Effects of Cinnamon (C. zeylanicum) Bark Oil Against Taxanes-Induced Damages in Sperm Quality, Testicular and Epididymal Oxidant/Antioxidant Balance, Testicular Apoptosis, and Sperm DNA Integrity

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ABSTRACT

The aim of this study was to investigate whether cinnamon bark oil (CBO) has protective effect on taxanes-induced adverse changes in sperm quality, testicular and epididymal oxidant/antioxidant balance, testicular apoptosis, and sperm DNA integrity. For this purpose, 88 adult male rats were equally divided into 8 groups: control, CBO, docetaxel (DTX), paclitaxel (PTX), DTX+PTX, DTX+CBO, PTX+CBO, and DTX+PTX+CBO. CBO was given by gavage daily for 10 weeks at the dose of 100 mg/kg. DTX and PTX were administered by intraperitoneal injection at the doses of 5 and 4 mg/kg/week, respectively, for 10 weeks. DTX+PTX and DTX+PTX+CBO groups were treated with DTX during first 5 weeks and PTX during next 5 weeks. DTX, PTX, and their mixed administrations caused significant decreases in absolute and relative weights of all reproductive organs, testosterone level, sperm motility, concentration, glutathione level, and catalase activity in testicular and epididymal tissues. They also significantly increased abnormal sperm rate, testicular and epididymal malondialdehyde level, apoptotic germ cell number, and sperm DNA fragmentation and significantly damaged the histological structure of testes. CBO consumption by DTX-, PTX-, and DTX+PTX-treated rats provided significant ameliorations in decreased relative weights of reproductive organs, decreased testosterone, decreased sperm quality, imbalanced oxidant/antioxidant system, increased apoptotic germ cell number, rate of sperm with fragmented DNA, and severity of testicular histopathological lesions induced by taxanes. In conclusion, taxanes cause impairments in sperm quality, testicular and epididymal oxidant/antioxidant balance, testicular histopathological structure, and sperm DNA integrity, and long-term CBO consumption protects male reproductive system of rats.

Introduction

Docetaxel (DTX) and paclitaxel (PTX) are the taxane class of drugs which are among the most effective chemotherapeutic agents (1). They have the same effect and exhibit their chemotherapeutic influences through polymerization of tubulin monomer, leading to mitotic arrest inducing phosphorylation of Bcl-2, a protein producing anti-apoptotic effects, and apoptotic cell death (2–4). PTX is a natural diterpene originally extracted from the bark of the Pacific yew tree, *Taxus brevifolia* (5). DTX, extracted from *Taxus baccata* (European yew tree), is a semisynthetic analog of PTX (6). Both PTX and DTX have a broad spectrum of antitumor activity and they are

widely used in the treatment of various types of cancers such as breast, non-small cell lung, advanced stomach, head and neck, and metastatic prostate cancer (2,7,8).

Nowadays, a high percentage of cancer patients are treated by using chemotherapeutic agents. Long-term exposure to chemotherapeutic agents leads to damage in reproductive system (9,10). Reproductive organs, especially testes, are the target organs for the damage resulting from chemotherapeutic agents. The testis produces mature gametes through spermatogenesis which is negatively affected when exposed to chemotherapeutic agents (9,11). Spermatogenesis is unfavorably affected by

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secretory substances of the tumor, such as hormones and cytokines and chemotherapeutic agents (12). Chemotherapeutic agents easily reach Leydig and Sertoli cells and spermatogonia. Many chemotherapeutic agents can penetrate the Sertoli cell barrier and lead to damage of the late-stage germ cells. Particularly, differentiating spermatogonia are highly vulnerable to cytotoxic agents (13). In animal studies, it has been shown that chemotherapies lead to mutations; later-stage spermatogenic cells (spermatocytes onward) are vulnerable to mutagenic injury—the mutated DNA in these cells can transmit to the next generations (13,14). Besides, long-term exposure to chemotherapeutic agents leads to the induction of germ cell apoptosis, long-lasting azoospermia, and infertility (9,10).

The testis is a very active organ which has prosurvival and proapoptotic systems. These systems work together to organize the germ cell apoptosis. Normally, the production of germ cells is regulated by physiological apoptosis in the seminiferous epithelium. However, on exposure to testicular toxicants (e.g., chemotherapeutic agents), apoptosis noticeably increases; the excessive apoptotic-activity-induced dysfunction in seminiferous epithelium results in severe injury in germ cells (15,16). It has been reported in many studies that chemotherapeutics lead to decreases in reproductive organ weights, impairment of antioxidant defense mechanisms, and increased free radicals (17–21) inducing testicular germ cell apoptosis (22–24). Chemotherapeutics, despite their strong antitumor activities, cause an increment in lipid peroxidation levels and reduction in antioxidant enzyme activities that prevent and/or protect testes against peroxidative damage (17–24). Normally, the generation of reactive oxygen species (ROS; hydrogen peroxide, superoxide anion, and hydroxyl radical) is a physiologic event in various organs, including the testes. However, the overproduction of ROS causes DNA fragmentation and impairs sperm function due to damaging effect of free radicals on the mitochondria and plasma membrane. Additionally, spermatozoa are especially vulnerable to oxidative stress, associated with high concentration of polyunsaturated fatty acids and low antioxidant capacity (25).

Nowadays, many free radical scavengers and antioxidant agents have been used in order to prevent damages in testes and spermatozoa induced by chemotherapeutics. For this purpose, some herbal antioxidants can be used to block testicular oxidative stress. Cinnamon is an herbal antioxidant and has also been used as a spice. *C. zeylanicum* bark and leaf oils, *C. cassia* (cassia oil) and *C. camphora* are the most important essential oils extracted from cinnamon (26). Cinnamon bark oil (CBO) has potent free radical scavenging and antioxidant activities (27–29). Additionally, long-term CBO consumption has been reported to provide increments in sperm quality and reproductive organ weights in healthy (28) and carbon tetrachloride toxicated rats (29) and reductions in toxicant-induced testicular apoptosis by decreasing lipid peroxidation level (28). However, there is no scientific evidence related to the effects of taxanes on sperm DNA integrity as well as impact of CBO on taxanes-induced structural and functional damages in male reproductive system. Therefore, this study was conducted to investigate whether CBO has protective effect on taxanes-induced adverse changes in sperm quality, testicular apoptosis and histopathological lesions, and sperm DNA fragmentation associated with the oxidative stress.

Materials and methods

CBO, drugs, and chemicals

CBO was purchased from a local store (Altınterim Co., Elazığ, Turkey). According to the manufacturer's procedure, *C. zeylanicum* barks were transported in polypropylene bags and dried to constant weight in room temperature. CBO was obtained by hydrodistillation method. The plant materials (about 100 g) were then ground into small pieces and placed in a flask (2 l) together with double distilled water (1.5 l). The mixture was boiled for 4 h. The extract was condensed in cooling vapor to collect the essential oil. The extracted oil was dried over anhydrous sodium sulfate. The CBO used in this study was previously analyzed using GC-MS by our study group, and the components of CBO were reported in a study by Şimşek et al. (30). In that study, the major components of CBO were reported to be cinnamaldehyde (88.2%), benzyl alcohol (8.1%), and eugenol (1.0%). The concentrations of other compounds were reported to be ≤0.5% (30). CBO was kept at 4°C until use. DTX (Taxotere®, 80 mg/2 ml) and PTX (Taxol®, 100 mg/17 ml) were purchased from Sanofi-Aventis Inc. (İstanbul, Turkey) and Bristol-Myers Squibb Inc. (İstanbul, Turkey), respectively. The other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental protocol

The experimental protocol was approved by the Animal Experimentations Local Ethics Committee of Erciyes University (Kayseri, Turkey). Eighty-eight healthy adult male Wistar albino rats, aged 2 months, were obtained and maintained from Erciyes University Experimental Research and Application Center (Kayseri, Turkey). The animals were housed in polycarbonate cages in a room

with a 12-h day/night cycle, temperature of 24 ± 3°C, and humidity of 45–65%. During the whole experimental period, animals were fed with a balanced commercial diet (Optima Food Co., Bolu, Turkey) ad libitum and fresh drinking water was given ad libitum.

CBO was administered by gavage at a dose of 100 mg/kg/day for 10 weeks. To equalize the total amount (1 ml) that each rat will receive in each application, CBO was further suspended in corn oil and the CBO ratio in 1 ml corn oil was adjusted according to the weight of each rat. The dose of CBO used in this study was selected based on the previous reports (28,29). DTX and PTX were intraperitoneally injected during the experimental period. Because the spermatogenesis is 7–8 weeks (31) and epididymal transit of spermatozoa is 1–2 weeks (32) in rats, the treatment period in this study was set out at 10 weeks to achieve the maximum effect. Each rat was weighed weekly, and dosing suspensions were adjusted for changes in body weights during experimental period. The rats were randomly divided into eight groups, 11 animals each.

The groups established in this study were as follows:
- Control; received weekly 0.5 ml isotonic saline (i.p.) + daily 1 ml corn oil (oral).
- CBO; treated with weekly 0.5 ml isotonic saline (i.p.) + 100 mg/kg/day CBO within 1 ml corn oil (oral).
- DTX; treated with weekly 5 mg/kg DTX in 0.5 ml isotonic saline (i.p.) + daily 1 ml corn oil (oral).
- PTX; treated with weekly 4 mg/kg PTX in 0.5 ml isotonic saline (i.p.) + daily 1 ml corn oil (oral).
- DTX+PTX; treated with weekly 5 mg/kg DTX (for the first 5 weeks) + weekly 4 mg/kg PTX (for the second 5 weeks) in 0.5 ml isotonic saline (i.p.) + daily 1 ml corn oil (oral).
- DTX+CBO; treated with weekly 5 mg/kg DTX in 0.5 ml isotonic saline (i.p.) + 100 mg/kg/day CBO in 1 ml corn oil (oral).
- PTX+CBO; treated with weekly 4 mg/kg PTX in 0.5 ml isotonic saline (i.p.) + 100 mg/kg/day CBO in 1 ml corn oil (oral).
- DTX+PTX+CBO; treated with weekly 5 mg/kg DTX (for the first 5 weeks) + weekly 4 mg/kg PTX (for the second 5 weeks) in 0.5 ml isotonic saline (i.p.) + 100 mg/kg/day CBO in 1 ml corn oil (oral).

Collection of samples

The rats were sacrificed using xylazine/ketamine anesthesia at the end of 10th week. The blood samples were collected using a sterile injector from heart. Testes, epididymides, seminal vesicles, and ventral prostate were removed, cleared from adhering connective tissue, and weighed. Absolute and relative (organ weight (g)/final body weight (g) × 100) reproductive organ weights were recorded. The collected blood samples were centrifuged at 3000 g for 10 min to obtain serum. One of the testis samples was fixed in Bouin's solution for histopathological examination. The other testis samples and blood sera were stored at −20°C for biochemical analyses. Testes were taken from a −20°C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a nine-fold volume of phosphate-buffered saline (PBS) (pH 7.4). For the enzymatic analyses, testes were minced in a glass and homogenized by a Teflon® glass homogenizer for 3 minutes in cold physiological saline on ice.

Serum testosterone assay

The serum testosterone level was measured by electrochemiluminescence immunoassay (ECLIA) method and commercial testosterone kit (Elecsys® Testosterone II, Roche Diagnostics Ltd., Rotkreuz, Switzerland) in the device of Cobas e 602 module. The testosterone level was expressed as ng/dL.

Determination of testicular and epididymal tissue oxidative stress markers

All analyses were performed with the aid of a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). Lipid peroxidation level was measured according to the concentration of thiobarbituric acid reactive substances, and the amount of malondialdehyde (MDA) produced was used as an index of lipid peroxidation. The MDA level at 532 nm was expressed as nmol/g protein (33).

Reduced glutathione (GSH) level was measured using the method described by Sedlak and Lindsay (34). The level of GSH at 412 nm was expressed as nmol/g protein. Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence and Burk (35). The GSH-Px activity at 340 nm was expressed as IU/g protein. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as k/g protein, where k is the first-order rate constant (36). Protein concentration was determined by the method of Lowry et al. (37).

Sperm analyses

The methods reported in the studies of Türk et al. (20,22) were used for all the sperm analyses. The sperm count in the right cauda epididymal tissue was determined with a hemocytometer. Freshly isolated left cauda epididymal

tissue was used for the analysis of sperm motility. The percentage of sperm motility was evaluated using a light microscope with a heated stage. To determine the percentage of morphologically abnormal spermatozoa, slides stained with eosin-nigrosin (1.67% eosin, 10% nigrosin, and 0.1 M of sodium citrate) were prepared. The slides were then viewed under a phase-contrast microscope at ×400 magnification. A total of 300 spermatozoa were examined on each slide (3300 cells in each group), and the head, tail, and total abnormality rates of spermatozoa were expressed as percentage.

Histopathological examination

Testis tissues were fixed in Bouin's solution for 48 h, they were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5-μm thicknesses, and stained with Mayer's hematoxylin and eosin. Twenty seminiferous tubules were randomly examined per section and the lesions were photographed. Diameters of 20 seminiferous tubules (DST) per section were measured using an ocular micrometer in a light microscope. Johnsen's testicular scoring (38) was done in 20 seminiferous tubules for each section, and a score between 1 (very poor) and 10 (excellent) was given to each tubule according to Johnsen's criteria.

Determination of apoptotic germ cell number

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay with the ApopTag® Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) was used to detect apoptotic germ cell number according to the manufacturer's instructions. To detect TUNEL+ apoptotic germ cell number, 20 seminiferous tubules of each section were randomly selected and examined at original magnification of ×200. TUNEL+ apoptotic germ cells were counted in the defined areas with the aid of Image J software program (version 1.41, Bethesda, MD, USA) for quantitative histomorphometric analysis and photographed.

Determination of sperm DNA fragmentation by comet assay

Diluted sperm samples extracted from epididymis were centrifuged at 300 g for 10 min at 4°C. The supernatant was removed and the remaining sperm cells were washed with Ca^{2+} and Mg^{2+} free PBS (39). Sperms with fragmented DNA were determined using the single-cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions (40). The images of 100 randomly chosen nuclei from sperm sample of each

animal were visually analyzed and sperms with fragmented DNA were counted. Observations were made at a magnification of ×400 using a fluorescent microscope (Olympus, Tokyo, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head causing a "comet" pattern, whereas whole sperm heads, without a comet, were not considered to be damaged (41).

Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). Nonparametric Kruskal-Wallis analysis of variance test was used to determine the differences between the groups, and nonparametric Mann-Whitney U test with "Bonferroni correction" for multiple comparison of the groups was used with respect to all parameters studied. In this case, P value (0.05) was divided into the number of groups and a P value of < 0.006 (0.05/8 = 0.006) was accepted as significant. All the analyses were carried out using the SPSS/PC software program (Version 22.0; SPSS, Chicago, IL, USA).

Results

Alterations in body and reproductive organ weights

DTX and mixed administration of DTX+PTX caused significant ($P < 0.001$) decreases in the final body weight compared to control group. These decreased values in DTX and mixed groups were brought to values near that of control group by CBO administration to chemotherapeutics-treated rats.

The mean data related to the absolute and relative reproductive organ weights are presented in Tables 1 and 2, respectively. The significant ($P < 0.001$) decreases
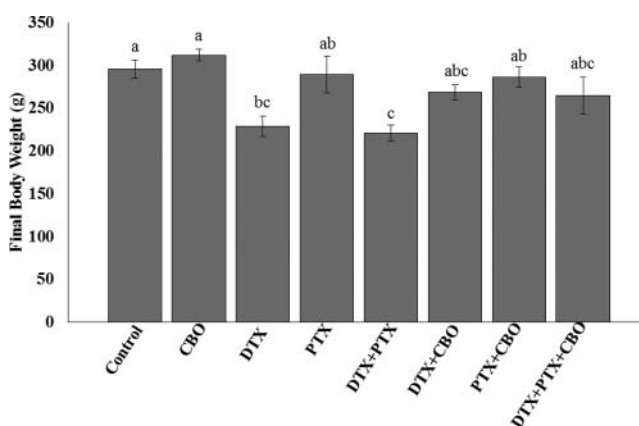

Figure 1. Alterations in final body weights in response to various treatments. CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel. Data are expressed as mean±SEM. The difference between the mean values that do not have the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

Table 1. Alterations in the absolute reproductive organ weights in response to various treatments.

Groups	Absolute reproductive organ weights (g)				
	Testis (Right+left/2)	Whole epididymis (Right+left/2)	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	1.263 ± 0.008[ab]	0.514 ± 0.007[a]	0.186 ± 0.003[ab]	1.126 ± 0.052[a]	0.550 ± 0.019[ab]
CBO	1.296 ± 0.007[a]	0.547 ± 0.013[a]	0.211 ± 0.012[a]	1.247 ± 0.061[a]	0.587 ± 0.030[a]
DTX	0.706 ± 0.054[ef]	0.307 ± 0.012[c]	0.113 ± 0.005[c]	0.657 ± 0.027[de]	0.283 ± 0.029[e]
PTX	0.980 ± 0.035[cd]	0.423 ± 0.007[b]	0.116 ± 0.009[c]	0.821 ± 0.026[cd]	0.339 ± 0.010[de]
DTX+PTX	0.623 ± 0.051[f]	0.297 ± 0.015[c]	0.097 ± 0.007[c]	0.619 ± 0.053[e]	0.266 ± 0.019[e]
DTX+CBO	0.933 ± 0.029[cd]	0.379 ± 0.025[b]	0.151 ± 0.012[b]	0.929 ± 0.035[bc]	0.393 ± 0.018[cd]
PTX+CBO	1.095 ± 0.047[bc]	0.486 ± 0.016[a]	0.166 ± 0.006[b]	1.069 ± 0.041[ab]	0.509 ± 0.029[ab]
DTX+PTX+CBO	0.829 ± 0.037[de]	0.420 ± 0.009[b]	0.170 ± 0.003[b]	0.825 ± 0.032[cd]	0.466 ± 0.017[bc]
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel.
Data are expressed as mean±SEM.
The difference between the mean values without the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

in the absolute weights of all reproductive organs were found in only chemotherapeutics-treated groups compared to control group. The lowest values of the absolute reproductive organ weights were obtained from DTX+PTX group. Besides, DTX had more damaging effect than PTX. However, significant improvements were determined in the absolute weights of whole epididymis, right cauda epididymis, seminal vesicles, and ventral prostate in all treatments with the combination of chemotherapeutics and CBO compared to only chemotherapeutic-treated groups ($P < 0.001$). DTX and DTX+PTX groups had significant reductions in the relative weights of testis and whole epididymis ($P < 0.001$). Only DTX+PTX administration caused a significant decrease in the relative weight of seminal vesicles ($P < 0.001$). All of the chemotherapeutic treatments significantly reduced the relative weights of right cauda epididymis and ventral prostate ($P < 0.001$) compared to control group. However, CBO consumption by chemotherapeutics-treated groups provided significant improvements in relative weights of reproductive organs ($P < 0.001$). The data related to relative weights of all

reproductive organs obtained from DTX+CBO, PTX+CBO, and DTX+PTX+CBO groups were found as near values to that in the control group due to the positive effects of CBO on organ weights.

Alterations in serum testosterone level

Serum testosterone levels of all the groups are given in Fig. 2. DTX, PTX, and their mixed administrations to rats significantly reduced the testosterone level ($P < 0.001$) compared to other experimental groups. CBO administration to chemotherapeutics-treated rats significantly enhanced the decreased testosterone level compared to only chemotherapeutics-treated groups ($P < 0.001$).

Alterations in testicular and epididymal tissue oxidative stress markers

The data related to the lipid peroxidation levels (MDA) and antioxidant markers (GSH, GSH-Px, and CAT) in testes and epididymides of all the groups are given in

Table 2. Alterations in relative reproductive organ weights in response to various treatments.

Groups	Relative reproductive organ weights [Absolute organ weight (g) / Final body weight (g) × 100]				
	Testis (Right+left/2)	Whole epididymis (Right+left/2)	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	0.430 ± 0.017[a]	0.175 ± 0.007[a]	0.063 ± 0.002[a]	0.381 ± 0.014[ab]	0.187 ± 0.009[a]
CBO	0.416 ± 0.007[a]	0.176 ± 0.005[a]	0.068 ± 0.004[a]	0.401 ± 0.022[a]	0.189 ± 0.012[a]
DTX	0.308 ± 0.020[bc]	0.136 ± 0.007[b]	0.050 ± 0.003[bc]	0.293 ± 0.019[bc]	0.123 ± 0.008[b]
PTX	0.348 ± 0.021[abc]	0.152 ± 0.011[ab]	0.042 ± 0.004[c]	0.293 ± 0.020[bc]	0.122 ± 0.010[b]
DTX+PTX	0.282 ± 0.019[c]	0.135 ± 0.007[b]	0.044 ± 0.003[c]	0.279 ± 0.018[ec]	0.123 ± 0.013[b]
DTX+CBO	0.382 ± 0.031[ab]	0.155 ± 0.007[ab]	0.060 ± 0.003[ab]	0.377 ± 0.031[ab]	0.158 ± 0.014[ab]
PTX+CBO	0.385 ± 0.016[ab]	0.171 ± 0.008[a]	0.058 ± 0.002[ab]	0.378 ± 0.021[ab]	0.180 ± 0.013[a]
DTX+PTX+CBO	0.311 ± 0.018[bc]	0.158 ± 0.005[ab]	0.064 ± 0.003[a]	0.310 ± 0.017[bc]	0.175 ± 0.008[a]
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel.
Data are expressed as mean ± SEM.
The difference between the mean values without the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).
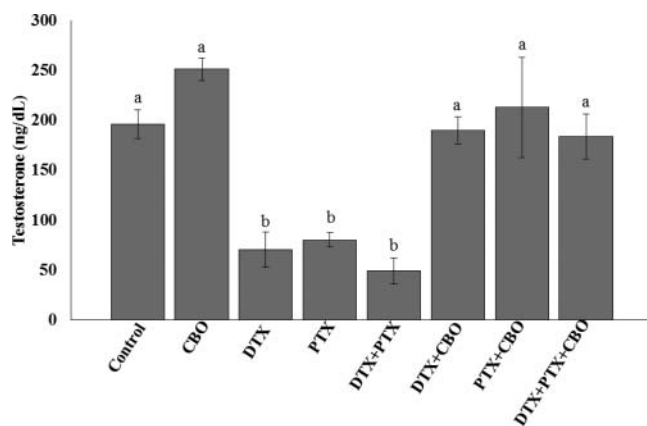
Figure 2. Alterations in testosterone levels in response to various treatments. CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel. Data are expressed as mean±SEM. The difference between the mean values that do not have the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

Table 3. All chemotherapeutic administrations resulted in significantly ($P < 0.001$) increased MDA level and significantly ($P < 0.001$) decreased GSH level and CAT activity in testicular and epididymal tissues compared to the control and CBO groups. The highest MDA level was recorded in the DTX+PTX group in testicular and epididymal tissues compared to the other chemotherapeutic treatment groups. With respect to data related to GSH-Px in testicular and epididymal tissues, no statistically significant difference was observed between all the experimental groups. CBO administration to PTX- and DTX+PTX-treated rats showed statistically significant decreases in MDA level in comparison with the alone PTX and DTX+PTX groups in testicular tissue ($P < 0.001$). However, in the epididymal tissue, CBO consumption by DTX-, PTX-, and DTX+PTX-treated rats significantly ($P < 0.001$) decreased the increments in

MDA levels in alone DTX, PTX, and DTX+PTX groups. With respect to testicular tissue GSH levels, the statistically significant improvements were recorded in the DTX+CBO, PTX+CBO, and DTX+PTX+CBO groups in comparison with the groups given only chemotherapeutics ($P < 0.001$). In epididymal tissue, only PTX+CBO group provided a significant increase in GSH level compared to PTX group ($P < 0.001$). Although a significant ($P < 0.001$) increase was observed in testicular CAT activities of DTX+CBO and DTX+PTX+CBO groups as compared to the DTX and DTX+PTX groups, respectively, simultaneous administration of CBO to DTX, PTX, and DTX+PTX groups had significant ($P < 0.001$) positive effect on epididymal CAT activity compared to chemotherapeutics-treated groups not consuming CBO.

Alterations in sperm parameters

Epididymal sperm motility, sperm concentration, and abnormal sperm rate in all groups are presented in Table 4. Significant decreases ($P < 0.001$) in sperm motility and concentration as well as significant increases ($P < 0.001$) in head, tail, and total abnormal sperm rates were observed in chemotherapeutics-treated groups compared to control group. Significant improvements were observed in sperm motilities of DTX+CBO, PTX+CBO, and DTX+PTX+CBO groups compared to only chemotherapeutics-treated groups ($P < 0.001$). Similarly, CBO consumption by PTX-treated rats significantly enhanced the decreased epididymal sperm concentration in comparison to PTX group ($P < 0.001$). Significant decreases ($P < 0.001$) in head and total abnormality rates of spermatozoa were recorded in DTX+CBO, PTX+CBO, and DTX+PTX+CBO groups compared to only chemotherapeutics-treated groups.

Table 3. Alterations in oxidative stress markers in testicular and epididymal tissues in response to various treatments.

Groups	Testis				Epididymis			
	MDA (nmol/g prot.)	GSH (nmol/g prot.)	GSH-Px (IU/g prot.)	CAT (k/g prot.)	MDA (nmol/g prot.)	GSH (nmol/g prot.)	GSH-Px (IU/g prot.)	CAT (k/g prot.)
Control	2.92 ± 0.26[d]	13.64 ± 0.37[b]	0.63 ± 0.18	63.76 ± 5.00[ab]	4.24 ± 0.25de	17.69 ± 0.99[ab]	1.29 ± 0.37	48.51 ± 6.22[a]
CBO	2.84 ± 0.30[d]	17.64 ± 1.10[a]	0.69 ± 0.17	70.96 ± 6.53[a]	2.73 ± 0.20e	19.97 ± 0.30[a]	1.49 ± 0.14	57.80 ± 6.42[a]
DTX	5.68 ± 0.51[bc]	6.45 ± 0.26[d]	0.17 ± 0.04	24.91 ± 3.96[de]	7.13 ± 0.22b	8.27 ± 0.61[de]	0.71 ± 0.21	20.69 ± 2.57[c]
PTX	5.26 ± 0.15[b]	8.80 ± 0.38[c]	0.35 ± 0.10	40.26 ± 1.81[cd]	6.10 ± 0.23bc	9.97 ± 0.43[de]	0.79 ± 0.24	27.79 ± 1.95[bc]
DTX+PTX	10.50 ± 0.49[a]	6.21 ± 0.33[d]	0.25 ± 0.08	16.61 ± 1.74[e]	11.49 ± 0.76a	7.80 ± 0.64[e]	0.57 ± 0.11	18.59 ± 1.83[c]
DTX+CBO	3.91 ± 0.35[cd]	10.72 ± 0.26[c]	0.53 ± 0.13	45.06 ± 5.27[bc]	5.04 ± 0.49cd	13.14 ± 0.94[bcd]	1.28 ± 0.16	41.43 ± 3.23[ab]
PTX+CBO	3.49 ± 0.15[d]	11.43 ± 0.35[b]	0.42 ± 0.07	52.04 ± 3.73[abcd]	4.30 ± 0.28de	15.53 ± 2.10[abc]	1.29 ± 0.12	46.67 ± 5.08[a]
DTX+PTX+CBO	5.29 ± 0.28[bc]	9.96 ± 1.06[c]	0.45 ± 0.13	42.58 ± 5.52[cd]	5.69 ± 0.29bcd	11.37 ± 0.66[cde]	1.08 ± 0.18	40.56 ± 4.01[ab]
Significance	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$

CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel; MDA: Malondialdehyde; GSH: Reduced Glutathione; GSH-Px: Glutathione peroxidase; CAT: Catalase; NS: Non-significant.
Data are expressed as mean ± SEM.
The difference between the mean values without the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

Table 4. Alterations in sperm parameters in response to various treatments.

	Sperm parameters		Abnormal sperm rate (%)		
Groups	Motility (%)	Concentration (million/right cauda epididymis)	Head	Tail	Total
Control	77.62 ± 1.40[a]	118.28 ± 5.95[a]	3.86 ± 0.74[f]	3.29 ± 0.42[e]	7.15 ± 0.77[f]
CBO	88.09 ± 0.68[a]	135.14 ± 3.86[a]	3.43 ± 0.57[f]	2.86 ± 0.40[e]	6.29 ± 0.78[f]
DTX	17.14 ± 3.89[de]	50.86 ± 1.25[cd]	28.14 ± 4.20[c]	15.14 ± 1.55[b]	43.28 ± 5.03[c]
PTX	22.92 ± 3.96[cde]	47.76 ± 1.97[d]	22.25 ± 1.25[cd]	11.13 ± 0.64[c]	33.38 ± 1.65[cd]
DTX+PTX	11.66 ± 0.82[e]	42.58 ± 0.71[cd]	56.00 ± 1.38[a]	19.86 ± 0.55[a]	75.86 ± 1.87[a]
DTX+CBO	33.33 ± 5.25[c]	74.86 ± 1.99[bc]	15.86 ± 0.63[de]	8.71 ± 0.42[cd]	24.57 ± 0.75[de]
PTX+CBO	55.41 ± 2.11[b]	91.50 ± 2.17[b]	13.00 ± 1.25[e]	7.75 ± 0.42[d]	20.75 ± 1.18[e]
DTX+PTX+CBO	26.19 ± 3.04[cd]	66.58 ± 1.09[bcd]	43.71 ± 2.93[b]	17.43 ± 0.95[ab]	61.14 ± 2.72[b]
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel.
Data are expressed as mean ± SEM.
The difference between the mean values without the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

CBO administration to DTX- and PTX-treated, but not DTX+PTX-treated, rats significantly decreased the abnormalities in sperm tail compared to only chemotherapeutics-treated groups ($P < 0.001$).

Alterations in testicular histological structure

Figure 3 demonstrates the changes observed in the testicular tissue histological structure of each group. The sections of control (Fig. 3A) and CBO (Fig. 3B) groups showed normal testicular architecture with normal germ cell polarity and regular seminiferous tubular morphology. The Sertoli cells between the germ cells were observed to be normal in control and CBO groups. However, many histopathological changes such as degeneration, desquamation, disorganization in germinal cells, interstitial edema, capillary congestion, multinucleated giant cell formation, and hemorrhage were observed in the testis sections of DTX (Fig. 3C), PTX (Fig. 3D), and DTX+PTX (Fig. 3E) groups compared to control group. Many of the seminiferous tubules of DTX, PTX, and DTX+PTX groups contained a great number of spermatogonia, but with a very few number of spermatocytes and spermatids due to the spermatogenic arrest. Some seminiferous tubules of chemotherapeutics-treated groups were exactly empty. In addition, DTX, PTX, and DTX+PTX administrations significantly ($P < 0.001$) decreased the data related to DST and Johnsen's testicular scoring when compared to control group (Table 5). Although most of the lesions observed in only chemotherapeutics-treated rats were detected in DTX+CBO (Fig. 3F), PTX+CBO (Fig. 3G), and DTX+PTX+CBO (Fig. 3H) groups, there was a trend in decreased severity of lesions by CBO administration to DTX-, PTX-, and DTX+PTX-treated rats in comparison to only chemotherapeutics-treated groups.

Alterations in the apoptotic germ cells and sperm DNA fragmentation

The microphotographic view of apoptotic germ cells and their numbers in all groups are presented in Fig. 4 and Table 5, respectively. There was no increase in TUNEL+apoptotic germ cell number in the testis tissues of control (Fig. 4A) and CBO (Fig. 4B) groups. TUNEL+apoptotic germ cell numbers in 20 seminiferous tubules in groups of DTX (Fig. 4C), PTX (Fig. 4D), and DTX+PTX (Fig. 4E) were statistically ($P < 0.001$) higher than that of the control group (Table 5). Statistically significant decreases ($P < 0.001$) were observed in TUNEL+apoptotic germ cell numbers of DTX+CBO (Fig. 4F), PTX+CBO (Fig. 4G), and DTX+PTX+CBO (Fig. 4H) groups compared to only chemotherapeutics-treated groups.

Microphotographic view of sperms with fragmented DNA and their numbers in all groups are presented in Fig. 5 and Table 5, respectively. Only CBO (Fig. 5B) administration significantly ($P < 0.001$) decreased the rate of sperm DNA fragmentation in comparison to the control (Fig. 5A) group. The rate of sperm DNA fragmentation was statistically ($P < 0.001$) higher in DTX (Fig. 5C), PTX (Fig. 5D), and DTX+PTX (Fig. 5E) groups than that of control group. Statistically significant decreases ($P < 0.001$) were observed in the rate of sperm DNA fragmentation in DTX+CBO (Fig. 5F), PTX+CBO (Fig. 5G), and DTX+PTX+CBO (Fig. 5H) groups compared to only chemotherapeutics-treated groups.

Discussion

Damaging effect of taxanes

The taxane class compounds (DTX and PTX) are the most important cancer chemotherapeutics. Both compounds have a similar mechanism of action and broad-
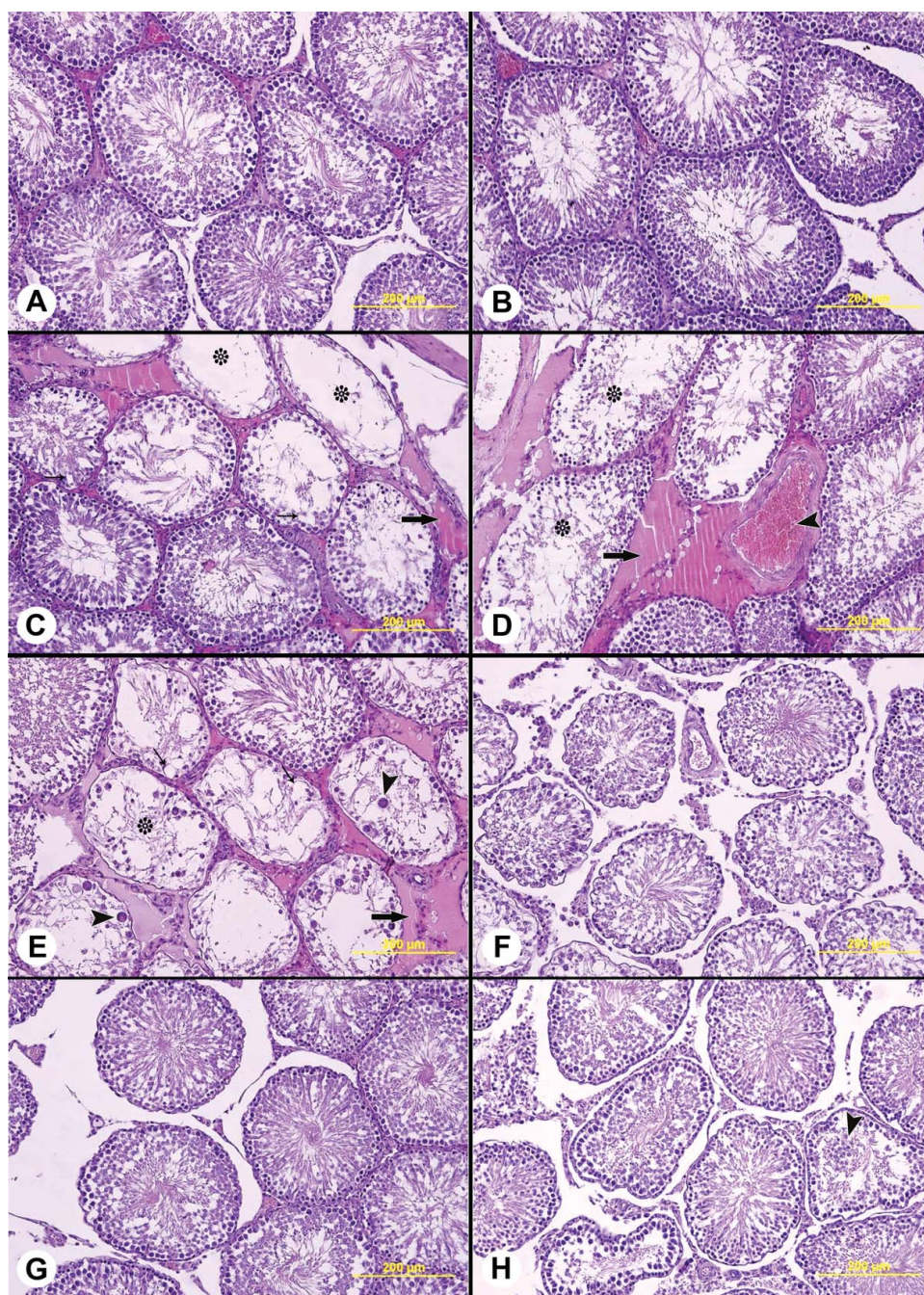
Figure 3. Microphtographic views of testicular histological structure in control (A), cinnamon bark oil (B), docetaxel (C), paclitaxel (D), docetaxel+paclitaxel (E), docetaxel+cinnamon bark oil (F), paclitaxel+cinnamon bark oil (G), and docetaxel+paclitaxel+cinnamon bark oil (H) groups (Hematoxylin and Eosin staining).

spectrum antitumor activity in the treatment of a wide variety of cancers (prostate, breast, ovary, and lung cancer). However, there are very limited numbers of scientific studies about the effects of both chemotherapeutics on male reproductive system. According to our knowledge, there is no evidence concerning the impact of these drugs on sperm DNA integrity. In the present study, the organs, tissues, and cells of male reproductive system

were examined in detail to observe the possible adverse effects of both drugs and the improvement effect of a protective agent, CBO.

Chemotherapeutics, despite their strong antitumor activities, also have toxic effects on the reproductive system as in many systems. It has been reported in many studies that chemotherapeutics (18–20,24) including DTX (42,43) and PTX (42,44) lead to a decrease in

Table 5. Alterations in diameter of seminiferous tubules, Johnsen's testicular scoring, TUNEL+apoptotic germ cell number, and sperm DNA fragmentation in response to various treatments.

Groups	Parameters			
	DST(μm)	Johnsen's testicular scoring	TUNEL+apoptotic germ cell number	Sperm DNA fragmentation (%)
Control	270.99 ± 4.42[ab]	9.58 ± 0.15[a]	0.26 ± 0.06[a]	3.23 ± 0.07[a]
CBO	279.47 ± 2.73[a]	9.35 ± 0.82[a]	0.45 ± 0.09[a]	1.10 ± 0.02[b]
DTX	218.56 ± 3.95[ef]	5.64 ± 0.13[cd]	4.97 ± 0.71[b]	11.11 ± 0.12[c]
PTX	229.53 ± 3.75[de]	5.88 ± 0.17[c]	4.75 ± 0.73[b]	10.81 ± 0.11[c]
DTX+PTX	205.21 ± 6.19[f]	5.17 ± 0.16[d]	5.28 ± 0.61[b]	11.51 ± 0.11[d]
DTX+CBO	230.71 ± 3.87[de]	6.80 ± 0.18[b]	1.29 ± 0.17[a]	8.53 ± 0.08[e]
PTX+CBO	255.68 ± 2.98[bc]	7.44 ± 0.14[b]	0.69 ± 0.14[a]	8.13 ± 0.08[f]
DTX+PTX+CBO	239.61 ± 3.02[cd]	6.90 ± 0.18[b]	1.64 ± 0.28[a]	9.32 ± 0.10[g]
Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

DST: Diameter of seminiferous tubules; CBO: Cinnamon bark oil; DTX: Docetaxel; PTX: Paclitaxel.
Data are expressed as mean ± SEM.
The difference between the mean values without the same superscript letter(s) within the same column is statistically significant ($P < 0.001$).

reproductive organ weights. In the present study, the final body weight (DTX and DTX+PTX) and absolute weights of all reproductive organs as well as relative weights of right cauda epididymis and ventral prostate of chemotherapeutics-treated rats were significantly reduced. Besides, in the present study, DTX, PTX, and their mixed administrations to healthy rats caused a significant reduction in the level of testosterone. According to the findings of our study, the measurement of reduced testosterone level confirms the detrimental effect of taxanes on testis functions. This finding is in agreement with the results of a previous study performed by Altintas et al. (43) in which a weak expression of testosterone was observed in the Leydig cells of DTX-treated rats. Additionally, many studies have also reported that chemotherapeutics exhibit a harmful effect on testosterone level (22,24). Decrease in testicular testosterone expression due to the Leydig cell impairment and increased lipid peroxidation may possibly be responsible for the reductions observed in body and reproductive organ weights in the present study because permanent testosterone secretion is needed for body metabolism, and growth and functions of the reproductive organs.

When the taxanes are used for the therapy, oxidative stress develops depending on overproduction of ROS, which also leads to decrease in the antioxidant enzyme level. This situation demonstrates that taxanes toxicity plays a detrimental role in liver tissue (45). The chemotherapeutics like cisplatin (17,18,20,23), adriamycin (19,24), and cyclophosphamide (22), which are frequently used in cancer treatment, have been reported to deteriorate testicular oxidant-antioxidant balance. However, only one study (43) has been conducted related to the damaging effect of DTX on redox balance (increased MDA level and decreased superoxide dismutase, GSH, and GSH-Px activities) of testicular tissue so far. There is no study about the effects of PTX or DTX+PTX administrations on testicular and epididymal lipid peroxidation and antioxidant enzyme activity. In the present study, DTX, PTX, and their mixed administrations resulted in significantly increased MDA level—an indicator of lipid peroxidation—and significantly decreased GSH level and CAT activity in testicular and epididymal tissues compared to control group. The highest MDA level in testicular and epididymal tissues was recorded in the mixed group compared to the other chemotherapeutic treatment groups. The probable reason for this imbalance in redox status of testicular and epididymal tissue is the increased free radicals after the administrations of taxanes.

Normally, small amounts of ROS are produced in spermatozoa, which play a beneficial role in spermatological process. However, spermatozoa are highly vulnerable to oxidative stress induced by excessive production of ROS because their cytoplasm has limited scavenging antioxidant enzyme and large amount of polyunsaturated fatty acids (46). It has been demonstrated in many studies (17–20,22,24) that different chemotherapeutics cause reductions in sperm count and motility, and increment in abnormal and death sperm rate. Besides, DTX (43) and PTX (44) administrations to rats have been reported to decrease sperm motility and count, and increase abnormal sperm rate. In this study, DTX, PTX, and their mixed administrations resulted in significantly decreased sperm motility and concentration as well as significantly increased total abnormal sperm rates compared to control group. The deteriorations in sperm parameters in the present study may be explained by testicular and epididymal oxidative stress, as evidenced by increased MDA and decreased GSH and CAT, and decreased testosterone level or their combined effects.

The testes are the target organs of chemotherapeutic agents and their use adversely affects the structure of testis and functionality of spermatogenesis by reducing the DST and the number of germ cells and by increasing
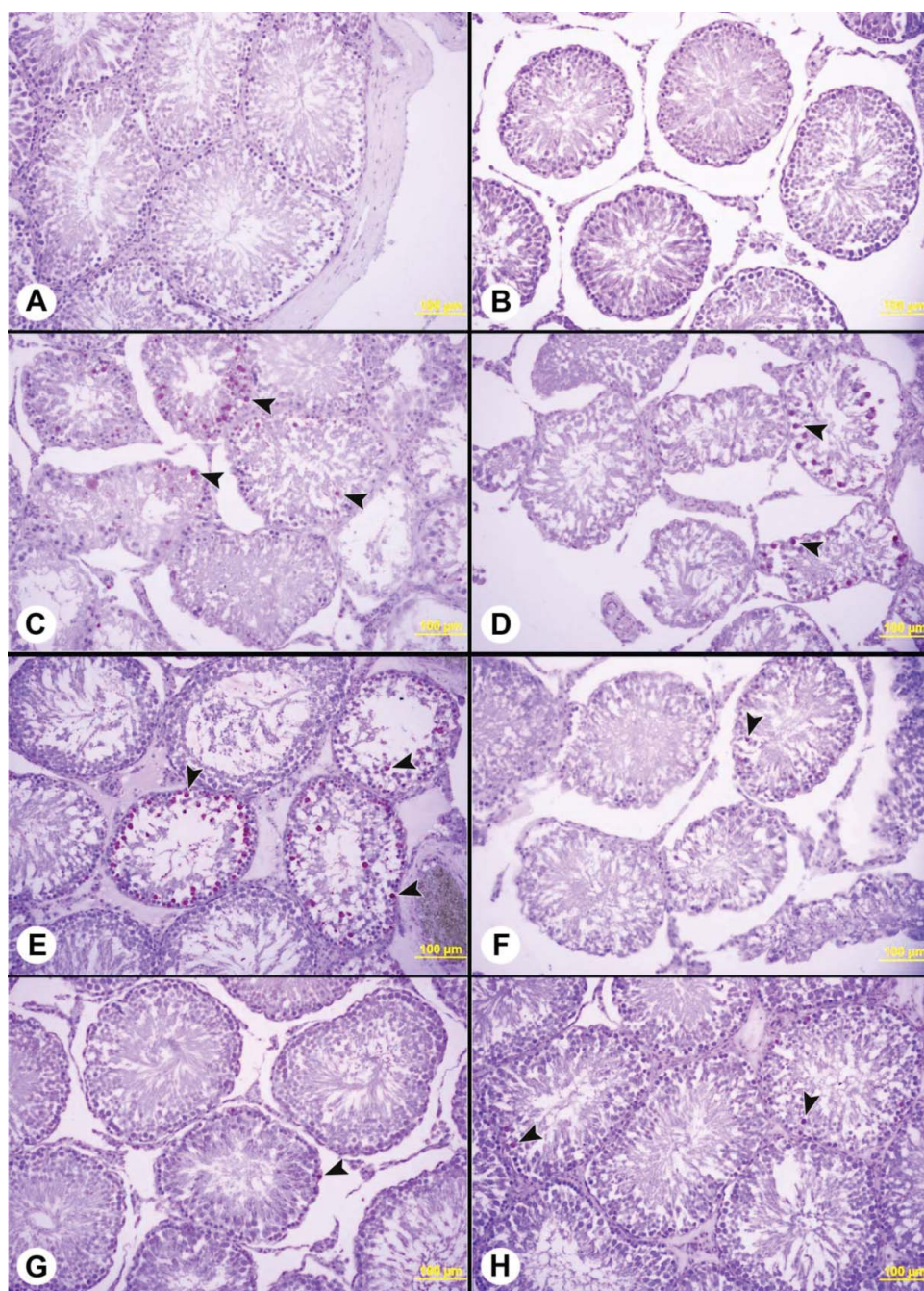
Figure 4. A: Microphotographic views of TUNEL+ apoptotic germ cells in testicular tissue of control, B: CBO, C: DTX, D: PTX, E: DTX+PTX, F: DTX+CBO, G: PTX+CBO, and H: DTX+PTX+CBO groups (TUNEL staining).

germ cell maturation arrest (17,19–21,24) and apoptosis (22,23) through oxidative stress. It has been demonstrated that DTX (43) and PTX (44) administrations to rats cause significant structural damages in testis, and DTX reduces the testicular testosterone receptors due to the Leydig cell impairment and oxidative stress. The most significant marker of DNA damage in the cells including spermatogenic cells is apoptosis, which resulted from overproduction of free radicals (47). The rat sperm nuclear chromatin is condensed by disulfide

bonds within the testis and especially during epididymal transit. Therefore, high condensation of chromatin makes nuclei of spermatozoa resistant to DNA damage induced by various agents (48). However, lipid peroxides formed during lipid peroxidation and other ROS cause damage to sperm DNA through oxidation of purine and pyrimidine bases of DNA (49). Exposure to many toxicants containing chemotherapeutics has been reported to result in increased testicular apoptosis (22,24). Besides, sperm DNA integrity is necessary for the
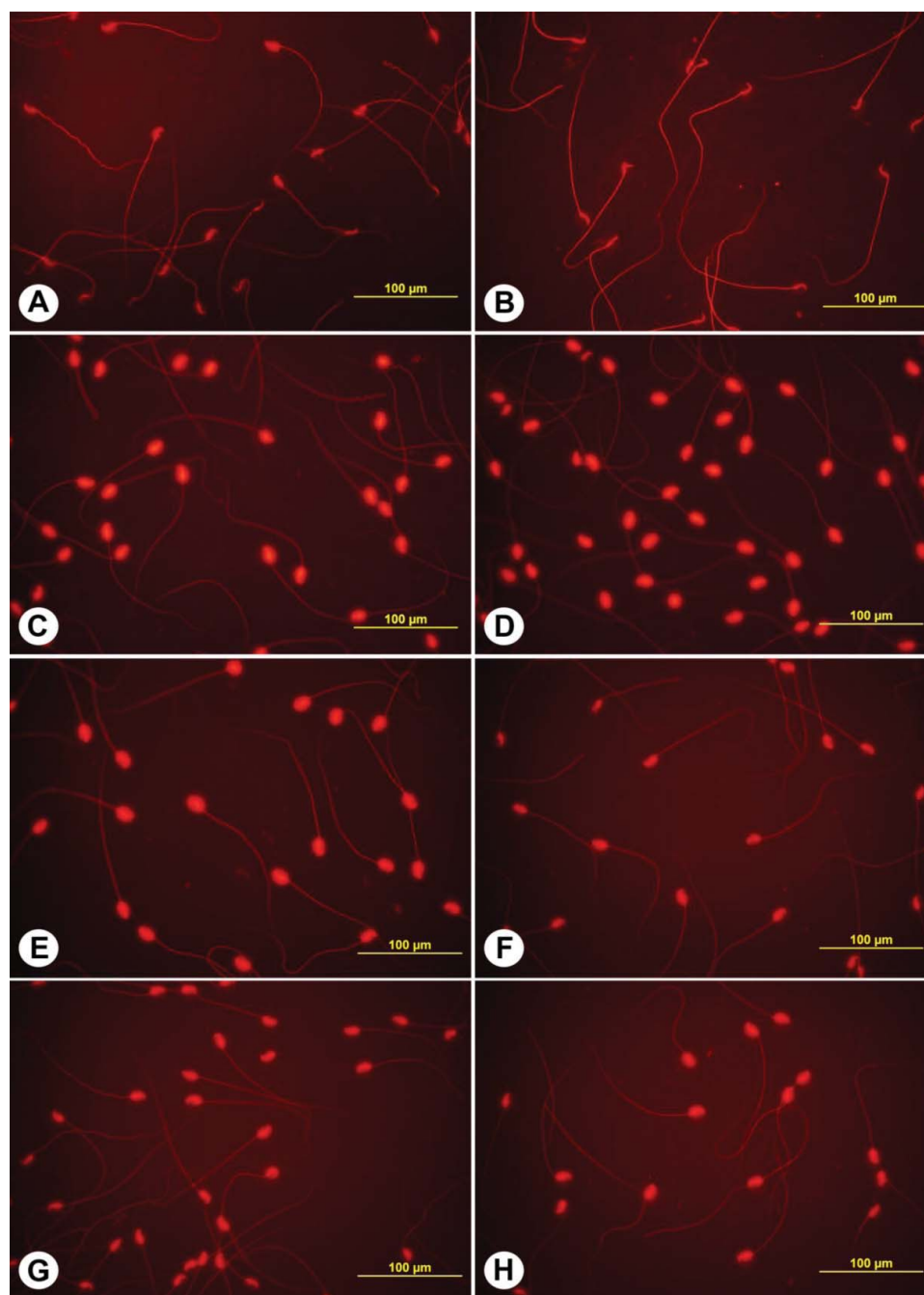
Figure 5. A: Microphotographic views of sperm DNA fragmentation in control, B: CBO, C: DTX, D: PTX, E: DTX+PTX, F: DTX+CBO, G: PTX+CBO, and H: DTX+PTX+CBO groups (Ethidium bromide staining).

transmission of genetic information to the future generations. Therefore, sperm DNA integrity is a more objective indicator of sperm function versus sperm parameters such as motility (50). However, there is no evidence related to the effects of DTX and PTX on testicular apoptosis and sperm DNA integrity. In the present study, aforementioned similar structural damages in testis histology, and increased testicular apoptosis and sperm DNA fragmentation were observed in the testis

sections of DTX, PTX, and DTX+PTX groups compared to control group. The possible reason of damages in the histological structure of testis and as well as elevation in apoptotic cell rates and sperm DNA fragmentation that were observed in the present study may be explained with the direct or indirect effects of DTX-, PTX- and their mixed application-induced lipid peroxidation, which is a chemical mechanism capable of disrupting the structure and function of testis.

Beneficial effect of CBO

The leaves and barks of *C. zeylanicum* are used as spices and for the production of volatile oils. CBO is one of the most important volatile oils. Although there is no evidence about the effect of cinnamaldehyde, which is the major component of CBO used in this study, on sperm parameters, testis structure, and sperm DNA integrity, it has been reported that ingestion of plant extract containing cinnamaldehyde effectively protects pig lymphocytes against oxidative DNA damage (51). Oil and other products of *C. zeylanicum* have been reported to result in decrease in testicular lipid peroxidation levels, and increases in LH, FSH, and testosterone (52,53) concentrations; reproductive organ weights; sperm count and motility; antioxidant activities (GSH, GSH-Px, and CAT); and DST in healthy animals (28). Besides, long-term CBO administration has been demonstrated to protect testes, epididymides, accessory sex organs, and spermatozoa, and to decrease testicular apoptosis against carbon-tetrachloride-induced reproductive toxicity by preventing oxidative stress (29). In this study, CBO administration to DTX-treated rats led to significant increases in the absolute weights of all reproductive organs, testosterone level, sperm motility, testicular GSH level, and epididymal CAT activity, and significant decreases in head, tail, and total abnormal sperm rates, epididymal MDA level, severity of testicular histopathological lesions, apoptosis, and sperm DNA fragmentation compared to only DTX-treated rats. When PTX+CBO group was compared to only DTX group, significant increases in the absolute weights of reproductive organs except testis, relative weights of right cauda epididymis and ventral prostate, testosterone level, sperm motility and concentration, testicular and epididymal GSH level, epididymal CAT activity, and significant decreases in head, tail, and total abnormal sperm rates, testicular and epididymal MDA level, severity of testicular histopathological lesions, apoptosis, and sperm DNA fragmentation were found in DTX+CBO group. Besides, significant increments in the absolute weights of all reproductive organs, relative weights of right cauda epididymis and ventral prostate, testosterone level, sperm motility, testicular GSH level, testicular and epididymal CAT activity, and significant decreases in head and total abnormal sperm rates, testicular and epididymal MDA level, severity of testicular histopathological lesions, apoptosis, and sperm DNA fragmentation were determined in DTX+PTX+CBO group in comparison to the only DTX+PTX group. The possible explanation for these ameliorations after CBO administration to DTX-, PTX-, or DTX+PTX-treated rats may be the prevention of imbalance in oxidant-antioxidant system by CBO.

In conclusion, the results of this study clearly suggest that DTX and PTX cause impairment in reproductive organs, tissues, and cells by decreasing reproductive organ weights, serum testosterone level, and sperm motility and concentration, and by increasing abnormal sperm rates, testicular apoptosis, and sperm DNA fragmentation rates as well as inducing testicular tissue histopathological lesions. These impairments in male reproductive system are potently related to taxanes-induced increment in lipid peroxidation level and reduction in antioxidant enzyme activities. In addition, the findings of the present study obviously indicate that long-term CBO consumption protects male reproductive organs, tissues, and cells of rats against structural and functional damages of taxanes by its antiperoxidative effect. However, additional physiologic and metabolic changes could not be observed, because inhibitory effects of taxanes on cancer growth were not examined in this study. In addition, it might not be revealed how the protective activity of CBO on gonads will be impressed because certain types of cancer can synthesize androgens and other steroids that have an impact on the sexual organs. Therefore, there is a need for further studies containing cancer induction.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported, and authors declare no financial or other conflict of interest.

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