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Evaluating the Impact of Doxorubicin on rat testicular tissue and the protective role of Resveratrol and Thymoquinone

Evaluación del impacto de la doxorrubicina en el tejido testicular de ratas y el papel protector del resveratrol y la timoquinona

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ABSTRACT

In this study, the harmful effects of doxorubicin on rat testicular tissue and the protective role of thymoquinone and resveratrol were investigated. The study employes 10 groups, each consisting of 8 male Wistar albino rats, for a total of 80 male rats. The groups were set up as follows: control (C), doxorubicin (D), resveratrol (R) (5 mg·kg^{-1}, 20 mg·kg^{-1}), thymoquinone (T) (5 mg·kg^{-1}, 20 mg·kg^{-1}), and combination groups (D + R 5 mg·kg^{-1}, D + R 20 mg·kg^{-1}, D + T 5 mg·kg^{-1}, D + T 20 mg·kg^{-1}). The C group received physiological saline by oral gavage every other day. Groups administered D at a dose of 15 mg·kg^{-1} intraperitoneally on day 10. Groups receiving T and R were given every other day for 21 days. At the end of the study period, the animals were euthanized by cervical dislocation under anesthesia. Testis and epididymis samples were collected with attention to sterility for spermatological, histopathological, and immunohistochemical analyses. The findings showed that sperm concentration decreased in all groups treated with D, motility and mitochondrial activity decreased, and acrosome and membrane integrity were impaired. Histopathological and immunohistochemical data also revealed significant decreases in germinal cell layer thickness, tubule diameter, Johnson's testicular score, and relative testicular weight, while the B-cell lymphoma 2 (Bcl-2) ratio decreased and Bcl–2–associated X protein (Bax) expression increased (P<0.05). Additionally, the T 5 mg·kg^{-1} group exhibited an adverse effecton sperm density, motility, membrane integrity, and mitochondrial activity. The T 20 mg·kg^{-1}, R 5 mg·kg^{-1}, and R 20 mg·kg^{-1} groups, on the other hand, yielded more positive results than the control group for many parameters. When considering the combined groups, the D + R 5 mg·kg^{-1} and especially the D + R 20 mg·kg^{-1} groups successfully prevented the toxicity caused by the D group in terms of both spermatological and histopathological and immunohistochemical parameters. In conclusion, R was found to have a stronger protection against D-induced testicular toxicity compared to thymoquinone.

Key words: Doxorubicin; resveratrol; thymoquinone; apoptosis; testis

RESUMEN

En este estudio se investigaron los efectos nocivos de la doxorrubicina sobre el tejido testicular de ratas y la protección que proporcionan la timquinona y el resveratrol. El estudio empleó 10 grupos, cada uno compuesto por 8 ratas macho albinas Wistar, para un total de 80 ratas macho. Los grupos se establecieron de la siguiente manera: control (C), doxorrubicina (D), resveratrol (R) (R 5 mg·kg^{-1}, R 20 mg·kg^{-1}), timoquinona (T) (T 5 mg·kg^{-1}, T 20 mg·kg^{-1}) y grupos combinados (D + R 5 mg·kg^{-1}, D + R 20 mg·kg^{-1}, D + T 5 mg·kg^{-1}, D + T 20 mg·kg^{-1}). El grupo C recibió solución salina fisiológica por vía oral cada dos días mediante sonda. Los grupos que recibieron D recibieron una dosis de 15 mg·kg^{-1} por vía intraperitoneal el día 10. Los grupos que recibieron T y R recibieron estos compuestos cada dos días durante 21 días. Al final del período del estudio, los animales fueron sacrificados mediante dislocación cervical bajo anestesia. Se recolectaron muestras de testículo y epidídimo, prestando atención a la esterilidad, para análisis espermatológicos, histopatológicos e inmunohistoquímicos. Los hallazgos mostraron que la concentración espermática disminuyó en todos los grupos tratados con D. Asimismo, la motilidad y la actividad mitocondrial se redujeron, y la integridad del acrosoma y de la membrana se vieron afectadas. Los datos histopatológicos e inmunohistoquímicos también revelaron disminuciones significativas en el grosor de la capa de células germinales, el diámetro de los túbulos, el puntaje testicular de Johnson y el peso testicular relativo, mientras que la proporción de proteínas del linfoma de células B2 disminuyó y la expresión de la proteína X asociada a Bcl–2 aumentó (P<0.05). Además, el grupo T 5 mostró un efecto adverso sobre la densidad espermática, la motilidad, la integridad de la membrana y la actividad mitocondrial. Por otro lado, los grupos T 20 mg·kg^{-1}, R 5 mg·kg^{-1} y R 20 mg·kg^{-1} mostraron resultados más positivos que el grupo control en muchos parámetros. Al considerar los grupos combinados, los grupos D + R 5 mg·kg^{-1} y especialmente D + R 20 mg·kg^{-1} lograron prevenir con éxito la toxicidad causada por el grupo D en términos de parámetros espermatológicos, histopatológicos e inmunohistoquímicos. En conclusión, se ha descubierto que R tiene una mayor capacidad protectora frente a la toxicidad testicular inducida por la D en comparación con la timoquinona.

Palabras clave: Doxorrubicina; resveratrol; timoquinona; apoptosis; testículo

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INTRODUCTION

Cancer has plagued humanity for centuries [1], and humanity has been struggling with cancer for many years. In the last few decades, various chemotherapeutic agents have been developed to treat malignancies. Among them, doxorubicin remains a cornerstone drug due to its efficacy against a broad spectrum of cancers, including breast, lung, bladder, thyroid, prostate, and testicular cancers, as well as Hodgkin lymphoma and Wilms tumor [2].

Doxorubicin exerts its chemotherapeutic effect by inhibiting DNA and RNA polymerases, altering the activity of topoisomerase II, and disrupting mitochondrial function. When these systems are disrupted, it leads to oxidative stress, DNA damage, and cell death [3]. However, doxorubicin has also been the subject of scientific studies due to its cytotoxic effect, which can damage both cancerous and healthy tissues.

Studies have indicated that doxorubicin has adverse effects on the testes and have revealed that chemotherapeutic agents cause testicular tissue degeneration in patients undergoing cancer treatment. It is also emphasized that the adverse effects of doxorubicin on testicular tissue are mainly due to oxidative stress and apoptosis [4, 5, 6].

Over the years, the concept of using antioxidants to reduce the systemic toxicity of doxorubicin has gained traction. Scientists are increasingly interested in the potential of antioxidants to neutralize reactive oxygen species (ROS) and maintain cellular homeostasis[7].

One such antioxidant is thymoquinone, a bioactive compound derived from *Nigella sativa*. Due to its potent antioxidant and anti-inflammatory properties, it has long been used in traditional medicine in the Mediterranean and West Asia [8]. Thymoquinone exerts its antioxidant effect by activating the Nrf2/HO-1 signaling pathway to suppress reactive oxygen species and prevent lipid peroxidation [9]. It also exerts its anti-inflammatory effects through its regulatory role on nuclear factor kappa B (NF-κB) signaling pathways [10].

Another well-known antioxidant, resveratrol (trans-3,5,4′-trihydroxystilbene), is a natural polyphenol found in abundance in grapes, peanuts, mulberries, and red wine [11]. Resveratrol exhibits numerous biological activities, including antioxidant, anti-inflammatory, cardioprotective, neuroprotective, and anticancer effects. These effects are primarily attributed to its ability to scavenge mitochondrial superoxide radicals, regulate coenzyme Q activity, and inhibit iron-induced lipid peroxidation [12, 13].

Resveratrol, which also plays a role in the elimination of free oxygen radicals, exerts this effect through the hydroxyl (OH·) groups located at the 3, 4′, and five positions of its molecular structure [14].

The non-targeted cytotoxic effects of doxorubicin and its detrimental impact on testicular tissue have been clearly demonstrated in numerous scientific studies. Consequently, the use of various protective agents to mitigate this damage in the testes has become a necessity.

Therefore, this study was designed to systematically evaluate the effects of doxorubicin on spermatological, histopathological, and apoptotic parameters, and to investigate whether resveratrol and thymoquinone exert protective effects against doxorubicin-induced testicular toxicity in an experimental rat model.

MATERIALS AND METHODS

Animals and experimental protocols

Eighty male Albino Wistar rats (*Rattus norvegicus*) of 8 weeks old, and ~400g body weight were obtained from the Experimental Medicine Research Center of Selcuk University, Konya, Türkiye with the precision balance (TEM, TS 200, Türkiye). Animals were housed under standard conditions (16/8 hour (h) light/dark, 22 ± 2°C, 60–65% humidity) in stainless steel cages with *ad libitum* access to food and water. The Ethics Committee of the Experimental Medicine Research Center at Selçuk University approved all procedures with the number 2018–38 on October 26, 2018.

Rats were randomly assigned to 10 groups (n = 8 each): control (C), doxorubicin (D: 15 mg·kg^{-1}), resveratrol (R5: 5 mg·kg^{-1}; R20: 20 mg·kg^{-1}), thymoquinone (T5: 5 mg·kg^{-1}; T20: 20 mg·kg^{-1}), and combination groups (D+R5, D+R20, D+T5, D+T20). The 21-day (d) study included a single intraperitoneal injection of doxorubicin (15 mg·kg^{-1}, Koçak Farma, Türkiye) on d 10. Required amounts of resveratrol (#70675, Cayman Chemical) and thymoquinone (#15039, Cayman Chemical) were dissolved in 1 mL of DMSO, diluted with saline to adjust the administration dose, and administered orally (1 mL) every other d from the first to the 21st d. Controls received 1 mL saline alone to control for sham effects. The doses used in the study were adjusted based on previous studies [15].

At the end of the study, rats were euthanized under anesthesia (10 mg·kg^{-1} Xylazine and 90 mg·kg^{-1} Ketamine) via cervical dislocation. The testes and epididymides were excised and separated. The testes were placed in Bouin's solution for histopathological and immunohistochemical evaluation. At the same time, the cauda epididymis was finely minced in phosphate buffer solution (PBS) and incubated for 15 min to release spermatozoa. The resulting sperm suspension was collected in 1.5 mL Eppendorf tubes and maintained at 37.5°C in a water bath (Memmert, WTB15, Germany) for further analysis.

Spermatological analyses

Epididymal sperm motility

For sperm motility assessment, 7.5 µL of semen was placed on a pre-warmed (35°C) microscope slide (Tokai-Hit, TPi-SQX, Japan). A coverslip was applied, and motility was evaluated in five randomly selected fields at 20× magnification under a phase-contrast microscope (Leica, DM1000, Germany) with a heated stage at 35°C. The total motility score for each group was calculated subjectively [16].

Epididymal sperm concentration

Epididymal sperm concentration was determined using the hemocytometric method. Semen was diluted 1:200 with Hayem solution and transferred to 1.5 mL Eppendorf tubes. Then, 7.5 µL of the diluted sample was loaded onto a Thoma counting chamber (Marienfeld, Germany), and sperm cells in both grids were counted under a light microscope at 200× magnification.

Abnormal spermatozoa

Morphologically abnormal spermatozoa were evaluated using the Eosin-Nigrosin staining method. A 7.5 μL semen sample was mixed with stain at a 1:2 ratio, and thin smears were prepared on clean slides. After air-drying, smears were examined under a phase-contrast microscope. At least 200 sperm were examined, and sperm abnormalities were recorded as a percentage [17].

Sperm membrane integrity

Sperm plasma membrane integrity was assessed using the SYBR-14/Propidium Iodide (PI) Sperm Viability Kit (Molecular Probes, L7011). SYBR-14 was diluted 1:10 in DMSO, and PI was diluted to 2 $mg \cdot mL^{-1}$ in distilled water; both were stored at –20°C (Arçelik, 566406 MI, Türkiye). A 600 μL semen sample (300 μL semen+300 μL PBS) was prepared, and 30 μL of the sample was mixed with 6 μL SYBR-14 and 2.5 μL PI. After gentle mixing, the sample was incubated at 37°C (İldam Lab, ILD-ICS-30, Türkiye) for 15 min. Following incubation, 10 μL of Hancock's solution was added for sperm immobilization. A 5 μL aliquot was placed on a slide, covered with a coverslip, and examined at 400× magnification. At least 200 spermatozoa were evaluated per sample. Green-fluorescing cells were considered as membrane-intact, while red-fluorescing cells were taken as membrane-damaged. All steps were performed under low-light conditions to prevent photobleaching [18].

Mitochondrial activity

Sperm mitochondrial activity was evaluated using JC-1 stain (T3168, Invitrogen). From the previously prepared 600 μL semen sample (300 μL semen + 300 μL PBS), 300 μL was mixed with 2.5 μL JC-1 and 2.5 μL PI in Eppendorf tubes, and incubated at 37°C for 15 min.

After incubation, 10 μL of Hancock's solution was added to stop the reaction and immobilize spermatozoa. A 5 μL aliquot was placed on a microscope slide, covered with a coverslip, and examined under a fluorescence microscope at 400× magnification. At least 200 sperm cells were evaluated per sample. Cells lacking fluorescence were classified as having inactive mitochondria, while those with light green or orange fluorescence indicated mitochondrial activity [19].

Acrosomal integrity

Sperm acrosomal integrity was assessed using fluorescein isothiocyanate (FITC) conjugated peanut agglutinin (FITC-PNA; Thermo Fisher Scientific). A 60 μL semen aliquot from the PBS-sperm mixture was mixed with 10 μL FITC-PNA and 2.5 μL PI in 1.5 mL Eppendorf tubes and incubated at 37°C for 15 min. After incubation, 10 μL of Hancock's solution was added to terminate staining and immobilize spermatozoa. A 5 μL aliquot was placed on a slide, covered with a coverslip, and examined under a fluorescence microscope (Olympus, BX50F4, Japan). At least 200 spermatozoa were evaluated per sample. Green fluorescence in the acrosomal region indicated acrosomal damage [19].

Pathological analyses

Relative testicular weight

Following euthanasia, each rat was weighed, and a complete necropsy was performed. Testes and epididymides were excised, and the testes were cleaned and weighed to determine relative organ weight. Relative testicular weight was calculated with precision balance (Weightlab, WL 303L, Türkiye) as: (testis weight × 100) / body weight.

Histopathological analyses

Testes tissue was fixed in Bouin's solution for 24–48 h, washed in running water for 24 h, and processed using a tissue processor (Leica, TP1020, Germany). Paraffin-embedded tissues were sectioned at 5μm (Leica, RM2125RT, Germany), incubated at 37°C for 24 h, stained with Hematoxylin-Eosin following routine procedure, and examined under a light microscope (Olympus, BX51, Japan).

The intra-testicular germinal layer thickness, seminiferous tubule diameter, and Johnson's testicular scores were recorded. The diameter of the seminiferous tubules was determined by averaging two perpendicular measurements from 10 randomly selected tubules at 20× magnification. Germinal layer thickness was assessed from the same tubules using two measurements per tubule. Johnsen's scoring system (1–10 scale) was applied to evaluate spermatogenic activity [20].

Epididymal sperm concentration was semi-quantitatively scored based on the estimated percentage reduction compared to the controls: 0–25% (score 1), 26–50% (score 2), 51–75% (score 3), and > 75% (score 4) [21, 22].

Immunohistochemical analyses

Immunohistochemical (IHC) analyses were conducted to assess Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2) expression in testicular tissue. Paraffin-embedded sections (5 μm) were stained using the Leica Bond-Max immunostainer and the Bond™ Polymer Refine Detection Kit (Leica DS9800), following the manufacturer›s protocol with anti-Bax (Abcam, ab32503) and anti-Bcl-2 (Abcam, ab59348) primary antibodies at room temperature (TABLE I). The IHC staining was evaluated semi-quantitatively. For each sample, 10 randomly selected seminiferous tubules (TSC) were scored for cell positivity: 0 = none, 1 = < 10%, 2 = 10–50%, 3 = 51–80%, 4 = > 80%; and staining intensity: 1 = weak, 2 = moderate, 3 = strong. The average staining index was calculated as follows: mean positivity × staining intensity ÷ 10.

This index was then multiplied by a prevalence score based on the percentage of positively stained tubules in the entire section: 0 = none, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = > 75%. The final IHC score was calculated as follows: mean positivity × staining intensity/10 × prevalence score [23, 24].

Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics v25., group means for sperm parameters, histopathological findings, and immunohistochemical scores were compared using one-way ANOVA. Significant differences were further analyzed

TABLE I
Immunohistochemical Staining Protocols according to Primary Antibodies

Primary Antibody	Dilution	Antigen Retriveal	Peroxidase Block	Protein Block	Antibody Incubation	Post Primer	Polymer	DAB	Hem.
Bax	1:250	HIER1–100°C 20 min	30 min	30 min	60 min	10 min	10 min	3 min	2 min
Bcl-2	1:100	HIER2–100°C 20 min	30 min	30 min	60 min	10 min	10 min	3 min	2 min

Abbreviations: HIER1: Heat-induced epitope retrieval, Citrate Buffer Ph:6.0, HIER2: Heat-induced epitope retrieval, EDTA, pH:9.0, Hem.: Hematoxylin, DAB: 3,3' Diaminobenzidine, Bcl-2: B-cell lymphoma 2 (antiapoptotic), Bax: Bcl-2–associated X protein (pro-apoptotic)

with Duncan's multiple range test. Variables that did not show a normal distribution, including Bax and Bcl-2, were logarithmically transformed before analysis. A *P*-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Sperm motility, concentration, and abnormal spermatozoa

When motility data were examined, it was observed that the D and T5 groups significantly reduced motility rates compared to the control group ($P<0.05$). In contrast, motility increased significantly in the T20, R5, and R20 groups ($P<0.05$). When the combined groups were examined, the D + T5 and D + T20 groups failed to prevent the decrease in motility caused by doxorubicin when compared to the D group; in contrast, the D + R5 and D + R20 groups succeeded in preventing this decrease and produced results close to the control group ($P<0.05$).

The results for epididymal sperm concentration were similar to those for motility. The D, T5, and T20 groups significantly reduced epididymal sperm concentration, whereas the R20 group significantly increased sperm concentration ($P<0.05$). All groups used in combination prevented the decrease in concentration observed in the D group; the results of the D + T20 and D + R20 groups in particular were significantly higher than those of the D group and were close to those of the control group ($P<0.05$).

Doxorubicin significantly increased the rate of abnormal spermatozoa ($P<0.05$). Only the groups containing antioxidants (T5, T20, R5, and R20) did not show a different abnormality rate from the control group. All groups used in combination with doxorubicin significantly reduced the doxorubicin-induced abnormal spermatozoa rate ($P<0.05$; FIG 1). It has been reported in the literature that sperm motility, concentration, and morphology are impaired in patients receiving doxorubicin therapy [25]. The toxic effect of the drug also adversely affects plasma membrane integrity, acrosomal integrity, and mitochondrial activity. These adverse changes typically result in the release of cytochrome c from the mitochondrial membrane, oxidative stress, apoptosis, and disruption of the Bax/Bcl–2 balance [26, 27].

Resveratrol, a potent polyphenol, has demonstrated promising results in reducing gonadotoxicity caused by chemotherapeutic drugs due to its antioxidant properties [28, 29]. Consistent with existing studies, this research showed that R preserved sperm motility, concentration, and mitochondrial function in rats exposed to D. The protective effect of R is generally linked to mechanisms such as scavenging ROS, stabilizing lipid membranes, and activating AMPK and SIRT1–PGC-1α signaling pathways [30, 31]. However, the absence of significant improvement in acrosomal integrity may be because of the relatively mild acrosomal damage observed in This study, compared to the more severe impairments reported in other studies [32].

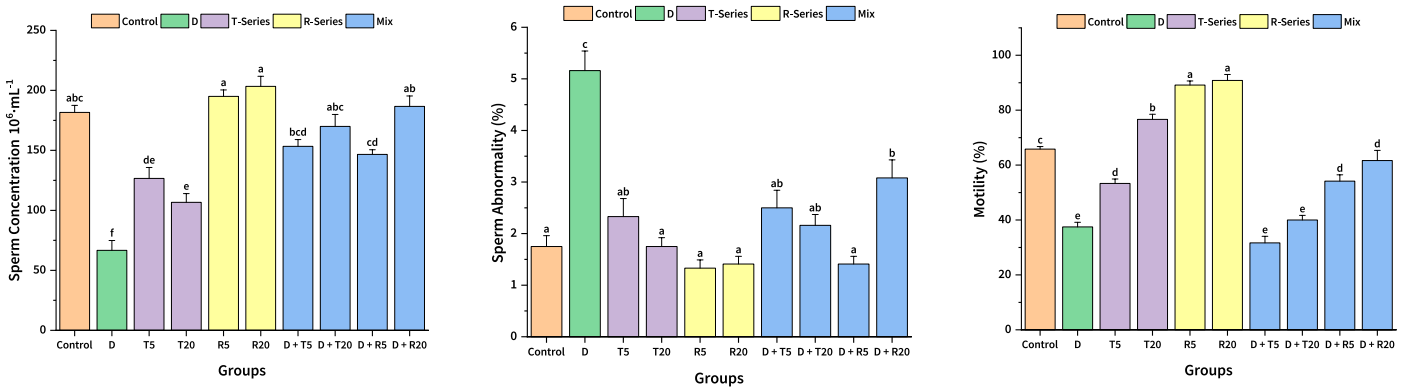


FIGURE 1. Epididymal sperm concentration (million·mL^{-1}), sperm abnormality rate, and motility levels in different experimental groups. Data are presented as mean ± standard deviation. Superscript letters indicate statistically significant differences from the control group only ($P<0.05$). Abbreviations: D: Doxorubicin (10 mg·kg^{-1}), T5: Thymoquinone (5 mg·kg^{-1}), T20: Thymoquinone (20 mg·kg^{-1}), R5: Resveratrol (5 mg·kg^{-1}), R20: Resveratrol (20 mg·kg^{-1}), D+T5: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (5 mg·kg^{-1}), D+T20: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (20 mg·kg^{-1}), D+R5: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (5 mg·kg^{-1}), D+R20: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (20 mg·kg^{-1})

When the results of the groups containing T were examined, a dose-dependent protective effect was found. In the study, the T5 group showed reduced motility, whereas the T20 group demonstrated a significant improvement. This is consistent with the biphasic effect reported in the literature [33, 34]. T has demonstrated protective properties, exhibiting antioxidant and cytoprotective effects in studies where it has been used [35, 36]. However, in the present study, T was dissolved in DMSO. It has been reported that DMSO in its pure form can exhibit toxic effects on various cell types [37, 38]. The adverse effect of low-dose T on motility may be attributed to the possible harmful effects of DMSO, used as a solvent, on testicular tissue and, indirectly, on spermatozoa. Accordingly, high doses of T and R may have compensated for DMSO-induced oxidative damage; however, the most likely reason is that the T5 group was insufficient in counteracting this toxic effect.

Sperm membrane integrity, mitochondrial activity and acrosomal integrity

Sperm plasma membrane integrity was significantly impaired in the D group compared to the C (P<0.05). T5 and T20 groups also showed reduced membrane integrity (P<0.05). While co-treatment groups significantly attenuated D-induced damage, integrity levels remained below those of the C (P<0.05; FIG. 2). Mitochondrial activity was markedly reduced in the D group relative to all others. Significant improvements were observed in the D + R5 and D + R20 groups, indicating a protective effect of R. Additionally, the T20, R5, and R20 groups showed significantly enhanced mitochondrial activity (P<0.05; FIG. 2). Acrosomal integrity was significantly reduced in all groups except for R20 compared to the C (P<0.05). Co-treatment groups did not provide significant protection against D-induced acrosomal damage (P>0.05; FIG 2).

Doxorubicin administration significantly decreased mitochondrial activity, acrosomal integrity, and membrane integrity. This decrease results from structural damage caused by drug-induced oxidative stress and lipid peroxidation. Similar findings have also been documented in the literature [39, 40]. A reduction in acrosome and membrane integrity was observed only in the groups treated with T and R. This is likely because, despite the strong antioxidant properties of these compounds, the doses used may not have been sufficient to fully stabilize the oxidative balance, and the potential toxic effects of DMSO as a carrier could have played a role [37, 38]. However, in the combined groups treated with D, this decrease was significantly reduced, with values approaching those of the C group. This aligns with studies indicating that both compounds support antioxidant defense systems, maintain lipid membrane stability, and limit free radical production when used together with various drugs [41, 42].

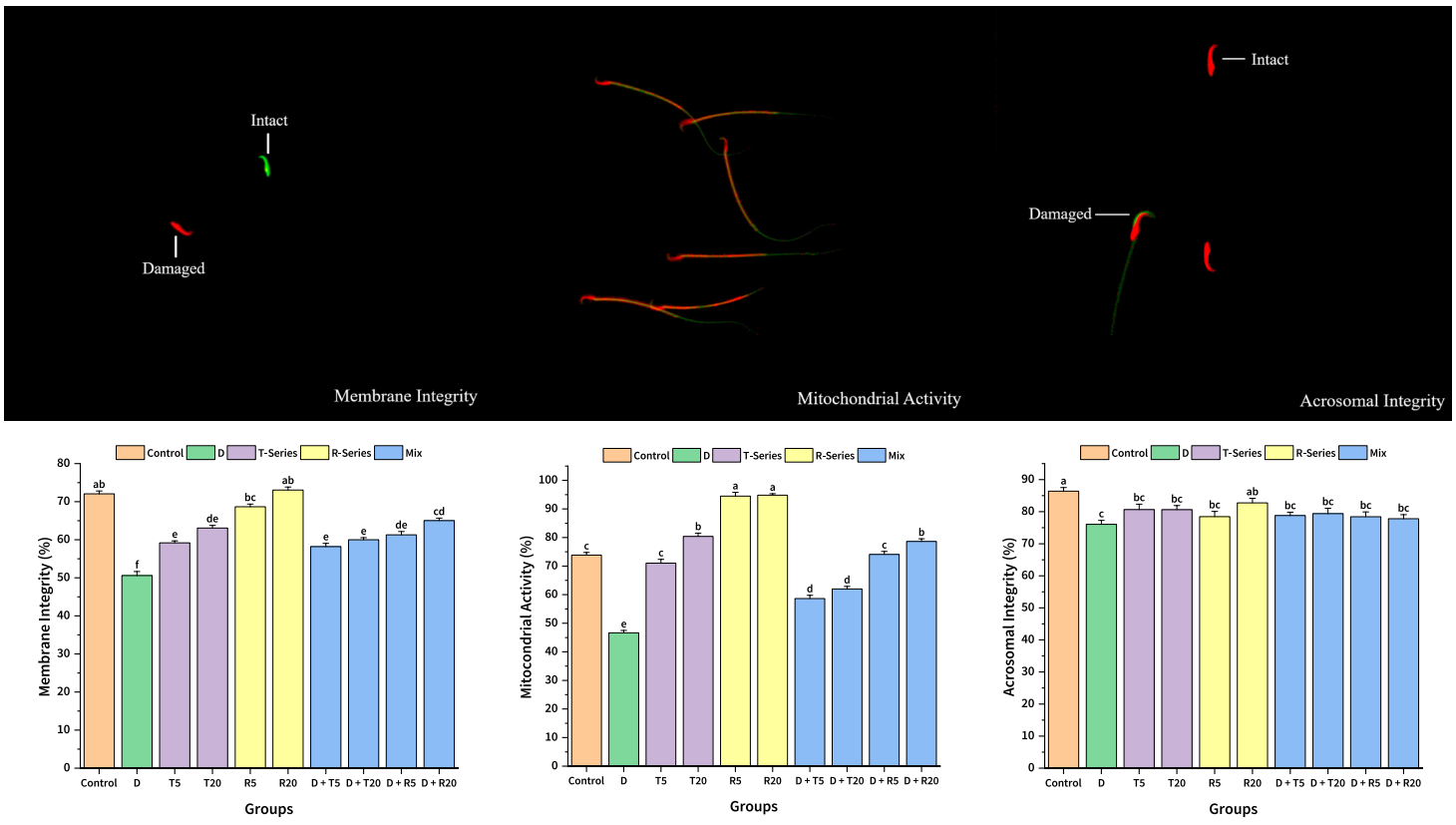


FIGURE 2. Membrane integrity, mitochondrial activity and acrosomal integrity in different experimental groups. Data are presented as mean ± standard deviation. Superscript letters indicate statistically significant differences from the control group only (P<0.05). Abbreviations: D: Doxorubicin (10 mg·kg^{-1}), T5: Thymoquinone (5 mg·kg^{-1}), T20: Thymoquinone (20 mg·kg^{-1}), R5: Resveratrol (5 mg·kg^{-1}), R20: Resveratrol (20 mg·kg^{-1}), D+T5: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (5 mg·kg^{-1}), D + T20: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (20 mg·kg^{-1}), D + R5: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (5 mg·kg^{-1}), D+R20: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (20 mg·kg^{-1})

Relative testicular weight and histopathological analyses

Regarding relative testicular weight (RTW), only the D group showed a decrease compared to the C group. All other groups had results similar to the C, and the D + T5, D + T20, D + R5, and D + R20 groups significantly reversed the reduction caused by the D group ($P<0.05$).

Germinal cell layer thickness was significantly reduced in the D group compared to the C ($P<0.05$). T5, T20, R5, and R20 groups maintained values comparable to the C ($P>0.05$). Although partial protection was observed in the D + T5, D + T20, and D + R20 groups, their thickness values remained significantly lower than C levels. Notably, the D + R5 group exhibited the closest values to the C, indicating the most substantial preservation effect ($P<0.05$).

Seminiferous tubule diameter was also significantly decreased in the D group ($P<0.05$). Partial improvement was observed in the D + T5 and D + T20 groups, though not statistically comparable to the C ($P>0.05$). In contrast, the D + R5 and D + R20 treatments effectively preserved tubule diameter, with values significantly closer to those of the C group ($P<0.05$; FIG. 3).

Johnson's score was highest in the C group and lowest in the D group. T5, T20, R5, and R20 groups showed scores similar to the C ($P>0.05$). Although all combination groups (D + R5, D + R20, D + T5, and D + T20) offered partial protection, none fully restored spermatogenic activity to C levels ($P<0.05$; FIGS. 3 and 4).

Doxorubicin significantly decreased RTW levels by inducing cellular apoptosis. This finding aligns with existing literature and is an expected outcome [43, 44]. However, when R or T was administered to rats treated with D, RTW levels in all groups reached those similar to the C group; this is consistent with results reported by Mendes *et al.* [45] and Mabrouk and Ben Cheikh [46]. Similar outcomes to RTW were also observed in germinal cell layer thickness and tubule diameter. Overall, it is evident that testicular tissue damage occurred in group D, but antioxidants successfully prevented this. Improvements in Johnson's testis score further indicated structural protection [33]. When histopathological images were evaluated, it was observed that group D reduced tubule diameter and caused germinative cell degeneration, while both antioxidants preserved germinal layer thickness and seminiferous tubule diameter, thus reversing D-induced structural damage. This aligns with data in the literature [36, 42].

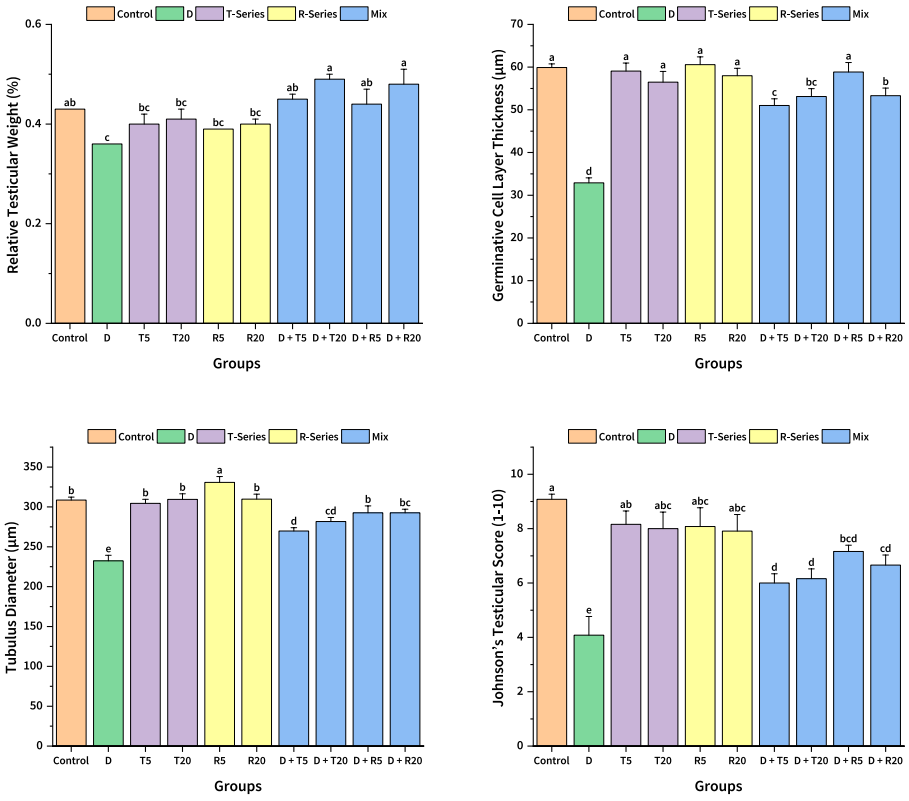


FIGURE 3. Comparison of relative testicular weight, germinative cell layer thickness, tubulus diameter, and Johnson's testicular score. Data were presented as mean ± standard deviation. Superscript letters indicate statistically significant differences from the control group only ($P<0.05$). Abbreviations: D: Doxorubicin (10 mg·kg^{-1}), T5: Thymoquinone (5 mg·kg^{-1}), T20: Thymoquinone (20 mg·kg^{-1}), R5: Resveratrol (5 mg·kg^{-1}), R20: Resveratrol (20 mg·kg^{-1}), D + T5: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (5 mg·kg^{-1}), D + T20: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (20 mg·kg^{-1}), D + R5: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (5 mg·kg^{-1}), D + R20: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (20 mg·kg^{-1})

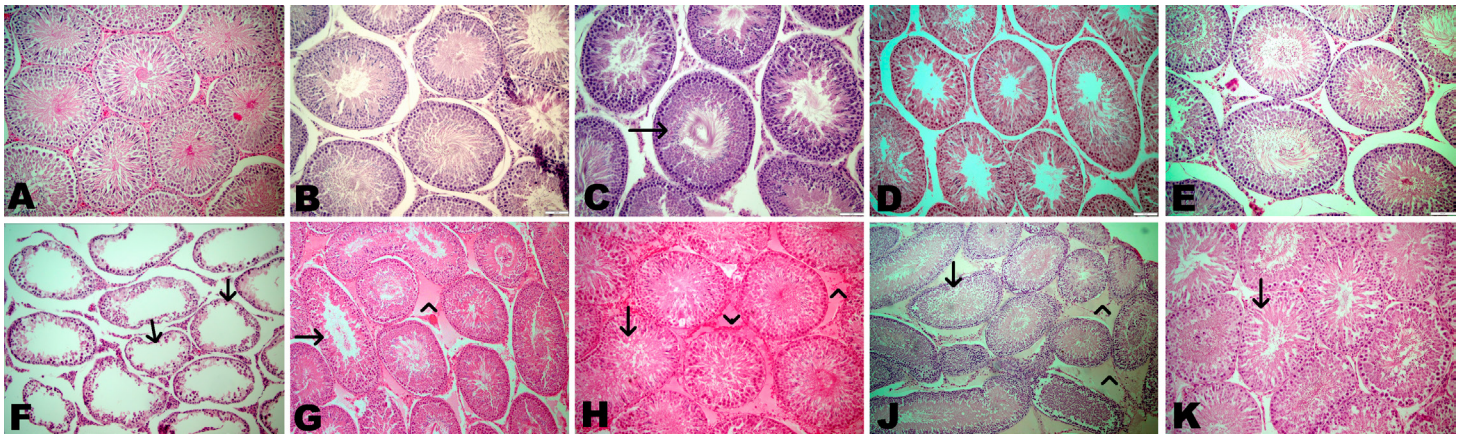


FIGURE 4. A: Normal histologic appearance of seminiferous tubules, Control; B, C, D, E: Normal histologic appearance, germinal cell layer thickness and spermatogenesis in seminiferous tubules. Respectively, T5, T20, R5, R20; F: Atrophy of seminiferous tubules, degeneration, desquamation and significant reduction of germinal cells, interstitial edema and hyperemia, Doxorubicin (arrow); G: Improvement in histologic appearance of seminiferous tubules, slight decrease in germinal cell thickness and interstitial edema, D + T5; G, H: Improvement in histological appearance of seminiferous tubules, slight decrease in germinal cell thickness (arrow), interstitial edema and hyperemia (arrow head), D + T5, D + T20; J,K: Improvement in histologic appearance of seminiferous tubules, normal germinal cell layer thickness (arrow) and interstitial edema (arrowhead), D + R5, D + R20, Hematoxylene-Eosin Staining, 20×, 500 µm. Abbreviations: D: Doxorubicin (10 mg·kg^{-1}), T5: Thymoquinone (5 mg·kg^{-1}), T20: Thymoquinone (20 mg·kg^{-1}), R5: Resveratrol (5 mg·kg^{-1}), R20: Resveratrol (20 mg·kg^{-1}), D + T5: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (5 mg·kg^{-1}), D + T20: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (20 mg·kg^{-1}), D + R5: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (5 mg·kg^{-1}), D + R20: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (20 mg·kg^{-1})

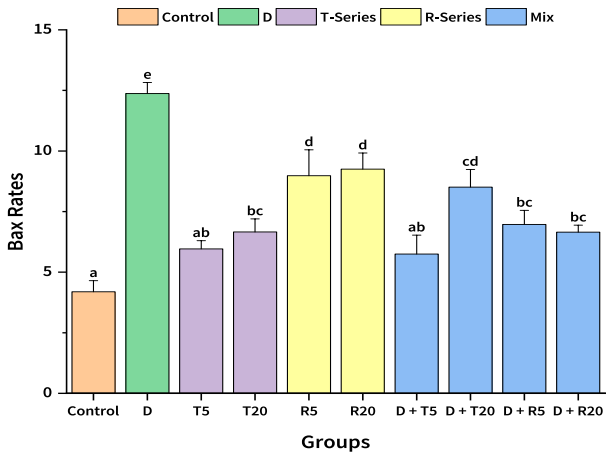
Immunohistochemical findings

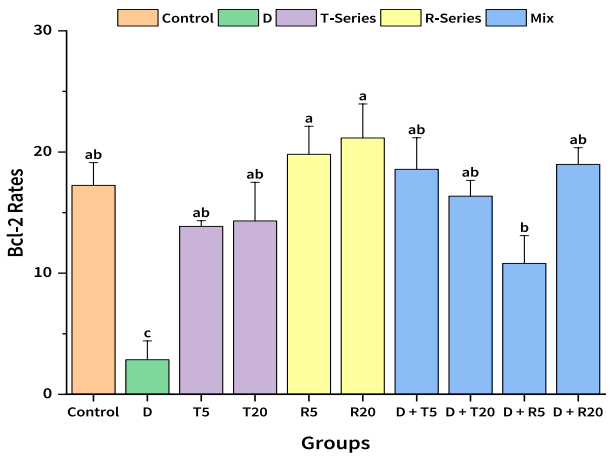
Bax expression was lowest in the C group and significantly increased in the D group, consistent with its pro-apoptotic effect (P<0.05). Among antioxidant-only groups, T20, R5, and R20 exhibited elevated Bax levels compared to C, whereas T5 did not. Notably, Bax expression in the D + T5 group was statistically similar to the C, indicating strong anti-apoptotic protection (P<0.05). Other co-treatment groups (D + T20, D + R5, D + R20) showed partial protection, with Bax levels lower than in the D group but still above C values (P<0.05; FIG. 5).

Bcl-2 expression was significantly reduced in the D group compared to all others (P<0.05). Antioxidant-only groups (T5, T20, R5, R20) showed Bcl-2 levels comparable to the C (P>0.05), indicating preserved anti-apoptotic activity. Similarly, all combination groups (D + T5, D + T20, D + R5, D + R20) displayed significantly higher Bcl-2 expression than the D group (P<0.05), suggesting a protective effect.

Immunohistochemical analyses have revealed that D increases Bax expression while decreasing Bcl-2 levels. This clearly indicates increased apoptosis in the D-treated groups. When administered alone, R and T were observed to slightly increase Bax levels and decrease Bcl-2; this is likely due to their transient pro-oxidant effects. However, when administered together with D, these effects were reversed, the Bax/Bcl-2 balance was restored, and their anti-apoptotic properties emerged [47, 48].

Taken together, the present findings clearly demonstrate that R and T effectively mitigate D-induced testicular damage by preserving spermatological parameters, mitochondrial function, and testicular histoarchitecture. These results support the view that targeting oxidative stress and apoptotic imbalance represents a rational strategy for reducing chemotherapy-associated gonadotoxicity. Although extrapolation to clinical settings requires caution, the consistent protective patterns observed in this experimental model underscore the potential of these antioxidants as supportive agents during chemotherapeutic regimens.





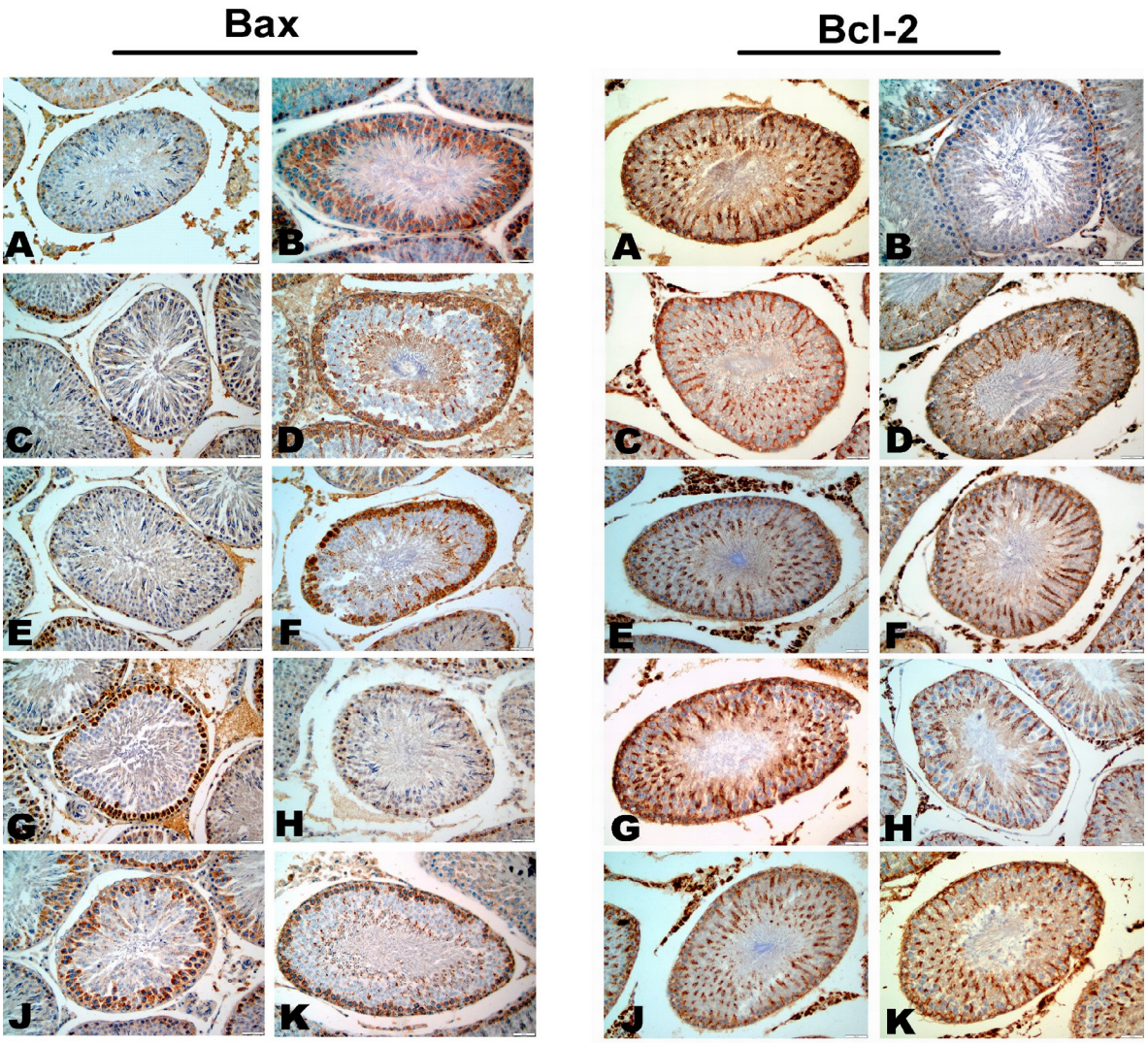


FIGURE 5. Comparison of Bax and Bcl-2 rates (*P*<0.05). Data are presented as mean ± standard deviation. Superscript letters indicate statistically significant differences from the control group, specifically for Bax and Bcl-2 immunoreactivity in the seminiferous tubules (spermatogonia, spermatocytes, Sertoli cells, and spermatids). For the immunohistochemical staining images, brown-colored staining is observed in the groups with high Bax and Bcl-2 activity, whereas in the groups with low activity, the staining appears blue·purple^{-1}. A: Control, B: Doxorubicin, C:T5, D (for images):T20, E:R5, F: R20, G:D + T5, H: D + T20, I: D + R5, J: D + R20, Immunohistochemistry, DAB-Hematoxylene,40×,500µm. Abbreviations: Bcl-2: B-cell lymphoma 2 (antiapoptotic), Bax: Bcl-2–associated X protein (pro-apoptotic), D (for graphics): Doxorubicin (10 mg·kg^{-1}), T5: Thymoquinone (5 mg·kg^{-1}), T20: Thymoquinone (20 mg·kg^{-1}), R5: Resveratrol (5 mg·kg^{-1}), R20: Resveratrol (20 mg·kg^{-1}), D + T5: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (5 mg·kg^{-1}), D + T20: Doxorubicin (10 mg·kg^{-1}) plus thymoquinone (20 mg·kg^{-1}), D + R5: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (5 mg·kg^{-1}), D + R20: Doxorubicin (10 mg·kg^{-1}) plus resveratrol (20 mg·kg^{-1})

CONCLUSIONS

In conclusion, both R and T exerted protective effects against D-induced testicular toxicity. While R demonstrated more consistent benefits across spermatological, histopathological, and mitochondrial parameters, T also provided significant protection, particularly at a dose of 20 mg·kg^{-1}. The dose – and context-dependent behavior of these antioxidants highlights the need for further research before clinical application. However, the results obtained are relevant and position these antioxidants as possible alternatives for reducing the side effects of chemotherapy in cancer patients.

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Conflict of interests

The authors declare that there are no financial, academic, or personal conflicts of interest related to this research work.

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