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Lycopene and ellagic acid prevent testicular apoptosis induced by cisplatin

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ABSTRACT

The aim of this study was to investigate the possible protective effects of lycopene (LC) and ellagic acid (EA) on cisplatin (CP)-induced testicular apoptosis in male rats. The control group was treated with placebo; LC, EA and CP groups were given alone LC, EA and CP, respectively; the CP + LC group was treated with a combination of CP and LC; and the CP + EA group was treated with a combination of CP and EA. Although CP significantly increased the number of Bax-positive (apoptotic) cells it had no effect on the number of Bcl-2-positive (antiapoptotic) cells compared with the control group. Administration of CP caused significant increase in lipid peroxidation and nonsignificant decrease in superoxide dismutase (SOD) activity along with some histopathological lesions in testicular tissue. However, combined treatments of LC or EA in addition to CP tended to prevent the CP-induced testicular apoptosis, histopathological lesions and lipid peroxidation.

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Introduction

Cisplatin (CP) is a highly effective antineoplastic DNA alkylating agent used to treat many types of cancer such as testicular, ovarian, bladder and lung. In spite of its therapeutic importance, a wide range of adverse effects including reproductive toxicity have been reported (Boekelheide 2005). Spermatogenesis couples genetic recombination and meiosis with germ cell proliferation and differentiation, culminating in the release of spermatozoa from the testis. Spermatogenesis is controlled by complex interactions between the Sertoli cells and germ cells of the seminiferous epithelium. One regulatory process that is required for functional spermatogenesis and orchestrated, in part, by this interaction is the death of germ cells via apoptosis. 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 spermatogonial population (Blanco-Rodriguez 1998). However, an increase in the frequency of germ cell apoptosis is seen because of the side effects of cisplatin (Amin et al. 2008; Lirdi et al. 2008) and the other chemoterapeutics on testis (Marcon et al. 2008; Türk et al. 2010) in experimental animals. In addition, decreased reproductive organ weights, astheno, azoo- and teratozoospermia (Ateşşahin et al. 2006a,b; Türk et al. 2008), atrophied seminiferous tubules and degenerated spermatogenic cells (Ateşşahin et al. 2006b; Türk et al. 2008) are some of the other detrimental effects induced by CP. The precise mechanism by which CP causes testicular toxicity and germ cell apoptosis is not fully known; however, numerous studies have shown that CP exposure can disrupt the redox balance of tissues, suggesting that biochemical and physiological disturbances may result from oxidative stress (Ateşşahin et al. 2006a,b; Amin et al. 2008; Türk et al. 2008). Reactive oxygen species (ROS) like singlet molecular oxygen ($^1O_2$), superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^\bullet OH$) and hydrogen peroxide (H_2O_2) are normally generated in subcellular compartments of testis, particularly mitochondria, which are subsequently scavenged by antioxidant defense systems of the corresponding cellular compartments (Agarwal et al. 2008). However, this balance can easily be broken by chemicals like CP, which disrupt the prooxidant–antioxidant balance, leading to cellular dysfunction (Ateşşahin et al. 2006b; Türk et al. 2008). It has been reported that H_2O_2 induces testicular germ cell apoptosis by extrinsic and intrinsic mechanisms as well other regulatory pathways (Maheshwari et al. 2009).

Lycopene (LC) is the most potent antioxidant among various common carotenoids. Among the various defense strategies, LC is most likely involved in the scavenging of two of the reactive oxygen species (ROS), singlet molecular oxygen ($^1O_2$), and peroxyl radicals. LC efficiently scavenges peroxyl radicals, especially at low oxygen tension, and contributes to the defense against lipid peroxidation (Stahl and Sies 2003). Ellagic acid (EA) has been receiving the most attention because of its wide array of biological properties, such as radical scavenging, chemopreventive (Ateşşahin et al. 2006a; Türk

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et al. 2008) and antiapoptotic (Türk et al. 2010). It has been reported that amifostine (Lirdi et al. 2008), ginger and roselle (Amin et al. 2008) have a protective effect on the CP-induced germ cell apoptosis. In our previous study (Türk et al. 2010) we observed that both LC and EA significantly decreased the frequency of testicular apoptosis induced by cyclophosphamide, a chemotherapeutic. The purpose of the present study was to investigate whether LC or EA has possible protective effects against CP-induced germ cell apoptosis associated with testicular tissue lipid peroxidation in rats.

Materials and methods

Chemicals

CP (10 mg ml^{-1}, code 1876A) was purchased from Faulding Pharmaceuticals (Warwickshire, UK); 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 protocol

Forty-eight healthy adult male Sprague–Dawley rats (8 weeks old) were used. The animals were obtained from Fırat University, Experimental Research Centre (Elazığ, Turkey) and were housed under standard laboratory conditions (24 ± 3 °C, 40–60% humidity, a 12-h light:12-h dark cycle). A commercial pellet diet (Elazığ Food Co., Elazığ, Turkey) and fresh drinking water were available *ad libitum*. The protocol for the animal use was approved by the Institutional Review Board of the National Institute of Health and Local Committee on Animal Research. The animals were randomly divided into six experimental groups of 8 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 10 mg kg^{-1} LC solution every other day; (3) the EA group, given 0.5 ml corn oil + 0.5 of 2 mg kg^{-1} EA solution every other day; (4) the CP group, given 0.5 ml of 2 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 2 mg kg^{-1} CP once a week + 0.5 ml of 10 mg kg^{-1} LC every other day; (6) the CP + EA group, given 0.5 ml of 2 mg kg^{-1} CP once a week + 0.5 ml of 2 mg kg^{-1} EA every other day. Although CP was administered by intraperitoneal injection, other chemicals were administered by gavage. The LC was suspended in corn oil, whereas EA, which is difficult to dissolve, was suspended in alkaline solution (0.01 M NaOH, 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 (Çeribaşı et al. 2010; Türk et al. 2010). Because the spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis, in rats is 48–52 days (Türk et al. 2010), 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. Both testes were removed, cleared of adhering connective tissue. One of the testicles was fixed in 10% neutral-formalin solution for histopathological and immunohistochemical examinations. The other testicle was stored at −20 °C until used for biochemical analyses. Testis tissues were taken from deep-freezer and weighed. They were than transferred to cold glass tubes and diluted with a ninefold volume of phosphate buffer (pH 7.4). For the enzymatic analysis, testicular tissues were minced and then homogenized used a Teflon-glass homogenisator at 16,000 × *g* for 3 min in cold physiological saline on ice (Türk et al. 2010).

Determination of apoptosis 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 solution (PBS) and were then incubated in 3% H_2O_2, prepared with PBS, for 10 min to inactivate endogenous peroxidase activity. Non-specific bindings were blocked by incubation with 1% untreated goat serum for 1 h. Testicular tissues were then incubated with primary rabbit polyclonal antibodies directed against Bax (a pro-apototic protein) and Bcl-2 (an anti-apoptotic protein) at dilutions 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-etilcarbazole (AEC) was used as color determining substrate. The reaction was stopped at the moment that color change occurred after 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 spermatid) were evaluated under 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.

Lipid peroxidation level

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 the test sample and two volume 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 1500 × *g* for 10 min and the absorbance of the supernatant was read on a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan) at 532 nm against a blank containing all reagents except test sample. The concentration of MDA was expressed as μM.

Superoxide dismutase (SOD) activity

Testicular SOD activity was measured using xanthine and xanthine oxidases 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

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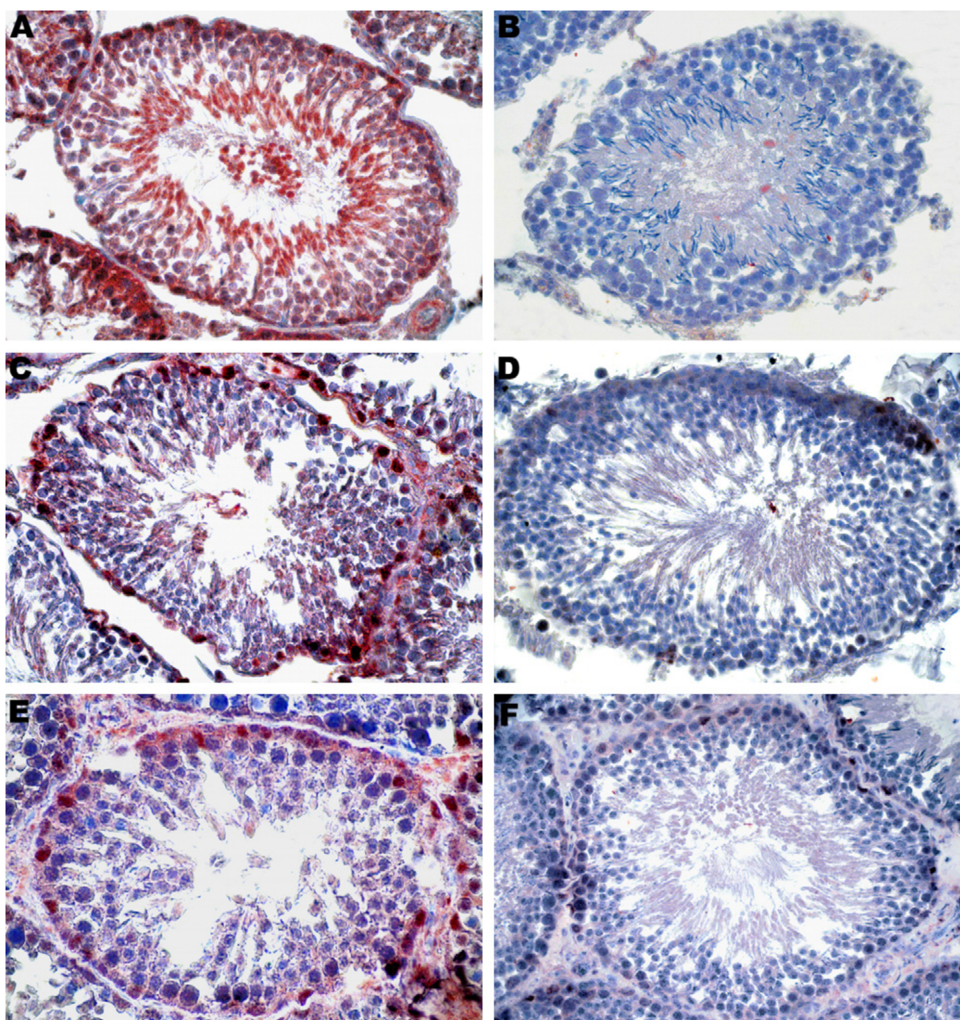

Fig. 1. Bax positive cells in seminiferous tubules from the CP treated alone (A); control (B); CP + LC treated (C); LC treated alone (D); CP + EA treated (E); EA treated alone (F) groups (200×).

was determined spectrophotometrically at 560 nm as the degree of inhibition and was expressed as U ml^{-1}.

Data analysis

All data are presented as mean ± 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-HSD test were used to evaluate differences between groups. $P < 0.05$ was considered significant.

Results

Apoptotic and antiapoptotic cell scores

Immunohistochemical analysis of Bax staining failed to demonstrate any significant difference between control (Fig. 1B), LC (Fig. 1D) and EA (Fig. 1F) groups. However Bax positive cells were observed more frequently in testis sections from CP-treated rats (Fig. 1A) 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. 1C) and CP + EA (Fig. 1E) 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 1), which was reduced by LC ($P < 0.05$) or EA (nonsignificant) treatments.

Immunohistochemical analysis of Bcl-2 staining failed to demonstrate any significant difference between the control (Fig. 2B), LC (Fig. 2D), EA (Fig. 2F), CP (Fig. 2A) and CP + LC (Fig. 2C) groups. However, significant intense staining was seen in almost all spermatogenic cells in CP + EA (Fig. 2E) group compared with the other groups except CP + LC group. Although CP administration itself did not affect the number of Bcl-2 positive cells compared with the control group (Table 1), concomitant administration of EA resulted in significant ($P < 0.05$) increase in the number of Bcl-2 positive antiapoptotic cells.

Histopathological changes

Histopathological analysis of testicular sections failed to demonstrate any significant difference between control (Fig. 3B), LC (Fig. 3D) and EA (Fig. 3F) groups. The histopathological lesions such as atrophy in seminiferous tubules; degeneration, desquamation, reduction and disorganisation in germinal cells; and interstitial oedema and capillary congestion were observed in alone CP group (Fig. 3A and Table 2). However, LC (Fig. 3C) and EA (Fig. 3E) administrations to CP-treated rats provided significant improvements in testicular histological view compared with

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Table 1
Mean ± SEM values of MDA, SOD, Johnsen's testicular score and immunohistochemical scores (LC = lycopene, EA = ellagic acid, CP = cisplatin).

Groups	Parameters				
	MDA (μM)	SOD (U ml^{-1})	Johnsen's testicular score (1–10)	Bax positive cell score (0–4)	Bcl-2 positive cell score (0–4)
Control	93.4 ± 12.1^{a}	1.7 ± 0.3^{a}	9.7 ± 0.2^{a}	0.33 ± 0.21^{a}	0.33 ± 0.21^{a}
LC	105.9 ± 15.4^{a}	1.6 ± 0.3^{a}	10.0 ± 0.0^{a}	0.17 ± 0.17^{a}	0.50 ± 0.22^{a}
EA	106.6 ± 8.9^{a}	8.5 ± 1.1^{b}	10.0 ± 0.0^{a}	0.67 ± 0.21^{a}	0.50 ± 0.22^{a}
CP	157.0 ± 24.5^{b}	1.3 ± 0.2^{a}	8.2 ± 0.3^{b}	1.83 ± 0.31^{b}	0.67 ± 0.21^{a}
CP + LC	138.3 ± 11.3^{ab}	1.3 ± 0.2^{a}	9.2 ± 0.2^{a}	1.00 ± 0.00^{c}	0.83 ± 0.17^{ab}
CP + EA	98.2 ± 17.4^{a}	4.7 ± 2.6^{c}	9.4 ± 0.1^{a}	1.33 ± 0.21^{bc}	1.00 ± 0.00^{b}

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

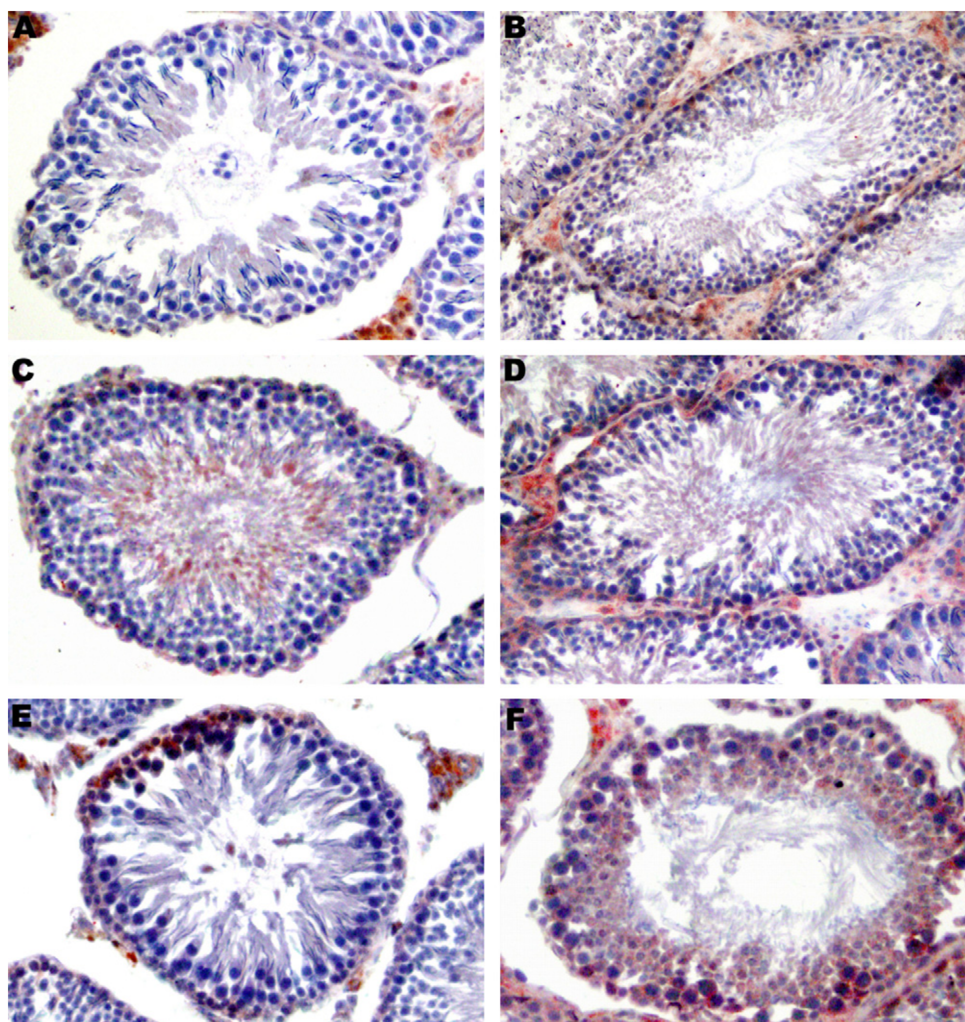

Fig. 2. Bcl-2 positive cells in seminiferous tubules from the CP treated alone (A); control (B); CP + LC treated (C); LC treated alone (D); CP + EA treated (E); EA treated alone (F) groups (200×).

Table 2
The existence of some pathological lesions in testicular tissues of different treatment groups (LC = lycopene, EA = ellagic acid, CP = cisplatin).

Lesions	Groups					
	Control	LC	EA	CP	CP + LC	CP + EA
Atrophy in seminiferous tubules	–	–	–	+	–	–
Degeneration in germinal cells	–	–	–	+	–	–
Desquamation in germinal cells	–	–	–	+	–	–
Reduction in germinal cell counts	–	–	–	+	–	+
Disorganisation in germinal cells	–	–	–	+	–	–
Interstitial oedema and capillary congestion	–	–	–	+	–	–

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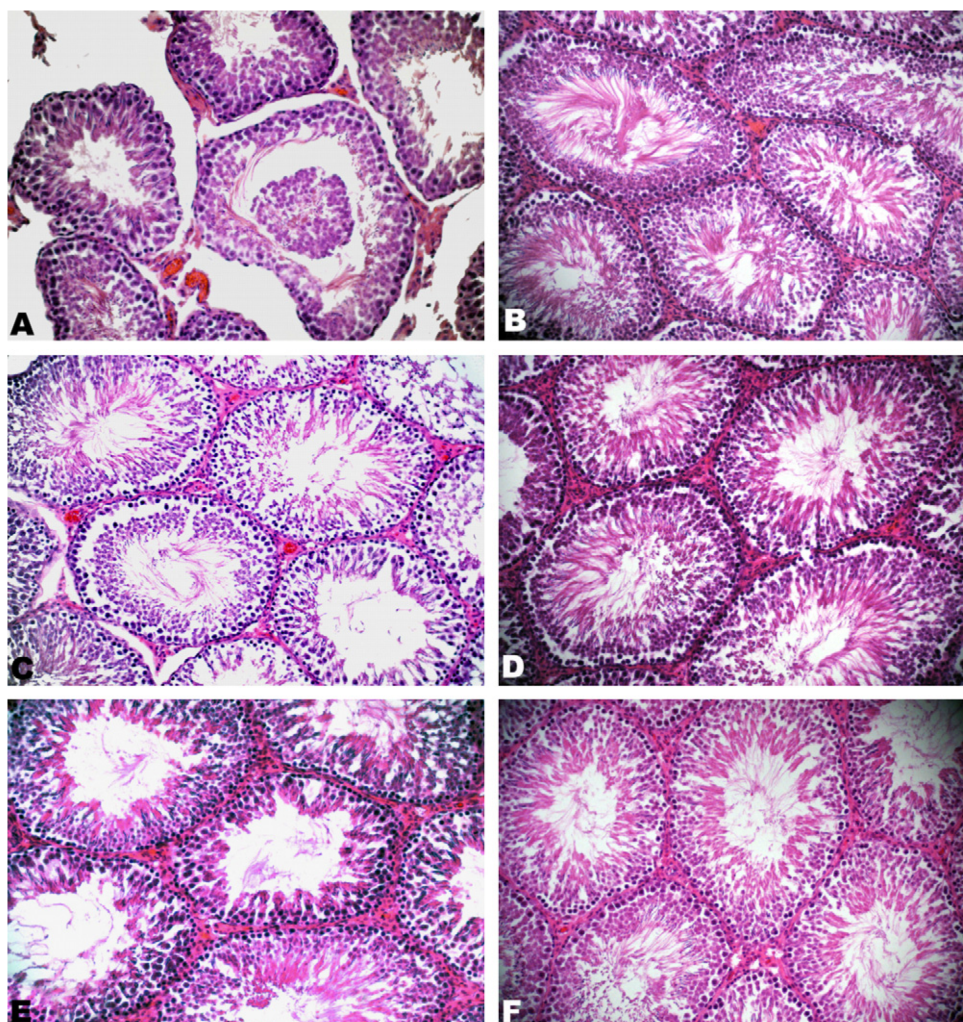

Fig. 3. (A) Atrophy in seminiferous tubules; degeneration, desquamation, reduction and disorganisation in germinal cells; and interstitial oedema and capillary congestion in alone CP-treated group. (B) Normal histological appearance of seminiferous tubules in control group. (C) Pivotal amelioration in testicular view and normal spermatogenesis in CP + LC treated group. (D) Normal histological appearance of seminiferous tubules in alone LC treated group. (E) Normal spermatogenesis along with reduction in germinal cells CP + EA treated group. (F) Normal histological appearance of seminiferous tubules in alone EA treated group (H&E, 100×).

the CP group. Although CP administration significantly ($P < 0.05$) decreased Johnsen's testicular score compared with the control group (Table 1), concomitant administration of LC and EA resulted in significant ($P < 0.05$) increase in this score compared with the alone CP group.

Lipid peroxidation level and SOD activity

Although treatment with CP alone significantly ($P < 0.05$) increased MDA levels compared with the control group, LC and in particular EA ($P < 0.05$) administration to CP-treated rats significantly reduced these increased MDA levels compared with levels in the CP-treated group. Although CP administration tended to decrease in SOD activity, the differences did not reach statistical significance. However, EA treatment alone and co-administration with CP provided a significant ($P < 0.05$) increase in SOD activity compared with the other groups (Table 1).

Discussion

CP-induced impaired spermatogenesis, azoospermi, lost of sperm motility, testicular tissue damages, and protective role of LC and EA against CP-induced these detrimental effects has been demonstrated in our previous studies (Ateşşahin et al. 2006a; Türk et al. 2008). In this study whether LC or EA have protective effects on the CP-induced germ cell apoptosis were also investigated.

Reproductive cells and tissues remain stable when ROS production and the scavenging antioxidants remain in balance. ROS can attack and inactivate or alter the biological activity of molecules such as lipids and proteins that are essential for cell function (Agarwal et al. 2008). It is generally accepted that the increased lipid peroxidation is one of the toxic manifestations of CP administration in testis. CP treatment results in increased MDA, by-product of lipid peroxidation, levels due to the excessive generation of free radicals, and decreased antioxidant enzyme activity (Ateşşahin et al. 2006a,b; Amin et al. 2008; Türk et al. 2008). Alone CP treatment caused significant increase in MDA level and nonsignificant decrease in SOD activity of testicular tissue in the present study. Increments in MDA can be attributed to the CP-induced excessive production of free radicals and consequently elevated lipid peroxidation. The nonsignificant decrease in SOD activity may be depended on the excessive utilisation of this antioxidant to scavenge ROS.

It has been reported that acute and chronic exposure to chemoterapeutics such as CP (Amin et al. 2008; Lirdi et al. 2008), cyclophosphamide (Türk et al. 2010), and doxorubicin (Hou et al. 2005) results in elevated apoptotic germ cell rates. In this study, Bax positive cells were observed more frequently in the

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alone CP-treated 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 spermatids) in alone CP-treated rat testis sections. Additionally, CP administration significantly elevated the number of Bax positive apoptotic cells compared to the control group. These findings are in agreement with the reports mentioned above. In terms of Bcl-2 positive staining and the number of Bcl-2 positive cells, there were no immunohistochemically significant differences between control and CP groups. It has been reported that H_2O_2 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 CP observed in this study may be explained by increased ROS and lipid peroxidation levels in testicular tissue and spermatogenic cells or direct DNA and chromatin damages of germ cells.

CP causes histopathologically reduction in size and number of the seminiferous tubules, degeneration and vacuolation in spermatogonia, spermatocytes and less number of germ cells, irregular seminiferous tubules, reduced seminiferous epithelial layers and significant maturation arrest (Ateşşahin et al. 2006b; Türk et al. 2008). In the present study, atrophy in seminiferous tubules; degeneration, desquamation, reduction and disorganisation in germinal cells, interstitial oedema and capillary congestion, and reduced Johnsen's testicular score were observed in alone CP group. The damages observed in the histological structure of testis in this work may be elucidated with the direct or indirect effect of CP which latter induces lipid peroxidation that is a chemical mechanism capable of disrupting the structure and function of testis.

LC and EA protect testes against cyclophosphamide-induced apoptosis (Türk et al. 2010) and histological lesions (Çeribaşı et al. 2010) by decreasing testicular and circulating levels of lipid peroxidation and increasing antioxidant enzyme activity. In this study, the decrease in Bax-positive and increase in Bcl-2 positive intense staining in almost all spermatogenic cells was observed in both CP + LC and CP + EA groups when compared to the alone CP group. Additionally, while LC or EA administrations to CP-treated rats decreased the increased number of Bax positive apoptotic cells, they increased the number of antiapoptotic Bcl-2 positive cells. These improvements after LC or EA administrations may be explained with partial or total modulation of CP-induced immunohistochemical damages, and decreased apoptotic cells, decreased lipid peroxidation and increased SOD activity.

It was observed that LC and especially EA administrations to CP-treated rats significantly reduced the increased MDA levels in comparison to the only CP group in this work. The decline of lipid peroxidation in testicular tissue apparently indicates that LC and EA potently scavenged the free radicals, and suppressed oxidative DNA damage. EA treatment alone and co-administration with CP but not LC provided a significant increase in SOD activity compared with the other groups in the present study. This status indicates that Ea has potent antioxidant activity.

In conclusion, this study apparently suggests that LC and EA have improvement effects against testicular apoptosis induced by CP. These improvement effects of LC and EA seem to involve suppression of lipid peroxidation. Therefore, LC or EA may be used in combination with CP in cancer patients to improve CP-induced germ cell apoptosis and lipid peroxidation.

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