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ORIGINAL ARTICLE

Alleviating doxorubicin-induced reproductive toxicity: protective and androgenic effects of drone larvae on sperm morphology and hormonal balance

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Abstract

Male infertility and compromised sperm quality are common side effects of Doxorubicin (DOX), a widely used chemotherapy drug. Its detrimental impact on male reproductive cells underscores the urgent need for effective protective measures. Lyophilized drone larvae (DL) from apitherapy have emerged as a potential solution due to their reported protective properties. By exploring DL's therapeutic potential, this research seeks to address the pressing need for strategies to protect male reproductive health during cancer treatment. The study aims to evaluate the protective effects of lyophilized DL from apitherapy against DOX-induced testicular damage in adult *Sprague–Dawley* rats. DOX negatively impacts male reproductive cells, leading to infertility and compromised sperm quality. Investigating DL's protective properties is crucial for understanding its therapeutic potential in mitigating such adverse effects. Forty rats were divided into four groups: control, DOX-treated, DL-treated, and DOX + DL-treated. Histopathological assessments, biochemical analyses (TAS, TOS, CAT, SOD, GPX), inflammatory marker measurements (TNF-α, IL-1β, IL-6), and comet assays for DNA damage were conducted on testicular tissue and blood samples. DOX induced histopathological alterations in the testis and epididymis, which DL mitigated. DL increased TAS levels, counteracted DOX-induced decreases in glutathione peroxidase (GPx), total protein, albumin, and increases in total cholesterol. DL also mitigated the rise in Follicle-Stimulating Hormone (FSH) levels caused by DOX, while increasing testosterone levels and lowering Luteinizing Hormone (LH) levels. Inflammatory markers remained unaffected. Tail moment measurements indicated a protective effect against DOX-induced DNA damage in erythrocytes with DL. DL protected sperm morphology, count, and Johnsen's score from DOX-induced reductions, suggesting its potential in mitigating cancer treatment side effects on male reproductive health. The findings suggest that DL, as an apitherapy product, holds significant promise in mitigating DOX's adverse effects on male reproductive systems. However, further investigations into its mechanisms and clinical applications in cancer therapy are warranted, emphasizing the need for continued research to fully understand DL's therapeutic benefits.

Keywords Apilarnil · Drone Larvae · Doxorubicin · Testicular damage · Male infertility · Reproductive health

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Introduction

Cancer, a non-communicable disease, remains a significant global health challenge, with high mortality rates despite advances in diagnosis, treatment, and prevention methods. In 2020, approximately ten million cancer-related deaths occurred worldwide [1], and projections suggest that the number of cancer cases will rise to nearly twenty-nine million by 2040 [2].

Doxorubicin (DOX) stands as a cornerstone in the treatment of various cancer types, yet its usage is limited by significant side effects [3]. Addressing and mitigating these adverse effects on non-target cells, tissues, and systems remains a critical aspect of cancer treatment [4]. During chemotherapy, normal cells in the fertility system experience oxidative stress, apoptosis, tissue damage, mitochondrial dysfunctions, and alterations in sexual hormone levels, leading to decreased fertility. Chemotherapeutic drugs, including DOX, exert harmful effects on reproductive organs, follicles, ovaries, sperm quality and quantity, as well as sexual hormones, posing challenges to reproductive health [5].

Drone larvae (DL), although less recognized compared to other bee products, have a long history in traditional medicine [6]. DL contains essential components such as sex hormones like testosterone and progesterone, fatty acids, vitamins, lipids, and proteins [6]. Notably, DL also has fatty acid methyl esters with androgenic effects[7]. DL has been attributed with anti-apoptotic, antioxidant, and anti-inflammatory properties, making it a recommended solution for fertility issues and libido enhancement in various regions, including Africa, South America, and Asia [8]. It has been traditionally used in China, Russia, and Transylvania for climate adaptation, sexual health, and elderly rehabilitation [9].

While existing research highlights DL's benefits for the reproductive system, its potential in mitigating DOX-induced male reproductive toxicity remains largely unexplored. The present study aims to address this gap by investigating the protective effects of DL against DOX-induced testicular toxicity. This investigation seeks to elucidate whether DL can protect against DOX-induced testicular damage and evaluate the role of its antioxidant and anti-inflammatory properties in alleviating toxicity. By comprehensively examining DL's impact on male reproductive health in the context of DOX treatment, the study aims to shed light on potential therapeutic strategies to mitigate chemotherapy-related side effects. The study's findings will contribute to our understanding of DL's role in male reproductive health and its potential as a protective agent against chemotherapy-induced toxicity. By elucidating the mechanisms underlying DL's effects, the research may pave the way for the development of novel interventions to improve cancer treatment outcomes while minimizing adverse effects on reproductive health.

In conclusion, this investigation represents a significant step towards harnessing the therapeutic potential of DL in mitigating chemotherapy-induced toxicity in male reproductive systems. The study's outcomes hold promise for the development of targeted interventions to enhance cancer treatment efficacy while preserving reproductive health.

Materials and methods

Experimental animals and treatments

Forty *Sprague–Dawley* male rats aged 3–4 months were used. Experimental animals were housed in 12L/12D photo period, with three rats in each cage and supplied to standard pellet feed and tap water ad libitum. Animal experiments were approved by The Animal Research Ethics Committee of Düzce University (protocol number: 2020/4/5). A total of four experimental groups were formed from randomly selected animals, with ten experimental animals in each group.

1. Control Group (C): Solvent / diluent of lyophilized drone larvae (distilled water) was given by oral gavage in a volume of 5 ml/kg for 21 days. On the 8th, 10th, 12th, 14th, 16th and 18th days of the administration, the solvent of DOX (physiological saline) was administered intraperitoneally (i.p.) with a one-day interval.
2. Drone Larvae Group (DL): Lyophilized DL were given at a dose of 1 g/kg and a volume of 5 ml/kg by oral gavage for 21 days. On the 8th, 10th, 12th, 14th, 16th and 18th days of the administration, the solvent of DOX (physiological saline) was administered i.p. [10].
3. Doxorubicin Group (D): Solvent of lyophilized DL was given by oral gavage in a volume of 5 ml/kg for 21 days. DOX was administered intraperitoneally (i.p.) at a dose of 3 mg/kg on the 8th, 10th, 12th, 14th, 16th and 18th days, with a one-day interval between each administration, resulting in a total dose of 18 mg/kg [11–13].
4. Drone larvae Group + Doxorubicin (DLD): Lyophilized DL were administered at a dose of 1 g/kg and a volume of 5 ml/kg by oral gavage for 21 days. On the 8th, 10th, 12th, 14th, 16th and 18th days of the treatment regimen, DOX was administered intraperitoneally (i.p.) at a dose of 3 mg/kg, resulting in a total dose of 18 mg/kg, with a one-day interval between each administration. On the 22nd day, blood samples were taken from the experimental animals via cardiac puncture for biochemical analysis, and the animals were euthanized by cervical dislocation method under anesthesia, and testis

and sperm samples, which are the main tissue of the hypothesis of this study, were taken.

Collection and lyophilization of drone larvae from hives

In the early spring period, ready drone combs were placed in the hives. When the larvae in the placed combs reached 3–5 days of age, they were collected in sterile containers with the larva collection device and transferred from the apiary to the laboratory without breaking the cold chain [14]. DL was placed in sterile petri dishes that were frozen at -20°C and stored until lyophilization process. Lyophilization of DL was carried out by freeze-drying at low pressure at −50 °C for 2–3 h, ground with the help of pestle and stored at + 4 °C in daily dosages to be applied to experimental animals [15].

Biochemical analysis

On the 22nd day, blood samples were taken from the animals to be used in biochemical measurements. The blood sample was centrifuged at 4000 rpm for 5 min at + 4°C. The obtained serum, sperm and testicular tissue samples were stored at −80°C until biochemical analyzes were measured.

Total antioxidant status

Total Antioxidant Status (TAS) were measured using commercially available kits (REL ASSAY, Cat No: RL0017, Türkiye). The novel automated method is based on the bleaching of characteristic color of a more stable ABTS (2,2′—Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol Trolox equivalent/L (mmol/L).

Total oxidant status

Total Oxidant Status (TOS) were measured using commercially available kits (REL ASSAY, Cat No: RL0024, Türkiye). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equivalent/L).

Oxidative stress index

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). For calculation, the resulting unit of TAS was converted to µmol/L, and the OSI value was calculated according to the following Formula: OSI (arbitrary unit) = TOS (µmol H_2O_2 equivalent/L) / TAC (µmol Trolox equivalent/L).

Super oxide dismutase

Super Oxide Dismutase (SOD) were measured using commercially available kits (REL ASSAY, Cat No: RLD0123, Türkiye). The role of superoxide dismutase is to accelerate the dismutation of the toxic radical, produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. the superoxide dismutase activity is then measured by the degree of inhibition of this reaction.

Catalase

Catalase (CAT) were measured using commercially available kits (REL ASSAY, Cat No: RL0253, Türkiye). This colorimetric assay involves two steps. Sample is first incubated with a known amount of hydrogen peroxide. Sample converts hydrogen peroxide to water and oxygen. The ratio is proportional to the concentration of catalase. The enzyme is stopped and the remaining hydrogen peroxide, following a fixed incubation period, is determined using a chromogen. The resulting absorbance is measured at 405 nm and the obtained results are expressed as U/L.

Glutathione peroxidase

Glutathione Peroxidase (GPx) were measured using commercially available kits (REL ASSAY, Cat No: RLD3465, Türkiye). This method is based on that of Paglia and Valentine. GPx catalases the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm is measured.

FSH, LH and testosterone

Blood samples were assessed for testosterone (Elabscience E-EL-0155), Follicle-Stimulating Hormone (FSH)

(Elabscience, E-EL-R0391) and Luteinizing Hormone (LH) (Elabscience E-EL-R0026) using ELISA kits following the manufacturers' protocols.

TNF-alfa, IL-6 and IL-1beta

Testicular tissue was assessed for Interleukin 6 (IL-6) (Elabscience, E-EL-R0015), Interleukin 1 Beta (IL-1B) (Elabscience, E-EL-H0149) and Tumor Necrosis Factor Alfa (TNF-α) (Elabscience, E-EL-H0109) using ELISA kits following the manufacturers' protocols.

Albumin, total protein, and cholesterol

Serum samples were used for analysis of Albumin, Total Protein and Cholesterol. Commercial kits of these parameters which are compatible with Mindray BS-120 automatic biochemistry analyzer was utilized. The device uses absorbance photometric method.

Preparation of sperm samples

The left epididymis was cut from the cauda (tail) part and taken into a petri dish containing 10 mL of saline. Afterwards, the petri dish was kept in a 37 °C incubator for 15 min, allowing the sperm to come out of the epididymis. After incubation, a homogeneous sperm suspension was obtained by gently mixing and pipetting. 0.5 mL of the suspension was taken into a Falcon tube containing 2 mL of saline and centrifuged at 1000 × g for 5 min. After discarding the supernatant part, the pellet part was dissolved in 1 mL of saline [16].

5 µL of the prepared suspension was placed in the "Makler Sperm Count Chamber" and placed under a phase contrast microscope. Counting started from the square in the top-left corner. Sperms that touched the left and upper margins of the squares were included in the count, while those extending to the right and lower margins were excluded. This procedure was repeated by three different researchers and the averages were taken. Sperm counts in each group were calculated [16, 17].

Sperm morphology analysis

Sperm were drawn on clean glass slides from the suspension prepared as described in the preparation of sperm samples. After these glasses were fixed with methanol, they were air-dried and stained with Giemsa dye for 35 min. It was washed under running tap water to remove excess dye. The dried slides were embedded in entellan [16]. 250 spermatozoa selected randomly were analyzed by three different researchers. Those with sperm anomalies were recorded and the abnormal sperm average was calculated [17].

Sperm vitality assessment

The epididymis separated from the testis was taken into a petri dish containing 2 mL of Tris buffer solution and cut into small pieces to allow the sperm to float in the liquid. In this solution containing sperm, sperm count, viability, motility, and morphology were evaluated on the same day. Semen sample was dropped on the slide and 1% eosin-y was added to it, mixed and the coverslip was closed. At least 100 sperm were evaluated for viability in different fields under a 40X light microscope. Sperm with a fixed head (in red) were considered dead, and sperm with an undetected head (in green) were considered alive [18].

Histological procedure

The testicles were removed from anesthetized animals, and the epididymis were separated from the testicles. Then, weight and volume of testis were measured. In the present study, the cauda epididymis region of the epididymis and the left testis were studied for histological evaluations. The testis and epididymis were fixed in Bouin's fixative for three days. Following routine histological procedures, the testis and epididymis were embedded in paraffin blocks. Tissues were cut by using a microtome (Thermo Scientific Shandon Finesse 325 microtome, United Kingdom) with a thickness of 5 µm and stained with hematoxylin and eosin (H&E).

In previous studies, criteria were determined by considering the pathological conditions that doxorubicin can cause on the testicles [19, 20]. According to the literature results, the testis and cauda epididymis tissue sections were evaluated for the presence of edema in the intertubular area, immature germinal cells in the lumen, spilled germinal epithelial cells in the lumen, irregularities in the basement membrane of the seminiferous tubule and scarcity of spermatozoa in the lumen. A scoring was determined according to the effects of the determined criteria on the tissue. During histopathological scoring, each sample was recorded as absent (0), minimal (+), moderate (+ +) or severe (+ + +) in 10 different areas. Evaluation with a light microscope (Olympus, BX53, digital camera: DP 80, Olympus and cellSens standard software version 1.17, Japan, X20 objective) was performed by a histologists blinded to the study groups [21].

Testicular volumes

The volume of testicular tissues can be measured using a variety of techniques, including stereology. There are numerous techniques for determining the testicular volume. The two that are most frequently applied among them are the Archimedes' principle and stereology [22] In the present study, Archimedes' principle was preferred because testicular tissues have regular borders, and it is an inexpensive, reliable, and easy-to-apply method. For this, testicular tissues were taken into a graduated cylinder filled with water. Testicular volumes were considered equal to the volume of water displaced and these values were recorded.

Evaluation of testicular seminiferous tubules damage

The preparations were examined under the microscope at different magnifications, and whether the germinal epithelium was smooth, the stages of spermatogenic activities and the diameters of the seminiferous tubules were examined. Considering these features, a scoring system was created, and the Johnsen score (Table 1) was used in this scoring [23].

Comet assay

The alkaline single cell gel electrophoresis analysis was performed as previously described with slight modifications [24]. Duplicated slides were prepared for each treated animal. 20 µl of whole blood was mixed with 100 µl of 0.8% LMA (low-melting-point agarose) and 80 µl of this mixture was applied to slides, which pre-coated with 1.0% NMA (normal-melting-point agarose). The slides covered with a microscope coverslip and incubated for 5 min at 4 ℃. After removed coverslips, the slides were immersed in lysing buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% N-sodium lauroyl sarcosinate, 1% Triton X-100, 10% DMSO, final pH 10.0) for 2 h at 4℃. The slides were then incubated in ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min, followed by electrophoresis at 25 V: 300 mA (1.25 V/cm), at 4 ℃ for 25 min. The slides were then neutralized with 0.4 M Tris–HCl buffer (pH 7.4) for least 5 min. and stained with SafeView™. One hundred cells per rat (50 cells analysed on each slide) were scored at 20X using a fluorescence microscope (Leica DMIL- LED Fluo, Germany). Images were analyzed using the TriTek CometScoreTM software (version 2.0, TriTek Corp., Sumerduck, VA, United States). The estimation of genomic damage determined by pixel density with this software is expressed in arbitrary units (AU). %DNA Tail, which are the most often used parameters, were used to evaluate DNA damage due to genotoxicity.

Statistical analysis

Statistical analysis was performed with SPSS 23.0 (IBM Inc.) package statistics program and GraphPad. The normality of the data was checked with Shapiro–Wilk. One-way analysis of variance (ONEWAY-ANOVA) was applied for normally distributed data. If the distribution between the groups was significant, first Levene's homogeneity test and then a post-hoc test suitable for the result of this test were selected. Data sets that did not show normal distribution were first subjected to the non-parametric Kruskal–Wallis test, and if the distribution between groups was significant, the Mann–Whitney U test was used. The statistical significance level was accepted as $p < 0.05$. The results are given as mean ± standard deviation.

Results

Body weight and metabolic changes

DOX and DL administration did not have a statistically significant effect on right-left testis volume and weight (Table 2). DOX administration significantly decreased body weight in group D when compared to group C ($p < 0.05$). DL administration did not cause a significant change in body weight in group DL compared to group C. In combination with DOX and DL was significantly found to reduce weight loss in the DLD group compared to the group D ($p < 0.05$).

Table 1 Johnsen score

Score	Spermatogenesis level
10	Germinal epithelium multi-row, numerous spermatozoa
9	Germinal epithelium disorganized and lumen aggregation, many spermatozoa
8	Germinal epithelium multi-row but less than 10 spermatozoa in lumen
7	No spermatozoa, many spermatids
6	No spermatozoa, less than 10 spermatids
5	No spermatozoa or spermatids, many spermatocytes
4	No spermatozoa, no spermatids, less than 5 spermatocytes
3	Presence of spermatogonia
2	Presence of Sertoli's cells
1	No cells

Table 2 Weight and volume of testis and body weight change

	C	D	DL	DLD
Weight (gr)				
Left testis	1.4 ± 0.3	1.4 ± 0.3	1.5 ± 0.2	1.2 ± 0.2
Right testis	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.2	1.4 ± 0.2
Body weight change	37.7 ± 35.6^{a}	-51.1 ± 40.6^{b}	42.2 ± 17.4$^{a''}$	-25.4 ± 18.1^{c}
Volume (ml)				
Left Testis	1.4 ± 0.3	1.6 ± 0.4	1.433 ± 0.4	1.267 ± 0.2
Right Testis	1.4 ± 0.4	1.4 ± 0.3	1.336 ± 0.4	1.314 ± 0.3

C control group, *D* doxorubicin group, *DL* drone larvae group, *DLD* drone larvae + doxorubicin group

Different letters indicate statistically significant differences at $p < 0.05$. Data are represented as mean ± SD

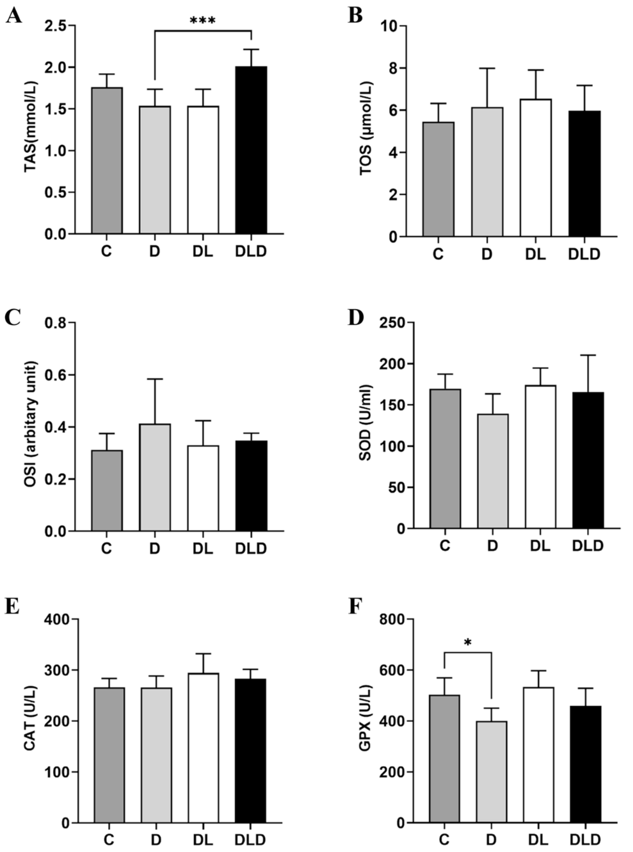

Fig. 1 Effect of Drone Larvae on Oxidative stress in testicular tissue. *C* Control, *D* Doxorubicin, *DL* Drone larvae, *DLD* Drone larvae + Doxorubicin, *TAS* total antioxidant status, *TOS* total oxidant status, *OSI* oxidative stress index, *CAT* catalase, *SOD* superoxide dismutase; *GPx* glutathione peroxidase. The data are represented as mean ± SD. *$p < 0.05$, ***$p < 0.001$ indicates significant difference

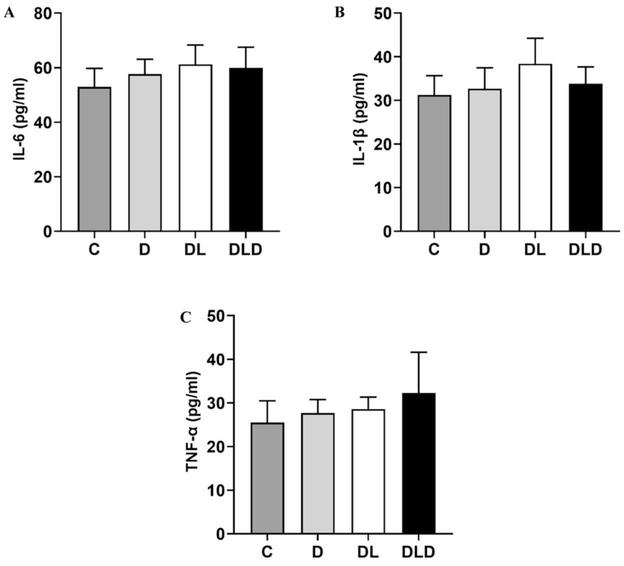

Fig. 2 Effect of Drone larvae on inflammatory markers in testicular tissue. *C* Control, *D* Doxorubicin, *DL* Drone larvae, *DLD* Drone Larvae + Doxorubicin, *IL-6* Interleukin 6, *IL-1β* Interleukin 1 Beta, *TNF-α* Tumor Necrosis Factor alpha. The data are represented as mean ± SD

Oxidative stress

To determine the effect of DL on oxidative stress in DOX-induced testis toxicity, we evaluated TAS, TOS, OSI, SOD, CAT and GPx. Compared to C group, TAS did not show significant difference in D and DL groups (Fig. 1a). However, DL treatment significantly increased TAS in DLD group in contrast to D and DL groups ($p < 0.05$). There were no significant differences in TOS, OSI, SOD and CAT levels among groups (Fig. 1b-e). GPx level was found to be significantly lower in group D group when compared to C group (Fig. 1f). DL treatment did not significantly alter GPx level in DL and DLD groups in contrast to C and D groups.

Inflammatory markers

We analyzed IL-6, IL-1β and TNF-α to evaluate whether DL exerts anti-inflammatory action in DOX-induced testis toxicity. The results showed that neither DL nor DOX significantly changed IL-6, IL-1β and TNF-α levels in testis (Fig. 2a-c).

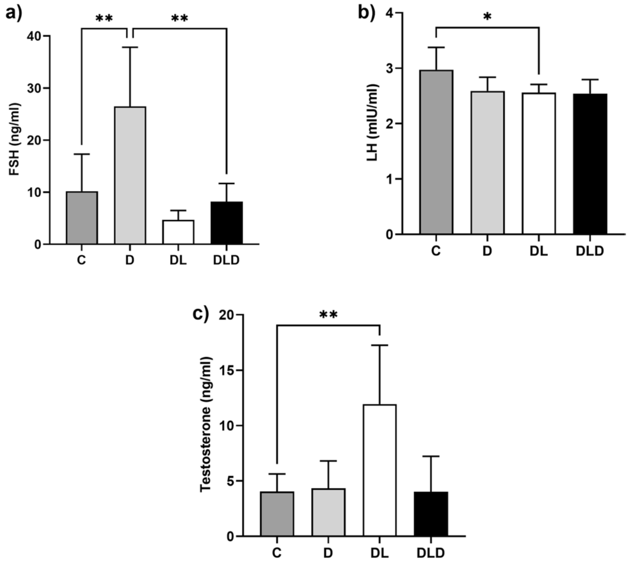

Fig. 3 Effect of Drone Larvae on FSH, LH and Testosterone levels in blood. *C* Control, *D* Doxorubicin, *DL* Drone larvae, *DLD* Drone larvae + Doxorubicin, *FSH* Follicle-Stimulating Hormone, *LH* Luteinizing hormone. The data are represented as mean ± SD. $*p < 0.05$, $**p < 0.01$ indicates significant difference

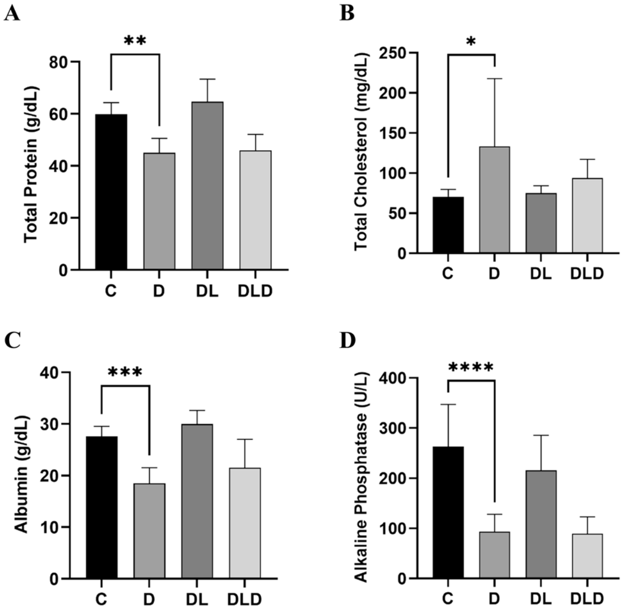

Fig. 4 The effect of Drone Larvae on total protein albumin and total cholesterol in blood. *C* Control, *D* Doxorubicin, *DL* Drone larvae, *DLD* Drone larvae + Doxorubicin. The data are represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.000$ indicates significant difference

FSH, LH and testosterone levels

FSH level was significantly increased in D group when compared to C group ($p < 0.01$, Fig. 3a). DL treatment significantly alleviated FSH level in DLD group in contrast to D group ($p < 0.01$). LH level was significantly decreased in DL group compared to C group ($p < 0.05$) although it was not changed in DLD group when compared to D group (Fig. 3b). In addition, DL treatment significantly increased testosterone levels in DL group in contrast to C group ($p < 0.01$).

Albumin, total protein, and cholesterol

DOX administration caused a statistically significant decrease in albumin, total protein, and alkaline phosphatase in D group compared to C group ($p < 0.05$, Fig. 4a-d). However, DOX significantly increased total cholesterol level in D group when compared to C group (Fig. 4c, $p < 0.05$).

Histopathological analysis

In C and DL group, normal seminiferous tubules, Leydig cells in the interstitial tissue and cells belonging to the spermatogenic series were observed, and no pathology was found (Fig. 5a and b). However, we observed irregularities in the basement membrane of the seminiferous tubule, immature and spilled germinal epithelial cells in the lumen, scarcity of spermatozoa in the lumen, and edema in the interstitial area in D group (Fig. 5c and d). When compared to D group, less irregularities in the seminiferous basement membrane, immature germinal epithelial cells in the lumen and spilled into the lumen, less spermatozoa in the lumen, and edema in the interstitial area were observed in DLD group ($p < 0.05$) (Fig. 5e and f) (Table 3).

In the histological examination of the Cauda Epididymis, the histological structures of the sperm were found to be normal in C and DL groups (Fig. 6a and b). However, immature germinal cells were observed in the epididymis lumens in D group (Fig. 6c). In DLD group, a small amount of immature germinal cells was found; however, sperm was present close to C group in general (Fig. 6d) (Table 3).

Seminiferous tubule structures were evaluated by Johnsen scoring system to determine testicular damage (Fig. 7). The result indicated that there was no statistical difference between the C and DL group. When D and C groups were compared, it was determined that the DOX significantly decreased Johnsen score compared to C group ($p < 0.01$). On the other hand, Johnsen score was found to be increased in DLD group compared to D group; however, it did not reach statistically a significant level (Table 3).

Comet assay evaluation

DOX-induced DNA damage, as evidenced by increased comet assay parameters, was attenuated by DL administration. These findings suggest a potential role for DL in protecting against chemotherapy-induced genotoxicity,

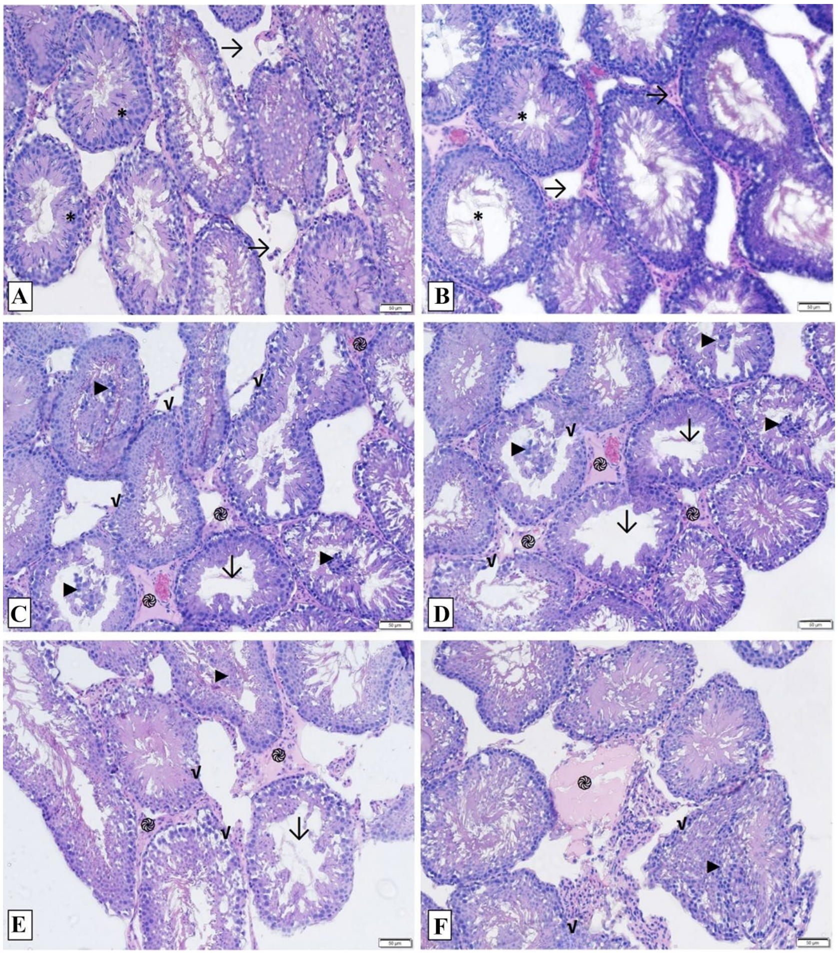

Fig. 5 Effect of drone larvae on testicular seminiferous tubules. Control (**a**), Drone Larvae (**b**), Doxorubicin (**c** and **d**), Drone Larvae + Doxorubicin (**e** and **f**). (**a** and **b**) Normal seminiferous tubules (*); normal interstitial connective tissue (→), (**c** and **d**) germinal cells that are immature and shed into the lumen (►), irregularities in the basement membrane of the seminiferous tubule (√), scarcity of spermatozoa in the lumen (↓), edema in the interstitial area (֍). (**e** and **f**) nearly normal seminiferous tubule (√) and and germinal cells (►), decrease in edema in the interstitial area (֍), and nearly few spermatozoa (↓) in the lumen (H&E, ×20)

Table 3 Histopathological evaluations of testicular tissues of all groups under light microscope (n = 10)

Pathologies features	Mean ± Standard deviation			
	Control groups	Drone Larvae groups	Doxorubicin groups	Drone larvae + Doxorubicin groups
Edema in the intertubular area	0.00 ± 0.00	0.00 ± 0.00	2.83 ± 0.41^{a}	1.83 ± 0.41^{a}
Immature germinal cells in the lumen	0.50 ± 0.55	0.00 ± 0.00	2.67 ± 0.52^{a}	1.67 ± 0.52^{a}
Spilled germinal epithelial cells in the lumen	0.00 ± 0.00	0.5 ± 0.55	2.67 ± 0.52^{a}	1.67 ± 0.52^{a}
Irregularities in the basement membrane of the seminiferous tubule	0.17 ± 0.41	0.17 ± 0.41	2.50 ± 0.55^{a}	1.50 ± 0.55^{a}
Scarcity of spermatozoa in the lumen	0.00 ± 0.00	0.33 ± 0.52	2.50 ± 0.55^{a}	1.83 ± 0.41^{a}
Johnsen score	9.67 ± 0.52	9.83 ± 0.41	5.33 ± 1.21^{a}	7.5 ± 0.55

Data presented as mean ± standard deviation

$^{a}p < 0.01$ compared to the control group

possibly through its antioxidant and DNA repair mechanisms. As shown in Fig. 8, there was no significant difference in DNA strand breaks between the C and DL group. In contrast, the DNA tail moment in D group was significantly different compared to C group ($p < 0.05$). Similarly, the difference between the D and DL group also showed a significant increase in DNA strand breaks. However, it was also found that DNA damage was significantly reduced in the DLD group compared to D group (Fig. 8).

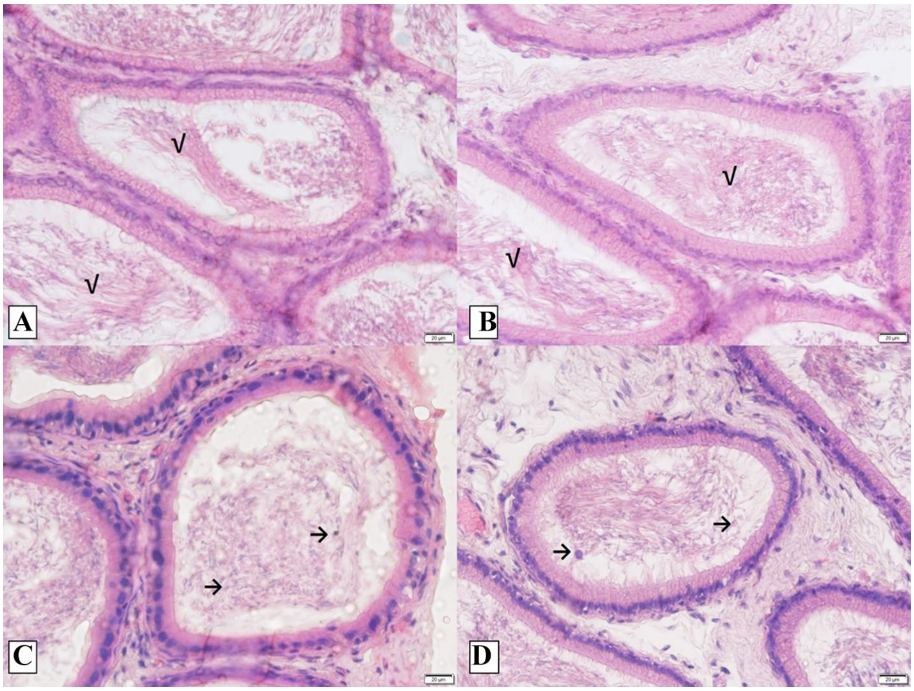

Fig. 6 Effect of Drone Larvae on Epididymis tissue. Control (**a**), Drone Larvae (**b**), Doxorubicin (**c**), Doxorubicin + Drone Larvae (**d**). Sperm in normal appearance (√); Germinal cells that have not completed maturation (→), (H&E, ×40)

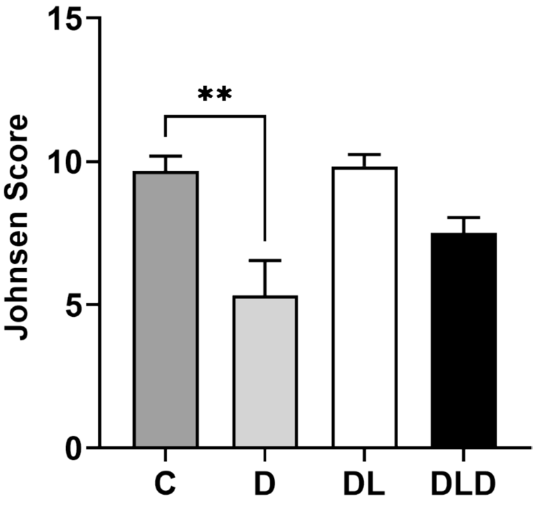

Fig. 7 Effect of Drone Larvae on Johnsen Score. *C* Control, *D* Doxorubicin, *DL* Drone Larvae, *DLD* Drone Larvae + Doxorubicin. The data are represented as mean ± SD. (n = 6) ** indicates significant difference at $p < 0.01$

Sperm morphology and count

In this study, three main morphological structures of sperm, namely curved tail, detached head, and multi-head and sperm count were examined to investigate the effects of DOX and DL on sperm morphology and anomalies.

DOX administration significantly decreased the percentage of sperm with normal morphology in D group compared to C group (Fig. 9a, $p < 0.0001$). Interestingly, DL administration resulted in a decrease in the percentage of sperm with normal morphology in DL group compared to C group ($p < 0.001$). However, DL administration significantly preserves normal sperm morphology in DLD group in contrast to D group. DOX administration caused a significant increase in the percentage of sperm with curled tails and multiheads in group D compared to group C (Figs. 9b and d, $p < 0.05$). There were no significant differences in the percentage of severed head among groups (Fig. 9c).

DOX administration caused a significant decrease in sperm count in group D compared to group C (Fig. 10, $p < 0.01$). Application of DL alone did not cause a significant change in sperm count in DL group compared to C group. Similarly, sperm count did not differ significantly in the DLD group compared to D and DL group.

Discussion

DOX has been utilized for several decades and is widely regarded as a powerful and efficient chemotherapy medication [25]. Despite its extensive usage in cancer treatment, it can lead to male infertility and/or diminished reproductive capacity, sperm quality, morphology and count due to non-target damage to healthy male reproductive cells [26]. Main results of the present study were (I) DOX decreased GPx, total protein, albumin and increased total cholesterol whereas DL increased only TAS when the animals subjected to DOX, (II) DOX increased FSH level; however, DL decreased FSH level (III) applying only DL decreased LH

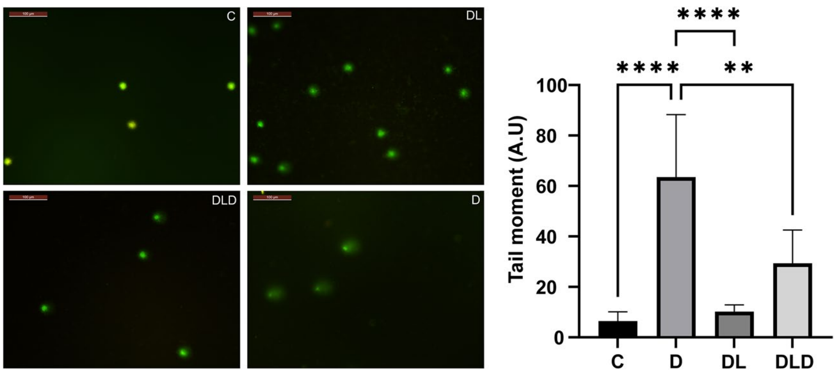

Fig. 8 Alkaline comet assay images of rat blood cells. *C* Control, *D* Doxorubicin, *DL* Drone Larvae, *DLD* Drone Larvae + Doxorubicin. Tail moment was defined as percentage of tail DNA × tail length and was quantified using the TriTek CometScore software (n = 5). The data are represented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.000$ indicates significant difference

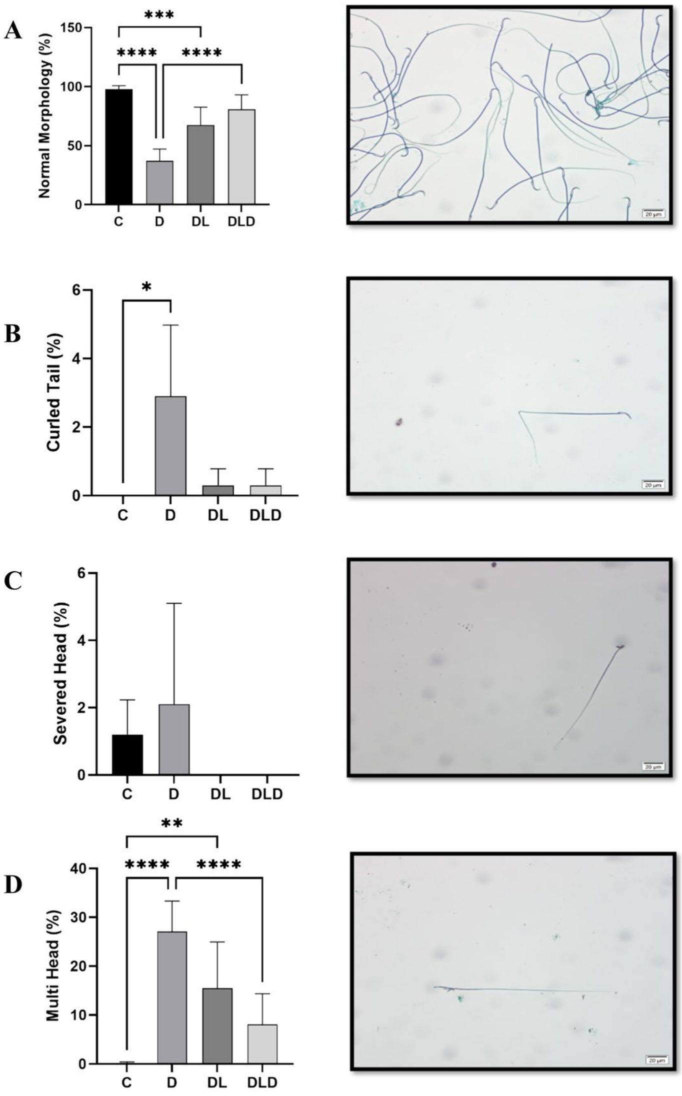

Fig. 9 Effect of Drone Larvae on sperm morphology. *C* Control, *D* Doxorubicin, *DL* Drone Larvae, *DLD* Drone Larvae + Doxorubicin. The data are represented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.000$ indicates significant difference

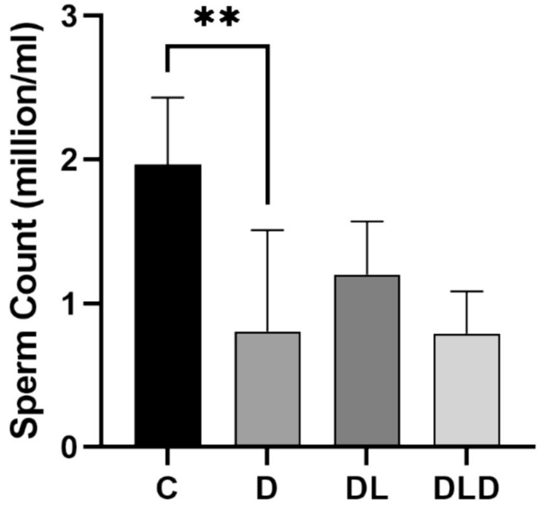

Fig. 10 Effect of Drone Larvae on sperm count. *C* Control, *D* Doxorubicin, *DL* Drone Larvae, *DLD* Drone Larvae + Doxorubicin. The data are represented as mean ± SD. ** indicates significant difference at $p < 0.01$

level whereas it increased testosterone level, (IV) neither DOX nor DL had effect on inflammatory markers, (V) DOX decreased Johnsen score, sperm count; however, DL failed to improve the score and the count, (VI) DOX decreased the percentage of sperm with normal morphology and increased the percentage of sperm with curled tail and multiheaded, nevertheless DL prevented DOX effects on sperm morphology including curled tail and multiheaded, (VII) DOX caused histopathological changes in the testis and epididymis; however, DL prevented the histopathological changes in the testis and epididymis, (VII) DOX increased genotoxicity by causing DNA strand breaks, but this effect was successfully reduced by DL.

DL, a little-known bee product utilized in ancient civilizations, has garnered attention for its potential health benefits. While its utilization remains limited e.g. protein source in European nations, it serves as a common nutritional supplement in countries such as Russia, Romania, China, Zambia, Senegal, and Ecuador [6, 27]. Characterized by its creamy consistency, DL boast a rich nutritional profile comprising proteins, lipids, fatty acids, carbohydrates, along with essential vitamins like A, B, E, and D, and vital minerals such as sodium, potassium, magnesium, zinc, and selenium. Notably, DL also serve as a notable source of sex hormones including testosterone, progesterone, estradiol, and prolactin, with varying concentrations corresponding to different developmental stages [6, 27]. Numerous scientific inquiries have underscored the therapeutic potential of DL in addressing pressing global health concerns, encompassing female ovarian dysfunction, male infertility, thyroid disorders, immune-related ailments, and combating malnutrition in children [6]. Despite the extensive exploration of its health benefits, literature review yielded no data regarding the potential impact of DL on DOX-induced testicular damage.

As limited information is available on undesired effect of DOX on the reproductive system [28], in our study, we tested whether DL application has a protective effect in reducing the negative effects of DOX on sperm parameters.

The result of the present study indicated that DL administration mitigated a decrease in DOX-induced weight loss. DOX induced weight loss in our study, which is consistent with previous reports [12, 29], can stem from many factors e.g. reduction of glucose uptake by skeletal muscles [30], inhibition of adipogenesis and lipogenesis [31], reduced food intake, increased energy expenditure and excess catabolism [32]. When the total protein amount in the blood is examined, DOX decreased total protein levels which was ultimately considered a cause of weight loss in our study. In other words, it can trigger weight loss by increasing protein breakdown [33]. However, it has shown that DL applications reversed this effect by preserving total protein level, which supports why DL reduced weight loss. All are endorsing our observed result. In our current study, DOX administration was found to cause a decrease in total protein levels. Long term usage of DL such as two months resulted in a decrease level of cholesterol in rats [34]. Similarly, birds feeding with DL showed low cholesterol level [35]. Our result suggests that DL administration alleviated increase of total cholesterol level on blood after DOX application. Interestingly, we didn't observe any changes in testicular weights. However, other studies in the literature indicate that DOX causes a decrease in testicular weight and volume [29, 36, 37]. Ateşşahin et al. found that DOX administration caused decreased testis and epididymis weight on their study which 10mg/kg single dose was used and finalized their study in 10 days [38]. Differences in durations of administration and dose of DOX may be the reasons for these contradictory results.

DOX is known to disturb or damage histological structures in testicular tissues, evidenced by degenerative changes in epithelial germinative cell layer, decreased tubular epithelial height, edema in intratubular area and epithelial height [11, 29, 39]. Comparison of our findings with those of other experimental studies confirms that DOX increased immature cells, interstitial edema and decreased leyding cells in seminiferous tubules and caudal epididymis. Our results also indicated that DOX administration caused loss in spermatogenic cells, decreased sperm count and Johnsen Score, and increased the percentage of sperm abnormalities, implicated by elevated the percentage of sperm with curled tails and multiheaded sperm morphology. These results are in accordance with recent studies showing that DOX decreased sperm viability, count, motility, Johnsen Score and increased abnormal morphology [29, 39, 40]. In the current study, DL caused a decrease in the germinal epithelial cells that were immature and spilled into the lumen, especially in the group with DL treated, suggesting that it positively affects spermatogenesis. Again, the findings lead us to conclude that DL is partially effective in the formation of damage to the seminiferous tubules. In our study, it is thought that damage to the seminiferous tubule structures of the testis tissues occurred in the DOX group, and DL administration reduced the damage to the seminiferous tubule structures. To our knowledge, it is the first study investigating the possible protective of DL on DOX-induced histopathological changes in testicular tissue. Given literature and our findings, the fact that most histopathological changes were not detected in DL administration [10], suggesting that DL ameliorate the adverse effects of DOX on sperm morphology and changes in histology of testicular tissues positively.

Serum levels of testosterone, FSH and LH could be evaluated for damage in seminiferous tubules and the Leydig cell in response to radiation and chemotherapy [41, 42]. Testosterone, LH, and FSH are important hormones in the regulation of spermatogenesis [43]. Testosterone is unique testicular hormone which is stimulated by LH to be released and also regulates LH secretion in negative feedback manner [44]. As known, LH stimulates Leydig cell to produce testosterone and FSH stimulates Sertoli cell to support and maintain metabolism [44]. Serum LH and FSH levels have increase in response to the damage on the male reproductive system [41, 42]. In experimental animal study, DOX treatment resulted in an increase serum FSH levels [45, 46]. Another study was found that serum testosterone levels were not significantly affected by DOX-treatment [47]. These are supported by our results presented here. The administration of DOX has been observed to result in a significant elevation of FSH levels, which may serve as a fundamental underlying factor contributing to the observed decline in both the normal morphology of spermatozoa and the overall sperm count in rats subjected to DOX treatments. The increase in serum FSH level negatively affects sperm morphology and number [48, 49]. DL administration into DOX-treated rats correlate with reduced FSH levels, suggesting a potential mitigating effect of DL on the hormonal imbalance induced by DOX. Besides, we showed that DL reduces the LH and increases the testosterone level in rats treated with only DL in our study. According to another study, it has been found that the use of DL increases sexual performance and testicular functions in older men, indicated by an increase in sperm number. Additionally, it has been determined to improve erectile problems [6]. In an experimental study, DL exerted androgenic effects, demonstrated by an increase in plasma testosterone levels in castrated rats via increasing the expression of Spot14-like androgen inducible protein [7]. Another study indicated that DL increased testosterone concentrations in male birds [35]. All these results show DL may induce secreting testosterone as LH does, thus, increased testosterone levels result in decreased LH levels via Hypothalamic-pituitary–gonadal axis feed-back mechanisms. A high concentration of testosterone within the testicular environment is critically essential not only for the proper progression of spermatogenic processes but also for the preservation of the structural integrity and functional physiology of the seminiferous tubules, which are vital components of the male reproductive system [50]. High level of testosterone level and good sperm quality can cause low level of LH reciprocally. All these results are interpreted as having an androgenic property and increasing sperm quality for DL. This phenomenon could also be supported with that the decrease in prevalence of multiheaded sperm morphology, alongside an increase in the prevalence of normal sperm morphology in DOX-treated rats receiving DL.

Interestingly, DL application wasn't found effective on sperm count. DL decreased the percentage of sperm with normal morphology and increased the percentage of sperm with multi-head in rats not treated with DOX. This finding is somewhat surprising given the fact that other research shows the protective or beneficial effect of DL in reproductive system. In addition, DL treatment did not change sperm counts significantly, which is contrary to a previous study suggesting that DL treatment resulted in an increased sperm count in Bisphenol A-induced testis damage [51]. It's important to remember that sperm counts were close to each other in all groups except for those who received DOX. The exact reasons for this inconsistency remain unknown. However, in accordance with our findings, it can be considered that DL has a positive effect on increasing quality of sperm instead of number against DOX side effects on the reproductive system. As, DL has a good effect to reverse DOX side effect, it should be stated that usage of DL in healthy condition should be investigated.

The underlying mechanisms responsible for DOX's pronounced antitumor efficacy have been attributed to its high affinity towards chromosomal DNA [52]. However, there is a growing number of research that increasingly indicates that these pharmacological agents also exert their effects on the mitochondria [53], thereby disrupting critical mitochondrial functions which are essential for cellular health and vitality. The detrimental consequences of DOX primarily arise from its tendency to generate free radicals as a result of its interactions with mitochondrial enzyme systems, and the concomitant suppression of vital antioxidant enzymes that play a crucial role in maintaining cellular redox balance [54]. Dysfunction of mitochondrial enzymes has been identified as an early biomarker for the induction of apoptosis and cytotoxicity that is mediated by DOX [55]. In fact, of research findings indicate that within a five-minute post-administration of DOX, a marked increase in the production of free radicals can be seen, which subsequently leads to a rapid decline in mitochondrial reduction potential. This phenomenon occurs approximately six hours prior to the onset of cell death. This timeline illustrates that the initial application of DOX causes mitochondrial dysfunction, which leads to the cellular process of apoptosis [56]. Moreover, investigative studies have revealed that myocytes treated with DOX undergo significant release of cytochrome c into the cytosol, alongside an increase in the activity of Caspase-3, an enzyme that plays a pivotal role in the execution phase of apoptosis. The activation of Caspase-3 has been linked to the induction of DNA damage, disruption of the cytoskeletal framework and injuries to the cell membrane, which are primarily attributed to the escalation of lipid peroxidation and the subsequent initiation of apoptotic pathways [53, 56]. Several reasons such as increased oxidative stress, inflammation, chromosomal abnormalities have been proposed to the negative effect of DOX on sperm count and morphology [28, 29, 40]. Oxidative stress is considered an imbalance between pro- and antioxidant species that results in molecular and cellular damage. Production of reactive species underlies the redox cycling profile of DOX [57]. Previous studies have shown that DOX causes a decrease in antioxidant enzyme activity (CAT, SOD, GST, GSH) and an increase oxidative stress and lipid peroxidation [58–60]. DOX creates free radicals and suppress antioxidant enzymes [38]. Since the structural composition of male germ cell membranes are rich in polyunsaturated fatty acids, these cells are particularly vulnerable to damage and cell death as a consequence of the exacerbated lipid peroxidation resulting in apoptosis and infertility that occurs in the presence of DOX [61, 62]. Correlation with literature is found that significant decrease of sperm counts of animal treated with DOX is detected in the current study. Also, DOX application caused a decrease in GPx level. However, this result can be reversed by DL. The research about DL stated that DL increases antioxidant enzymes levels and decreases Malondialdehyde (MDA), Xanthine Oxidase (XOD) and testisan-1 levels in brain damage induced by sepsis [63]. Similarly, DL administration has been reported to cause an increase in SOD and CAT levels in lipopolysaccharide-induced liver damage in rats [10]. Analyzes of DL have shown that it has a strong antioxidant effect in terms of total phenolic content, antioxidant activity and anti-radical inhibition level [64]. It was shown that the administration of lipopolysaccharide (LPS) increased the expression of NF-κB, TNF-a, and iNOS and the production of excessively reactive oxygen species. [65] The induction of iNOS in LPS depends mainly on the transcription factor NF- κB, and there is evidence that sepsis is also associated with ROS production[66] LPS binds to TLR4 in Kupffer cells and causes ROS generations and proinflammatory cytokines [67]. Doganyigit et.al. showed that DL suppressed proinflammatory cell production by suppressing the signal pathway of TLR4/HMBG-1/NF- κB induced by LPS and the production of iNOS [10]. These findings indicate that DL exhibits significant anti-inflammatory and antioxidative effects. The observed increase in TAS in rats administered DOX corroborates existing literature. Elevated TAS levels, in conjunction with a balanced hormonal status, may mitigate the morphological alterations and reduced cell counts induced by oxidative stress.

Inflammation is part of the complex biological response of body tissues to harmful stimuli such as pathogens, damaged cells, or irritants and is a protective response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell damage, clear necrotic cells and damaged tissues from the original damage and inflammatory process, and initiate tissue repair [68]. Wang et. al. showed that a significant increase in the levels of pro-inflammatory cytokines such as IL-1 and TNF-α following DOX administration [69]. Similarly, in a recent study, an increase in markers such as TNF-α and IL-1β was observed in groups administered DOX [70]. Contrary to these studies, Reis-Mendes et al. did not find differences in the levels of TNF-a, IL-1β, p38 mitogen-activated protein kinase (p38 MAPK) or NF- κB p52 subunit amongs DOX-treated infant and adult mice and their control groups [71]. When they focused on the levels of nuclear factor erythroid-2 related factor 2 (Nrf2) which inhibits NF-κB activation and induce antioxidant system, they observed significant increase the levels of Nrf2 in DOX-treated infant mice on the other hand there was no changed in DOX-treated adult mice. Parallel to the study, it was found that DOX administration did not cause a statistically significant changes in IL-6, IL-1β and TNF-α in testicular tissue in the current study. Our results may stem from Nrf2 expression on the rats which were at same age and culled at the same time point. There is strong evidence in existing literature that DOX can induce apoptosis via extrinsic mechanisms and the application of DL has been shown to significantly mitigate the elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [72]. The observed discrepancies in our findings regarding TNF-α, IL-1β, and IL-6 in comparison to existing literature may be attributed to the organ-specific effects of DOX as well as variations in dosing regimens and administration methods, including the choice between cumulative and single-dose protocols. To achieve a more comprehensive understanding of these phenomena, further elucidation of the underlying molecular pathways is warranted, especially considering the lack of observed increases in inflammatory cytokines.

The Alkaline comet assay was employed to evaluate the impact of DOX and DL on DNA damage. DOX-treated rat blood samples exhibited the highest levels of DNA damage, consistent with previous findings [73, 74]. In our study, DOX application significantly reduced tail percentage DNA, tail length, and tail moment, indicating DNA damage induction. These results corroborate existing literature [73, 74]. Our results indicate that DL is effective against DNA damage induced by DOX. DL administration led a significant decrease in tail percentage DNA, tail length, and tail moment, markers of DOX-induced DNA damage. These findings suggest that DL may offer protection against genotoxic chemicals such as DOX.

All in all, the present research explores, for the first time, the effects of DL on DOX induced testicular damage. Findings of the current study provide some initial evidence that suggests possible protective effect of DL against damage induced by DOX in testis, sperm morphology and count. However, it should be stated that further research is needed to shed light on the cellular and molecular mechanisms underlying the effects of DL on testicular toxicity induced by DOX and acquire more benefits for health.

In conclusion, these findings provide important insights into the role of DL as an apitherapy product in alleviating the undesired side effects of anti-cancer drug DOX in male reproductive systems. DL administration improved DOX-induced histopathology and genotoxicity. It also caused significant improvement in sperm morphology in DOX treated rats. In addition, the androgenic effect of DL was demonstrated in our current study, with an increase in testosterone level and a decrease in LH level. Based on the findings of the present study, it could be suggested that DL usage is a good strategic approach to mitigate the side effects of the anti-cancer drug DOX in male reproductive systems. DL is thought to have a beneficial effect on reversing the side effects of DOX without alleviating anticancer effect of DOX. It is recommended that it needs to be investigated for use in healthy conditions and the unexpected effects of DL treatment on sperm morphology.

Author contributions Kagan Agan: project administration, funding acquisition, review & editing, original draft, investigation, conceptualization. Salih T. Kaya: project administration, formal analysis, review & editing, writing—original draft, investigation, visualization, conceptualization. Aydan F. Agan: review & editing, methodology, investigation conceptualization. Pınar A. Yoldas: methodology, comet investigation. Taner Yoldas: methodology, comet investigation. Ayse I. Keles: methodology, histopathology. Tugce Caprazli: methodology, drone larvae production. Elif Arica: methodology, sperm investigation. Meral Kekecoglu: Review & editing.

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Data availability The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declarations

Conflict of interest The authors affirm that they have no conflicts of interest to disclose.

Ethical approval The Animal Research Ethics Committee of Düzce University approved the present study (protocol number: 2020/4/5). Experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

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