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EXPLORING THE PROTECTIVE EFFECTS OF CHINAR (*PLATANUS ORIENTALIS* L.) LEAF ON TESTES DAMAGE INDUCED BY ETHANOL IN RAT MODEL

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ABSTRACT

The aim of this study was to investigate the protective effects of an infusion obtained from dried Chinar (*Platanus orientalis* L.; PO) leaves against ethanol (EtOH)-induced damage in rat testes. Thirty male Wistar rats were randomly and equally divided into 5 groups, as the Control, EtOH 20%, EtOH 20% + Silymarin (10 mg/kg/day), EtOH 20% + PO-20 mg/mL infusion, and EtOH 20% + PO-60 mg/mL infusion. Histopathologically, treatment with PO-60 leaf infusion resulted in improvements in EtOH-induced damage in the testes by inhibiting the depletion of germ cells and loss of spermatozoa. Immunohistochemically, an increase in the expression of endothelial nitric oxide synthase (eNOS) and caspase-3 was observed in the EtOH group. Treatment with the PO extract markedly reduced the EtOH-induced expression of eNOS and caspase-3 in the EtOH 20% + PO-60 group. According to the biochemical results, both of the PO infusion treatments caused a decrease in the malondialdehyde levels compared to the EtOH group. On the other hand, the glutathione contents in the treatment groups were significantly higher than ethanol group. Fluctuations in antioxidant defense enzyme activities were determined. PO leaf might have a protective role against EtOH-induced testicular damage in rats by inducing apoptosis via the upregulation of eNOS expression and preventing lipid peroxidation.

Keywords: *Platanus orientalis*; eNOS; caspase-3; Ethanol; Immunohistochemistery

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INTRODUCTION

Ethanol (EtOH) has been described as a testicular toxin resulting in fertilization abnormalities such as low sperm counts and impaired sperm motility in men (Maneesh *et al*., 2006). EtOH causes the formation of reactive oxygen species (ROS) in testicular tissues (Quintans *et al*., 2005) and in the liver, where it is actively oxidized (Bailey and Cunningham, 2002; Dey and Cederbaum, 2006). Thus, oxidative damage occurs in proteins and DNA (Bailey and Cunningham, 2002; Quintans *et al*., 2005; Dey and Cederbaum, 2006). The direct effect of alcohol on the testicles is due to its transformation into acetaldehyde, its metabolite (Cicero *et al*., 1980).

Nitric oxide (NO) is a highly reactive gas molecule that plays a decisive role in the regulation of multiple functions in both the female and male reproductive systems (Burnett *et al*., 1995; Chamness *et al*., 1995; Rosselli *et al*., 1998). Although normal NO levels help to maintain the physiological functions of the male reproductive system, higher NO levels negatively affect various sperm parameters (Doshi *et al*., 2012) and cause testicular tissue damage (Turner and Lysiak, 2008). NO is generated from L-arginine by the NO synthase (NOS) family (Burnett *et al*., 1995; Chamness *et al*., 1995). There are 3 isoforms of NOS: inducible (i) NOS, endothelial (e) NOS, and neuronal (n) NOS (Doshi *et al*., 2012). Increasing levels of NOS expression cause excessive NO production (Taneli *et al*., 2005). eNOS is expressed in many tissues, including the testes. The expression of eNOS within a broad range in the testicles indicates that it may have an important role in both normal and abnormal testicular function (Chen *et al*., 2007; Shen *et al*., 2014). It has been stated that eNOS may mediate the testicular damage caused by alcohol (Sönmez *et al*., 2010).

Silymarin is a flavonoid compound isolated from milk thistle (*Silybum marianum* (L.) Gaertn.). Hepatoprotecitve, antioxidant, anticancer and antiinflammatorry roles of slymarin have been reported (Firenzuoli *et al.*, 2004; Dogan and Anuk, 2019). In

addition, treatment with silymarin resulted in an increase in the number of spermatidis and spermatozoa cells in rats (Abedi *et al.*, 2016). Moreover, silymarin has a protective role against oxidative stress induced damage in the testis (Eskandari and Momeni, 2016; Mazhari *et al.*, 2018; Khoei *et al.*, 2019).

Chinar (*Platanus orientalis* L.; PO), also known as Old World sycamore or Oriental plane, is one of n species of the Platanaceae family (Carpenter *et al.*, 2005). Additionally, it is one of the largest and longest-living trees in the Eastern Mediterranean (Hajhashemi *et al*., 2011). Proanthocyanidin glycosides (Nishanbaev *et al*., 2010), flavonol glycosides (El-Alfy *et al*., 2008), phenolic substances (Mitrokotsa *et al*., 1993), fatty acids (Khidyrova *et al*., 1995), phytol derivatives (Abdullaev *et al*., 1994), and flavonoids (Haider *et al*., 2012) have been identified as the plant's main chemical components. The leaves, fruits, seeds, and other structures of this species are used in public health (Dogan and Anuk, 2019). Leaf infusions, extracts, and isolated compounds have been reported to be used directly or indirectly as anti-HIV, anticancer (Bastos *et al*., 2007), antiseptic, and antiinflammatory (Haider *et al*., 2012) agents.

Alcohol has harmful effects on male reproductive system (Chen *et al.*, 2019). There is no any study investigation of the protective role of chinar plant in male reproductive system. The aim of this study was to explore the protective effects of a PO leaf infusion against EtOH-induced testicular damage in rats by histopathological, immunohistochemical, and biochemical methods.

MATERIALS AND METHODS

Chemicals: All of the chemicals used in this study were of technical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Antioxidant enzyme kits were purchased from Randox Laboratories Ltd. (Crumlin, UK). The anti-eNOS kit (76198) was purchased from Abcam (Waltham, MA, USA), and the caspase-3 kit (PA5-16335) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Plant material and preparation of the infusion: Leaves of the PO plant were collected from Haci Hamza hamlet, Dargeçit, Mardin, in the southeastern Anatolian region of Turkey, (GPS coordinates: 37°33′19.7″N; 41°47′43.3″E) in August, 2017. Plant materials were identified and confirmed by Dr. Abdullah Dalar at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Van Yuzuncu Yil University, Turkey. A voucher specimen was deposited in the university's herbarium (Herbarium code: 340 and Collector No: A.D-761, Van Yuzuncu Yil University Faculty of Pharmacy Herbarium). The fresh PO leaves were dried at room temperature at shadow after washing under tap water to remove dust and contaminants. The leaves were powdered using a blender. The powdered PO leaves were weighed and incubated in boiling water for 15 min to prepare the infusions. The infusions were cooled and filtered through a hydrophilic filter (0.45 µm, Millipore) using an injector following a gauze cloth (rough-hew) filtration.

Animals: The Experimental Animal Research Centre at Van Yuzuncu Yil University (Van, Türkiye) provided 30 male rats (*Wistar albino*) that were approximately 2 months old with an average weight of 200 g. The rats were randomly divided into 5 groups, with 6 in each group (n = 6). They were housed at 25 ± 2 °C and a daily light/dark photoperiod of 10:14. The rats were fed with a wheat-soybean-based diet *ad libitum* in stainless steel cages. Humane care according to the criteria expressed in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health, were followed throughout the experiment period. The ethics regulations followed were in accordance with national and institutional guidelines for the protection of animal welfare during the experiments. The local ethics committee of Van Yüzüncü Yıl University Animal Experiments approved this study under decision number 2022/07–16.

Experimental design: The 30 male rats were randomly divided into 5 groups, with each containing 6 rats. The experiment was conducted for 28 days. Absolute EtOH was diluted with water to obtain 20% of EtOH solution for the EtOH group. The dose of EtOH was selected on the basis of a 20% concentration that was administered orally instead of drinking water to induce oxidative stress (Yaylacı *et al.*, 2014). Additionally, absolute EtOH was diluted with PO infusion to prepare treatment solutions as a final concentration of 20% EtOH + 20 mg/mL PO and 20% EtOH + 60 mg/mL PO (Dogan and Anuk, 2019). The rats were grouped as shown in Table 1.

Histopathological examination: At the end of the experiment, i.e. 28 days, animals were anesthetized using ketamine (50 mg/kg) (Arion Pharmacy, İstanbul, Turkey) and xylazine (10 mg/kg) (Mefar Pharmacy, İstanbul, Turkey). Testis tissues were taken from the euthanized rats and fixed in 10% neutral buffered formalin and embedded in paraffin using routine methods. Sections measuring 5 µm thick were cut for histological examination. The histopathological findings were evaluated on hematoxylin-eosin (H&E)-stained tissue slides using a light microscope (E-400; Nikon, Corp., Minato City, Tokyo, Japan) equipped with a DS-Ri2 video camera (DS-U3; Nikon Corp) (Yaman *et al*., 2018).

Immunohistochemical staining: The streptavidin-peroxidase method (avidin–biotin peroxidase complex; ABC) was used to stain the sections. The slides were deparaffinized and rehydrated. After quenching the

endogenous peroxidase activity with 3% H_2O_2 (v/v) for 20 min, the slides were washed twice in 0.01 M phosphate buffered saline (PBS) for 5 min. Heat-induced antigen retrieval was performed with citrate buffer (pH 6.0) for 30 min at 95 °C using a water bath and then cooling for 20 min. Before adding the primary antibodies, the slides were incubated with blocking serum (Histostain Plus Bulk Kit, Zymed Laboratories Inc., Oxnard, CA, USA) for 15 min to block nonspecific binding. The sections were incubated with anti-eNOS (1:1000 dilution, 76198; Abcam) and caspase-3 (1/100 dilution, PA5-16335; Thermo Fisher Scientific Inc.) primary antibodies and kept at 4 °C overnight in a humidified chamber. After incubation, the slides were washed 4 times in 0.01 M PBS for 5 min, incubated with biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) for 20 min at room temperature (20-25°C), and then washed 4 times in 0.01 M PBS for 5 min. After incubation with the secondary antibody, the sections were incubated with streptavidin-peroxidase (HRP) conjugate (Histostain Plus Bulk Kit, Zymed) for 20 min, and then washed 4 times in 0.01 M PBS for 5 min, followed by enzymatic incubation. To visualize the reactions, the sections were incubated for 5–15 min with diaminobenzidine (DAB). After the development of the DAB reactions, the sections were counterstained with Gill's hematoxylin. Then, the sections were passed through alcohols and xylene and mounted directly with Entellan mounting medium. Negative controls were used to verify the staining. The slides incubated with PBS instead of primary antibodies served as negative controls to verify the immunostaining (Yaman and Aydemir, 2021; Yaman *et al*., 2021).

Immunohistochemical findings were evaluated subjectively according to the intensity of the staining in the tissue as negative (-), mild (+), moderate (++) and intense (+++), using a light microscope (E-400; Nikon Corp.) equipped with a DS-Ri2 video camera (DS-U3, Nikon Corp.). Two blinded researchers (T.Y. and O.F.K.) evaluated the positively stained cells, and the mean scores were calculated. In the testes sections, the caspase-3 and anti-eNOS expressions were measured at X100, X200, and X400 objective magnification.

Measurement of the malondialdehyde and antioxidant parameters: The malondialdehyde (MDA) concentrations in the testis tissue were determined using the method described by Jain *et al*. (1989). The reduced glutathione (GSH) levels were measured according to the method of Beutler *et al.* (1963). Superoxide dismutase (SOD) activity was determined at 505 nm by calculating inhibition percentage of the formazan dye formation (MacCord and Fridovich, 1969). The glutathione peroxidase (GPx) activity was assessed in accordance with the method described by Paglia and Valentine (1967). Catalase (CAT) activity was determined using Aebi's method (1974). Glutathione-S transferase (GST) activity was measured by monitoring the glutathione conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, as described by Guthenberg and Mannervik (1981).

Statistical analysis: The mean±standard deviation (SD) was used to express all of the data. The Minitab 13 packet program for Windows was used to conduct the statistical analyses. The one-way analysis of variance (ANOVA) and Tukey tests were used to compare the means of the experimental groups, and statistical significance was set at $P < 0.05$.

RESULTS

Histopathological results: Normal testicular histology was observed in the tissue sections of rats in the Control group (Fig. 1a). In the EtOH group, EtOH exposure caused histopathological changes such as degeneration of the seminiferous tubules, enlargement of the interstitial spaces due to tubular atrophy, degeneration and depletion of the germ cells, and irregular arrangement of the spermatogenic cell lines (Fig. 1b). The extent of these changes was significantly reduced in the EtOH + Silymarin group (Fig. 1c). Although histopathological findings decreased in the EtOH + PO-20 group, there was no significant difference compared with the EtOH group (Fig. 1d). Significant improvement in all of the histopathological parameters was observed in the EtOH + PO-60 group compared with the EtOH group (Fig. 1e).

Immunohistochemical results: The intensity of anti-eNOS and caspase-3 immunoreactivity in the testicular tissues are shown in Table 2. The eNOS expression was extremely low in the Control group (Fig. 2a). In the EtOH group, intense (+++) immunoreactivity was detected in the interstitial areas, Leydig cells, Sertoli cells, and spermatids (Fig. 2b). Moderate (++) staining was observed in the EtOH+ Silymarin (Fig. 2c) and EtOH + PO-20 (Fig. 2d) groups. Mild (+) immunoreactivity was observed in tissues in the EtOH + PO-60 group (Figure 2e).

Caspase-3 expression was not observed in the tissues in the Control group (Fig. 3a). In the EtOH group, immunoreactivity was observed in the Leydig and Sertoli cells, and it was more intense in the primary spermatocytes (Fig. 3b). Moderate (++) staining was observed in the EtOH + Silymarin group (Fig. 3c) and in the EtOH + PO-20 group (Fig. 3d). Mild (+) immunoreactivity of caspase-3 was detected in the tissues in the EtOH + PO-60 group (Fig. 3e).

Biochemical results: The MDA and GSH levels and antioxidant enzyme activities for all groups are shown in Table 3. While EtOH caused a significant increase in the MDA content in the testicular tissues, treatment with

silymarin and PO leaf infusion prevented lipid peroxidation. A significant decrease in the GSH level in the EtOH group was observed compared to all groups. On the other hand, treatment with silymarin and PO leaf infusions resulted in a significant increase in the GSH content. The increase in GPx enzyme activity in the ethanol group was significant compared to the other groups. In addition, the CAT activity was significantly higher in the EtOH + PO-20 group than in the EtOH + Silymarin group (Table 3).

Table 1. Animal groups

Group	Treatment	Route of Administration
1. Control group	Drinking water	Only offered, by mouth, ad libitum
2. EtOH group	20% EtOH	Only offered, by mouth, ad libitum
3. EtOH + Silymarin group (Positive control)	20% EtOH + silymarin (10 mg/kg/day)	Silymarin via gavage (Lee *et al.,* 2020)
4. EtOH + PO-20 group	20% EtOH + 20 mg/mL PO	Only offered, by mouth, ad libitum
5. EtOH + PO-60 group	20% EtOH + 60 mg/mL PO	Only offered, by mouth, ad libitum

A standard pellet diet *ad libitum* was available during the experiment.

Table 2. Intensity of the eNOS and caspase-3 immunoreactivity in the testes tissue.

		Groups				
Anibody	Cells with reactivity	Control	EtOH	EtOH + Silymarin	EtOH + PO- 20 mg/mL	EtOH + PO-60 mg/mL
Anti-eNOS	Leydig	+	+++	++	++	+
	Sertoli	-	+++	++	++	+
	Spermatocytes	-	-	-	-	-
	Spermatids	+	+++	++	++	+
Caspase-3	Leydig	-	+++	++	++	+
	Sertoli	-	+++	++	++	+
	Spermatocytes	-	+++	++	++	+
	Spermatids	-	-	-	-	-

Table 3. Effects of the silymarin and PO-leaf infusion on antioxidant enzymes against EtOH-induced oxidative stress in the rat testis.

			Groups				
Tissue	**Enzyme**	**Units**	**Control**	**EtOH**	**EtOH + Silymarin**	**EtOH + PO-20 mg/mL**	**EtOH + PO-60 mg/mL**
Testis	MDA	nmol/g	6.26 ± 1.20^{b}	10.46 ± 1.09^{a}	7.67 ± 1.39^{b}	7.61 ± 1.30^{b}	7.59 ± 1.37^{b}
	GSH	µmol/mg	6.40 ± 0.56^{b}	4.01 ± 0.36^{a}	6.64 ± 0.62^{b}	5.66 ± 1.35^{b}	6.19 ± 1.37^{b}
	SOD	U/g	84.56 ± 15.68^{a}	73.68 ± 17.12^{a}	81.31 ± 11.30^{a}	80.88 ± 16.89^{a}	75.69 ± 7.27^{a}
	GPx	U/g	10.31 ± 2.52^{b}	14.59 ± 1.03^{a}	6.24 ± 1.11^{c}	6.60 ± 0.86^{c}	10.05 ± 2.13^{b}
	CAT	nmol/g	0.40 ± 0.07^{ab}	0.44 ± 0.07^{ab}	0.34 ± 0.08^{b}	0.46 ± 0.10^{a}	0.42 ± 0.10^{ab}
	GST	nmol/g	29.23 ± 5.23^{a}	30.50 ± 2.77^{a}	28.21 ± 2.69^{a}	25.48 ± 7.39^{a}	27.37 ± 1.76^{a}

Data were expressed as the mean ± SD. One-way ANOVA followed by the Tukey test, when appropriate (n = 6 rats in each of the 5 groups)

Different lowercase (a ,b, c) letters in the same line indicate statistical significance ($P < 0.05$).

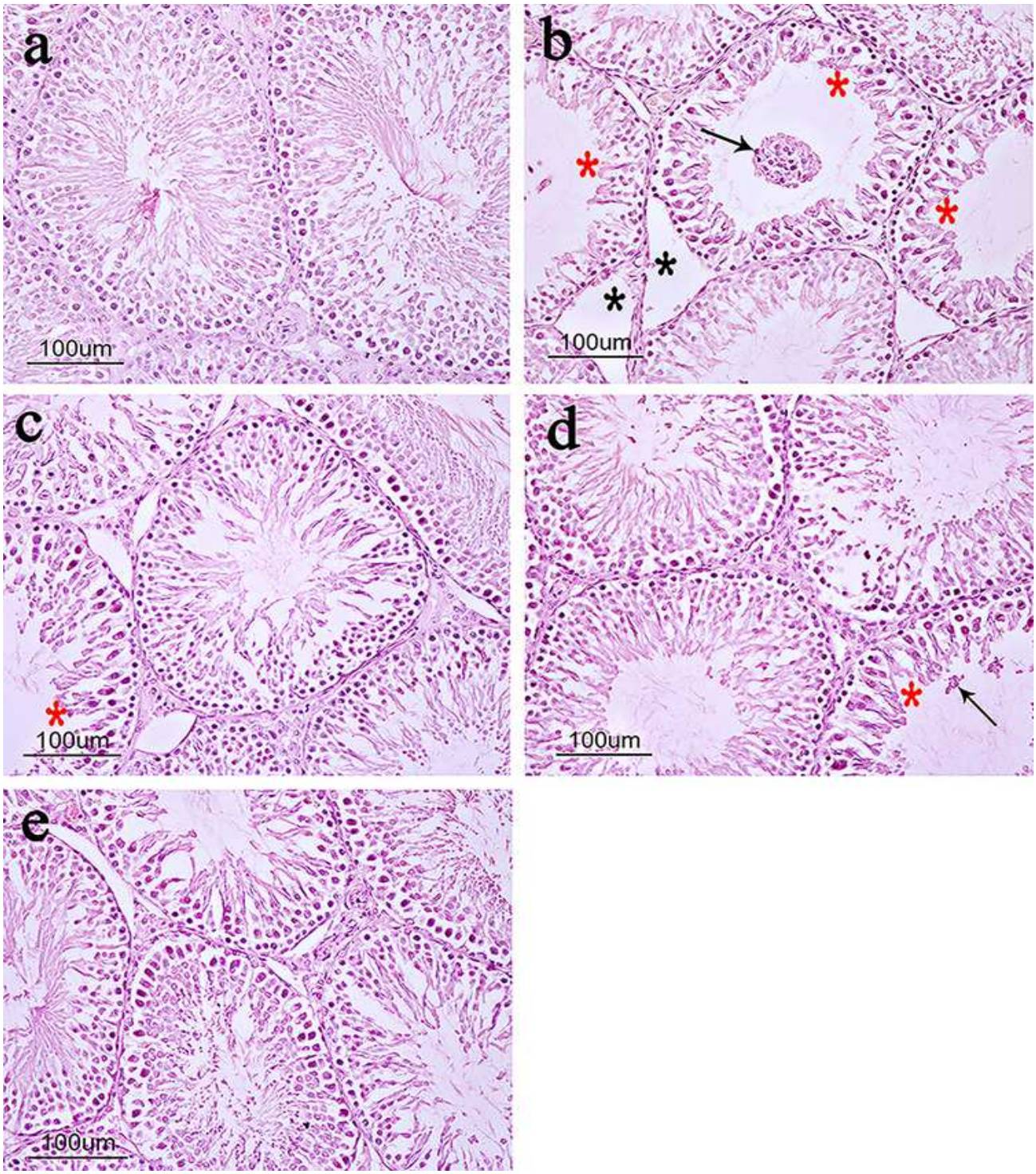

Fig. 1. Cross-section of the testes stained with H&E. (a) Control group: normal spermatogenesis and normal cell arrangement in the seminiferous tubules. (b) EtOH group: degeneration of the seminiferous tubules and loss of germ cells (red stars), desquamated immature germ cells in the lumen of the seminiferous tubules (arow), and enlargement of the interstitial spaces (black stars). (c) EtOH + Silymarin group: mild degeneration of the seminiferous tubules (red star). (d) EtOH + PO-20 group: degeneration of the seminiferous tubules (red star) and desquamated immature germ cells in the lumen of the seminiferous tubules (arow). (e) EtOH + PO-60 group: mild degeneration of the seminiferous tubules.

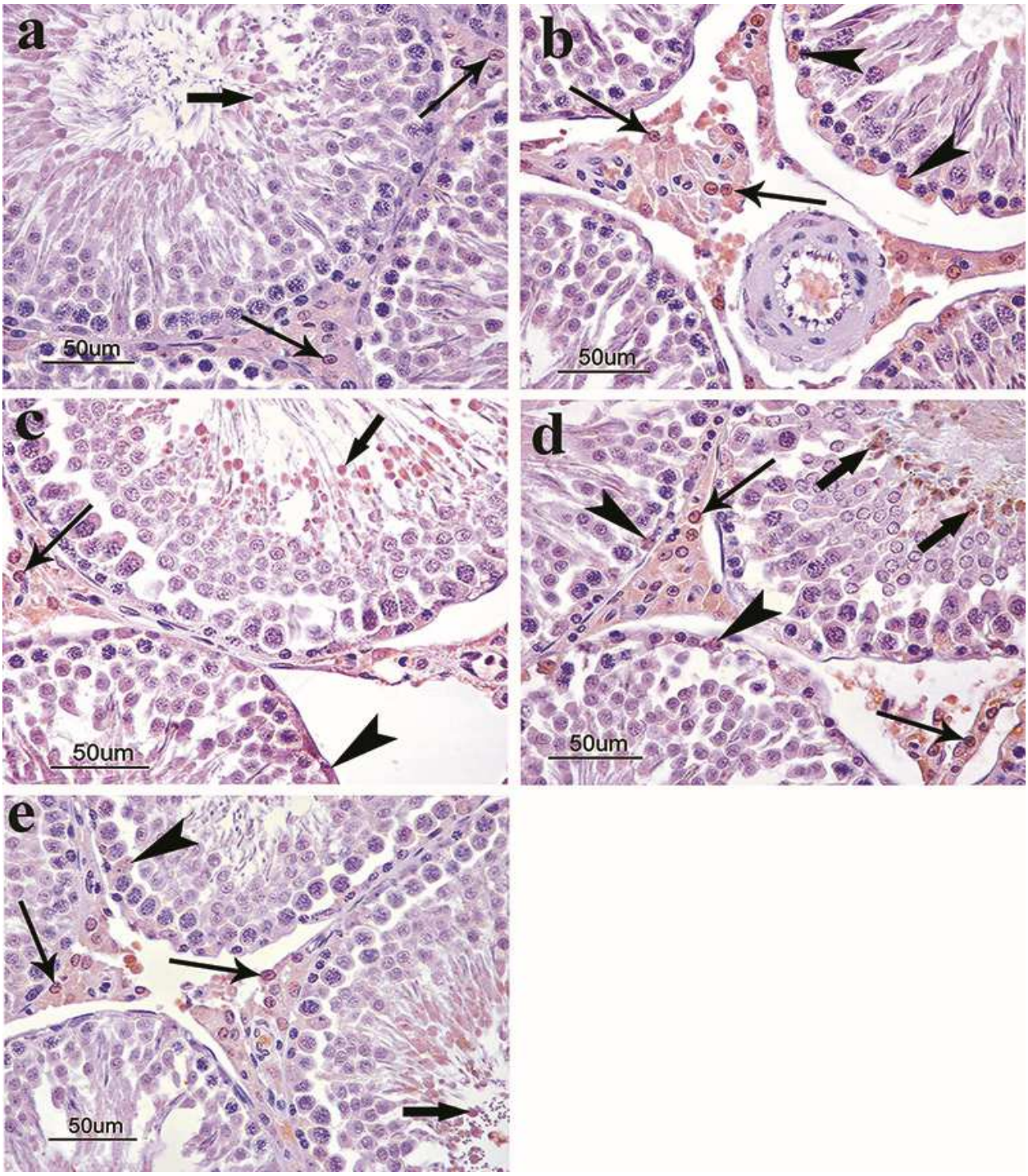

Fig. 2. Immunolocalization of eNOS in cross-sections of rat the testes. (a) Control group: minimal expression of eNOS in the Leydig cells (thin arrows) and spermatids (thick arrow). (b) EtOH group: intense immunoreactivity of eNOS in the Leydig cells (arrows) and Sertoli cells (arrowheads). (c) EtOH + Silymarin group: moderate immunostaining of eNOS in the Leydig cells (thin arrow), Sertoli cells (arrowhead), and spermatids (thick arrow). (d) EtOH + PO-20 groups: moderate immunostaining of eNOS in the Leydig cells (thin arrows), Sertoli cells (arrowheads), and spermatids (thick arrows). (e) EtOH + PO-60 group: mild (+) immunoreactivity in the Leydig cells (thin arrows), Sertoli cells (arrowhead), and spermatids (thick arrow). ABC method counterstained with Gills's haematoxylin.

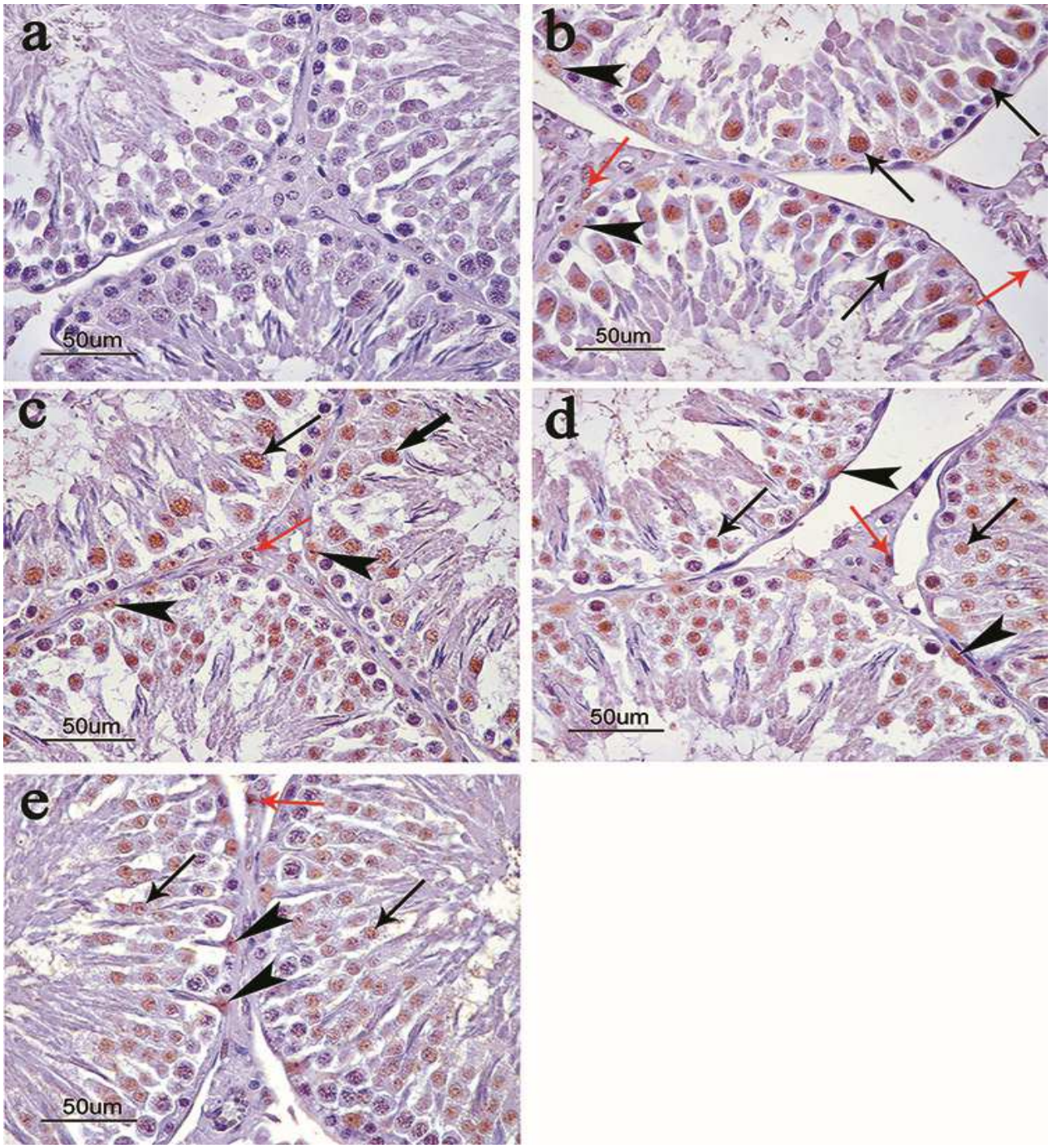

Fig. 3. Immunolocalization of caspase-3 in cross-sections of the rat testes. (a) Control group: no caspase-3 immunoreactivity in the testicular tissues. (b) EtOH group: intense caspase-3 immunoreactivity in the Leydig cells (red arrows), Sertoli cells (arrowheads), and primary spermatocytes (black arrows). (c) EtOH + Silymarin group: moderate immunostaining of caspase-3 in the Leydig cells (red arrow), Sertoli cells (arrowheads), primary spermatocyte (thin arrow), and secondary spermatocyte (thick arrow). (d) EtOH + PO-20 groups: moderate caspase-3 immunostaining in the Leydig cells (red arrow), Sertoli cells (arrowheads), and secondary spermatocytes (black arrows). (e) EtOH + PO-60 group: mild (+) immunoreactivity in the Leydig cells (red arrow), Sertoli cells (arrowhead), spermatids (thick arrow), and primary spermatocytes (black arrows). ABC method counterstained with Gills's hematoxylin.

DISCUSSION

EtOH is considered a toxin (Badger *et al*., 2003) because it can penetrate almost all tissues and cause changes in important multisystemic pathophysiological outcomes (Molina *et al*., 2014). EtOH reacts with cellular components and causes the oxidation of proteins and peroxidation of lipids in the cell membrane. Thus, it causes the formation of ROS (Han *et al*., 2015). Oxidative stress is a condition associated with an increased rate of cellular damage caused by ROS. In normal situations, plasma antioxidants suppress these ROS and provide protection against potential cellular damage (Maneesh *et al*., 2005). ROS are suppressed or eliminated by cellular antioxidant defense systems that have both enzymatic and nonenzymatic mechanisms. In the event of an excessive increase in ROS, a deficiency of antioxidants occurs. It has been stated that this deficiency can often be exacerbated by inadequate dietary or supplemental antioxidant intake (Zondervan *et al*., 1996). Natural antioxidant sources and active substances with higher antioxidant capacity are effective in preventing the damage caused by EtOH exposure (Yaylacı *et al*., 2014; Dogan and Anuk, 2019).

Epidemiological studies have shown that excessive alcohol consumption is associated with impaired testosterone production and testicular atrophy (Adler, 1992; Emanuele and Emanuele, 1998). Similar findings were observed in rats administered EtOH (Priya *et al*., 2014). Histopathologically, extensive separation of the seminiferous tubules as a result of edematous fluid (El-Ashmawy *et al*., 2007), a reduction in the diameter of the seminiferous tubules (Uygur *et al*., 2014), degeneration and atrophy of the seminiferous tubules, and sloughing of the germ cells (Tangsrisakda and Iamsaard, 2020; Agbodjento *et al*., 2021), as well as the presence of vacuoles in the tubules (Siervo *et al*., 2015; Tangsrisakda and Iamsaard, 2020; Agbodjento *et al*., 2021) were reported as result of EtOH administration in rats. In the present study, similar findings were observed in the EtOH group. The PO leaf infusion treatment preserved the seminiferous tubule structure and reduced germ cell loss.

NOS is expressed in the male reproductive tract under physiological conditions. It is required for spermatogenesis and the maintenance of androgen concentration in the testes (Burnett *et al*., 1995; Zini *et al*., 1996), and may play a crucial role in the normal biosynthesis and secretion of steroid hormones (Sengoku *et al*., 1998). However, an excess of NOS induces the production of large amounts of NO metabolites (Rosselli *et al*., 1995; O'Bryan *et al*., 2000). High NO expression reduces testosterone production in the testes (Ducsay and Myers, 2011). eNOS has a unique localization in human spermatozoa. However, abnormal patterns of eNOS expression in spermatozoa have been reported to be associated with decreased sperm motility, possibly through the production of excessive cytotoxic oxidants (O'Bryan *et al*., 1998).

The cellular localization of NOS in the testis was previously determined by immunohistochemistry, immunofluorescence microscopy, and/or immunoblotting in human Sertoli cells, Leydig cells, myofibroblasts, endothelial cells and spermatozoa (Middendorff *et al*., 1997; O'Bryan *et al*., 1998; Fujisawa *et al*., 2001), as well as in mouse spermatozoa (Herrero *et al*., 1997) and the Sertoli cells, germ cells, Leydig cells, and endothelial cells of rats (Zini *et al*., 1998; Zini *et al*., 1999; Meroni *et al*., 2000; Lee *et al*., 2003). eNOS immunoreactivity was observed in rats mainly in early and late-round spermatids, spermatogonia, Sertoli cells, and Leydig cells (Alpcan *et al*., 2014). The intensity of immunohistochemical staining for eNOS was more pronounced in peritubular myoepithelial cells, Leydig cells, and the cytoplasm of spermatids in rats (Yiğit *et al*., 2016). The expression of eNOS was demonstrated in both Sertoli and Leydig cells at all stages of spermatogenesis (Zini *et al*., 1996). Sönmez and Tascioglu (2016) demonstrated mild eNOS immunostaining in the germinal cells of the seminiferous tubules and moderate immunostaining of Leydig cells and vascular endothelial cells in rat testis. In the present study, the expression of eNOS was detected in similar areas. The expression of eNOS was increased in the EtOH group. On the contrary, immunoreactivity for eNOS was reduced in the germ cells and Leydig cells of the testes tissue from PO-treated animals compared to the EtOH group, which is evidence of the beneficial effects of PO against testicular tissue injury.

NO is involved in the control of programmed cell death (Li and Billiar, 1999; Kim *et al*., 2001). Low concentrations of NO prevent apoptosis via the inhibition of caspase-3 and -8, the major mediators of cell death, while high concentrations are toxic through the formation of reactive products such as peroxynitrite and dinitrogen trioxide and cause degeneration of germ cells via apoptosis (Kim *et al*., 2001; Lee, Cheng, 2004). eNOS, which is an essential cell-protective mediator under various conditions (Chen *et al*., 2007; Shen *et al*., 2014), plays a role in germ cell apoptosis (Yu *et al*., 2014). eNOS localizes with apoptotic germ cells undergoing DNA fragmentation but not with normal germ cells, suggesting its role in germ cell degeneration (Zini *et al*., 1996; Zini *et al*., 1998; Zini *et al*., 1999). Pathogenic agents cause a significant increase in eNOS activity, leading to oxidative stress, and thus increasing apoptotic activity in germ cells (Zini *et al*., 1998). An increase in eNOS staining was detected in degenerated seminiferous tubules with alcohol treatment (Sönmez *et al*., 2010). An increase in caspase-3 expression was reported as a result of EtOH administration (La Vignera *et al*., 2013). In the present study, the caspase-3 immunoreactivity in the

groups showed parallelism with eNOS immunoreactivity, especially in the Leydig and Sertoli cells. The similarity in the intensity of the eNOS and caspase-3 immunostaining suggests that eNOS may play a role in caspase-3-mediated apoptosis.

There is a correlation between EtOH consumption and increased oxidative stress (Yaylacı *et al*., 2014). In EtOH-induced oxidative stress, ROS generation and lipid peroxidation increase, while antioxidant defense enzyme activities decrease, such as SOD, GPx, and GST (Dogan and Celik, 2012). Treatment with a combination of EtOH and a plant extract (*Arctium lappa*) showed a protective effect on testicular damage by alterations in the SOD activity and hydrogen peroxide and MDA contents (Yari *et al*., 2018). The administration of PO leaf infusion at doses of 20 and 60 mg/mL was previously shown to have protective effects against EtOH-induced liver and kidney damage in rats (Dogan and Anuk, 2019). According to the findings in the present study, PO leaf infusion dosages of 20 and 60 mg/mL had a protective effect against EtOH-induced testicular injury in rats, lowered the rate of lipid peroxidation, increased the GSH level, and caused fluctuations in antioxidant defense enzyme activities. The protective effect of PO leaf extract on EtOH-induced testicular damage is due to the phytochemical compounds in the PO. PO plant leaves are rich in kaempferol and various fatty acids (Dogan and Anuk, 2019). Kaempferol has a testicular protective effect against EtOH toxicity in rats (Mbaveng *et al*., 2014).

Conclusion: This study is the first to explore the protective effects of PO leaf infusion against EtOH-induced damage in the testes of rats. In light of the histopathological, immunohistochemical, and biochemical findings, it was determined that EtOH caused damage in the testes of rats. As a conclusion, the orally administration of PO leaf infusion has protective role in testicular tissues against EtOH-induced damage by inducing eNOS expression, preventing lipid peroxidation and activating antioxidant defense system. This protective effect could have been related to the phytochemicals found in the chinar leaves. Isolating active substances and investigating their protective role against EtOH toxicity could be considered in further studies.

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Authors' contributions: T. Yaman drafted the manuscript and the performed histopathological and immunohistochemical studies. O.F. Keles performed the histopathological and immunohistochemical studies. A. Dogan was the main moderator of the study. F. Donmez, A. Battal and O.O. Anuk performed the biochemical investigation and treatment in the study. All of the authors have read and approved final version of the manuscript.

Ethical statement: The protocol was approved by Van Yuzuncu Yil University Animal Research Local Ethic Committee (Decision number: 2022/07–16).

Conflict of Interest: The authors declare that there are no conflicts of interest.

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