RESEARCH ARTICLE

Doxorubicin-Induced Reproductive Dysfunction in Wistar Rats: Biochemical, Oxidative Stress, Enzymatic and Spermatological Evaluation with Hesperidin Amelioration

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Abstract

Doxorubicin (DOX) is a widely used chemotherapeutic agent whose clinical application is limited by its adverse effects on male reproductive function. The present study was undertaken to evaluate the protective effect of hesperidin against DOX-induced reproductive toxicity in 40 adult male Wistar rats. The rats were randomly allocated to five groups, each of 8 animals, comprising a control group, a DOX-treated group, a DOX plus benazepril group, and DOX plus hesperidin (100 and 200 mg/kg) groups. Treatments were administered for 28 days, after which serum biochemical parameters, testicular oxidative stress markers, lactate dehydrogenase activity and epididymal sperm characteristics were evaluated. Doxorubicin administration caused significant reproductive dysfunction, evidenced by increased serum alkaline phosphatase and testicular malondialdehyde levels, decreased testosterone, superoxide dismutase and testicular LDH activities, and marked deterioration in sperm count, motility, viability and morphology. Co-administration of hesperidin significantly ameliorated these alterations in a dose-dependent manner, with the higher dose (200 mg/kg) restoring most parameters close to normal values and exhibiting greater protective efficacy than benazepril. These findings demonstrate that hesperidin effectively attenuates doxorubicin-induced reproductive toxicity through its antioxidant and gonadoprotective actions and may serve as a promising adjunctive agent for preserving male reproductive function during doxorubicin-based chemotherapy.

Keywords: Doxorubicin, Hesperidin, Oxidative stress, Reproductive toxicity, Serum biochemical assay.

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Introduction

Doxorubicin (DOX), an anthracycline antibiotic derived from *Streptomyces peucetius var. caesius*, is one of the most effective and widely used chemotherapeutic agents for a broad spectrum of malignancies in humans and veterinary species, including solid tumours and haematologic cancers (Minotti *et al*., 2004; Carvalho *et al*., 2009). Despite its established antineoplastic efficacy, its clinical utility is constrained by cumulative dose-dependent toxicity affecting multiple organ systems. Among these, DOX-induced testicular toxicity is of increasing clinical relevance, as male patients of reproductive age undergoing DOX-based chemotherapy are at significant risk of developing oligospermia, azoospermia and permanent male infertility (El-Maddawy *et al*., 2019).

The pathophysiological basis of DOX-induced reproductive dysfunction is multifactorial. Redox cycling of the quinone moiety of DOX generates superoxide anions, hydrogen peroxide and hydroxyl radicals through the Haber-Weiss reaction (Licata *et al*., 2000). The resulting oxidative stress impairs the antioxidant enzyme superoxide dismutase (SOD) and drives lipid peroxidation, measurable as elevated malondialdehyde (MDA), in spermatogenic and Leydig cells (Guleş *et al*., 2022). Doxorubicin further disrupts the hypothalamo-pituitary-gonadal axis and inhibits steroidogenic enzyme activity, resulting in reduced serum testosterone, impaired Sertoli cell function and depletion of the spermatogenic cell population, collectively reflected in reduced testicular lactate dehydrogenase (LDH-X) activity and deteriorated sperm parameters (El-Maddawy *et al*., 2019; Mustafa *et al*., 2022).

Serum alkaline phosphatase (ALP), a phosphohydrolase expressed in Sertoli cells, germ cells and hepatocytes, serves as an indicator of cellular membrane destabilisation and

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oxidative tissue damage (Swamy *et al*., 2012; Soliman *et al*., 2014). Elevated serum ALP following DOX exposure reflects broad systemic oxidative membrane injury extending to testicular tissue. Testicular LDH, specifically the germinal cell-associated LDH-X (LDH-C4) isoenzyme, is essential for anaerobic energy metabolism in developing spermatocytes and spermatids, and its reduction is a sensitive indicator of germ cell depletion (Rizk *et al*., 2014; Mustafa *et al*., 2022).

Hesperidin, a bioflavonoid glycoside (3,5,7-trihydroxyflavanone 7-rhamnoglucoside) naturally abundant in citrus fruits, possesses well-documented antioxidant, anti-inflammatory and anti-apoptotic properties (Garg *et al*., 2001; Khamis *et al*., 2023). Hesperidin's hydroxyl group-mediated free radical scavenging capacity and suppression of lipid peroxidation position it as a promising gonadoprotective adjunct in the context of chemotherapy-induced reproductive toxicity. Benazepril, a non-sulfhydryl ACE inhibitor, was included as a pharmacological reference comparator to assess indirect gonadoprotection through angiotensin II-mediated NADPH oxidase inhibition. The present study was designed to evaluate the dose-dependent ameliorative efficacy of hesperidin against doxorubicin-induced biochemical, oxidative stress, enzymatic and spermatological dysfunction in male Wistar rats.

Materials and Methods

The study was approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari (No. 149-VCN-VPP-2025; dt 21/06/2025). Forty adult male Wistar rats (6-8 weeks; 200-350 g), procured from Aarsh Research and Development Centre, Daman, were housed under controlled laboratory conditions (22 ± 3°C; 50-60% relative humidity; 12 h light/dark cycle) with *ad libitum* access to standard pelleted diet (M/S VRK Nutritional Solutions, Pune) and filtered RO water. Animals were acclimatised for seven days before commencement of the experimental period.

Experimental Design

Forty rats were randomly divided into five equal groups (n = 8). Group I served as the vehicle control and received 0.5% carboxymethyl cellulose (CMC) orally once daily. Group II received doxorubicin (2.5 mg/kg b.wt., i.p.) once weekly for four weeks. Group III received doxorubicin along with benazepril (10 mg/kg b.wt., orally, once daily). Groups IV and V received doxorubicin in combination with hesperidin at doses of 100 and 200 mg/kg b.wt., respectively, administered orally once daily. All treatments were continued for 28 days. Doxorubicin (Pfizer, India) was administered as a 2 mg/mL injectable solution, while benazepril and hesperidin (Sigma-Aldrich, USA) were suspended in 0.5% CMC immediately before administration. Oral treatments were initiated concurrently with the first doxorubicin injection and continued throughout the experimental period.

Serum Biochemical Assays

Blood was collected from the retro-orbital plexus under light anaesthesia at the end of the 28-day experimental period. Serum was separated by centrifugation (1500 × g for 15 min) and stored at –20°C until analysis. Serum ALP activity was estimated spectrophotometrically using a commercially available ALP diagnostic kit (Beacon Diagnostics Pvt. Ltd., Gujarat, India) using a semi-automated biochemistry analyser (Micro Lab RX-50V). Serum testosterone was estimated using a commercially available competitive ELISA kit (G Biosciences, USA) and a microplate reader (Multiscan Go, Thermo Fisher Scientific).

Testicular Tissue Processing and Oxidative Stress Markers' Assay

One testis from each rat was frozen at –20°C immediately after necropsy and subsequently used for biochemical analysis. Testicular tissue was accurately weighed, homogenised in chilled 0.1 M phosphate buffer (pH 7.4) at a concentration of 10% w/v using a micro-pestle on ice, and centrifuged at 10,000 × g for 20 min at 4°C (Patil and Balaraman, 2009). The resultant clear supernatant was used for all enzyme and lipid peroxidation assays. Total protein was estimated by the Lowry method. Superoxide dismutase (SOD) activity was estimated spectrophotometrically using the pyrogallol auto-oxidation method and expressed as U/mg protein. Malondialdehyde (MDA) concentration, as an index of lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) method measuring absorbance at 532 nm and expressed as nmol/mg protein.

Testicular tissue was homogenised in ice-cold 0.1 M Tris buffer (pH 8.1) and centrifuged at 10,000 × g for 45 min at 4°C. LDH activity in the supernatant was estimated using a commercially available LDH assay kit (Diatek Healthcare Pvt. Ltd., India) using a semi-automated analyser (Micro Lab RX-50V) (Rizk *et al*., 2014).

Epididymal Semen Analysis

The left cauda epididymis was excised immediately after necropsy and placed in 2 mL of pre-warmed (37°C) phosphate-buffered saline (PBS) in a sterile petri dish. The tissue was minced with a sterile blade to release spermatozoa, forming a homogeneous sperm suspension (Hafez, 1970), which was maintained at 37°C throughout all analyses.

A drop of freshly prepared sperm suspension was placed on a pre-warmed clean glass slide, covered with a pre-warmed coverslip and examined promptly under a light microscope (40× objective) while maintaining physiological temperature conditions. Spermatozoa exhibiting progressive or non-progressive forward movement were classified as motile. Motility was calculated across multiple random fields and expressed as a percentage, following the method of Slott *et al*. (1991). The sperm suspension was appropriately diluted using a WBC diluting pipette and loaded into a Neubauer

haemocytometer. After allowing spermatozoa to settle for a few minutes, counting was performed under a light microscope using a low-power objective. Total sperm count was calculated using the standard haemocytometric formula accounting for dilution factor, chamber depth and number of squares counted (Hari Priya *et al*., 2024), and expressed as $\times 10^6$/mL.

Smears were prepared from the sperm suspension, air-dried, stained using a standard differential eosin–nigrosin staining method and examined under a light microscope (40× objective). Spermatozoa with any head, midpiece or tail abnormality were classified as morphologically abnormal and results were expressed as percentage (Wyrobek and Bruce, 1975). In the same smear, live spermatozoa with intact plasma membranes remained unstained or faintly stained, while dead spermatozoa absorbed eosin and appeared pink against the dark nigrosin background, thus viability was expressed as a percentage.

Statistical Analysis

All data are expressed as Mean ± Standard Error (SE). Inter-group differences were analysed by one-way ANOVA followed by Duncan's post-hoc test using SPSS v20.0 (IBM Corp., Armonk, NY). A probability value of $p \leq 0.05$ was considered statistically significant.

Results and Discussion

The present study provides comprehensive biochemical and spermatological evidence that doxorubicin at 2.5 mg/kg i.p. once weekly for 28 days induces significant, reproducible and multi-parameter functional reproductive toxicity in male Wistar rats, and that hesperidin co-treatment produces dose-dependent and mechanistically coherent amelioration of all evaluated endpoints (Tables 1-3).

Serum ALP and Testosterone

Serum alkaline phosphatase (ALP) activity and testosterone levels recorded for different experimental groups are shown in Table 1. Group I demonstrated a mean ALP value of 236.33±0.98 U/L, representing the physiological baseline in healthy adult male Wistar rats. Group II exhibited a statistically significant ($p<0.05$) elevation to 281.25±1.43 U/L following DOX administration, representing a 19.0% increase above the control value. Group III recorded 258.25±1.13 U/L, significantly lower than Group II ($p<0.05$) but significantly higher than Group I ($p < 0.05$), indicating partial but incomplete protection by benazepril. Group IV demonstrated 241.75±1.24 U/L and Group V recorded 239.71±1.31 U/L, both significantly lower than Group II ($p<0.05$). Group V was not significantly different from Group I ($p>0.05$), indicating near-complete normalisation of serum ALP at the higher hesperidin dose. A clear dose-dependent reduction in serum ALP with increasing hesperidin dose was evident between Groups IV and V. The significant elevation of serum ALP in Group II reflects systemic oxidative membrane damage extending to both hepatic and testicular cellular membranes. Although primarily a hepatic enzyme, ALP is expressed in testicular Sertoli cells and developing germ cells, where it participates in membrane transport and

Table 1: Serum ALP and testosterone across experimental groups (Mean ± SE; n=8)

Parameter	Group I	Group II	Group III	Group IV	Group V
Serum ALP (U/L)	236.33a ± 0.98	281.25d ± 1.43	258.25c ± 1.13	241.75b ± 1.24	239.71ab ± 1.31
Testosterone (ng/mL)	2.48c ± 0.13	2.03a ± 0.08	2.17ab ± 0.09	2.20abc ± 0.04	2.34bc ± 0.09

Means bearing different superscripts in a row differ significantly ($p \leq 0.05$). ALP = alkaline phosphatase.

Table 2: Testicular oxidative stress markers and LDH activity across experimental groups (Mean ± SE; n=8)

Parameter	Group I	Group II	Group III	Group IV	Group V
SOD (U/mg protein)	41.89c ± 1.14	34.11a ± 0.68	37.17b ± 1.18	39.51bc ± 0.88	40.59c ± 1.32
MDA (nmol/mg protein)	0.89a ± 0.13	1.75b ± 0.09	1.41bc ± 0.12	1.25ab ± 0.12	1.01ab ± 0.24
LDH (U/L)	209.75d±1.34	144.00a ±1.73	184.00b ±1.25	203.00c ±1.39	206.13cd ±1.56

Means bearing different superscripts in a row differ significantly ($p \leq 0.05$). SOD = superoxide dismutase; MDA = malondialdehyde; LDH = lactate dehydrogenase.

Table 3: Epididymal semen analysis parameters across experimental groups (Mean ± SE; n=8)

Parameter	Group I	Group II	Group III	Group IV	Group V
Sperm count ($\times 10^6$/mL)	41.73d ± 1.38	29.76a ± 0.68	34.43b ± 1.03	36.45bc ± 1.54	39.11cd ± 1.49
Sperm motility (%)	80.38d ± 1.07	45.69a ± 1.16	64.00b ± 1.47	75.81c ± 1.18	77.63cd ± 1.34
Sperm viability (%)	90.13d ± 0.74	52.50a ± 1.31	71.94b ± 0.88	86.75c ± 0.90	88.50cd ± 0.47
Abnormal sperm (%)	19.38a ± 0.99	45.75d ± 1.00	28.94c ± 0.84	23.88b ± 1.43	21.81ab ± 1.36

Means bearing different superscripts in a row differ significantly ($p \leq 0.05$).

spermatogenic differentiation. Elevated serum ALP following doxorubicin exposure therefore reflects broad cellular membrane destabilisation consequent to ROS-mediated lipid peroxidation. These findings are consistent with Swamy *et al.* (2012), Soliman *et al.* (2014) and Ahmed *et al.* (2019), who documented similar ALP elevations following doxorubicin administration. The dose-dependent reduction in serum ALP by hesperidin, with Group V not significantly different from Group I, is attributable to the flavonoid's hydroxyl group-mediated radical scavenging, inhibition of lipid peroxidation and stabilisation of cellular membranes, consistent with Hozayen *et al.* (2014) and Laila *et al.* (2023). Benazepril's partial ALP reduction reflects its capacity to attenuate angiotensin II-mediated NADPH oxidase activation and ROS generation, thereby partially preserving membrane integrity.

Furthermore, Group I demonstrated a mean testosterone level of 2.48±0.13 ng/mL, consistent with normal Leydig cell steroidogenic function in healthy adult male Wistar rats. Group II exhibited a statistically significant ($p<0.05$) reduction to 2.03±0.08 ng/mL, indicating impaired androgenic function consequent to doxorubicin-induced gonadotoxicity. Group III recorded 2.17±0.09 ng/mL, which was significantly higher than Group II ($p<0.05$), suggesting partial restoration of Leydig cell steroidogenesis by benazepril. Group IV demonstrated 2.20±0.04 ng/mL and Group V recorded 2.34±0.09 ng/mL, both significantly higher than Group II ($p<0.05$), confirming dose-dependent restoration of Leydig cell steroidogenic function by hesperidin. The significant reduction in serum testosterone in Group II over Group I reflects both direct and indirect gonadotoxic mechanisms of doxorubicin on the hypothalamo-pituitary-gonadal (HPG) axis and testicular steroidogenesis. Direct mechanisms include ROS-mediated damage to Leydig cell mitochondria and endoplasmic reticulum, disrupting the cholesterol transport protein StAR and steroidogenic enzyme CYP11A1, thereby impairing the first and rate-limiting step of testosterone biosynthesis. Indirect mechanisms involve doxorubicin-induced suppression of gonadotrophin secretion and alteration of Sertoli cell paracrine signalling. These findings are in accordance with Patil and Balaraman, (2009), Mohamed *et al.* (2015), El-Maddawy *et al.* (2019) and Guleş *et al.* (2022). The dose-dependent testosterone restoration in Groups IV and V reflects hesperidin's capacity to protect Leydig cell mitochondrial and steroidogenic enzyme function from doxorubicin-induced oxidative damage, consistent with Hozayen (2012), Tekin and Celebi (2022) and Hari Priya *et al.* (2024).

Testicular Oxidative Markers and LDH

Testicular tissues oxidative markers, *viz.,* SOD, MDA and LDH activities found in rats under different experimental groups are presented in Table 2. Group I demonstrated a mean testicular superoxide dismutase (SOD) and malondialdehyde (MDA) activity of 41.89±1.14 U/mg protein and 0.89±0.13 nmol/mg protein, respectively, reflecting a robust enzymatic antioxidant defence in healthy adult male Wistar rats. Group II exhibited a statistically significant ($p<0.05$) reduction of SOD to 34.11±0.68 U/mg protein and elevation of MAD to 1.75±0.09 nmol/mg protein, indicating significant doxorubicin-induced lipid peroxidation and impairment of the primary enzymatic antioxidant defence within testicular tissue. Group III recorded significantly higher SOD 37.17±1.18 U/mg protein and lower MDA 1.41±0.12 nmol/mg protein than Group II ($p<0.05$) but significantly inverse trend than in Group I ($p<0.05$), indicating partial attenuation of lipid peroxidation and restoration by benazepril. Group IV & V demonstrated SOD 39.51 ± 0.88 and 40.59±1.32 U/mg protein, and MDA 1.25±0.12 and 1.01±0.24 nmol/mg protein, SOD in both groups significantly higher, while MDA significantly lower than Group II ($p<0.05$). Group V was not statistically different from Group I, confirming near-complete normalisation of testicular SOD activity at the higher hesperidin dose. A clear dose-dependent reduction of testicular lipid peroxidation with improvement in SOD was evident across Groups II through V, Group V showing the greatest approach toward the normal control value.

The significant reduction in testicular SOD activity in Group II over Group I and the reciprocal significant elevation of MDA collectively confirm severe doxorubicin-induced oxidative stress and lipid peroxidation in testicular tissue, consistent with Trivedi *et al.* (2011), Rizk *et al.* (2014), Mohamed *et al.* (2015), Owumi *et al.* (2020) and Guleş *et al.* (2022). Doxorubicin's quinone moiety undergoes enzymatic one-electron reduction to semiquinone radicals, which are oxidised back by molecular oxygen, generating a continuous flux of superoxide anions. The resulting hydrogen peroxide participates in iron-catalysed Fenton reactions, producing highly reactive hydroxyl radicals that overwhelm testicular antioxidant defences and initiate lipid peroxidation of germ cell membranes. The dose-dependent restoration of testicular SOD and suppression of MDA in Groups IV and V by hesperidin is attributable to its catechol-type B-ring hydroxyl groups, which directly scavenge superoxide, hydroxyl and peroxyl radicals, chelate redox-active iron ions, and upregulate endogenous antioxidant enzyme expression through activation of the Nrf2/HO-1 signalling pathway. These mechanisms are consistent with Kaya *et al.* (2015), Afolabi *et al.* (2018) and Hari Priya *et al.* (2024).

Further, Group I demonstrated a mean Lactate Dehydrogenase (LDH) activity of 209.75±1.34 U/L, reflecting robust spermatogenic enzyme function in healthy adult male Wistar rats. Group II exhibited a statistically significant ($p<0.05$) reduction to 144.00±1.73 U/L, indicating severe impairment of testicular energy metabolism and spermatogenic cell function (Table 2). This reduction corresponds to a 31.3% decline from the control value. Group III recorded 184.00±1.25 U/L, which was significantly higher than Group II ($p<0.05$) but significantly lower than Group I ($p<0.05$), indicating partial restoration of testicular LDH activity by benazepril. Group IV demonstrated 203.00±1.39 U/L and Group V recorded 206.13±1.56 U/L, both significantly higher than Group II ($p<0.05$) and not

statistically different from Group I, confirming near-complete normalisation of testicular LDH activity in both hesperidin-treated groups, with a clear dose-dependent trend.

The significant reduction in testicular LDH activity in Group II than in Group I reflects DOX-induced depletion of the LDH-expressing spermatogenic cell population, primarily spermatocytes and spermatids, which depend on anaerobic glycolysis for ATP generation during the later stages of spermatogenesis. Testicular LDH-C4 (LDH-X), the germinal cell-specific isoenzyme, serves as an essential metabolic enzyme supporting sperm energy metabolism and capacitation. Its reduction is therefore a sensitive and specific indicator of spermatogenic cell depletion and functional impairment of the spermatogenic process. These findings are consistent with Rizk *et al.* (2014) and Mustafa *et al.* (2022). The near-complete restoration of testicular LDH in Groups IV and V by hesperidin, with values not significantly different from Group I, reflects preservation of spermatogenic cell populations through antioxidant and anti-apoptotic protection, consistent with Olayinka and Adewole (2021).

Epididymal Semen Analysis

The results of epididymal semen analysis for all experimental groups are presented in Table 3. Doxorubicin administration in Group II resulted in significant impairment ($p<0.05$) of all four semen parameters in comparison to Group I. Group III demonstrated partial but statistically significant recovery across all parameters in comparison to Group II. Groups IV and V showed dose-dependent and statistically significant improvement in all semen parameters, with Group V values approaching or not significantly different from the normal control.

Sperm motility & count: Group I demonstrated a mean sperm motility of 80.38±1.07% and sperm count of 41.73±1.38 $\times10^6$/mL, reflecting the high proportion of progressively motile spermatozoa and total count in healthy adult male Wistar rats. Group II exhibited a statistically significant ($p<0.05$) reduction in motility to 45.69±1.16% and sperm count to 29.76±0.68 $\times10^6$/mL, indicating severe impairment of spermatogenic cell production and sperm flagellar function. Group III recorded 64.00 ± 1.47% sperm motility, and 34.43±1.03 $\times10^6$/mL sperm count, significantly higher than Group II ($p<0.05$), indicating partial restoration by benazepril co-treatment. Group IV & V demonstrated 75.81±1.18% and 77.63±1.34% motility, and 36.45±1.54 $\times10^6$/mL and 39.11±1.49 $\times10^6$/mL sperm count, both significantly higher than Group II ($p<0.05$). Groups IV and V were not significantly different from each other ($p>0.05$) but both were significantly higher than Group II ($p<0.05$), reflecting a clear dose-dependent improvement in sperm motility. Group V values were most closely approaching the normal control, suggesting near normalisation of spermatogenic output and sperm dynamics at the higher hesperidin dose.

Sperm morphology & viability: Group I demonstrated a mean abnormal sperm percentage of 19.38±0.99% and sperm viability of 90.13±0.74%, reflecting the baseline optimal level of naturally occurring morphological abnormalities and viability in healthy adult Wistar rats. Group II exhibited a statistically significant ($p<0.05$) increase in sperm abnormality to 45.75±1.00% and reduction in sperm viability to 52.50±1.31%, indicating severe morphological impairment of spermatozoa and sperm membrane integrity and cellular viability with doxorubicin treatment. Group III demonstrated 28.94 ±0.84% abnormal spermatozoa and 71.94 ± 0.88% sperm viability, *i.e.,* significantly lower and higher, than in Group II ($p<0.05$), indicating partial attenuation/restoration of sperm function by benazepril. Group IV & V recorded 23.88±1.43% and 21.81±1.36% abnormal spermatozoa, and 86.75±0.90% and 88.50±0.47% viability, respectively, both values of abnormality significantly lower and viability higher than Group II ($p<0.05$), with Group V not statistically different from Group I ($p>0.05$), indicating near-complete normalisation of sperm of sperm viability and morphological quality at the higher hesperidin dose.

The significant and comprehensive impairment of all four semen parameters in Group II represents the cumulative functional consequence of all upstream biochemical and oxidative pathological events. Reduced sperm count reflects doxorubicin-induced depletion of the spermatogenic progenitor cell pool through ROS-mediated apoptosis, consistent with Trivedi *et al.* (2011) and El-Maddawy *et al.* (2019). Impaired sperm motility reflects disruption of the structural and bioenergetic determinants of flagellar function, including axonemal protein oxidation and mitochondrial ATP generation deficits, consistent with Turedi *et al.* (2015) and El-Maddawy *et al.* (2019). Reduced sperm viability reflects ROS-mediated oxidative damage to the plasma membrane and mitochondrial membrane of spermatozoa, impairing the electrochemical gradients essential for viability, consistent with El-Maddawy *et al.* (2019) and Guleş *et al.* (2022). The increase in morphologically abnormal spermatozoa reflects doxorubicin's genotoxic disruption of spermiogenesis, causing abnormal head morphology, midpiece defects and flagellar anomalies, consistent with Trivedi *et al.* (2011), Turedi *et al.* (2015) and El-Maddawy *et al.* (2019).

The dose-dependent restoration of all four semen parameters by hesperidin in Groups IV and V, with Group V values approaching the normal control across all endpoints, reflects the bioflavonoid's comprehensive gonadoprotective mechanism encompassing antioxidant preservation of spermatogenic cell populations, anti-apoptotic protection of developing germ cells, membrane-protective effects on sperm plasma and mitochondrial membranes, and testosterone-restorative effects that support the hormonal milieu essential for normal spermatogenesis. The improvement in sperm motility reflects hesperidin-mediated preservation of mitochondrial function and flagellar structural integrity. The reduction in morphological abnormalities reflects its anti-genotoxic and cytoprotective effects on spermatids during spermiogenesis. These observations are consistent

with Afolabi *et al.* (2018), Tekin and Celebi (2022) and Hari Priya *et al.* (2024), who documented significant improvements in semen parameters following hesperidin supplementation across various reproductive toxicity models.

The internal coherence of the findings across all evaluated parameters is notable. The upstream oxidative stress (low SOD, high MDA) and hormonal disruption (low testosterone, elevated ALP) provide a mechanistically consistent explanation for the downstream spermatogenic failure (reduced LDH, impaired semen parameters). Hesperidin's dose-dependent modulation of all upstream biochemical and oxidative endpoints directly predicts and accounts for the dose-dependent improvement in semen parameters, creating a complete and internally consistent mechanistic narrative. The partial protection by benazepril, evident across all parameters, reflects the ACE inhibitor's capacity to reduce angiotensin II-mediated NADPH oxidase activation and thereby partially attenuate testicular ROS generation, consistent with Eid *et al.* (2016) and Elsaeed *et al.* (2023).

Conclusion

The present study conclusively demonstrates that doxorubicin administration at 2.5 mg/kg b.wt. intraperitoneally once weekly for 28 days induces significant, reproducible and mechanistically coherent functional reproductive toxicity in male Wistar rats, encompassing enzymatic & hormonal disruption (elevated serum ALP, reduced testosterone), testicular oxidative stress (reduced SOD, elevated MDA), impaired spermatogenic energy metabolism (reduced LDH) and comprehensive semen quality deterioration (reduced count, motility, viability and increased morphological abnormalities). The observed biochemical and spermatological abnormalities form a mechanistically coherent cascade: doxorubicin-induced ROS generation overwhelms testicular antioxidant defences, drives lipid peroxidation and oxidative DNA damage, disrupts Leydig cell steroidogenesis and depletes spermatogenic cell populations, collectively producing the documented functional reproductive impairment. Hesperidin co-administration @ 100 and 200 mg/kg, b.wt., produced dose-dependent, significant and comprehensive amelioration of all evaluated endpoints, with the 200 mg/kg dose demonstrating superior protective efficacy. Benazepril provided only partial protection. These findings strongly indicate that hesperidin possesses significant multi-targeted gonadoprotective properties and may serve as an effective adjunctive therapy for safeguarding male reproductive structure and function during doxorubicin-based chemotherapy.

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