, others (Satoh *et al.* 2002; Fukushima *et al.* 2005) have reported no such effect. In the present study, CP did not change bodyweight or absolute and relative organ weights compared with the control group. Our findings are in agreement with the results of Satoh *et al.* (2002) and Fukushima *et al.* (2005). The apparent discrepancies reported in the literature may be due to differences in the doses used, the duration of treatment, the route of CP administration and the sensitivity of the animals used in the different studies.

Because the sperm plasma membrane contains large quantities of polyunsaturated fatty acids and sperm cytoplasm contains low concentrations of scavenging enzymes, spermatozoa are particularly susceptible to the damage induced by excessive ROS (Aitken and McLaughlin 2007; Agarwal *et al.* 2008c). The increase in free radicals in cells can induce lipid peroxidation by oxidative breakdown of polyunsaturated fatty acids in the membranes of these cells. Obviously, peroxidation of sperm lipids destroys the structure of the lipid matrix in the plasma membranes and it is associated with rapid loss of intracellular ATP, leading to axonemal damage, decreased sperm viability and, in extreme cases, even complete inhibition of spermatogenesis (Türk *et al.* 2007, 2008). Selvakumar *et al.* (2006) and İlbey *et al.* (2009) have reported that treatment of male rats with CP results in a significant decrease in

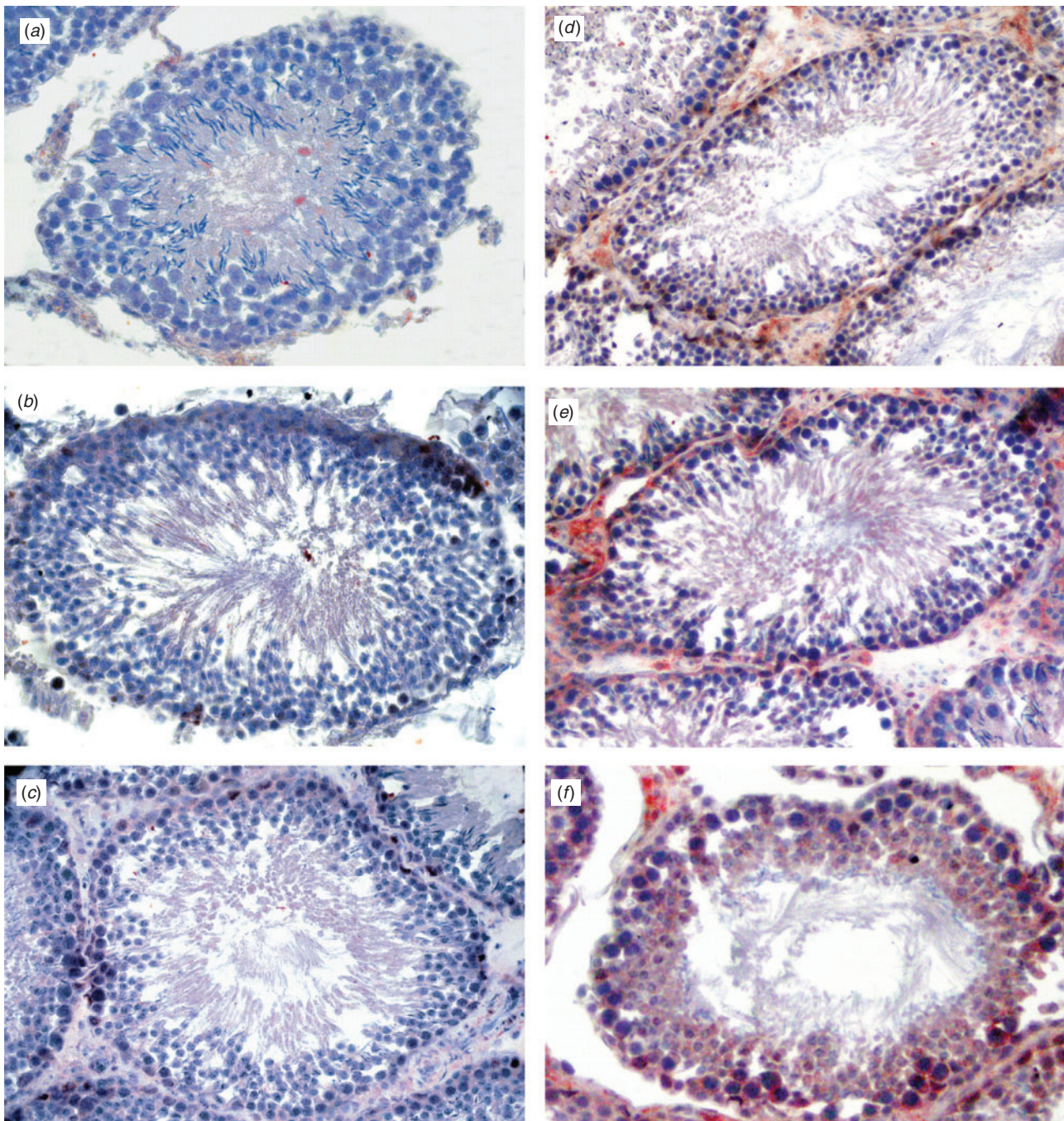


Fig. 1. (a–c) Bax- and (d–f) Bcl-2-positive cells in seminiferous tubules from the control group (a, d), the lycopene (LC)-treated group (b, e) and the ellagic acid (EA)-treated group (c, f). There were eight rats in each group. (Original magnification $\times 200$.)

sperm concentration and motility. In the present study, it was observed that CP administration resulted in significant decreases in sperm motility and concentration compared with the control group. Our findings are in agreement with those previous studies. The negative changes observed in sperm quality after CP exposure in the present study may be attributed to the peroxidation of polyunsaturated fatty acids in the plasma membranes of the

spermatozoa, damage to the flagellum, which is important for sperm motility, direct impairment of spermatogenic cell development, impaired maturation or spermiation and damaged to sperm DNA.

The pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins exist when apoptosis reached a high point after the onset of cellular stress. The ratio of these molecules has been implicated as a

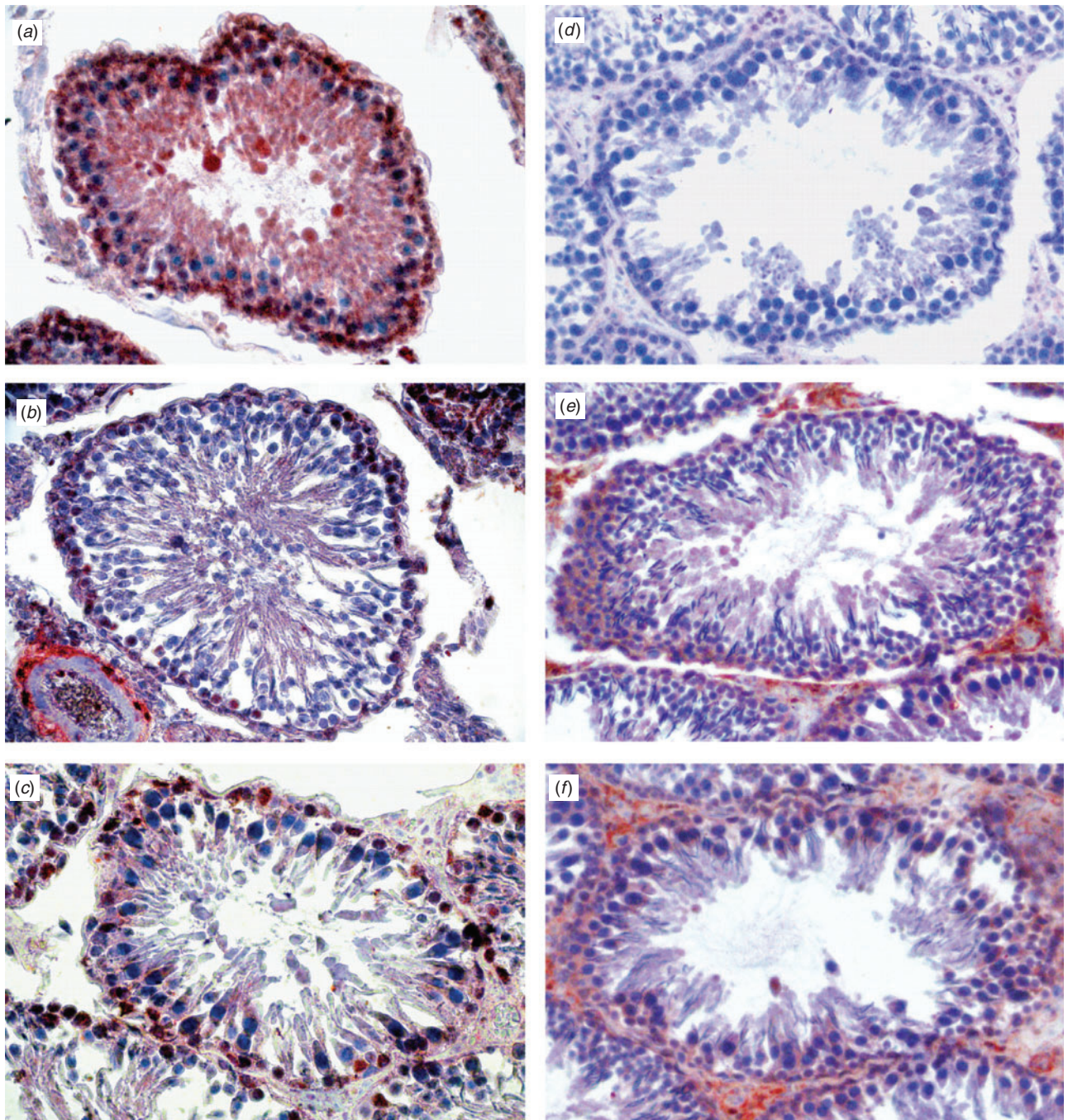


Fig. 2. (a–c) Bax- and (d–f) Bcl-2-positive cells in seminiferous tubules from rats treated with cyclophosphamide (CP) alone (a, d), with CP+lycopene (b, e) or with CP+ellagic acid (c, f). There were eight rats in each group. (Original magnification $\times 200$.)

critical determinant of cell fate, such that elevated Bcl-2 favours extended survival of cells and increased levels of Bax accelerate cell death (Sinha Hikim and Swerdloff 1999). It has been reported that acute and chronic exposure to chemotherapeutics, such as CP (Cai *et al.* 1997; He *et al.* 2006), cisplatin (Zhang *et al.* 2001) and doxorubicin (Hou *et al.* 2005), results in elevated apoptotic germ cell rates. In the present study, Bax-positive cells

were observed more frequently in testis sections from CP-treated rats than in the control group. Intense staining was observed in almost all spermatogenic cell types (from spermatogonia to elongated spermatids) in testis sections from CP-treated rats. In addition, CP administration significantly elevated the Bax-positive apoptotic cell score compared with the control group. In terms of Bcl-2-positive staining and Bcl-2-positive cell scores,

there were no immunohistochemically significant differences between the control and CP-treated groups. These findings are in agreement with the reports mentioned above. H_2O_2 , a type of ROS, induces testicular germ cell apoptosis by extrinsic and intrinsic mechanisms, as well as through other regulatory pathways (Maheshwari *et al.* 2009). The elevated apoptotic cell rates after exposure to CP observed in the present study may be explained by increased ROS and lipid peroxidation levels in testicular tissue and spermatogenic cells or direct DNA and chromatin damage in germ cells.

The use of oxygen during normal metabolism produces ROS, some of which are highly toxic and deleterious to cells and tissues. The most abundant ROS formed during the course of cellular metabolism is $\bullet O_2^-$ (Halliwell 1991). Dismutation of $\bullet O_2^-$ or the direct action of oxidase enzymes gives rise to H_2O_2 . This molecule is not a free radical itself but, in the presence of transition metals (e.g. Cu^{2+} , Fe^{2+}), it is rapidly converted via the Fenton reaction to the hydroxyl radical ($\bullet OH$). It is widely accepted that $\bullet OH$ is the most damaging ROS produced by cells (Halliwell and Gutteridge 1992). It is generally accepted that increased lipid peroxidation is one of the toxic manifestations in the testis following CP administration. Treatment with CP results in elevated MDA levels, a by-product of lipid peroxidation, due to the excessive generation of free radicals (Ghosh *et al.* 2002; Selvakumar *et al.* 2005a, 2006; İlbey *et al.* 2009). In the present study, treatment with CP alone resulted in a significant increase in MDA levels in testicular tissue. This can be attributed to the CP-induced excessive production of free radicals and the subsequent increase in lipid peroxidation.

Cells have anti-oxidant mechanisms to combat ROS production partially or totally. The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of $\bullet O_2^-$ when they are converted to H_2O_2 and diatomic oxygen by the enzyme SOD. Two types of enzymes participate in the removal of H_2O_2 from the cellular environment, namely peroxidases and catalase. The most abundant peroxidase is GPx. This enzyme uses GSH as a substrate (Matés 2000). It has been demonstrated that CP reduces GSH levels, as well as GPx, catalase and SOD activity, in the testis (Ghosh *et al.* 2002; Selvakumar *et al.* 2005a, 2006; İlbey *et al.* 2009). However, Salvemini *et al.* (1999) 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) suggested that, under conditions of oxidative stress, there may be positive regulation of GSH biosynthesis, resulting in increased levels of GSH. Catalase activity may increase in cells under oxidative stress to compensate for the lower activity of GSH reductase or NADPH (Pande and Flora 2002). Although CP administration in the present study caused a significant increase in catalase activity, it did not significantly increase GSH levels or decrease GPx and SOD activity compared with the control group. The non-significant increase in GSH levels after CP treatment in the present study may be attributed to the adaptive process of GSH. The significant increase in catalase activity in testicular tissue induced by CP may be explained as a compensatory mechanism for the lower GPx and SOD activity. The non-significant effects of CP on GPx and SOD activity in the present study may be attributed to the excessive use of these anti-oxidants to scavenge the free

radicals. The activity of microsomal steroidogenic enzymes (i.e. 3β -hydroxysteroid dehydrogenase (HSD) and 17β -HSD) in the testis is reduced in the presence of elevated free radical products (MDA) induced by CP (Ghosh *et al.* 2002). In the present study, although CP administration tended to decrease plasma testosterone concentrations, the effect was not statistically significant. This may be correlated with the inhibition of testicular steroidogenesis and impairment of Leydig cells.

It is known that LC is the most effective anti-oxidant among the carotenoids. It is a highly efficient scavenger of 1O_2 and other excited species. During 1O_2 quenching, energy is transferred from 1O_2 to the LC molecule, converting it to the energy rich triplet state. In contrast, trapping of other ROS, such as $\bullet OH$, NO_2 or peroxy nitrite, leads to oxidative breakdown of the LC molecule. Thus, LC may protect *in vivo* against the oxidation of lipids, proteins and DNA (Stahl and Sies 2003). It has been reported that EA, a naturally occurring plant-derived polyphenol (Soong and Barlow 2004), exhibits anti-oxidative properties both *in vivo* (Yüce *et al.* 2007; Türk *et al.* 2008) and *in vitro* (Seeram *et al.* 2005). It also chelates metal ions and prevents iron- and copper-catalysed formation of ROS. In earlier studies, we found that LC and EA protect the testes and spermatozoa against cisplatin- and adriamycin-induced toxicity (Ateşşahin *et al.* 2006a, 2006b; Türk *et al.* 2008). Yu *et al.* (2005) have reported that EA reduces oxidative stress and apoptosis in hyperlipidaemic rabbits. In the present study, the administration of LC and EA to CP-treated rats resulted in significant increments in sperm motility, but non-significant increases in sperm concentration, compared with the CP-treated group. A decrease in Bax-positive and an increase in Bcl-2-positive staining in almost all spermatogenic cells was observed in both the CP+LC and CP+EA groups compared with that in the CP group. In addition to decreasing the CP-induced increase in Bax-positive apoptotic cell scores, LC and EA also increased the anti-apoptotic Bcl-2-positive cell scores. These improvements in testicular tissue and sperm quality after LC or EA administration may be explained by the partial or total modulation of CP-induced immunohistochemical damage, as well as the tendency for increased sperm concentrations in the epididymis, decreases in the number of apoptotic cells and lipid peroxidation and non-significant increases in GPx and SOD activity.

It has been reported that, in the testis, LC and EA decrease MDA levels and increase GSH levels and GPx and catalase activity (Ateşşahin *et al.* 2006a, 2006b; Türk *et al.* 2008). In the present study, it was observed that LC and in particular EA administration to CP-treated rats significantly reduced the CP-induced increase in MDA levels. The decline in lipid peroxidation in testicular tissue apparently indicates that LC and EA potentially scavenge free radicals and suppress oxidative DNA damage. Although LC and EA administration to CP-treated rats increased the CP-induced decrease in GPx activity (by 48.4% and 95.5%, respectively), these increments did not reach statistical significance. No significant changes were observed in catalase activity and GSH and testosterone concentrations between the CP, CP+LC and CP+EA groups. That is, LC and EA have no marked effects on the activity of endogenous anti-oxidant enzymes and plasma testosterone concentrations.

In conclusion, the results of the present study suggest that LC and EA have modulatory effects against testicular and spermatozoal toxicity induced by CP. These modulatory effects of LC and EA seem to involve the suppression of lipid peroxidation and apoptosis. According to the findings of the present study, although LC and EA have no marked effects on the activity of endogenous anti-oxidant enzymes, they do have potent anti-oxidant and anti-apoptotic actions because they inhibit lipid peroxidation and apoptosis. Therefore, LC or EA may be used in combination with CP in the treatment of cancer patients, those with autoimmune diseases and after transplantation to improve CP-induced damage to sperm quality and to prevent apoptosis and lipid peroxidation.

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