



Impact of ellagic acid on adriamycin-induced testicular histopathological lesions, apoptosis, lipid peroxidation and sperm damages

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ABSTRACT

The aim of the present study was to investigate whether ellagic acid (EA) has protective effect on adriamycin (ADR)-induced testicular and spermatozoal toxicity associated with the oxidative stress in male rats. Thirty-two healthy 8-week-old male Sprague–Dawley rats were equally divided into four groups. The first (EA) group was treated with EA (2 mg/kg/every other day) by gavage. The second (ADR) group received ADR (2 mg/kg/once a week) intraperitoneally, while the combination of ADR and EA was given to the third (ADR + EA) group. The fourth (control) group was treated with placebo. At the end of the 8-week treatment period, reproductive organ weights, epididymal sperm parameters, histopathological changes and apoptosis via Bax and Bcl-2 proteins, testicular tissue lipid peroxidation, and antioxidant enzyme activities, were investigated. ADR administration was determined to cause significant decreases in reproductive organ weights, epididymal sperm concentration and motility, plasma testosterone concentration, diameter of seminiferous tubules, germinal cell layer thickness, Johnsen's testicular score and Bcl-2 positive antiapoptotic cell rate, whereas it caused significant increases in level of lipid peroxidation and glutathione, catalase activity, abnormal sperm rates and Bax positive apoptotic cell rates along with degeneration, necrosis, immature germ cells, congestion and atrophy in testicular tissue when compared with the control group. EA administration to ADR-treated rats provided significant improvements in ADR-induced disturbed oxidant/antioxidant balance, decreased testosterone concentration, testicular apoptosis and mild improvements in the histopathological view of the testicular tissue. However, EA failed to improve decreased reproductive organ weights and deteriorated sperm parameters due to ADR administration. It is concluded that while ADR has direct or indirect (lipid peroxidation) negative effects on sperm structure and testicular apoptosis in rats, EA has protective effects on ADR-induced testicular lipid peroxidation and apoptosis.

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1. Introduction

Adriamycin (ADR, also named doxorubicin) is an anthracycline antibiotic with potent anticancer activity against a wide range of tumors. Its use is severely circumscribed due to the adverse effects including testicular toxicity (Lui et al., 1986). ADR treatment is associated with decreased spermatogenic activity that characterized with damaged quality and quantity of spermatozoa. It has been reported that ADR treated rats have shown depletion in the number of spermatogonia, decrease in the percentage of motile sperm, and increase in sperm morphological abnormalities (Kato et al., 2001; Ateşşahin et al., 2006), apoptosis at specific stages of seminiferous epithelial cycle (Sjoblom et al., 1998; Shinoda et al., 1999; Endo

et al., 2003) and decrease in testosterone concentrations (Ateşşahin et al., 2006) in rats. In addition, ADR causes severe degenerative changes, shrunken seminiferous tubules with decreased germ cells in testicular tissue (Ateşşahin et al., 2006). The preferential target of ADR is the DNA of dividing cells; the drug intercalates within DNA strands causing cell cycle blockage in the G2 phase, single-strand breaks (Konopa, 1988) and inhibition of the activity of some nuclear proteins, such as DNA and RNA-polymerase and DNA-topoisomerase II (Speth et al., 1988). However, it has been reported that ADR-induced lipid peroxidation is also responsible for its testicular toxicity (Prahalthan et al., 2004; Ateşşahin et al., 2006). Increased lipid peroxidation in the membranes can be detrimental to male fertility (Sikka, 1996).

Ellagic acid, a member of flavanoids (EA; C₁₄H₆O₈; MW: 302.202; 3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) has been receiving the most attention because it has potent antioxidant activity, radical scavenging

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capacity, chemopreventive (Ateşşahin et al., 2006; Türk et al., 2008, 2010a; Çeribaşı et al., 2010) and antiapoptotic (Türk et al., 2010a,b) properties. It contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage (Pari and Sivasankari, 2008). Berries are the most EA rich fruits (Wedge et al., 2001) and they are highly consumed by humans in the worldwide. EA is a very stable compound and is readily absorbed through the gastrointestinal system in mammals, including humans (Falsaperla et al., 2005). Additionally, berries and flavanoids have been showed between top ten antioxidants by many nutritional website. In our previous studies we observed that EA significantly improved the damages in sperm parameters, oxidant/antioxidant balance and testicular apoptosis induced by chemotherapeutics such as cisplatin (Türk et al., 2008, 2010b) and cyclophosphamide (Türk et al., 2010a). On the basis of the potential antioxidant activity of flavanoids, the present study was designed to investigate whether EA has possible protective effect against ADR-induced deteriorated epididymal sperm characteristics, damaged oxidant/antioxidant balance and testicular apoptosis in rats.

2. Materials and methods

2.1. Chemicals

Adriamycin (Adriblastina) was purchased from Carlo Erba (İstanbul, Turkey). EA was supplied from Fluka (Steinheim, Germany) and the other chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Animals and experimental design

Thirty-two healthy 8-week-old male Sprague–Dawley rats were divided into four equal groups. The animals were obtained from Experimental Research Centre of Firat University (Elazığ, Turkey) and were housed under standard laboratory conditions (temperature $24 \pm 3^\circ$, humidity 40–60%, a 12-h light: 12-h dark cycle). A commercial pellet diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water were given *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.

ADR was dissolved in isotonic saline and injected intraperitoneally to the animals at the dose of 2 mg/kg once a week. EA is hardly dissolved under natural condition. Therefore, it was dissolved in alkaline solution (0.01 N NaOH; approximately pH 12). pH of the final solution after the addition of EA was approximately 8. This final solution (pH $\approx$ 8) was administered to the animals by gavage at the dose of 2 mg/kg/every other day. All the treatments were lasted for 8 weeks. Owing to the fact that rats need a period of 48–52 days for the exact spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis (Çeribaşı et al., 2010; Türk et al., 2010a,b), the administration period was set at 8 weeks. The control group was injected 0.5 ml isotonic saline once a week and gavaged with 0.5 ml slightly alkaline solution every other day. EA group was injected 0.5 ml isotonic saline once a week and gavaged with 0.5 ml slightly alkaline solution containing 2 mg/kg EA every other day. ADR group was injected 0.5 ml isotonic saline containing 2 mg/kg ADR and gavaged with 0.5 ml slightly alkaline solution every other day. ADR+EA group was injected 0.5 ml isotonic saline containing 2 mg/kg ADR and gavaged with 0.5 ml slightly alkaline solution containing 2 mg/kg EA every other day. The doses of EA (Çeribaşı et al., 2010; Türk et al., 2010a,b) and ADR (Ateşşahin et al., 2006) were determined based on the reports of previous studies.

2.3. Sample collection and homogenate preparation

The rats were killed under slight ether anaesthesia at the end of the treatment period (eight weeks). Testes, epididymides, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. Collected blood samples were centrifuged at $3000 \times g$ for 10 min to obtain plasma. One of the testes was fixed in 10% neutral-formalin solution for histopathological and immunohistochemical examinations. The other testes and plasma samples were stored at -20°C until biochemical analyses. Testes tissues were taken from deep-freezer and weighed and then, they were immediately transferred to the cold glass tubes. For the enzymatic analyses, testicular tissues were minced in a glass and homogenized by a teflon-glass homogenisator at $16,000 \times g$ for 3 min in cold physiological saline on ice. Then, the tissues were diluted with a 9-fold volume of phosphate buffer (pH 7.4) (Türk et al., 2010a).

2.4. Tissue preparation for histopathological and immunohistochemical evaluation

The testicular tissues were fixed in 10% neutral-formalin, embedded in paraffin, sectioned at $5 \mu\text{m}$ and were stained with hematoxylin and eosin (Bancroft and Stevens, 1990). Light microscopy was used to measure diameters of seminiferous tubules (DST) and germinal cell layer thicknesses (GCLT) and to evaluate the damages in testicular tissue. Johnsen's testicular score (Johnsen, 1970) was performed for the control and treatment groups. All cross sectioned tubules were evaluated, 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.

Avidin–Biotin–Peroxidase method was used for the immunohistochemical analyses (Jahnukainen et al., 2004). Testes tissues, which were embedded in paraffin and sectioned at $4 \mu\text{m}$, were deparaffinised with xylene and dehydrated with alcohol series. Testicular sections were incubated in 0.01 M Na-citrate for 20 min to bring into the open the antigenic receptors. They were washed with phosphate buffer solution (PBS) and were then incubated in 3% H_2O_2 , which was 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. After that testicular tissues were incubated with primary rabbit polyclonal antibodies directed against Bax (proapoptotic protein) and Bcl-2 (antiapoptotic 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 at the rate of 1:1000 in PBS containing 0.1% goat serum (secondary biotinylated goat anti-rabbit IgG) for 30 min and 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 color determining substrate. The period of time elapsed after substrate addition to the testicular tissues. At the last stages, testicular tissues were washed with tap water for 2 min after they were stained with Mayer's hematoxylin for 15 s. Stained tissues were covered with immune-mount and then Bax- and Bcl-2 positive spermatogenic cells (from spermatogonia to elongated spermatid) were evaluated under light microscope and scored as follows (Kandi Coşkun and Çobanoğlu, 2005).

- Score 0: Negative stained cells.
- Score 1: <25% positive stained cells.
- Score 2: 26–50% positive stained cells.
- Score 3: 51–75% positive stained cells.
- Score 4: >75% positive stained cells.

Table 1Mean $\pm$ SEM values of absolute reproductive organ weights (EA = ellagic acid, ADR = adriamycin).

Groups	Parameters			
	Testis weight (g)	Epididymis weight (g)	Seminal vesicles weight (g)	Prostate weight (g)
Control	1.345 $\pm$ 0.03 ^a	0.454 $\pm$ 0.009 ^a	0.902 $\pm$ 0.03 ^a	0.455 $\pm$ 0.03 ^a
EA	1.348 $\pm$ 0.04 ^a	0.456 $\pm$ 0.006 ^a	0.985 $\pm$ 0.05 ^a	0.483 $\pm$ 0.01 ^a
ADR	0.418 $\pm$ 0.04 ^b	0.280 $\pm$ 0.020 ^b	0.435 $\pm$ 0.10 ^b	0.223 $\pm$ 0.01 ^b
ADR + EA	0.499 $\pm$ 0.04 ^b	0.290 $\pm$ 0.046 ^b	0.520 $\pm$ 0.04 ^b	0.235 $\pm$ 0.05 ^b

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

2.5. Biochemical analyses

The lipid peroxidation 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 lipid peroxidation. 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). The GSH-Px activity was expressed as IU/g protein. The 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). The superoxide dismutase (SOD) activity was spectrophotometrically measured using xanthine and xanthine oxidases to generate superoxide radicals which react with nitroblue tetrazolium (NBT) and expressed as U/ml (Flohe and Otting, 1984). The plasma testosterone level was measured by ELISA method using DRG Elisa testosterone kit (ELISA EIA-1559, 96 Wells kit, DRG Instruments, GmbH, Marburg, Germany) according to the kit manufacturer's instructions and expressed as ng/dl.

2.6. Sperm analyses

The epididymal sperm concentration in the right cauda epididymal tissue was determined with a hemocytometer using a modified method (Türk et al., 2008). Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. The percentage sperm motility was evaluated using a light microscope with heated stage (Sönmez et al., 2005). To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared. The slides were then viewed under a light microscope at 400 $\times$ magnification. A total of 300 spermatozoa were examined on each slide (2400 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage (Türk et al., 2008).

2.7. Statistical analysis

All values were presented as mean $\pm$ 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 the groups. The SPSS/PC program (Version 10.0; SPSS, Chicago, IL) was used for the statistical analyses.

3. Results

3.1. Reproductive organ weights

The values of absolute reproductive organ weights are given in Table 1. The EA treatment alone did not affect the absolute organ

weights. On the other hand, ADR administration alone caused significant ($P < 0.05$) decreases in all reproductive organ weights when compared with the values in the control group. However, the treatment with the combination of EA and ADR failed to increase the decreased reproductive organ weights due to ADR administration.

3.2. Testicular histopathology and immunohistochemistry

Histological appearances of testicular tissues of the control (Fig. 1A) and EA (Fig. 1B) groups were normal. The histopathological changes such as necrosis, degeneration, desquamation, disorganisation and reduction in germinal cells, atrophy in tubules, interstitial connective tissue proliferation, hyperplasia in Leydig cells, vacuolisation in Sertoli cells, thickening in basal layer of seminiferous tubules, interstitial oedema and capillary congestion were observed in the ADR and ADR + EA groups (Table 2). ADR induced prominent morphological changes in the testis. Almost all germ cells disappeared from the seminiferous tubules. The main loss of germ cells was in spermatocytes and round spermatids, additionally elongated spermatids and spermatozoa were not observed in some seminiferous tubules. Widening of the interstitial space and severe vacuolisation were also observed in Sertoli cells. Additionally, the seminiferous tubules showed severe vacuolisation and were displaced by some fibrinoid debris. Seminiferous tubules were contained only spermatogonia cells in the ADR group. However, a few numbers of elongated spermatids in addition to two or three layers of spermatocytes were observed in seminiferous tubules in the ADR + EA group. These damages were observed to be more severe in the ADR (Fig. 1C) group than the ADR + EA (Fig. 1D) group. In other words, EA administration to ADR-treated rats provided a mild improvement in testicular histological view when compared to the ADR group. Significant ($P < 0.05$) decreases in DST, GCLT and Johnsen's testicular score were observed in the ADR group when compared to the control group. Although, EA administration to ADR-treated animals prevented the ADR-induced decreases in these parameters significantly ($P < 0.05$), these improvements were not close to the control values (Table 3).

There were no immunohistochemically significant differences between the control (Fig. 2A) and EA (Fig. 2B) groups in terms of Bax positive staining. However, Bax positive cells were observed to be more frequent in the ADR (Fig. 2C) group than the control group. The intense staining was observed in almost all the spermatogonia in the ADR-group. Significant decrease was observed in intense staining in the ADR + EA (Fig. 2D) group when compared with the ADR group. ADR administration increased the Bax positive apoptotic cell counts significantly ($P < 0.05$) when compared to the control group. However, EA administration provided significant decreases in increased Bax positive apoptotic cell counts due to ADR treatment (Table 3).

With respect to Bcl-2 positive staining, there were no immunohistochemically significant differences between the control (Fig. 3A) and EA (Fig. 3B) groups. Significant decreases were observed in Bcl-2 immunopositive stainings in the ADR (Fig. 3C) group when compared with the control group. However, signifi-

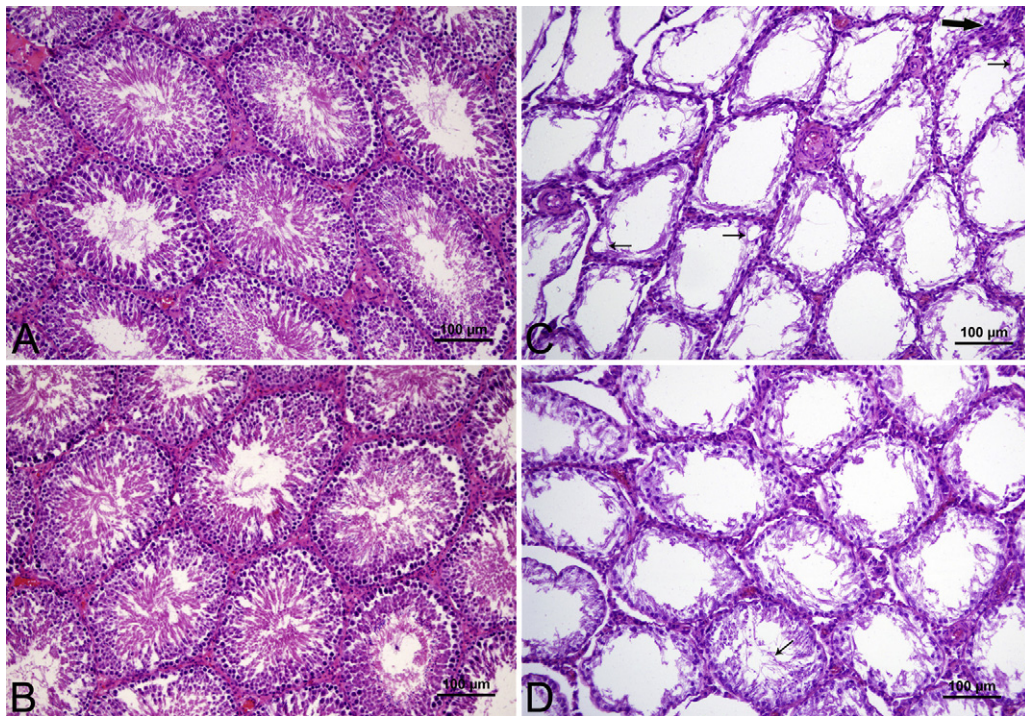


Fig. 1. (A) Normal histological appearance of seminiferous tubules in control group H&E. (B) Normal histological appearance of seminiferous tubules in alone EA group H&E. (C) Severe testicular degeneration; germinal cells necrosis, tubular atrophy, vacuolisation of sertoli cells (small arrows) and interstitial connective tissue proliferation (big arrow) in alone ADR group H&E. (D) Moderate testicular degeneration; germinal cells necrosis, tubular atrophy but elongated spermatids (arrow) and two or three layers of germinal epithelium were seen in some seminifer tubules in ADR + EA group H&E.

Table 2

The existence of some histopathological lesions (EA = ellagic acid, ADR = adriamycin).

Parameters	Groups			
	Control	EA	ADR	ADR + EA
Necrosis in germinal cells	–	–	+	+
Atrophy in seminiferous tubules	–	–	+	+
Thickening in tubule basal membrane	–	–	+	+
Degeneration in germinal cells	–	–	+	+
Desquamation in germinal cells	–	–	+	+
Hyperplasia in Leydig cells	–	–	+	+
Vacuolisation in Sertoli cells	–	–	+	+
Reduction in germinal cell counts	–	–	+	+
Disorganisation in germinal cells	–	–	+	+
Interstitial oedema and capillary congestion	–	–	+	+

cant increase was obtained in Bcl-2 immunopositive stainings in the ADR + EA (Fig. 3D) group when compared with the ADR group. ADR administration caused statistically significant ($P < 0.05$) decreases in the Bcl-2 positive antiapoptotic cell scores when compared with the control group. EA administration to ADR-treated rats caused significant ($P < 0.05$) increases in this parameter when compared with the ADR group (Table 3).

3.3. Biochemical parameters

Testicular tissue lipid peroxidation levels, antioxidant enzyme activities and plasma testosterone levels are presented in Table 4. Although ADR administration alone increased the MDA levels significantly ($P < 0.05$) when compared to the control group, EA administration to ADR-treated rats reduced the increased MDA

Table 3

Mean $\pm$ SEM values of DST, GCLT, Johnsen's testicular and immunohistochemical scores (DST = diameter of seminiferous tubules, GCLT = germinal cell layer thickness, EA = ellagic acid, ADR = adriamycin).

Groups	Parameters				
	DST (μ m)	GCLT (μ m)	Johnsen's testicular score (1–10)	Bax positive cell score (0–4)	Bcl-2 positive cell score (0–4)
Control	223.6 $\pm$ 2.20 ^a	76.40 $\pm$ 0.98 ^a	9.67 $\pm$ 0.21 ^a	0.33 $\pm$ 0.21 ^a	0.33 $\pm$ 0.21 ^a
EA	224.5 $\pm$ 1.90 ^a	74.73 $\pm$ 0.99 ^a	10.00 $\pm$ 0.00 ^a	0.67 $\pm$ 0.21 ^a	0.50 $\pm$ 0.22 ^a
ADR	129.3 $\pm$ 1.88 ^b	17.80 $\pm$ 0.63 ^b	2.17 $\pm$ 0.31 ^b	3.67 $\pm$ 0.21 ^b	1.00 $\pm$ 0.00 ^{ab}
ADR + EA	140.0 $\pm$ 1.29 ^c	37.07 $\pm$ 0.53 ^c	5.00 $\pm$ 0.25 ^c	3.00 $\pm$ 0.26 ^b	1.50 $\pm$ 0.22 ^b

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

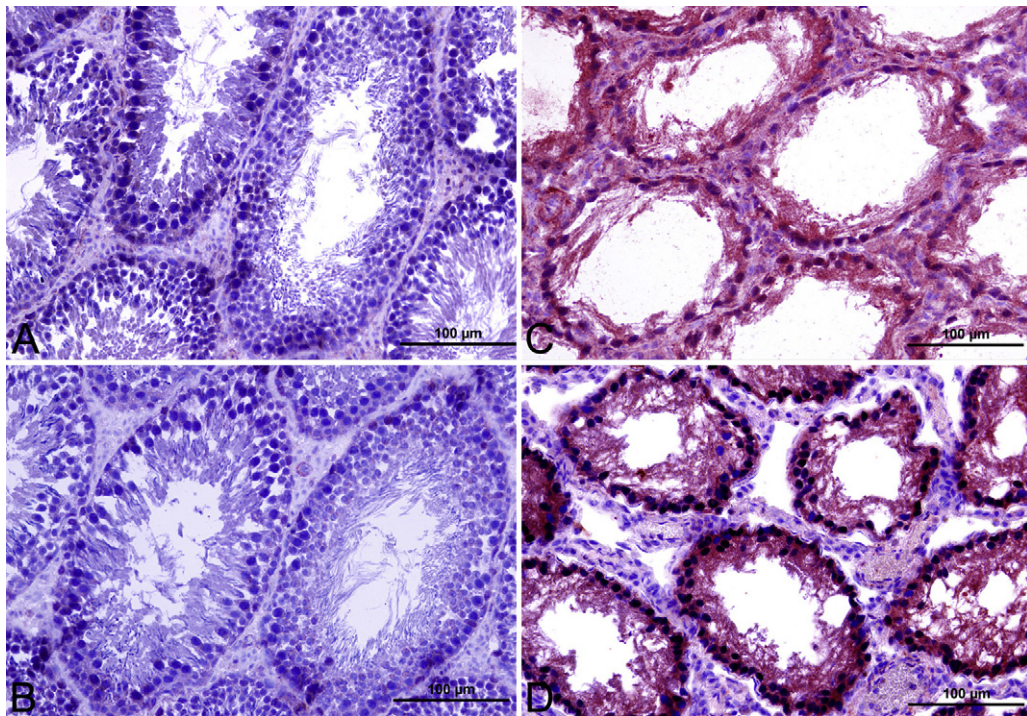


Fig. 2. (A) Bax immunopositive stainings in seminiferous tubules in control group. (B) Bax immunopositive stainings in seminiferous tubules in alone EA group. (C) Intensive Bax immunopositive stainings in seminiferous tubules in alone ADR group. (D) Intensive Bax immunopositive stainings in seminiferous tubules in ADR + EA group.

levels significantly ($P < 0.05$) when compared to the ADR group. A significant ($P < 0.05$) increase in GSH levels was observed in both ADR and ADR + EA groups compared to the control group. ADR treatment had no significant effect on GSH-Px activity when compared to the control group. However, EA administration to ADR-treated rats provided significant ($P < 0.05$) increase in GSH-Px activity when compared with the values in ADR and other treatment groups. The

CAT activities of ADR and ADR + EA groups were found significantly ($P < 0.05$) higher than the control and EA groups. Similarly, a significant ($P < 0.05$) increase was observed in CAT activity of ADR + EA group when compared with the ADR group. No significant difference was found between the treatment groups in terms of SOD activity. ADR administration decreased the plasma testosterone levels significantly ($P < 0.01$) when compared to the control group.

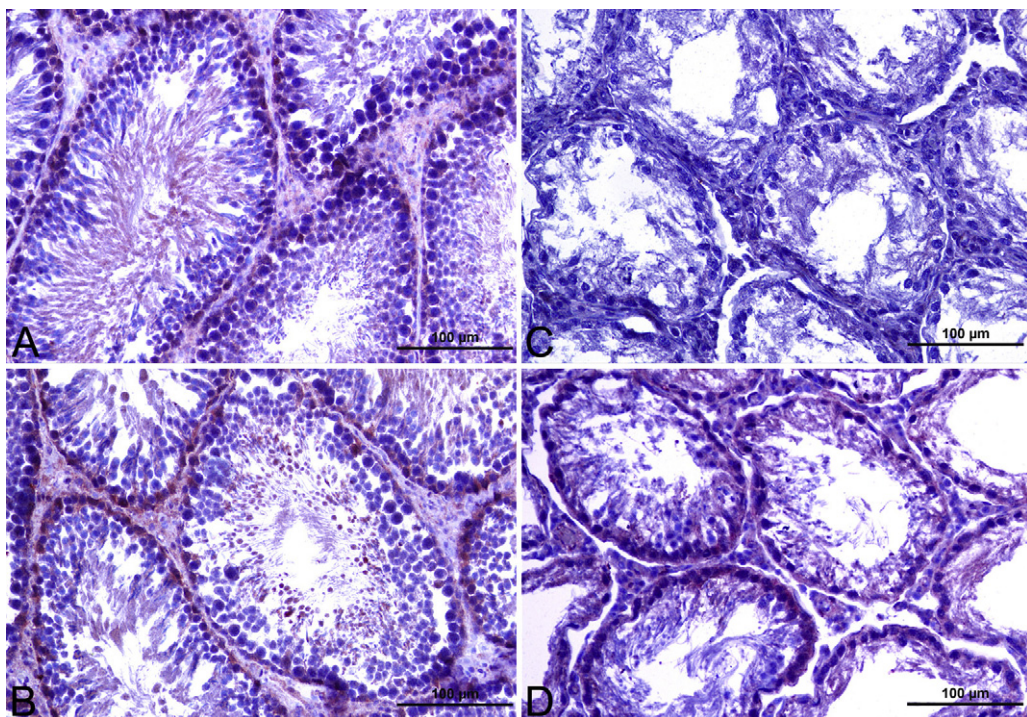


Fig. 3. (A) Bcl-2 immunopositive stainings in seminiferous tubules in control group. (B) Bcl-2 immunopositive stainings in seminiferous tubules in alone EA group. (C) Bcl-2 immunopositive stainings was not seen in seminiferous tubules in alone ADR group. (D) Bcl-2 immunopositive stainings in seminiferous tubules in ADR + EA group.

Table 4

Mean $\pm$ SEM values of testicular tissue malondialdehyde (MDA), glutathione (GSH) levels and glutathione-peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD) activities and plasma testosterone levels (EA = ellagic acid, ADR = adriamycin).

Groups	Biochemical parameters					
	MDA (nmol/ml)	GSH (nmol/ml)	GSH-Px (IU/g protein)	CAT (kU/g protein)	SOD (U/ml)	Testosterone (ng/dl)
Control	93.4 $\pm$ 12.0 ^a	6.5 $\pm$ 1.2 ^a	10.2 $\pm$ 1.0 ^a	6.2 $\pm$ 0.4 ^a	1.7 $\pm$ 0.3	392 $\pm$ 65 ^A
EA	105.9 $\pm$ 15.4 ^a	8.7 $\pm$ 0.7 ^{ab}	10.2 $\pm$ 2.4 ^a	6.5 $\pm$ 0.3 ^a	1.6 $\pm$ 0.3	354 $\pm$ 73 ^A
ADR	141.7 $\pm$ 7.8 ^b	10.1 $\pm$ 0.9 ^b	16.2 $\pm$ 2.6 ^a	16.7 $\pm$ 0.6 ^b	1.9 $\pm$ 0.2	139 $\pm$ 29 ^B
ADR + EA	38.8 $\pm$ 6.7 ^c	10.3 $\pm$ 0.8 ^b	51.9 $\pm$ 12.0 ^b	23.3 $\pm$ 2.1 ^c	2.4 $\pm$ 0.6	295 $\pm$ 124 ^A

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

However, this decrease was elevated significantly ($P < 0.01$) by EA administration.

3.4. Epididymal sperm characteristics

Epididymal sperm concentration, sperm motility, and abnormal sperm rates are presented in Table 5. Although, ADR treatment caused significant ($P < 0.01$) decreases in sperm concentration and motility, it increased head, tail and total abnormality rates of sperm significantly ($P < 0.01$) when compared with the values of the control group. Although concomitant administration of EA with ADR tended to increase the values of sperm concentration and motility, and to decrease abnormal sperm rates, these improvements were not statistically significant when compared with the values of the ADR group.

4. Discussion

Many drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organs. Treatment with cancer chemotherapy is associated with significant gonadal damage in the male reproductive organs. Spermatogenic cells are targeted by cytotoxic agents because of their high mitotic activity. Damages in spermatogonia result in prolonged sterility or oligozoospermia (Howell and Shalet, 2001; Endo et al., 2003). The chance of recovery of spermatogenesis following cytotoxic insult, and also the extent and speed of recovery, are related to the agent used and the dose received. ADR is one of the widely used cytotoxic agents known to disturb spermatogenesis and testicular functions (Howell and Shalet, 2005).

The most sensitive indicators to detect male reproductive toxicity induced by ADR are testicular weight and histopathological findings in the testes. It has been reported that exposure to ADR decreases reproductive organ weights (Kato et al., 2001; Ateşşahin et al., 2006), but according to Endo et al. (2003) ADR had no significant effect on these parameters. In this study, the ADR administration alone caused significant decreases in all reproductive organ weights when compared with the values in the control group. This may be the result of severe parenchymal atrophy in the seminiferous tubules, spermatogenic damages in testicular tissue, and significant decrease in sperm count due to ADR treatment. The dif-

ferentiation of spermatozoa is maintained through the function of Leydig cells which secrete testosterone. ADR-induced increased reactive oxygen species (ROS) levels or its direct effects lead to Leydig cell impairment and decrease in testosterone production (Howell and Shalet, 2001; Endo et al., 2003; Ateşşahin et al., 2006). The reason of the ADR-induced decreased testosterone concentration observed in this study was mainly due to the damages in Leydig cells. A plausible explanation for the reduction in weights of seminal vesicle and prostate in this study might be the decreased secretion of this organ as testosterone production was diminished by direct or indirect effect of ADR.

Histopathologically, ADR causes reduction in size of the seminiferous tubule, number of the seminiferous tubules, degeneration and vacuolation in spermatogonia, spermatocytes, less number of germ cells, irregular seminiferous tubules, reduced seminiferous epithelial layers, significant maturation arrest, perivascular fibrosis and hyalinization of intertubular tissue (Ateşşahin et al., 2006). Nambu and Kumamoto (1995) reported that spermatogenic disorder was generated by interaction with impaired DNA synthesis in stem cells and Sertoli cell dysfunction both of which were directly produced by ADR. Kato et al. (2001) reported that the morphological degeneration of Sertoli cells was noted in the males treated with ADR at 2.0 mg/kg. Necrosis, degeneration, desquamation, disorganisation and reduction in germinal cells, atrophy in tubules, interstitial connective tissue proliferation, hyperplasia in Leydig cells, vacuolisation in Sertoli cells, thickness of basal layers of ST, interstitial oedema and congestion, reduced DST, GCLT and Johnsen's testicular score were observed in histological structure of ADR-treated rats in the present study. These findings are in agreement with the previous reports. The damages observed in the histological architecture of testis in this work may be elucidated with the direct or indirect effect of ADR; the latter induces lipid peroxidation that is a chemical mechanism capable of disrupting the structure and function of testis.

A proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins exist in culmination of apoptosis after the onset of cellular stress. The ratio of these molecules has been implicated to be a critical determinant of cell fate, such that elevated Bcl-2 favors extended survival of cells and increasing levels of Bax expression accelerates cell death (Sinha Hikim and Swerdloff, 1999). It has been reported that acute and chronic exposure to chemotherapeutics such as ADR (Hou et al.,

Table 5

Mean $\pm$ SEM values of sperm parameters (EA = ellagic acid, ADR = adriamycin).

Groups	Parameters				
	Sperm motility (%)	Epididymal sperm concentration (million/g tissue)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	77.77 $\pm$ 2.94 ^a	347.5 $\pm$ 11.5 ^a	2.28 $\pm$ 0.31 ^a	3.78 $\pm$ 0.78 ^a	6.06 $\pm$ 2.01 ^a
EA	82.76 $\pm$ 3.15 ^a	351.3 $\pm$ 11.5 ^a	1.89 $\pm$ 0.41 ^a	2.55 $\pm$ 0.31 ^a	4.44 $\pm$ 0.72 ^a
ADR	14.43 $\pm$ 4.60 ^b	66.80 $\pm$ 8.2 ^b	44.28 $\pm$ 7.12 ^b	17.56 $\pm$ 1.56 ^b	61.84 $\pm$ 7.47 ^b
ADR + EA	20.83 $\pm$ 4.17 ^b	80.85 $\pm$ 6.2 ^b	35.78 $\pm$ 6.89 ^b	19.61 $\pm$ 1.47 ^b	55.39 $\pm$ 8.21 ^b

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a and b: $P < 0.01$).

2005), cyclophosphamide (Türk et al., 2010a) and cisplatin (Türk et al., 2010b) results in elevated apoptotic germ cell rates. In this study, ADR administration caused significant increase in Bax positive intense staining and Bax positive apoptotic cell scores, and significant decrease on Bcl-2 positive intense staining and Bcl-2 positive antiapoptotic cell rate compared to the control group. These findings are in agreement with the abovementioned reports. H_2O_2 , which is one of 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 ADR observed in this study may be explained by increased ROS and lipid peroxidation levels in testicular tissue or direct DNA and chromatin damages of germ cells.

ROS, including superoxide anion ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$) radical, peroxy ($ROO\cdot$) and alkoxy ($RO\cdot$) radicals, as well as non-radical species such as 1O_2 , ozone (O_3) and H_2O_2 , are produced during use of oxygen in normal metabolism and are required for some physiological evidences in male reproductive system. However, overproduction of ROS leads to the lipid peroxidation, resulting in oxidative stress. Cells have antioxidant mechanisms to decrease ROS production partially or totally. Antioxidant enzymes such as SOD and CAT react with radicals $O_2^{\cdot-}$ and H_2O_2 , respectively. GSH-Px scavenges alkyl ($R\cdot$), $RO\cdot$ and $ROO\cdot$ radicals that may be formed from oxidized membrane components, and it uses GSH as a substrate (Agarwal et al., 2008a,b). It is generally accepted that the increased lipid peroxidation is one of the toxic manifestations of ADR administration in testis. It has been reported that (Ateşşahin et al., 2006) ADR treatment results in elevated MDA levels due to the excessive generation of free radicals. ADR treatment alone caused significant increase in MDA level of testicular tissue in the present study. This increment can be attributed to the ADR-induced excessive production of free radicals and consequently elevated lipid peroxidation. Salvemini et al. (1999) reported that GSH synthesis may be induced in cells exposed to oxidative stress as an adaptive process. Similarly, Yılmaz et al. (2006) suggested that under oxidative stress conditions, there may be positive regulation in the GSH biosynthesis, resulting in the increased level of GSH contents. Pande and Flora (2002) reported that CAT activity may increase in cells under oxidative stress to compensate lower activity of GSH-reductase or NADPH. In this study, ADR administration alone increased the GSH level and CAT activity significantly, but had no significant effect on GSH-Px and SOD activity compared to the control group. This finding is consistent with our previous study (Türk et al., 2010a) in which cyclophosphamide caused insignificant increase in testicular GSH level and significant increase in testicular CAT activity. Significant increase in GSH level after exposure to ADR observed in the present study can be attributed to adaptive process of GSH. Significant increase in CAT and insignificant increase in GSH-Px and SOD activities of testicular tissue observed in this study may be explained by excessive production of these antioxidants in order to scavenge the overproduction of free radicals under oxidative stress induced by ADR.

The present study showed that treatment with ADR resulted in significant decrease in sperm concentration and motility, and significant increase in abnormal sperm rates. Because spermatozoa plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes, they are particularly susceptible to the damage induced by excessive ROS (Aitken and McLaughlin, 2007). ROS can attack to the unsaturated bonds of the membrane lipids in an autocatalytic process, with the genesis of peroxides, alcohol and lipidic aldehydes as by-product of the reaction. Thus, the increase of free radicals in cells can induce the lipid peroxidation by oxidative breakdown of polyunsaturated fatty acids in membranes of cells. Obviously, peroxidation of sperm lipids destroys the structure of lipid matrix in the membranes of spermatozoa, and it is

associated with rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases (Türk et al., 2008, 2010a). It has been reported that ADR-induced direct DNA fragmentation (Suominen et al., 2003) and chromosomal aberrations (Au and Hsu, 1980), and oxidative stress (Prahalthan et al., 2004, 2005; Ateşşahin et al., 2006) causes to decrease in sperm count and motility, and increase in dead and abnormal sperm rates. The negative changes observed in sperm quality after ADR exposure in the present study may be attributed to the peroxidation of polyunsaturated fatty acids in membranes of spermatozoa, damaged flagellum which is important machinery for the sperm motility, directly impairing of spermatogenic cell development, impaired maturation or spermiation and damaged sperm DNA.

EA is a naturally occurring plant-derived polyphenol that exhibits antioxidative and antiapoptotic (Türk et al., 2010a,b) properties and chelates metal ions and prevent iron- and copper-catalysed formation of ROS. Researches in cell cultures and laboratory animals have demonstrated that EA is an effective antimutagen and anticarcinogen phytotherapeutic agent that prevents carcinogens binding to DNA and strengthens connective tissue and thus may keep cancer cells from spreading, inhibits cancer onset and tumor proliferation (Smith et al., 1998; Smith and Gupta, 1999) and protects healthy cells during chemotherapy (Smith et al., 1998; Smith and Gupta, 1999; Türk et al., 2008, 2010a,b). This mechanism is partly induced by stimulating various glutathione-S-transferase isoforms involved in cytodetoxifying processes (Barch et al., 1995), free radical scavenger action and inhibition of correlated lipoperoxidative damage (Türk et al., 2008, 2010a,b). While this is promising, at this time there is no reliable evidence available from human clinical studies showing that EA can prevent or treat cancer. In a human clinical study, it has been reported that EA seems to reduce some side effects, in particular neutropenia, of chemotherapy in men with advanced prostate cancer, although it does not slow disease progression or improve survival (Falsaperla et al., 2005). In our earlier study, we observed that EA protected cisplatin-induced testicular and spermatozoal toxicity by decreasing lipid peroxidation and increasing scavenging enzymes (Ateşşahin et al., 2006). Türk et al. (2010a,b) have reported that EA reduced testicular apoptotic cell rates induced by chemotherapeutics (cyclophosphamide and cisplatin) in rats. In the present study, administration of EA to ADR-treated rats provided significant improvements in testicular MDA levels, activities of GSH-Px and CAT, testosterone concentration, and also in testicular histopathological measurements and Bax and Bcl-2 staining and cell rates, but failed to improve the ADR-induced damages in reproductive organ weights and sperm quality parameters when compared to the ADR group. However, significant decrease in the intensity of the ADR-induced testicular lesions according to the Johnsen's criteria and increase in DST and GCLT measurements were determined in ADR+EA group when compared to the ADR group. The findings related to reproductive organ weights and sperm parameters are not in agreement with our previous reports. This may be due to the use of different chemotherapeutics. ADR-induced direct DNA intercalating, cell cycle blockage in the G2 phase, single-strand breaks (Konopa, 1988) or increased ROS levels are responsible for its reproductive toxicity (Ateşşahin et al., 2006). Vendramini et al. (2010) reported that amifostine, which has cytoprotector and ROS scavenger activity, does not affect the decreased testes weights elicited by ADR over 60 days experimental period in rats. The findings related testes weights are in agreement with results of Vendramini et al. (2010). The lack of effect of EA on decreased reproductive organ weights exerted by ADR although ADR-induced disturbed oxidant/antioxidant balance was alleviated by EA administration may be explained that antioxi-

dant potency of EA is not enough for the improvement of direct toxic effect of ADR on reproductive organ weight and sperm quality parameters over the 8 weeks long experimental period. However, further investigations are needed for the explanation of this status. Improvements observed in testicular oxidant/antioxidant balance, testosterone concentration, testicular architecture and apoptosis after EA administration may be explained by potential free radical scavenging activity of EA.

In conclusion, this study apparently suggests that EA has potent antiperoxidative and antiapoptotic effect, but has no significant protective effect on damages in reproductive organ weights and sperm quality parameters against ADR-induced testicular toxicity.

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