Improvement of cisplatin-induced injuries to sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid

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Objective: To investigate whether ellagic acid (EA) has a possible protective effect against cisplatin (CP)-induced negative changes in epididymal sperm characteristics and the histologic structure of testis and prostate associated with oxidative stress in rats.

Design: Experimental study.

Setting: Fırat University Medical School Experimental Research Center, Elazığ, Turkey.

Patient(s): Eight-week-old adult male Sprague Dawley rats (n = 24).

Intervention(s): Cisplatin was administered to rats at a single dose of 7 mg/kg IP. Ellagic acid was administered both separately and simultaneously with CP by gavage daily for 10 days at the dose of 10 mg/kg.

Main Outcome Measure(s): Reproductive organ weights, epididymal sperm characteristics, and histopathologic structure of testes and ventral prostate were determined along with malondialdehyde (MDA) and glutathione (GSH) levels and glutathione-peroxidase (GSH-Px) and catalase (CAT) activities of plasma, sperm, and testicular tissue.

Result(s): Ellagic acid ameliorated the CP-induced reductions in weights of testes, epididymides, seminal vesicles, and prostate along with epididymal sperm concentration and motility. Additionally, EA decreased the CP-induced increments in abnormalities of sperm. Whereas CP increased the MDA levels of plasma, sperm, and testicular tissue, it decreased the GSH-Px and CAT activities in the study samples compared with the control group. The administration of EA to CP-treated rats decreased the MDA level and increased GSH-Px and CAT activities in these samples. Cisplatin caused degeneration, necrosis, interstitial edema, and reduction in germinative cell layer thickness and rarely reduction in spermatogenic activity in some seminiferous tubules. The CP-induced changes in histopathologic findings of testis were partially reversed by treatment with EA. No significant changes were observed in the histopathologic structure of the prostate among any of groups.

Conclusion(s): Ellagic acid has a protective effect against testicular toxicity caused by CP. This protective effect of EA seems to be closely involved with the suppressing of oxidative stress. (Fertil Steril® 2008;89:1474–81. ©2008 by American Society for Reproductive Medicine.)

Key Words: Cisplatin, ellagic acid, oxidative stress, sperm characteristics, testis, rat

Cytotoxic chemotherapy has improved the survival rates in many conditions, particularly testicular malignancies. Treatment is, however, associated with significant morbidity, and testicular dysfunction is among the most common long-term side effects of this therapy (1). The introduction of alkylating agent cisplatin (CP; cis-diamminedichloroplatinum-II), an antineoplastic drug, into clinical practice has led to extraordinary improvement in the curability of testicular, ovarian, and bladder cancers (2, 3). Currently, treatment of testicular germ cell cancer with CP is often successful. However, receiving a high cumulative dose of CP (4) and/or other chemotherapeutics (5) may cause genotoxicity and infertility.

Cisplatin-based chemotherapy for testicular cancer may also result in impaired spermatogenesis (6), chromosomal abnormalities in sperm (7), and temporary or permanent azoospermia (8).

Fertilization and pregnancy are dependent on a series of functional sperm parameters, which are affected by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and/or hydroxyl radical ($\cdot OH$) (9). The production of ROS is a normal physiologic event in various organs, including the testis. However, the overproduction of ROS stimulates DNA fragmentation and can be detrimental to sperm function, associated with peroxidative damage to the mitochondria and plasma membrane. Additionally, spermatozoa are especially susceptible to peroxidative damage, because of high concentration of polyunsaturated fatty acids and low antioxidant capacity (10, 11). Lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and defects

of membrane integrity (12, 13). Cisplatin causes an increment in lipid peroxidation and reduction in antioxidant enzyme activities that prevent and/or protect against peroxidative damage in testis tissue.

The administration of some antioxidants, such as melatonin (14), vitamin E (15), vitamin C (16), and lycopene (17), may have a protective effect against CP-induced damage. Flavonoids, which are polyphenolic antioxidants, occur naturally in vegetables and fruits. Ellagic acid (EA; 2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) is a naturally occurring phenolic constituent in certain fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel (18, 19), and pomegranate (20–22). Ellagic acid has a variety of biologic activities, including potent antioxidant (23, 24), anticancer (25), antiproliferative (26), antimutagen (27), antiatherogenic, apoptotic (28), and estrogen receptor modulator (29) properties. Although the exact mechanism of EA effects is unknown, its potent scavenging action on both ˙OH and O_2^- might be responsible for these effects (30). Therefore, the present study was designed to investigate whether EA has a protective effect against CP-induced negative changes in epididymal sperm characteristics and histologic structure of testis and prostate associated with oxidative stress in rats.

MATERIALS AND METHODS
Chemicals
Cisplatin (10 mg/10 mL, code 1876A) was obtained from Faulding Pharmaceuticals (Warwickshire, U.K.). Ellagic acid and the other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Animals and Treatment Design
Twenty-four healthy adult male Sprague-Dawley rats (8 weeks old, 249 ± 7.2 g body weight) were used in this study. The animals were obtained from Fırat University School of Medicine Experimental Research Center, Elazığ, Turkey. They were maintained at $24 \pm 3°C$ with a 12-hour light/dark cycle and given a commercial pellet diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water ad libitum. The animal use protocol was approved by the National Institutes of Health and the local Committee on Animal Research.

The rats were randomly divided into four groups, each group containing 6 rats. Cisplatin was intraperitoneally (IP) injected to animals at the single dose of 7 mg/kg. The dose and administration period of CP was selected according to previous studies that demonstrated significant damage in sperm parameters of rats (14, 17). Ellagic acid was suspended in corn oil at the concentration of 5 mg/mL and administered to animals by gavage at the dose of 10 mg/kg/day. Isotonic saline (1.0 mL) and corn oil (0.5 mL) were the vehicles for administering CP and EA, respectively. The first group of rats served as control and were administered corn oil for 10 days after a single-dose IP injection of isotonic saline. The second group of rats were treated with EA for 10 days after a single-dose IP injection of isotonic saline. The third group received corn oil for 10 days after a single-dose IP injection of CP. The fourth group of rats were treated with EA for 10 days after a single-dose IP injection of CP.

Sample Collection
The rats were killed with ether at the end of 10 days. Blood samples were collected from vena cava via sterile injector containing EDTA and centrifuged at $3000g$ for 10 minutes. Plasma was separated and then stored at $-20°C$ until analysis.

Testes, epididymides, seminal vesicles, and ventral prostate were removed, cleared of adhering connective tissue, and weighed. One of the testes and the prostate were fixed in 10% formalin for histopathologic examination. Plasma and the other testis samples were stored at $-20°C$ until biochemical analysis.

Evaluation of Sperm Parameters
The epididymal sperm concentration was determined with a hemocytometer using a modified method described by Türk et al. (31). Briefly, the right epididymis was finely minced by anatomic scissors in 1 mL of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2 minutes and then allowed to incubate at room temperature for 4 hours to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue–fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid was diluted at the rate of 1:200 with a solution containing 5 g sodium bicarbonate, 1 mL formalin (35% v/v), and 25 mg eosin per 100 mL distilled water using a pipette designed for counting red blood cells. Approximately 10 μL of the diluted sperm suspension was transfered to counting chambers (Improved Neubauer Depth 0.1 mm; Labart, Darmstadt, Germany) and allowed to stand for 5 minutes. The sperm cells in two chambers were counted with the help of light microscope at $\times 200$ magnification. The remainder of supernatant fluid of each rat was stored at $-20°C$ to determine the lipid peroxidation level and antioxidant enzyme activities in spermatozoa.

The percentage of forward progressive sperm motility was evaluated using a light microscope with heated stage as described by Sönmez et al. (32). For this process, a slide was placed on a light microscope with a heated stage warmed to 37°C, and then several droplets of Tris buffer solution [3.63 g tris (hydroxymethyl) aminomethane, 0.50 g glucose, 1.99 g citric acid, and 100 mL distilled water] were dropped on the slide and a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris buffer solution and mixed by a cover-slip. The percentage of forward progressive sperm motility was evaluated visually at $\times 400$ magnification. Motility estimations were performed

from three different fields in each sample. The mean of the three successive estimates was used as the final motility score. To determine the percentage of morphologically abnormal spermatozoa, slides stained with eosin-nigrosin (1.67 g eosin, 10 g nigrosin, and 2.9 g sodium citrate per 100 mL distilled water) were prepared. After preparation, the slides were viewed under a light microscope at ×400 magnification. A total of 300 sperm cells were examined on each slide (1800 cells in each group), and the head, tail, and total abnormality rates of spermatozoa were expressed as percentages (31).

Biochemical Studies

Lipid peroxidation level Testes were homogenized in Teflon-glass homogenizer with buffer containing 1.5% potassium chloride to obtain 1:10 (w/v) whole homogenate to determine lipid peroxidation and antioxidant enzyme activity in testicular tissue. The plasma, sperm, and testicular tissue lipid peroxidation levels were measured according to the concentration of thiobarbituric acid reactive species (33). The amount of produced malondialdehyde (MDA) was used as an index of lipid peroxidation. Briefly, one volume of the test sample and two volumes of stock reagent (15% w/v trichloroacetic acid in 0.25 N HCl and 0.375% w/v thiobarbituric acid in 0.25 N HCl) were mixed in a centrifuge tube. The solution was heated in boiling water for 15 minutes. After cooling, the precipitate was removed by centrifugation at 1500g for 10 minutes, and then absorbance of the supernatant was read at 532 nm against a blank containing all reagents except test sample on a spectrophotometer (2R/UV-visible; Shimadzu, Tokyo, Japan). The MDA level was expressed as nmol/mL for plasma and sperm and as nmol/g protein for testicular tissue.

Glutathione level and glutathione peroxidase activity The reduced glutathione (GSH) contents in plasma, sperm and testicular tissue were measured at 412 nm using the method of Sedlak and Lindsay (34). The samples were precipitated with 50% trichloracetic acid and then centrifuged at 1000g for 5 minutes. The reaction mixture contained 0.5 mL supernatant, 2.0 mL Tris-EDTA buffer (0.2 mol/L, pH 8.9) and 0.1 mL of 0.01 mol/L 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 minutes and then read at 412 nm on the spectrophotometer. The levels of GSH were expressed as nmol/mL for plasma and sperm and as nmol/g protein for testicular tissue. The glutathione peroxidase (GSH-Px) activity was determined according to the method of Lawrence and Burk (35). The reaction mixture consisted of 50 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L sodium azide (NaN$_3$), 0.2 mmol/L reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/mL oxidized GSH reductase, 1 mmol/L GSH, and 0.25 mmol/L H$_2$O$_2$. Enzyme source (0.1 mL) was added to 0.8 mL of the above mixture and incubated at 25°C for 5 minutes before initiation of the reaction with the addition of 0.1 mL of peroxide solution. The absorbance at 340 nm was recorded for 5 minutes on a spectrophotometer.

The activity was calculated from the slope of the lines as micromoles NADPH oxidized per minute. The blank value (with enzyme replaced by distilled water) was subtracted from each value. The GSH-Px activity was expressed as IU/g protein for plasma, sperm, and testicular tissue. The protein concentration was also measured by the method of Lowry et al. (36).

Catalase activity The plasma and sperm catalase (CAT) activity was measured as previously described by Góth (37). Briefly, 0.2 mL of plasma sample was incubated in 1.0 mL substrate (65 μmol/L hydrogen peroxide in 50 mmol/L phosphate buffer, pH 7.0) at 37°C for 60 seconds. The enzymatic reaction was terminated with 1.0 mL 32.4 mmol/L ammonium molybdate. The color after this reaction was read on a spectrophotometer at 405 nm against blank containing all the components except the enzyme. The plasma and sperm CAT activity was measured according to the decrease in H$_2$O$_2$ level and expressed as kU/L, where k is the first-order rate constant and U is the unit. The testicular tissue CAT activity was determined by measuring the decomposition of hydrogen peroxide at 240 nm, according to the method of Aebi (38), and was expressed as k/g protein.

Histopathologic Examination

Fixed testes and prostate tissue samples in 10% formalin were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Light microscopy was used for the evaluations. The diameter and germinal cell layer thickness of the seminiferous tubules (ST) from five different areas of each testicle were measured using an ocular micrometer, and averages were calculated.

Data Analysis

Data are presented as mean ± standard error of means. One-way analysis of variance and post hoc Tukey-HSD test was used to determine the differences between the groups in terms of all studied parameters using the SPSS/PC computer program (version 10.0; SPSS, Chicago, IL); a value of $P<.05$ was considered to be significant.

RESULTS

Table 1 demonstrates the changes in reproductive organ weights, epididymal sperm concentration, sperm motility, and abnormal sperm rate in response to various treatments for 10 days. Cisplatin caused statistically significant decreases in weights of testes ($P<.01$), epididymides ($P<.05$), and seminal vesicles ($P<.01$) and insignificant ($P>.05$) reduction in prostate weight compared with the control group. Administration of EA to CP-treated rats resulted in statistically significant increments ($P<.01$) in testes weight and insignificant ($P>.05$) increases in weights of epididymides, seminal vesicles, and prostate compared with the CP-alone group.

Although CP treatment significantly decreased sperm concentration ($P<.01$) and sperm motility ($P<.05$) and increased

the percentage of head ($P<.05$), tail ($P<.01$), and total ($P<.01$) abnormality of sperm compared with the control group, the administration of EA to CP-treated rats significantly (including concentration [$P<.01$], motility [$P<.05$], and abnormality [$P<.01$]) prevented the CP-induced negative effects in sperm quality, including concentration, motility, and abnormality, compared with the CP group.

The MDA and GSH levels and GSH-Px and CAT activities of all the treatment groups are shown in Figure 1. Cisplatin caused significant increases in MDA levels in plasma ($P<.01$), sperm ($P<.001$), and testicular tissue ($P<.001$) compared with the control group. Significant decreases ($P<.001$) were observed in CP + EA rats compared with the CP-alone group with respect to plasma, sperm, and testicular tissue MDA levels.

Cisplatin did not significantly affect the GSH levels compared with the control and CP + EA groups. Only CP treatment caused significant decreases in GSH-Px activities of plasma ($P<.05$), sperm ($P<.01$), and testicular tissue ($P<.001$) compared with the control group. Administration of EA to CP-treated rats prevented the CP-induced decreases in GSH-Px activities. Although decreases in the CAT activities of plasma (insignificant; $P>.05$), sperm (insignificant; $P>.05$), and testicular tissue (significant; $P<.01$) were found in the CP group compared with the control group, it was observed that administration of EA to the CP-treated group could bring the plasma, sperm, and testicular tissue activity of this enzyme back to the level of the control group.

Upon histopathologic examination of testis, degeneration, necrosis, and interstitial edema were detected in CP-treated group compared with the control group. Desquamative germinal cells and the reductions in spermatogenic activity were seen in lumen of some ST of CP-treated rats (Fig. 2A). Although CP treatment caused a significant ($P<.001$) decrease in germinal cell layer thickness of the testis compared with the control group, its effect was absent on the diameter of the ST (Table 1). The CP-induced changes in histopathologic findings of testis were partially reversed by treatment with EA (Fig. 2B). No significant changes were observed in the histopathologic structure of the prostate among any of treatment groups.

DISCUSSION

Many drugs used for chemotherapy, particularly alkylating agents, have gonadotoxic effects, and their gonadotoxicity or spermitoxicity is associated with variables such as antineoplastic agent group, number of chemotherapeutic agents used, their total doses, treatment duration, and individual sensitivity (1, 7). The present aim of all multiagent chemotherapeutic protocols is to achieve a balance between highest care results and the smallest side effects. Cisplatin is an effective alkylating chemotherapeutic agent using for the treatment of testicular, ovary, head, neck, and cervix cancer types (3, 8).

Germinal epithelial damage, resulting in oligo- or azoospermia, has long been a recognized consequence of

Malondialdehyde (MDA), glutathione (GSH) levels, and glutathione peroxidase (GSH-Px), catalase (CAT) activities in plasma, sperm, and testicular tissue samples. The mean differences between the bars bearing values with different superscript letters are statistically significant (a and b: $P<.05$; A and B: $P<.01$; x, y, and z: $P<.001$).
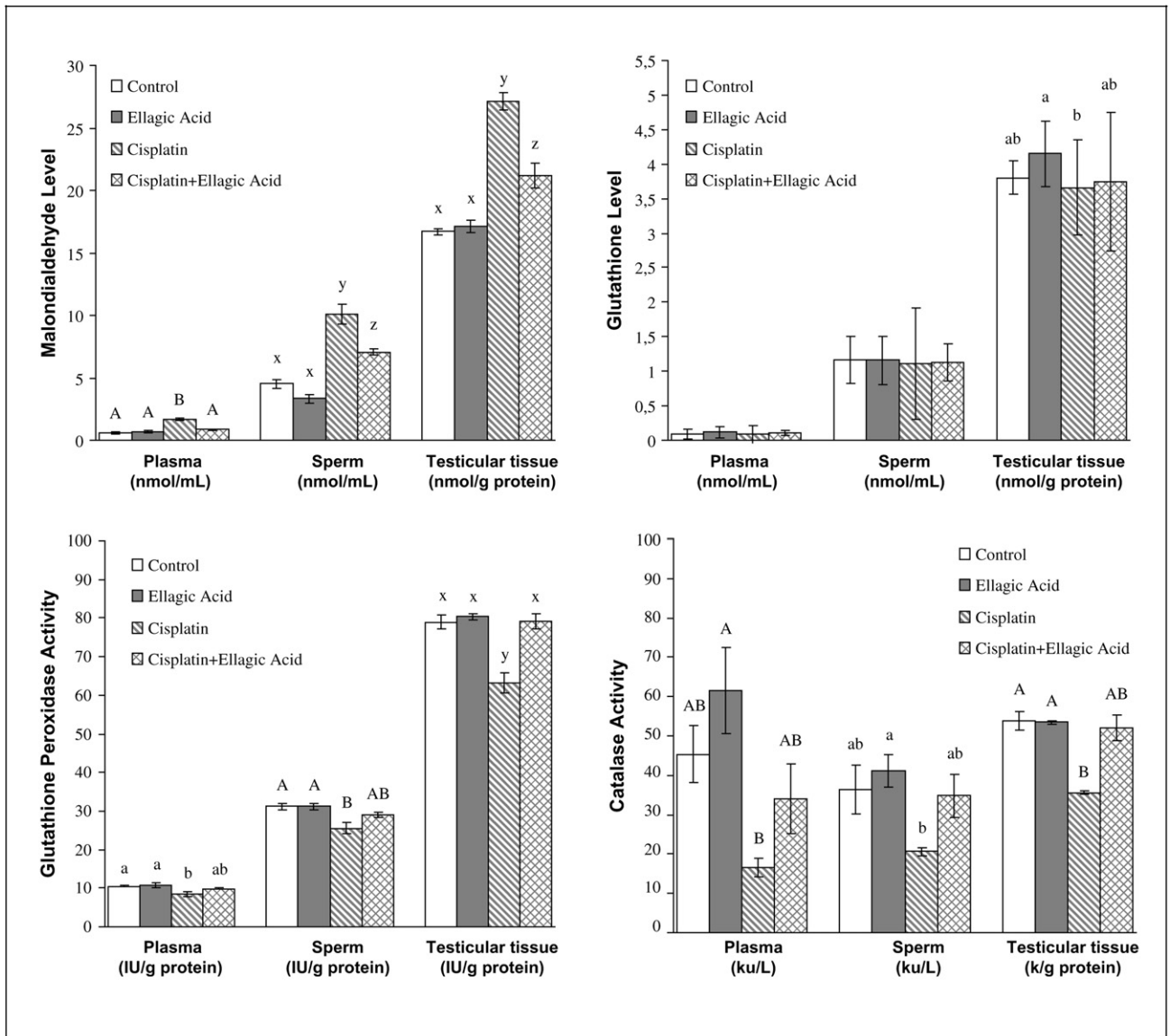
Türk. Ellagic acid protects testis from CP toxicity. Fertil Steril 2008.

treatment with chemotherapeutic agents (6, 14, 17). Chemotherapeutic regimen–induced testicular damage is drug specific and dose related (1). In the present study, CP caused decreases in weights of testes, epididymides, seminal vesicles, and prostate compared with the control group. These findings are confirmed by our previous reports, which clearly demonstrated that the use of CP (14, 17) or adriamycin (39) caused decreases in the relative weight of the reproductive organs of healthy male rats. The reductions in testis and epididymidis weights are due to the marked parenchymal atrophy in germinal cell layer thickness and the other deteriorated histopathologic findings in testis, along with decreased sperm

concentration, which were observed in CP-treated rats in the present study. The secretory activity of accessory glands is dependent on testosterone produced by the interstitial cells. In the present study, the reduction in accessory reproductive organ weights may be explained that the secretion of these organs likely decreased because testosterone levels were diminished, as suggested by our previous study in which a significant decrease was observed in plasma testosterone level of CP-treated rats (17).

The cellular/biochemical mechanisms by which CP causes reproductive toxicity is poorly understood; however, CP has

Light microscopy of testis tissue from rat treated with cisplatin alone (**A**) and cisplatin + ellagic acid (**B**) (hematoxylin and eosin, ×20). Seminiferous tubules show degeneration, necrosis, and interstitial edema in the cisplatin-alone group. These damages were reversed by ellagic acid administration, and seminiferous tubules show nearly normal structure in the cisplatin + ellagic acid group.
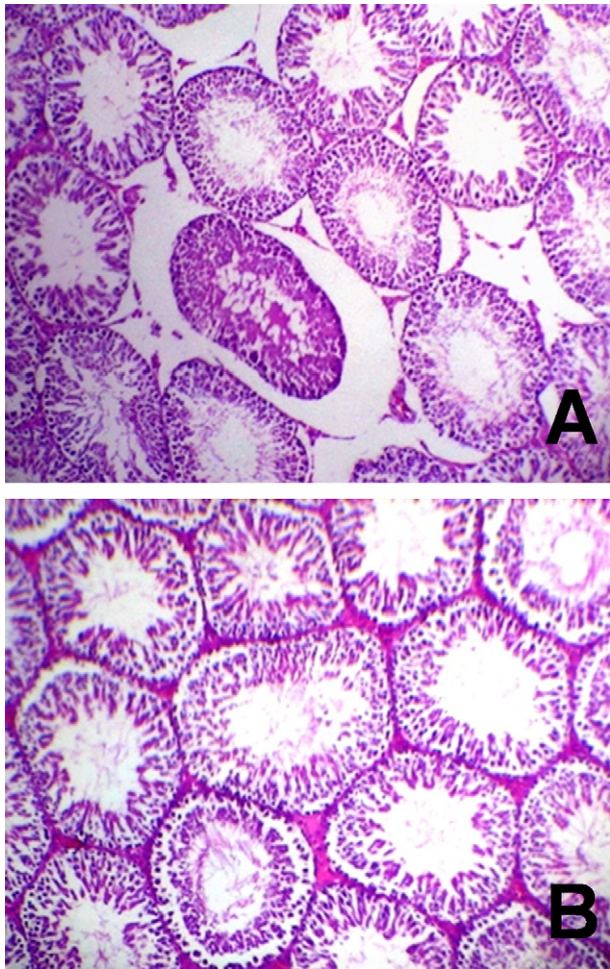
Türk. Ellagic acid protects testis from CP toxicity. Fertil Steril 2008.

physiologic side effects leading to mutations and other genotoxic changes in DNA of nontumor cells (40), and CP exposure enhances intracellular ROS production (16). These highly reactive substances, which exhibit half-life times in the nanosecond ($\cdot$OH) to milli-second range (O_2^-), are very strong oxidants and are physiologically produced in any living cell during respiration (9). However, the overproduction of ROS stimulates DNA fragmentation and can be detrimental to sperm function associated with oxidative stress to the mitochondria and plasma membrane. Additionally, because of the extraordinarily high content of polyunsaturated fatty acids in the plasma membrane, which is an essential require-

ment for the male germ cell to maintain sperm functions, and owing to the very low content of protective systems, spermatozoa are highly susceptible to oxidative stress. The lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa and thus causes the loss of motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, defects of membrane integrity, and increased morphologic defects (12, 13). The negative changes observed in sperm quality in the present study may be attributed to the peroxidation of polyunsaturated fatty acids in plasma membranes of spermatozoa, mutations, and other genotoxic effects caused by CP administration.

The increments in plasma, sperm, and testicular tissue MDA levels determined in the present study are due to the increases in peroxidation of lipids, which is supported by earlier studies of ours (14, 17) and of other researchers (15, 16), and in cellular peroxidative DNA damage caused by CP regimen. Glutathione is the major cellular sulfhydryl compound that serves as both a nucleophilic and an effective reductant by interacting with numerous electrophilic and oxidizing compounds. It can act as a nonenzymatic antioxidant by direct interaction of sulfhydryl group with ROS. However, in the present study GSH content does not appear to be altered, as confirmed by other studies (14, 17, 41), and this may be attributed to the direct conjugation of CP with free or protein-bound sulfhydryl groups, thereby interfering with the antioxidant functions. The decreases in the activities of the antioxidant enzymes might predispose the spermatozoa to increased ROS damage. Glutathione peroxidase and CAT have been considered to be the primary scavengers of H_2O_2 (42, 43). In the absence of adequate GSH-Px or CAT activity, more H_2O_2 could be converted to toxic $\cdot$OH radicals and may contribute to the oxidative stress of CP toxicity. The decline observed in the activities of these antioxidant enzymes in the present study may be elucidated with their inactivation caused by excessive ROS production. Therefore, the balance of this enzyme system is essential to dispose the O_2^- and peroxides generated in the spermatozoa. The reduction in the activities of these enzymes and increase in MDA could reflect the adverse effects of CP on this finely balanced antioxidant system in the epididymal sperm of rats.

Various agents have been attempted to protect from and/or prevent the side effects of many chemotherapeutics. Flavonoids are among these agents and are found in almost all food categories, with fruits and vegetables being the main source. Flavonoids have many functions, such as phenolic antioxidants, scavengers of free radicals, chelating agents, and modifiers of various enzymatic and biologic reactions (44). Ellagic acid is a naturally occurring plant polyphenol (18, 19) that exhibits antioxidative properties both in vivo (23, 24) and in vitro (26). In fact, EA has been shown to exert a potent scavenging action on both O_2^- and $\cdot$OH, as well as lipid peroxidation (45). Administration of EA to CP-treated rats resulted in a statistically significant increment in testes weight and insignificant increases in weights of epididymides,

seminal vesicles, and prostate. Additionally, the CP-induced decreases in germinal cell layer thickness, and the other deteriorated histopathologic findings of testis were partially ameliorated by EA administration to CP-treated rats. This status may be explained with partially attenuation of CP-induced degeneration, reduction in germinal cell layer thickness, and possibly enhancement of sperm concentration in the epididymis and increment in fluids of accessory glands due to the decreased lipid peroxidation and increased antioxidant enzyme activities caused by EA administration.

The declining of lipid peroxidation in all samples studied apparently indicates that EA potently scavenged the free radicals (O_2^- and $\cdot OH$), and suppressed oxidative DNA damage. The antioxidant activity of EA and mitigation of ROS-induced depletion of GSH-Px and CAT activities also show that EA has strong antioxidant activity.

In conclusion, this study apparently suggests that EA has a protective effect against testicular toxicity caused by CP. This protective effect of EA seems to be closely involved with the suppression of oxidative stress. Therefore, EA may be used combined with CP in chemotherapeutic treatments to improve CP-induced injuries in sperm quality and oxidative stress parameters.

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