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Protective effect of propolis on the antioxidant enzymes activities, characteristics of epididymal spermatozoa and histopathological structure of testis from rats treated with cyclophosphamide

Efecto protector del propóleo sobre las actividades de las enzimas antioxidantes, las características de los espermatozoides epididimarios y la estructura histopatológica de los testículos de ratas tratadas con ciclofosfamida

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ABSTRACT

The aim of this study was to evaluate the possible therapeutic effect of propolis on cyclophosphamide (CP)–induced testicular lipid peroxidation and on the associated changes in spermatological parameters in epididymal spermatozoa and histopathological structure of rat testis. Rats were randomly separated into 4 groups with 7 rats in each group. Groups were formed as; 1st group: Control group (untreated rats), 2nd group: Propolis–treated group, 3rd group: CP–treated group, and 4th group: CP+Propolis–treated group. Propolis was administered to the rats at dose of 200 mg·kg bw^{-1} by gavage for 7 days (d). CP was administered to the rats at a single dose of 150 mg·kg bw^{-1} intraperitoneally. Propolis administration was started 2 d before CP administration and continued for 7 d. Malondialdehyde (MDA) and reduced glutathione (GSH) levels, catalase (CAT), glutathione peroxidase (GSH–Px), glutathione S–transferase (GST), and superoxide dismutase (SOD) activities, spermatological parameters, the weight of the reproductive organs, and the histopathological structure were determined. Compared to the control group, MDA levels and SOD activities significantly increased; while CAT and GST activities decreased, it was not found changed in GSH levels and GSH–Px activities in CP group. In the CP–treated group, there was decreased in sperm motility in epididymal spermatozoa, sperm density in epididymal spermatozoa, and weight of testis, prostate, epididymis and vesicula seminalis; while there was increased in abnormal sperm ratio compared to the control group in epididymal spermatozoa. Propolis normalized biochemical and spermatological parameters in epididymal spermatozoa. The histopathological examination of testicular tissue showed that the most significant histopathological change such as cell debris, invagination and degeneration occurred in CP group. In the pathogenesis of CP–induced testicular toxicity may play role the deterioration in oxidant–antioxidant balance and propolis may reduce severe side effects of CP–induced alterations.

Key words: Antioxidant; cyclophosphamide; malondialdehyde; oxidative stress; propolis

RESUMEN

El objetivo de este estudio fue evaluar el posible efecto terapéutico del propóleo sobre la peroxidación lipídica testicular inducida por ciclofosfamida (CP) y sobre los cambios asociados en los parámetros espermatológicos en los espermatozoides epididimarios y la estructura histopatológica de los testículos de rata. Las ratas se separaron aleatoriamente en 4 grupos con 7 ratas en cada grupo. Se formaron grupos como; 1er grupo: grupo control (ratas no tratadas), 2do grupo: grupo tratado con propóleo, 3er grupo: grupo tratado con CP y 4to grupo: grupo tratado con CP+propóleo. Se administró propóleo a las ratas en una dosis de 200 mg·kg pc^{-1} mediante alimentación forzada durante 7 días (d). Se administró CP a las ratas en una dosis única de 150 mg·kg pc^{-1} por vía intraperitoneal. La administración de propóleo se inició 2 d antes de la administración de CP y continuó durante 7 d. Niveles de malondialdehído (MDA) y glutatión reducido (GSH), actividades de catalasa (CAT), glutatión peroxidasa (GSH–Px), glutatión S–transferasa (GST) y superóxido dismutasa (SOD), parámetros espermatológicos, peso de los órganos reproductivos. y se determinó la estructura histopatológica. En comparación con el grupo de control, los niveles de MDA y las actividades de SOD aumentaron significativamente; Si bien las actividades CAT y GST disminuyeron, no se encontraron cambios en los niveles de GSH y las actividades GSH–Px en el grupo CP. En el grupo tratado con CP, hubo una disminución en la motilidad de los espermatozoides del epidídimo, la densidad de los espermatozoides en los espermatozoides del epidídimo y el peso de los testículos, la próstata, el epidídimo y la vesícula seminal; mientras que hubo un aumento en la proporción de espermatozoides anormales en comparación con el grupo de control en los espermatozoides epididimarios. El propóleo normalizó los parámetros bioquímicos y espermatológicos en los espermatozoides epididimarios. El examen histopatológico del tejido testicular mostró que los cambios histopatológicos más significativos, como restos celulares, invaginación y degeneración, ocurrieron en el grupo CP. En la patogénesis de la toxicidad testicular inducida por la PC puede desempeñar un papel el deterioro del equilibrio oxidante–antioxidante y el propóleo puede reducir los efectos secundarios graves de las alteraciones inducidas por la PC.

Palabras clave: Antioxidante; ciclofosfamida; malondialdehído; estrés oxidativo; propóleos

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INTRODUCTION

Chemotherapy is the administration of antineoplastic drugs to slow down, regress or stop the progression of neoplastic disease in many cancer treatments. The most carcinogenic antineoplastic drugs are those of the alkylating type. The most important factor limiting the use of alkylating drugs in chemotherapy applications is that they cause multiple organ toxicities. This prevents these types of drugs from providing a strong therapeutic efficacy [1]. Cyclophosphamide (CP) is one of the most widely used antineoplastic agents today. It is used for the treatment of chronic and acute leukemias, myelomas, lymphomas and bone marrow transplantation. In addition, CP is thought to have highly potent immunosuppressive activity [2]. Apart from its tumor-selective activity, it also has many toxic side effects [3].

Cyclophosphamide, a cytotoxic drug, undergoes hydroxylation in the liver and turns into its metabolites phosphoramide mustard and acrolein. CP exerts its antineoplastic effects with phosphoramide mustard. Phosphoramide suppresses cell division by binding to nucleic acids in the mustard organism and helps the antitumor effects of CP. The main toxic effect of CP is known to be due to its metabolite acrolein [4]. Metabolites alkylate Deoxyribonucleic acid (DNA) and proteins, leading to cytotoxicity and the formation of cross-links in DNA [5]. Their cytotoxic effects are caused by the irreversible combination of the electrophilic alkyl radical in their structure and the nucleophilic part of the target macromolecules [6]. Acrolein interacts with the antioxidant system in cells and tissues and causes the formation of free radicals. These metabolites interact with systems that produce reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{-}\cdot$). In addition, increased ROS causes DNA damage and disruption of the oxidant/antioxidant balance, leading to cellular dysfunction [7].

Because testicular germinal epithelial cells have high mitotic activity, chemotherapy drugs can cause temporary or permanent infertility by affecting the functions of the reproductive organs and reducing the sperm count. Chemotherapeutics cause lipid, cholesterol and protein peroxidation and oxidative stress, causing adverse effects such as DNA damage, apoptosis, decreased sperm quality, infertility and sterility, especially in spermatogenic cells, in the male genital system [8]. This effect is mostly caused by cytotoxic chemotherapeutics. Chief among these is CP. Alkylating agents (CP) are particularly harmful to the germinal epithelium and are effective by disrupting the DNA repair mechanism in the late spermatogenesis stage [9]. Although the exact mechanism underlying the toxicity of CP to testicles and other organs is unknown, a number of studies have demonstrated that tissue redox balance can be upset by CP exposure, raising the possibility that oxidative stress is the cause of biochemical and physiological abnormalities [10, 11, 12]. Long-term azoospermia may occur in 90–100% of patients with lymphoma treated with chemotherapeutic agents containing CP. It also takes about 2 years for spermatogenesis to return to normal [13].

Propolis is a sticky, resinous substance that is collected and deposited from the leaves and stems of different plant buds by the honey bee (*Apis mellifera L.*) and protects the hive from external factors, microorganisms and other pests [14]. Numerous biological properties of propolis have been reported, including antioxidant, cytostatic, antimutagenic and immunomodulatory, antimicrobial, antiviral, free radical scavenger, anti-inflammatory, local anesthetic, hepatoprotective, antitumoral, and immunostimulating. These properties of propolis are based on its rich, flavonoid, phenolic acid and terpenoid contents. For these reasons, propolis is widely used in apitherapy and medical applications [15, 16].

This study was aimed to investigate the effects of propolis application against testicular toxicity that may be caused by CP by examining malondialdehyde (MDA), reduced glutathione (GSH) levels, antioxidant enzyme activities, spermatological parameters in epididymal spermatozoa and some reproductive organ weights and the histopathological structure of the testis.

MATERIALS AND METHODS

Animals and working order

In this study, Wistar-Albino male rats *(Rattus norvegicus)* (3-month-old) weighing 250–300 g, obtained from the Firat University Laboratory Animals Breeding Unit, were used. This study was conducted with the ethical approval of the Firat University Animal Experiments Local Ethics Committee (Protocol No: 2014/10). The rats were kept in air-conditioned rooms with a fixed temperature of 25±2°C and 60–65% humidity, with a 12/12h dark/light cycle, under standard conditions. They were fed on standard rat food (pellet) and tap water ad libitum throughout the experimental practices. Experimental practices on rats were performed in the Firat University Experimental Research Center.

Experimental protocol

In this study, with seven rats apiece, four groups of rats were formed: Groups were formed as; 1st group: Control group (untreated rats), 2nd group: Propolis-treated group, 3rd group: CP-treated group, and 4th group: CP+propolis-treated group. Propolis was administered to the rats at dose of 200 mg·kg bw^{-1} by gavage for 7 days (d) [16, 17]. The CP was administered to the rats at a single dose of 150 mg·kg bw^{-1} intraperitoneally [16, 18]. Propolis administration was started 2 d before CP administration and continued for 7 d. Propolis is prepared daily by dissolving in 40% ethanol. In the testis tissue, the levels of MDA and GSH, as well as the activity of antioxidant enzymes like catalase (CAT), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST) and superoxide dismutase (SOD) were measured spectrophotometrically.

Biochemical analyses

When the experiment was finalized, the rats in control and experimental groups were sacrificed by decapitation under ether anesthesia. Testis tissue samples were removed immediately. Until biochemical analysis, tissue samples were kept at -80 °C in a freezer (ilShin Deep Freezer, DF8517, NID0218, Maxwellstraat 11, 6716 BX Ede, Holland). Physiological saline solution (0.9% w/v NaCl) was used to wash the tissue samples, and they were subsequently diluted with distilled water at a weight-to-volume ratio of 1:10 and homogenized in a Potter-elvehjem homogenizer (CAT R50D, Germany). Centrifuging (NUVE NF800R, Turkey) was done on homogenates at +4°C for 15 min at 3,000 G for MDA, GSH levels, CAT, GST, SOD activities analysis, and for 55 min at 13,000 G for GSH-Px activity analysis.

Malondialdehyde and GSH levels and the activities of CAT, GSH-Px, GST, and SOD were analyzed in testis tissues by spectrophotometrically (Thermo Scientific, Genesys 10S UV-VIS Spectrophotometer, USA). The MDA level was measured by spectrophotometry as per the method of Placer *et al*. [19]. This method was based on the reaction of MDA with TBA, one of the aldehyde products of lipid peroxidation. GSH level was determined by the method of Ellman *et al*. [20]. This

method was a spectrophotometric method based on the formation of highly stable yellow color of sulfhydryl groups when 5,5'dithiobis–2–nitrobenzoic acid (DTNB) was added. CAT activity was evaluated by using Aebi's method [21]. CAT activity was determined by measuring the resolution of H_2O_2 at 240 nm. GSH–Px activity was measured by the Beutler method [22]. GSH–Px catalyzes the oxidation of GSH to oxide glutathione (GSSG) using H_2O_2. The rate of formation of GSSG was measured by the GR reaction. GST activity was measured by the method of Habig *et al*. [23]. Enzyme activity was determined with spectrophotometry by measuring the amount of enzyme catalyzing 1 µmol of 1–(S–gluthathionyl)–2,4 dinitrobenzene formed at 340 nm at 37°C per min using GSH and 1–chloro–2,4–dinitrobenzene (CDNB). SOD activity was measured by using xanthine and xanthine oxidases to generate $O_2^{-\cdot}$, reacting with nitroblue tetrazolium [24]. The protein concentration was determined based on the method of Lowry *et al.* [25].

Sperm analyses

Epididymal spermatozoon motility

A glass slide was put on the microscope's stage, and the microscope was heated to 37°C by setting it on a heating table. A dropper was placed on the left cauda epididymis, and a few drops of the Tris buffer solution —which contains tris (hydroxymethyl) aminomethane 3.63 g, glucose 0.50 g, citric acid 1.99 g, and distilled water 100 mL— were dropped onto the slide. By cutting a piece of the left cauda epididymis, a tiny drop of semen was extracted and placed on the slide. To guarantee homogeneity, the resultant sample was mixed by pressing a coverslip on it. After that, three separate fields were scrutinized at 400× magnification, and a subjective calculation of the motility rate (%) was made [26].

Epididymal spermatozoon density

For every animal, the right cauda epididymis was taken apart and weighed. After that, it was cut and broken up using a knife and forceps in 1 mL of 0.9% NaCl for a 2 min period in a petri dish. To enable the spermatozoa in the epididymal tissue to pass into the solution, the tissue was left at room temperature for 4 h. After being collected, the spermatozoa–containing supernatant was diluted 1:200. In order to allow the spermatozoa in the solution to uniformly distribute over the entire area, 10 µL of the diluted mixture was transferred to a Thoma slide, covering both areas. The slide was then incubated for 5 min. By counting the spermatozoa in each square in both counting areas under a light microscope with a 200x magnification, the spermatozoon density was determined [26].

Abnormal spermatozoon rate

A tiny drop of semen suspension extracted from the left cauda epididymis was combined with a few drops of Tris buffer solution and applied to a dry, clean, and previously heated (37°C) slide. The froti was painted using a Diff Quik painting set. Initially, the slide was covered with a fixation solution (fast green stain) and incubated for 30 s. Eosin Y solution was added to cover the slide and incubated for 20 s after the excess solution was removed. After removing any leftover dye, Thiazine dye was applied to the slide and it was incubated for an additional 30 s. After being cleaned with distilled water, the painted slide was allowed to dry. Using a light microscope (Olympus CX21, CX21FS, Tokyo, Japan) with a 400x magnification, the smears were examined. 200 spermatozoa in all were analyzed in the smear, and the percentages of abnormal spermatozoa in the head, tail, and total were reported [26]. For all spermatological analyses, a phase–contrast microscope (Nikon E 200, Tokyo, Japan) was used at 37°C.

The absolute weight of the reproductive organs such as V. seminalis, prostate, right and left testis, and epididymis were determined by using a precision weighing device (Precisa BJ 410C, Precisa Instruments AG, CH–Dietikon, TYP 160–9422–050/P, Switzerland).

Histopathological examination

The tissues were fixed in Bouin's solution for 8 h, then moved to 50% ethanol and dehydrated in a succession of ethanol concentrations that steadily increased. The tissues were cleaned in xylene and then embedded in paraffin wax after being incubated in 100% alcohol. Haematoxylin and eosin was used to stain the tissue blocks, which were sliced into slices that were 5 µm thick [27].

Statistical analyses

Using the SPSS 22 software (Version 22.0; SPSS, Chicago, Illinois, USA), the statistical significance between various groups was assessed. To determine if the raw values of all the measured parameters showed a normal distribution, the Shapiro–Wilk normality test was employed. The test's results showed that all of the parameter values did. One–way analysis of variance (ANOVA) was used to evaluate group differences based on the results of this test, and the post hoc Tukey test was employed to compare the two groups. The mean and standard error of the mean (mean ± SEM) were used to derive all values. The findings obtained in this study were represented by the mean and standard error. *P*–values less than 0.05 were considered significant statistically.

RESULTS AND DISCUSSIONS

Biochemical results

In the testis tissue of the control and experimental groups, the levels of MDA and GSH as well as the activities of antioxidant enzymes such CAT, GSH–Px, GST, and SOD are shown in TABLE I. The information showed that CP group levels of MDA in the testis tissue were considerably greater than those of the control group ($P<0.001$). When compared to the CP group, the MDA level appeared to normalize during treatment with CP+propolis group. When all groups were compared, no statistical change was detected in GSH levels ($P>0.05$). No change was detected in GSH levels when both the control and CP+propolis groups, compared to CP applied group. While CAT activities decreased in the CP group compared to the control group ($P=0.024$), no change was detected when compared to the CP group and CP+propolis ($P=0.516$). When all groups were compared, no statistical change was detected in GSH–Px levels ($P=0.644$). No change was detected in GSH–Px levels when both the control and CP+propolis groups, compared to CP applied group. While GST activities decreased in the CP group compared to the control group ($P=0.035$), no change was detected when compared to the CP group and CP+propolis ($P=0.784$). A statistical increase in SOD activities was determined in the CP group compared to both the control group ($P=0.014$) and the CP+propolis group ($P=0.020$). There is no statistical difference between the propolis applied group and the control group in all biochemical parameters.

TABLE I
The effect of propolis supplementation on rat testis MDA and GSH levels and CAT, GSH–Px, GST, and SOD activities

	MDA (nmol·mL^{-1})	GSH (μmol·mL^{-1})	CAT (kg·g prot.$^{-1}$)	GSH–Px (U·g prot.$^{-1}$)	GST (U·mg prot.$^{-1}$)	SOD (U·mg prot.$^{-1}$)
Control	0.46±0.04	15.94±0.20	6.58±0.25	124.72±2.95	59.92±2.15	20.60±0.51
Propolis	0.51±0.10	16.59±0.50	7.61±0.15	134.13±3.03	57.99±1.55	21.54±0.23
CP	0.76±0.02*[a]	16.22±0.82	5.14±0.31*	137.30±3.67	51.20±1.12*	23.45±0.32*[a]
CP+Propolis	0.52±0.01	15.14±0.66	5.97±0.27	130.43±1.24	54.51±1.78	21.04±0.51

*: It means that it is statistically different compared to the control group. [a]: It means that it is statistically different compared to the CP+propolis group. *P*–values less than 0.05 were considered significant statistically. MDA: Malondialdehyde, GSH: Reduced Glutathione, CAT: Catalase, GSH–Px: Glutathione Peroxidase, GST: Glutathione S–Transferase, SOD: Superoxide Dismutase

Spermatological results

TABLES II and III demonstrate the differences between the control and experimental groups in terms of spermatological characteristics in epididymal spermatozoa and reproductive organ weight.

The total abnormal sperm ratio increased whereas epididymal sperm concentration and sperm motility were considerably lower in the CP group in epididymal spermatozoa (*P*<0.001). Significant increases in sperm concentration and motility as well as a decrease in the overall rate of abnormal sperm were seen in the CP+propolis group compared to the CP group (*P*<0.001). In comparison to the control group, there were no statistically significant differences in the epididymal sperm concentrations, sperm motility, or overall abnormal sperm rate between the propolis group and the CP+propolis group (TABLE II).

TABLE II
The effect of propolis supplementation on sperm motility, sperm concentration and abnormal sperm rate in epididymal spermatozoa

	Control	Propolis	CP	CP+Propolis	*P*
Motility (%)	82.0±2.49[a]	80.0±0.10[a]	41.43±3.45[b]	60.0±4.95[ab]	*P*<0.05
Concentration (million/right cauda epididymis)	122.0±6.56[a]	124.0±5.77[a]	91.71±6.15[b]	122.75±6.72[a]	*P*<0.05
Abnormal sperm rate (%)	6.60±0.34[b]	5.0±0.57[c]	10.14±0.34[a]	7.50±0.28[b]	*P*<0.05

Within rows, means with different letters ([a, b]) are significantly different (*P*<0.05)

Compared the control group, significant decreases were observed in the absolute weight of the right and left testis in CP group (*P*<0.001). When compared with CP group, increases were observed in the absolute weight of the right and left testis in CP+propolis group (*P*<0.001) but there were no statistically significant changes in left testis weight (TABLE III).

There were no statistically significant changes in the absolute weight of prostate in other groups compared to the control group. Compared the control group, significant decreases were observed in the absolute weight of the right and left epididymis and seminal vesicles in CP group but there were no statistically significant changes at between CP group and CP+propolis (TABLE III)

TABLE III
The effect of propolis supplementation on testis, epididymis, v. seminalis and prostat weights

	Control	Propolis	CP	CP+Propolis	*P*
Testis (g)					
Right	1.99±0.08[a]	1.94±0.08[a]	1.49±0.02[c]	1.71±0.06[ab]	*P*<0.05
Left	1.91±0.12[a]	1.96±0.05[a]	1.45±0.02[b]	1.69±0.06[ab]	
Epididymis Weight (g)					
Right	0.60±0.03[ab]	0.65±0.01[a]	0.52±0.01[c]	0.54±0.01[bc]	*P*<0.05
Left	0.60±0.02[ab]	0.63±0.01[a]	0.49±0.01[c]	0.53±0.02[bc]	
V. Seminalis Weight (g)	1.64±0.11[a]	1.46±0.01[ab]	1.13±0.06[c]	1.22±0.05[abc]	*P*<0.05
Prostat Weight (g)	47.0±0.03	47.0±0.04	45.0±0.02	35.0±0.05	*P*>0.05

Within rows, means with different letters (a, b and c) are significantly different (*P*<0.05)

Histopatological results

Testicular tissue was observed in normal structure in the control and only propolis applied groups. In the CP–administered group, cell debris of the immature spermatogenic series in the lumen of some seminiferous tubules (ST), invaginations in the tubule basal membrane, and occasional interstitial edema were distinguished. Degeneration was detected in the epithelium of some ST. In the CP+propolis group, the epithelium of the ST was observed close to the control group. In this group, cell debris belonging to the immature spermatogenic series was distinguished in a few tubules, but it was determined that propolis had a very protective effect against CP toxicity (FIG. 1).

The basis of cancer chemotherapy; to stop the growth and proliferation of tumor cells or, if possible, to destroy them without damaging the patient's normal cells. Because there is not much qualitative difference between malignant cell and normal cell; the difference is more quantitative. Therefore, most cancer drugs have side effects on normal cells and blood tissue. In cancer patients, the toxic side effects of cancer drugs have been the subject of increasing studies [28].

Antitumoral activity of CP, a chemotherapeutic drug with wide clinical use, proven to be effective in the treatment of cancer and non–malignant diseases (such as lupus erythematosus, Behçet's disease, vasculitis, immune diseases, systemic connective tissue disease, rheumatoid arthritis, autoimmune hemolytic anemia, nephrotic syndrome), attributed to its use in high doses. While high–dose alkylating (affecting DNA) agents such as CP destroy cancer cells, they adversely affect normal tissues [29].

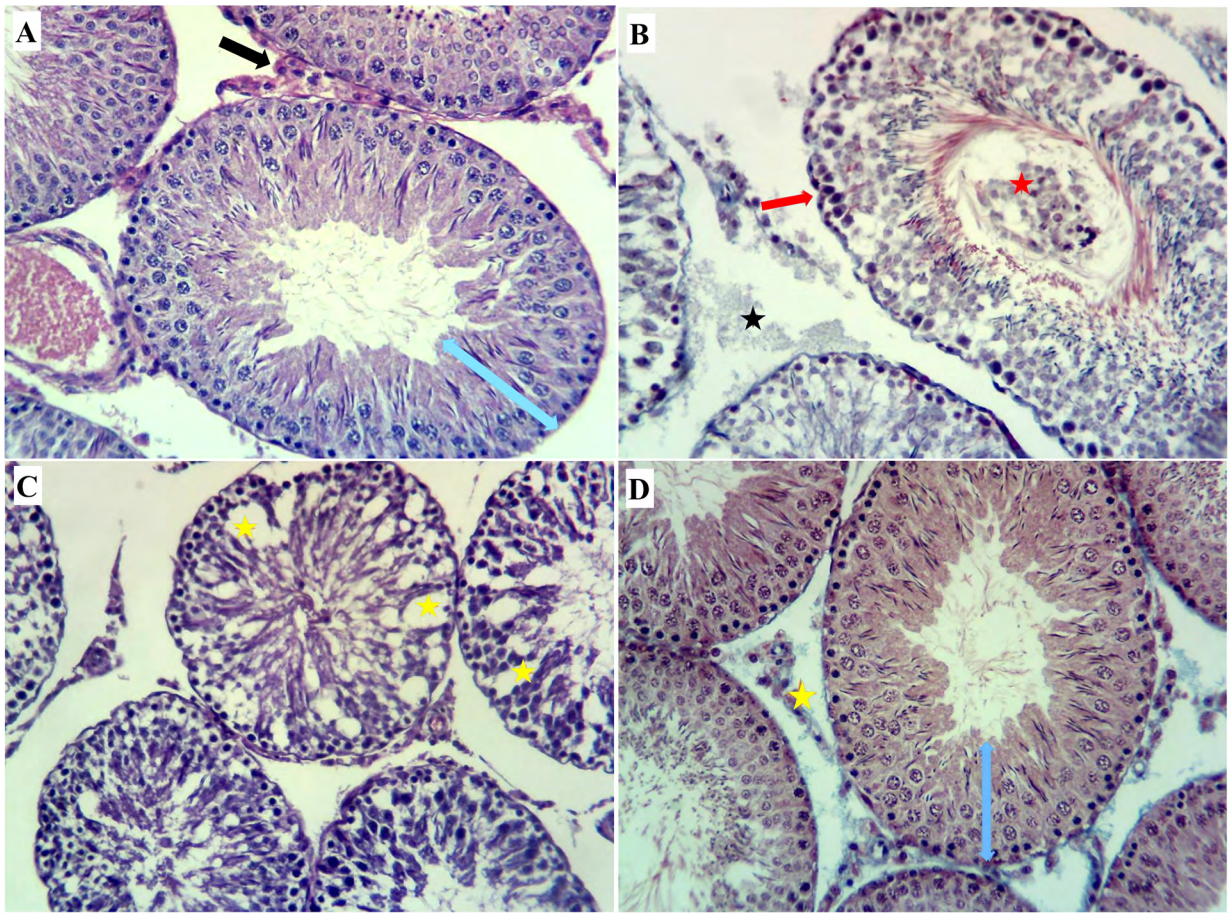

FIGURE 1. Control group (A): Seminiferous tubule epithelium (blue arrow) and interstitial Leydig cells (black arrow) are observed in normal structure. Hematoxylin & Eosin 200×; CP group (B): Immature cells spilled into the lumen of the seminiferous tubule (red star), invaginations of the basement membrane of the seminiferous tubule (arrow) and interstitial edema (black star). Triple painting of Masson 200×; (C): Degeneration of the seminiferous tubule epithelium (*) in CP group. Hematoxylin & Eosin 200×; CP+Propolis group (D): Seminiferous tubule epithelium (arrow) and interstitial Leydig cells (*) are distinguished in normal structure. Triple painting of Masson 200×

Cyclophosphamide is metabolized by cells to the active metabolites acrolein and phosphoramide mustard [30]. Acrolein, one of these metabolites, is cytotoxic and causes peroxide production by activating intracellular ROS and nitric oxide production [31]. Oxidative stress and increased production of ROS are known to play important roles in CP-induced testicular toxicity [32, 33]. Free radicals formed by CP can attack lipids and cause serious changes in membrane structure and function [34]. In addition, conjugation of acrolein with GSH causes depletion of intracellular GSH level and thus oxidative stress [35]. The obtained results showed that Propolis reduces free radical damage and oxidative stress and improves the antioxidant system, including SOD, CAT, GPx and GSH. This indicates that Propolis protects against CP-induced oxidative stress and lipid peroxidation in testicular tissue. The free radical scavenging properties of the phenols in the structure of Propolis show strong antioxidant effects through different pathways such as metal chelation and enzymatic activity modulation (regulation). Quercetin, which is among the flavonoid compounds in the structure of propolis, is the component with the most effective radical scavenging feature and quercetin is also the phenolic compound with the highest anti-inflammatory effect. The results obtained in this study are consistent with previous studies on the antioxidant properties of Propolis [36, 37, 38, 39].

Male germ cells are highly sensitive to chemotherapeutic agents. Many chemotherapeutic agents can cross the blood-testicular barrier and cause permanent damage to germ cells (with hyalinization and fibrosis of interstitial tissues). The damage is closely related to the dose and duration. In addition, the maximum threshold doses at which each chemotherapeutic agent can harm are also different. Among the CP alkylators, they are the chemotherapeutics with the highest gonadal toxicity [40]. CP, which has a very strong toxic effect on the germinal epithelium, can reduce the fertility potential of children treated with this drug in the prepubertal period by up to 50% in adulthood [41]. Similarly, in children treated with CP for cancer, azoospermia that persists for a period of 20 years [42, 43] and even permanent sterility [44] is claimed to be due to failures in sperm regeneration [45].

In experimental rat studies with CP, decreased sperm concentration and motility, apoptotic germ cell count, increase in the ratio of dead and abnormal sperm, severe testicular structure disorders [32, 46], Testosterone [46, 47] decreases in type a spermatogonia, preleptotene and pahiten spermatocytes and spermatids [10] and at the same time, sperm nuclear matrix profile [48], changes in sperm chromosome structure and simple proteins [49], and double strand breaks in spermatocyte DNA were also detected [50]. Many of the

reproductive side effects of CP in rats are also observed in albino mice (*Mus musculus*) [51, 52].

Whether the intervention of antioxidants during cancer chemotherapy affects the efficacy of treatment or reduces undesirable side effects is a subject of intense research. If ROS production by a cancer chemotherapeutic agent plays an important role in its cytotoxicity, it is likely that antioxidants interfere with the drug's anti-neoplastic activity. However, if ROS is primarily responsible for the drug's side effects, antioxidants can actually reduce the severity of such effects without affecting its effectiveness. Therefore, it is important to distinguish between the ability of a drug to induce oxidative stress in biological systems and the role of free radicals in the mechanism of action of the drug under investigation. CP is an alkylating agent and its tumor cell killing activity is mainly due to DNA alkylation. However, free radical production by acrolein is often associated with undesirable toxic effects [53].

Çeribaşi *et al.* [32], in their study examining the effects of lycopene and ellagic acid against CP toxicity, found a increases in tail and total abnormality of sperm, plasma MDA level and erythrocyte SOD activity, and decreases in erythtocyte CAT activity. Sadeghzadeh *et al.*, [33] in their study investigating the antioxidant effects of Ceratonia extract in improving the toxicity caused by CP on spermatogenesis, found a significant increase in the average MDA level in the CP group compared to the control group after CP application, while sperm motility and number, serum and tissue total antioxidant amount. and observed significant decreases in serum testosterone levels. Researchers have attributed these changes to changes in redox balance that CP may cause.

The degree of ROS produced in the environment is directly correlated with the severity of oxidative stress. Significant oxidative stress, cellular damage, and necrosis are brought on by an excess of ROS in response to CP. These effects are mediated by a number of processes, including protein denaturation, membrane lipid peroxidation, and DNA damage [54]. Numerous studies show that CP exposure raises the creation of ROS inside cells, and that the resulting physiological and biochemical abnormalities may be the result of the emerging oxidative stress. CP has been demonstrated to function as an oxidant precursor; by causing oxidative stress and raising lipid peroxidation in critical organs, it lowers the activity levels of various antioxidant enzymes [16, 32, 33, 34, 35].

Malondialdehyde is one of the often used techniques for predicting the degree of LPO [55]. This is based on the observation that the loss of one hydrogen atom from the unsaturated fatty acid chains caused by ROS during the LPO process results in elevated levels of MDA [16]. In the current investigation, the markedly elevated MDA concentrations in the testis tissue of the CP-treated rats appear to be the consequence of elevated ROS levels brought on by the stress the rats' CP poisoning caused. The administration of Propolis, however, restored the elevated MDA levels to those observed in the control group. This suggests that Propolis may squelch free radicals, impede the LPO process, and avert oxidative damage to the rat testis's membrane lipids. The persistent generation of free radicals and the compromised defense mechanisms against antioxidants are thought to be linked to the elevated MDA levels in CP-treated animals. One theory for the cause could be that conditions that encourage the generation of free radicals eventually target the cell membrane, specifically, setting off a series of events that cause fatty acids to peroxide [16]. The oxidative damage caused by CP may have OH radicals as the initiating species. The protective effects of GSH and GST on tissues against CP's harmful effects are significant. GSH can interact directly as a cofactor or coenzyme with the –SH moiety, just like ROS can. Because biological membranes are vulnerable to peroxidation, it aids in their protection [16]. The current study's observed decline in GST activity may indicate that the cell is used more GST to potentially combat ROS generation during CP metabolism.

In this investigation, it was observed a decrease in CAT activity in the CP-administered groups, this shows that the CAT enzyme is affected by CP application by separating H_2O_2 into water and oxygen in testicular cells after CP application. Under CAT-deficit conditions, H_2O_2-derived hydroxyl radicals (OH·) are produced. Excessive OH· production exacerbates oxidative stress as well. Interference with antioxidant processes may be the cause of the decrease in CAT activity in the testis tissue, which may be an adaptive response to elevated levels of oxidative stress. One of the first enzymes produced as a line of defense against ROS is the antioxidant enzyme GSH-Px, and following CP administration, no change was detected in GSH-Px enzyme activity and its substrate GSH level. ROS-induced inhibition of enzymatic activities is the main cause of decreased GSH-Px activity in CP-induced oxidative stress. ROS binding to proteins and subsequent structural alterations that lead to protein oxidation could be a secondary cause. However, no statistically significant change was detected in this study. The reason for this may be that the change in the level of GSH, which is the substrate of the enzyme, remains insufficient.

Cengiz *et al.* [54], in their study aiming to determine the protective effects of boron on CP (200 mg·kg^{-1})-induced testicular toxicity, determined a decrease in Bcl-2, TAC and GSH levels, and an increase in TOC, OSI, MDA, Bax and Caspase-3 levels. Accordingly, they concluded that CP application may cause damage to the testicle. Alkhalaf *et al.* [56], after CP (200 mg·kg^{-1} ip single dose) application, evaluated sperm counts, motility, viability and abnormalities, testosterone, luteinizing hormone and follicle stimulating hormone levels, as well as parameters such as MDA, nitric oxide and total antioxidant capacity. In their study, they showed that CP disrupts the redox balance in testicular tissues and therefore disrupts testicular functions by negatively disrupting sperm characteristics, hormonal levels and testicular histology. They also concluded that CP disrupts the oxidative balance. Many studies such as these have emphasized that CP may cause oxidative damage in testicular tissue at different doses and cause negativities in spermatological markers [16, 32, 33, 34, 35]. In common with all of them, it is emphasized that CP may cause these effects by causing a deterioration in the redox balance in the testicular tissue. In addition, in the aforementioned studies, the effects of some substances with high antioxidant activity against the negativities caused by the effect of CP by showing effects in different ways were also mentioned. CP results in irregular ST, reduced seminiferous epithelial layers, significant maturation arrest, perivascular fibrosis, hyalinization of intertubular tissue, and histopathological reduction in the size and number of ST. It also causes degeneration and vacuolation in spermatogonia, spermatocytes, and less number of germ cells [57]. Çeribaşi *et al.* [32], in their study examining the effects of lycopene and ellagic acid against CP toxicity, found a decrease in the diameter of the ST and the thickness of the germinal cell layer in the testicular tissue after CP application, as well as degeneration, necrosis, immature germ cells, congestion and atrophy. In the current study, conditions such as the immature cells spilled into the lumen of the ST, invaginations of the basement membrane of the ST, interstitial edema, and degeneration of the ST epithelium were encountered. In the current study, it was observed that the Propolis it was used

against the effects of CP could alleviate significant damage (according to biochemical, spermatological and histopathological results).

In the present literature review, which is not tissue and organ specific, it was encounter Propolis applications in many tissues (such as liver, kidney, heart) against CP toxicity in different doses and durations [16, 36, 37, 39]. The fact that no study has been found in which the effects of propolis on the testicular tissue of CP in rats was evaluated using biochemical, spermatological and histopathological data increases the originality of this study.

Many studies examining the positive effects of Propolis on testicular tissue have concluded that Propolis reduces tissue damage due to its strong antioxidant activity. In some studies conducted on rats, Propolis was used against methotrexate toxicity [58] (Propolis 100 mg·kg^{-1}), against cyclosporine toxicity [59] (Propolis 100 mg·kg^{-1}), against excessive copper toxicity [60] (Propolis 100 mg·kg^{-1}), against aluminum chloride toxicity [61] (Propolis 50 mg·kg^{-1}), against the toxicity of the insecticide chlorpyrifos [62] (Propolis 50 mg·kg^{-1}), against the toxicity of cisplatin [63] (Propolis 100 mg·kg^{-1}), against the toxicity of doxorubicin [17] (Propolis 200 mg·kg^{-1}) and positive effects were obtained. What is common in all studies is that propolis corrects the negative effects of Propolis on sperm quality and testicular tissues against different toxic substance exposures by increasing antioxidant activities and/or scavenging free radicals.

It is reported that flavonoids and some other components found in Propolis are important compounds that prevent the effects of free radicals. Some flavonoids can affect the initial stage of LPO by interacting with the peroxy radicals of unsaturated fatty acids. The antioxidant effects of flavonoids are thought to be related to their activities in removing peroxide ions, H_2O_2, lipid peroxide and singlet oxygen radicals. In addition, it is reported that flavonoids have antioxidant effects by inhibiting lipoxygenase and cyclooxygenase enzymes [16, 38, 64].

CONCLUSIONS

The improvement in antioxidant biomarkers with propolis application can be explained by the ability of propolis to prevent oxidative stress and free radicals by limiting the production of free radicals. It can be said that Propolis, in addition to its protective role of the cell membrane by inhibiting LPO, can reduce the toxic side effects of cytotoxic agents such as CP, thanks to its interaction with antioxidants.

However, in order to make a more accurate evaluation in spermatological studies in rats or other species, spermatogenesis times according to animal species should be taken into account. In this study, it was tried to talk about the effects on the last stage of spermatogenesis and/or spermatozoa in epididymal spermatozoa.

Conflict of interests

No financial conflicts of interest for all authors are declared.

Ethics approval and consent to participate

The Firat University Animal Studies Local Ethics Committee accepted the experiments (Protocol No: 2014/10), which were carried out strictly in compliance with the Experimental Animal Ethics Committee's Guiding Principles.

Disclosure statement

The authors state that there are no interests at odds with one another.

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