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Paper

Isorhamnetin: a flavonoid, attenuated doxorubicin-induced testicular injury via regulation of steroidogenic enzymes and apoptotic signaling gene expression in male rats

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Background. Male reproductive damage is one of the most adverse side effects of doxorubicin (DOX). Isorhamnetin is a natural flavonoid, which displays remarkable antioxidant potential.
Objective. The current research was designed to assess the protective effects of Isorhamnetin against DOX-instigated testicular damages.
Methods. Adult male Wistar rats (n=32) were divided into 4 groups: control, DOX (3 mg/kg i.p. 3 doses each after 1 week), DOX + Isorhamnetin (3 mg/kg 3 doses each after 1 week +10 mg/kg i.p. daily for 28 days, respectively), and Isorhamnetin (10 mg/kg i.p. per day). After 28 days of treatment, biochemical, spermatogenic, steroidogenic, hormonal, proapoptotic, antiapoptotic, and histopathological parameters were estimated.
Results. DOX exposure significantly decreased the activity of acid phosphatase, lactate dehydrogenase, and gamma-glutamyl transferase. Furthermore, DOX substantially decreased the activities of antioxidant enzymes, i.e. catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase along with protein content, whereas it increased the malondialdehyde level. It also reduced sperm progressive motility, viability, the number of hypoosmotic tail swelled spermatozoa, and epididymis sperm count and increased the sperm morphological anomalies (head, midpiece, and tail). Besides, it decreased the levels of follicle-stimulating hormone, luteinizing hormone, and plasma testosterone and lowered the expression of steroidogenic enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and steroidogenic acute regulatory protein) and testicular antiapoptotic marker (B-cell lymphoma 2) but increased the expression of proapoptotic markers (BCL2-associated X protein and caspase-3) along with histopathological impairments. However, isorhamnetin prevented all the damages caused by DOX.
Conclusion. Conclusively, Isorhamnetin can be used as a powerful mitigating agent to avert DOX-induced testicular damages.

Key words: doxorubicin; oxidative stress; testicular damage; isorhamnetin; antioxidant.

Introduction

Chemotherapy is one of the most efficient cancer treatments, which uses anticancerous drugs to target rapidly dividing cells.[1] Chemotherapy affects all cells that grow and divide quickly in the body. This includes cancer as well as normal cells, i.e. such as proliferating blood cells in the bone marrow or the cells in the stomach, skin, and reproductive organs.[2] Modern therapies using multiple combinations of chemotherapeutic drugs reduce the cytotoxicity of these drugs to normal tissues and increase their survival rates.[3] However, even after using the safe doses of these drugs, children and young patients exposed to chemotherapy in the prepubertal phase can yet show irreversible impairment or loss of fertility status.[4] Among various antineoplastic agents, at present, almost 60% of children with cancer receive anthracyclines, commonly doxorubicin (DOX).[5] DOX is an anthracycline antibiotic with potent chemotherapeutic activity against a variety of cancers.[6] It is widely used to treat esophageal, lung, and prostate cancers, including malignancies, such as leukemia, sarcoma, and lymphoma.[1] However, the risk of renal, hematological, testicular, pulmonary, and cardiac injuries limits its effective and extensive use in clinical oncology.[2]

The core mechanism of anthracyclines includes the generation of reactive oxygen species (ROS) that defeats the cellular antioxidant defense, which results in cytotoxicity.[3] In the testes, spermatogonia are the main target of DOX, as observed after etoposide treatment, because of their intense and continuous proliferative

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activity. So, adults encountering chemotherapy endure the risk of transient, long-term, or perpetual gonadal impairment.[7] Extensive injuries, such as decreased spermatogonia, degeneration of early spermatocytes, reduction of epididymal sperm count, and sperm motility, have been observed in rats followed by DOX treatment.[8]

Cancer research is facing problems in finding nontoxic and cytoprotective agents that may protect normal tissues during chemotherapy. In recent years, many compounds, especially natural antioxidants, are attaining much attention due to their potential curative effects against various diseases.[9] For this reason, research is being carried out on natural or synthetic antioxidant agents to counter the potential toxicity.[4,10] Isorhamnetin is a natural bioflavonoid compound, which is known to exhibit cardiovascular protection,[11] antioxidant,[12] antiapoptotic,[13] antidiabetic,[14] and antitumor[12] effects. It is present in the leaves, flowers, and fruits of *Hippophae rhamnoides* L., *Ginkgo biloba* L., and in some other plants.[15] Based on these suggested protective effects, the present study was aimed to examine the ameliorative effects of isorhamnetin against DOX-induced testicular dysfunctions.

Materials and methods

Chemicals

DOX was purchased from Dalian Meilun Biotech Co., Ltd, China. Isorhamnetin was purchased from Aktin Labs Phytochemicals Division of Aktin Chemicals, Inc., China.

Animals

This study was accomplished on 32 Wistar adult male rats (250 ± 20 g). They were kept in steel cages at standard temperature (22–25 °C), humidity (45 ± 5%), and 12-h light/dark cycle in the animal house of Agriculture University, Faisalabad. Tap water (H_2O) and food chaw were provided ad libitum. Animals were treated in compliance with the protocol of the European Union for the Animal Care and Experimentation (CEE Council 86/609), which was approved by the ethical board of the University of Agriculture, Faisalabad, Pakistan. Guidelines of the national institute of animal health (NIH guidelines) were strictly followed to perform experiments.

Experimental protocol

Rats were separated into 4 different groups (n = 8/group); group I or the control group, group II or DOX-treated group; DOX dose was selected according to the previous literature.[10] DOX group was administered 3 mg/kg. BW. of DOX intraperitoneally, on the seventh, 14th, and 21st days of the experiment. Group III or DOX + Isorhamnetin-treated group (3 mg/kg BW. of DOX [3 doses each after 1 week] and 10 mg/kg. BW. of Isorhamnetin daily for 21 days) and group IV or Isorhamnetin-only treated group (10 mg/kg BW. i.p. daily for 3 weeks). Isorhamnetin was dissolved in .1% DMSO. The whole experiment lasted for 28 days. After 4 weeks of the study, rats were sacrificed, and blood was drawn from the heart and collected in sterile tubes, which were later on centrifuged for 10 min at 3,000 r/min. Testes were removed from the animals, and the right testis (treated with liquid nitrogen) were kept at −80 °C for biochemical examinations, and the left testis was fixed in 10% formalin for histopathological studies.

Assessment of testicular marker enzymes

Testicular acid phosphatase (ACP) and lactate dehydrogenase (LDH) activities were determined using Randox diagnostic kits. ACP activity was determined by the method of Tietz et al.[16] The p-nitrophenol formed by the hydrolysis of p-nitrophenyl phosphate confers yellowish color on the reaction mixture and its intensity can be monitored at 405 nm to give a measure of enzyme activity. The determination of testicular LDH activity was based on the method of Cabaud and Wróblewski.[17] The level of gamma-glutamyl transferase (γ-GT) in the testes was determined by the kinetic method utilizing the glutamyl-p-nitroanilide as substrate. Experiments were carried out in triplicate.

Biochemical evaluation

Analysis of catalase

The activity of catalase (CAT) was assessed according to Afsar and colleagues' procedure.[18] Frozen testicular tissues from each rat were homogenized in ice-cold phosphate buffer (KCl: 140 mmol/L, phosphate: 20 mmol/L, pH 7.4) and were centrifuged at 14,000 × g for 10 min. CAT activity was ascertained by measuring the exponential disappearance of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as U/mg protein

Analysis of superoxide dismutase

The activity of superoxide dismutase (SOD) was carried out by the method of Afsar et al.[19] The reaction mixture was comprised of 1.2 mL of sodium pyrophosphate buffer (pH 7) and 0.1 mL of phenazine methosulphate. After centrifuging the 0.3 mL of supernatant (1,500 × g for 10 min followed by 10,000 × g for 15 min), the homogenate was poured into the reaction mixture. After that, 0.2 mL of NADH was added to initiate an enzymatic reaction, which was later on terminated by adding the 1 mL of glacial acetic acid. With the help of a spectrophotometer, the chromogen amount was determined by recording absorbance at 560 nm. The results were stated in U/mg protein.

Analysis of glutathione peroxidase

Glutathione peroxidase (GPx) activity was assessed by Lawrence and Burk's method.[20] Testicular homogenates were centrifuged, and the supernatant was used to measure GPX activity. The 0.25 mM of H_2O_2 was used as the substrate for the GPX assay. GPX activity was measured as U/mg protein.

Analysis of glutathione reductase

The activity of glutathione reductase (GSR) was ascertained according to the protocol of Carlberg and Mannervik,[21] with some modifications. The change in absorbance was estimated at 340 nm. NADPH was used as a substrate. An extinction coefficient of 6.22 × 103/M/cm was used for calculations. The values obtained were displayed as nM NADPH oxidized/min/mg tissue.

Analysis of total protein

The evaluation of total protein content was carried out by the protein kit (Cat No. BR5202-S, AMEDA Labordiagnostik GmbH, Krenngasse, Graz, Austria). Results were calculated by plotting the absorbance of standard versus sample absorbance on the graph. Final results were presented in mg/g of tissues.

Analysis of malondialdehyde levels

Malondialdehyde (MDA) level was measured according to the method of Afsar et al.[22] The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3 tetra methoxy propane. The values of MDA were expressed as nmol/mg protein. Every sample was assayed in duplicate, and the assay coefficients of variation for MDA were <3%.

Evaluation of progressive motility, viability, count, and the structural abnormality of sperm

The caudal piece of epididymis was isolated to retrieve the sperm samples. Initially, the epididymal part was finely minced in 5 mL of physiological-saline and was incubated for 30 min at 37 °C for spermatozoa releasing of the epididymal ducts. Sperm progressive motility percentage was noted through the phase-contrast microscope at 400×.[23] Sperm viability was assessed, by eosin or nigrosin staining, accompanied by microscopic evaluation. Moreover, a hemocytometer was employed to count epididymal sperm in the suspension.[24] Furthermore, morphological anomalies of head, tail, and midpiece of sperm were determined in percentage using the method of Filler.[25] The apparent abnormal characteristics included (i) the size and shape of spermatozoa heads (big or small heads) with lighter and emphasized curvature; (ii) intermediary pieces' defects that result in untied heads; and (iii) defects of tails (short, multiple, folded, and broken tails).

Hypo-osmotic swelling test

The integrity of the sperm plasma membrane was assessed by the hypo-osmotic swelling (HOS) test according to a protocol described by Correa and Zavos.[26] HOS test was carried out by placing 20 μL of semen in 180 μL of fructose solution and maintaining the osmotic pressure at 80 mosm/L for about 20 min. Subsequent incubating and processing, the sperm were stained with eosin or nigrosin. Finally, 200 spermatozoa with swollen and nonswollen tails were examined using a light microscope (40×).

Hormonal analysis

Quantitative EIA kits were used for the measurement of testosterone (BioCheck Inc., US Catalog No. BC-1115), luteinizing hormone (LH; BioCheck Inc., US Catalog No. BC-1031), and follicle-stimulating hormone (FSH; BioCheck Inc., US Catalog No. BC-1029) concentrations in the tissues and the assays were performed by the instructions with the kits. The intraassay coefficient of variation for testosterone, FSH, and LH were 3.9, 3.6, and 5.2%, respectively. All the assays were repeated with both inter- and intravariations for precise results.[27]

Ribonucleic acid extraction and real-time quantitative reverse transcription-polymerase chain reaction

Expression of the steroidogenic 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), and steroidogenic acute regulatory protein (StAR), BCL2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), and caspase-3 were determined by RT-qPCR. TRIzol (Invitrogen) reagent (Life Technologies, New York, United States) was employed to isolate total ribonucleic acid (RNA), while RNA concentrations were assessed by Nano-Drop 2000c spectrophotometer. Total RNA was selected with A260/A280 ratio between 1.8 and 2.2 was used in reverse transcriptase PCR. Reverse transcription of RNA transformed it into complementary deoxyribonucleic acid (DNA) by using total RNA by Fast Quant RT kit (Takara, China). The RT-qPCR was carried out in 25 μL of reaction volume using the SYBR Green. No template control and minus reverse transcriptase control were used as a negative control. Alterations in the expression of these steroidogenic enzymes and apoptotic markers were observed by $2^{-\Delta\Delta CT}$ and by considering β-actin as the internal control.[28] Primer sequences of β-actin and target genes are displayed in Table 1, as reported previously.[29]

Histopathology

For the assessment of testicular histopathology, testicular tissues from the left testis were fixed for about 48 h in a 10% formaldehyde solution, dehydrated in ascending grades of alcohol, and embedded in paraffin wax. Then, 5-μm thickness sections were cut transversely, stained with hematoxylin–eosin, and 100 tubular sections per testis were randomly analyzed under a light microscope (Nikon, 187842, Japan). Leica-LB (Olympus Optical Co. LTD, Japan) was used for capturing the photographs of specimens. Stereological analyses, such as interstitial spaces, tunica propria thickness, the diameter of seminiferous tubules, the epithelial height of seminiferous tubules, and tubular lumen, were performed with the help of Image-J2X software by using photographs, whereas spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids were identified based on their morphology.

Table 1. Primers sequences for RT-qPCR.

Gene	Primers 5′ -> 3’	Accession number	Amplicon length	Citation
3β-HSD	Forward: GCATCCTGAAAAATGGTGGC Reverse: GCCACATTGCCTACATACAC	NM_001007719	135	[35]
17β-HSD	Forward: CAGCTTCCAAGGCTTTTGTG Reverse: CAGGTTTCAGCTCCAATCGT	NM_054007	161	[35]
StAR	Forward: AAAAGGCCTTGGGCATACTC Reverse: CATAGAGTCTGTCCATGGGC	NM_031558	113	[35]
Bax	Forward: GGCCTTTTTGCTACAGGGTT Reverse: AGCTCCATGTTGTTGTCCAG	NM_017059.2	119	[35]
Bcl-2	Forward: ACAACATCGCTCTGTGGAT Reverse: TCAGAGACAGCCAGGAGAA	NM_016993.1	103	[35]
Caspase-3	Forward: ATCCATGGAAGCAAGTCGAT Reverse: CCTTTTGCTGTGATCTTCCT	NM_012922.2	233	[35]
β-Actin	Forward: TACAGCTTCACCACCACAGC Reverse: GGAACCGCTCATTGCCGATA	NM_031144	135	[35]

Table 2. The score for evaluating spermatogenesis (JS).

Score	Level of spermatogenesis
10	Full spermatogenesis
9	Slightly impaired spermatogenesis
8	<5 spermatozoa per tubule
7	No late spermatids; many early spermatids
6	Few early spermatids; arrest of spermatogenesis at the spermatid stage
5	
4	Many spermatocytes
3	Few spermatocytes; arrest of spermatogenesis at the primary spermatocyte stage
2	
1	Spermatogonia only

Spermatogenesis was also assessed according to the Johnsen score (JS) method (Table 7). For this, the JS (scale of 1–10 based on the level of spermatogenesis) was evaluated for each seminiferous tubule (Table 2). Then, the JS of each sample was assessed.[30]

Statistical analysis

The results were shown as mean ± SEM. The Kolmogorov–Smirnov test was used to check the normal distribution of the data. Tukey's test was applied for the entire data after using the 1-way analysis of variance. Chi-square analysis was applied for the JS analysis. $P < 0.05$ was considered to be statistically significant.

Results

Effect of Isorhamnetin on testicular marker enzymes

Testicular marker enzymes ACP, LDH, and γ-GT in testis were assessed to interpret the damage in testicular functions. In the testis of DOX administrated rats, the ACP, LDH, and γ-GT levels were significantly ($P < 0.05$) lower than that of the control group. Whereas the treatment of Isorhamnetin with DOX significantly ($P < 0.05$) increased their levels. Isorhamnetin-alone-treated group showed the normal levels of these testicular marker enzymes which were comparable to control (Table 3).

Effect of Isorhamnetin on biochemical parameters (CAT, SOD, GPx, GSR, protein content, and MDA level)

DOX administration significantly ($P < 0.05$) lowered the CAT, SOD, GPx, GSR activities, and total protein content, while it elevated the MDA level in comparison to the control group. Instead, Isorhamnetin supplementation in the cotreated group caused substantial ($P < 0.05$) elevation in CAT, SOD, GPx, and GSR activities, protein content along with a reduction in MDA level in comparison to the DOX group. Isorhamnetin-alone treatment displayed the normal activity of antioxidant enzymes and MDA levels near to the control group (Table 4).

Effect of Isorhamnetin on sperm motility, viability, count, and HOS test of sperm

DOX exposure significantly ($P < 0.05$) decreased sperm progressive motility, viability, epididymal sperm count, and the number of HOS coil-tailed sperm, while morphological sperm abnormalities (sperm head, midpiece, and tail) were significantly ($P < 0.05$) raised in the DOX-administered group in comparison to the control group. Conversely, Isorhamnetin treatment significantly ($P < 0.05$) protected all these sperm abnormalities in the cotreated group as compared to the DOX group. Besides, no substantial change was detected between the mean values of Isorhamnetin-alone and the control groups. Table 5 shows the mean values of spermatogenic indices.

Effect of Isorhamnetin on the steroidogenic enzymes (3β-HSD, 17β-HSD, and StAR) expression

Figure 1 represents the relative alterations in the steroidogenic enzyme expressions. DOX administration considerably ($P < 0.05$) reduced the StAR, 3β-HSD, and 17β-HSD expressions as contrasted with the control group. However, Isorhamnetin + DOX treatment significantly ($P < 0.05$) prevented the reduction in steroidogenic enzyme expressions in the cotreated group compared to the DOX group. Isorhamnetin-alone treatment displayed

Table 3. Mean ± SEM of testicular marker enzymes in control, DOX-treated, cotreated, and Isorhamnetin groups.

	Control	DOX	DOX + Isorhamnetin	Isorhamnetin
ACP (U/g)	182.7 ± 5.25^{a}	38.51 ± 3.27^{b}	133.9 ± 5.44^{c}	196.3 ± 6.59^{a}
LDH (U/g)	2395 ± 77.8^{a}	768.9 ± 41.7^{b}	1900 ± 77.4^{c}	2528 ± 95.7^{a}
γ-GT (U/g)	38.72 ± 2.02^{a}	$9.25 \pm .83^{b}$	33.28 ± 1.00^{c}	40.16 ± 1.85^{a}

Results are represented in mean ± SEM (n = 8 rats/group). Values having different superscripts in a same row are significantly different from other groups.

Table 4. Mean ± SEM of biochemical parameters in the testicular tissues of control, DOX-treated, cotreated, and Isorhamnetin groups.

	Control	DOX	DOX + Isorhamnetin	Isorhamnetin
CAT (U/mg protein)	$8.96 \pm .54^{a}$	$4.36 \pm .22^{b}$	$7.51 \pm .27^{c}$	$8.84 \pm .31^{a}$
SOD (U/mg protein)	$5.94 \pm .16^{a}$	$2.07 \pm .16^{b}$	$4.96 \pm .09^{c}$	$6.03 \pm .26^{a}$
GSR (nm NADPH oxidized/min/mg tissue)	$3.64 \pm .13^{a}$	$1.30 \pm .10^{b}$	$3.05 \pm .13^{c}$	$3.55 \pm .19^{ac}$
GPx (U/mg protein)	$13.64 \pm .13^{a}$	$7.12 \pm .15^{b}$	$10.54 \pm .18^{c}$	$14.4 \pm .46^{a}$
Total protein content (mg/g of tissues)	268.0 ± 5.30^{a}	110.5 ± 4.21^{b}	224.1 ± 3.92^{c}	277.0 ± 4.76^{a}
MDA (nmol/mg protein)	$0.76 \pm .16^{a}$	$2.23 \pm .12^{b}$	$1.27 \pm .07^{c}$	$0.80 \pm .16^{a}$

Results are represented in mean ± SEM (n = 8 rats/group). Values having different superscripts in a same row are significantly different from other groups.

Table 5. Mean ± SEM of spermatogenic parameters in control, DOX-treated, cotreated, and Isorhamnetin groups.

	Control	DOX	DOX + Isorhamnetin	Isorhamnetin
Motility (%)	$73.47 \pm .98^{a}$	38.17 ± 1.49^{b}	61.37 ± 1.12^{c}	$73.97 \pm .91^{a}$
Dead sperms (%)	$18.71 \pm .77^{a}$	64.49 ± 1.56^{b}	27.38 ± 1.26^{c}	$19.85 \pm .81^{a}$
Head abnormality (U/mg protein)	$4.69 \pm .03^{a}$	$10.68 \pm .60^{b}$	$5.86 \pm .15^{c}$	$4.72 \pm .14^{a}$
Midsperm abnormality (%)	$0.83 \pm .06^{a}$	$6.08 \pm .18^{b}$	$1.68 \pm .10^{c}$	$0.78 \pm .07^{a}$
Tail abnormality (%)	$2.33 \pm .12^{a}$	$9.35 \pm .33^{b}$	$4.02 \pm .10^{c}$	$2.28 \pm .20^{a}$
Hypo-osmotic swelled sperm count (%)	68.86 ± 1.79^{a}	33.59 ± 1.34^{b}	59.52 ± 1.13^{c}	69.74 ± 1.64^{a}
Epididymal sperm count (million/mL)	23.15 ± 1.18^{a}	$12.56 \pm .54^{b}$	$19.44 \pm .48^{c}$	22.80 ± 1.21^{a}

Results are represented in mean ± SEM (n = 8 rats/group). Values having different superscripts in a same row are significantly different from other groups.

normal steroidogenic enzyme expressions with no significant changes as compared to the control group.

Effect of Isorhamnetin on hormonal levels (LH, FSH, and plasma testosterone)

DOX exposure substantially ($P < 0.05$) reduced the FSH, LH, and plasma testosterone levels in DOX administration rats as compared to the control group. Whereas Isorhamnetin + DOX treatment remarkably ($P < 0.05$) prevented the reduction in hormonal levels in the cotreated group as matched with the control group. Treatment with Isorhamnetin-alone displayed normal hormonal levels with no significant changes. Table 6 shows the mean values of the hormonal levels in the treated groups.

Effect of Isorhamnetin on the gene expression of antiapoptotic (Bcl-2) and apoptotic (Bax and caspase-3) markers

DOX administration significantly ($P < 0.05$) reduced the gene expression of the antiapoptotic-marker (Bcl-2), whereas the expressions of apoptotic-markers (caspase-3 and Bax) were notably ($P < 0.05$) enhanced in the DOX exposed group as compared to the control group. Instead, cotreatment with Isorhamnetin substantially ($P < 0.05$) prevented the downregulation of the expression of antiapoptotic and the upregulation of the expression of apoptotic markers compared to the DOX-administered group. Isorhamnetin-alone treatment displayed normal expressions of antiapoptotic and apoptotic markers with no remarkable changes as matched with the control group. Figure 2 displays the mean values of antiapoptotic and apoptotic marker gene expressions.

Effect of Isorhamnetin on histopathological parameters

The histopathological alterations following DOX and Isorhamnetin treatment are shown in Table 7 and Fig. 3. The outcomes of the study showed that DOX exposure significantly ($P < 0.05$) reduced the diameter and lowered the epithelial height of seminiferous tubules. DOX exposure significantly ($P < 0.05$) reduced the height of tunica propria. Furthermore, it scaled up the luminal diameter of tubules. DOX treatment also significantly ($P < 0.05$) reduced the spermatogonia, primary-secondary spermatocytes, and spermatids in contrast to the control group. But, the treatment with Isorhamnetin significantly ($P < 0.05$) protected all these structural damages as well as the germ cell count in testicles of cotreated groups as compared to the DOX group.

The present study revealed that the JS was significantly ($P < 0.05$) decreased in the DOX-administered group in contrast to the control group. The JS of the DOX + Isorhamnetin was prevented from decrease as compared to the DOX group. No significant difference was noted between the Isorhamnetin-only treated group and the control group (Table 7).

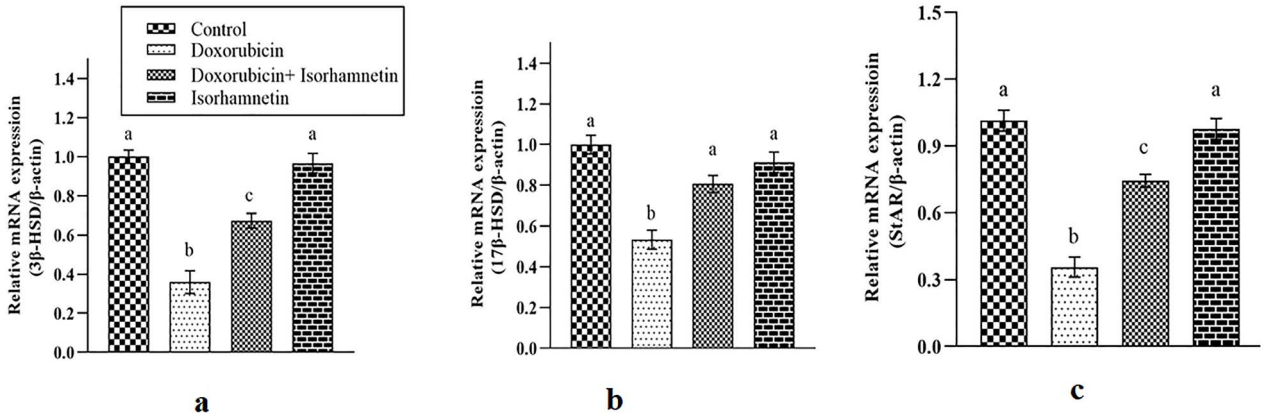

Fig. 1. Outcomes of the DOX and Isorhamnetin exposure on the expression of a) 3β-HSD, b) 17β-HSD, and c) StAR. Verticle bars are presented on the base of mean ± SEM values (n = 8/group). Different superscripts on the bars are presenting significant ($P < 0.05$) difference.

Table 6. Mean ± SEM of hormonal levels in control, DOX-treated, cotreated, and Isorhamnetin groups.

	Control	DOX	DOX + Isorhamnetin	Isorhamnetin
LH (mlU/mL)	2.73 ± .08^a	1.20 ± .09^b	2.07 ± .10^c	2.62 ± .09^a
FSH (mlU/mL)	3.58 ± .14^a	1.37 ± .09^b	3.02 ± .10^a	3.46 ± .12^a
Plasma testosterone (ng/mL)	4.75 ± .12^a	2.02 ± .12^b	3.59 ± .13^c	4.67 ± .14^a

Results are represented in mean ± SEM (n = 8 rats/group). Values having different superscripts in a same row are significantly different from other groups.

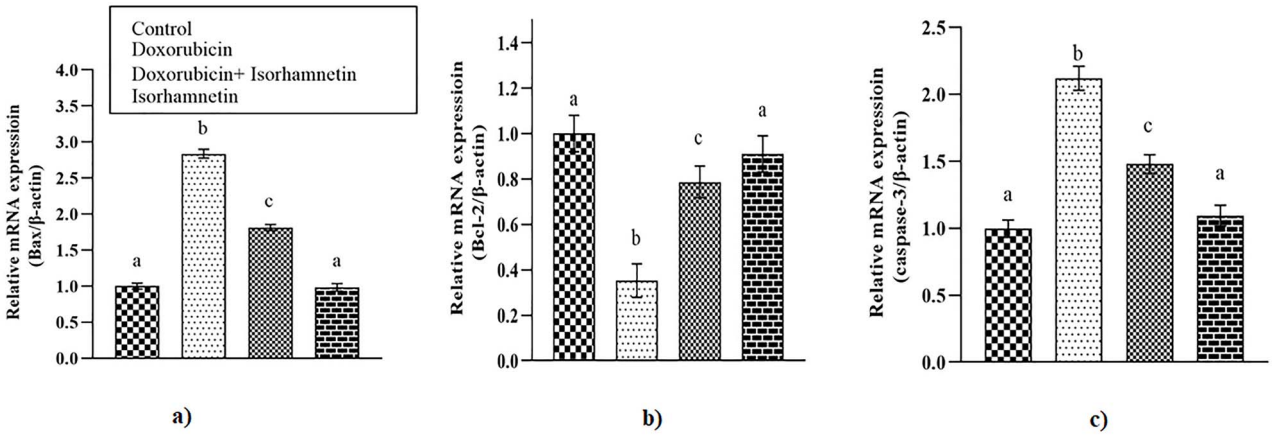

Fig. 2. Outcomes of the DOX and Isorhamnetin exposure on the expression of a) Bax, b) Bcl-2, and c) caspase-3. Verticle bars are presented on the base of mean ± SEM values (n = 8/group). Different superscripts on the bars are presenting significant ($P < 0.05$) difference.

Table 7. Mean ± SEM of histomorphometry of rat testis in control, DOX-treated, cotreated, and Isorhamnetin groups.

	Control	DOX	DOX + Isorhamnetin	Isorhamnetin
Interstitial spaces (μm)	7.83 ± .21^a	19.63 ± .98^b	12.07 ± .51^c	7.96 ± .38^a
Tunica propria (μm)	38.37 ± 1.18^a	14.96 ± .75^b	31.16 ± .79^c	38.08 ± 1.56^a
Diameter of tubules (μm)	300.29 ± 6.07^a	145.29 ± 6.61^b	207.51 ± 3.86^c	288.94 ± 5.24^a
Seminiferous tubule epithelial height (μm)	89.07 ± 4.20^a	46.24 ± 2.53^b	75.9 ± 1.16^c	84.53 ± 2.78^a
Tubular lumen (μm)	54.10 ± 1.55^a	93.29 ± 3.09^b	65.67 ± .97^c	55.48 ± 1.31^a
Spermatogonia (n)	57.82 ± 1.00^a	31.14 ± 1.27^b	44.11 ± 1.51^c	59.62 ± .99^a
Primary spermatocytes (n)	44.95 ± 1.08^a	24.42 ± 1.18^b	38.17 ± .66^c	45.07 ± 1.46^a
Secondary spermatocytes (n)	36.96 ± 1.18^a	15.36 ± .80^b	25.58 ± 1.24^c	36.01 ± 1.68^a
Spermatids (n)	51.45 ± .99^a	20.65 ± 1.26^b	40.66 ± .96^c	49.74 ± 1.64^a
JS	10(10–9)^a	5(4–6)^b	8(6–8)^c	10(10–9)^a

Results are represented in mean ± SEM or median (range; n = 8 rats/group). Values having different superscripts in a same row are significantly different from other groups.

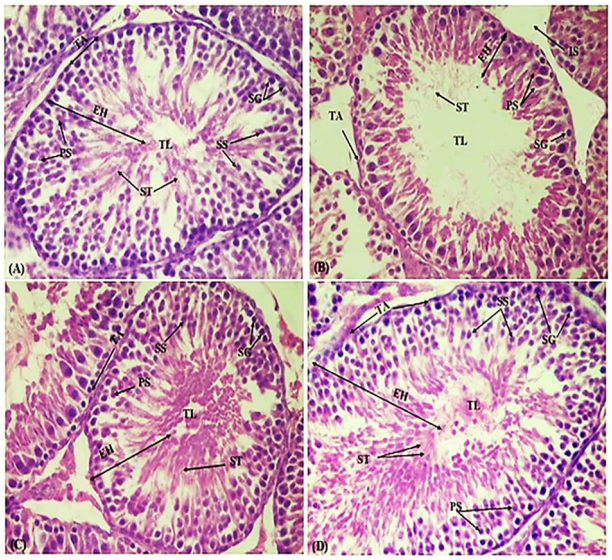

Fig. 3. Histopathological alterations by DOX and Isorhamnetin in testicular tissues. a) Control group presenting standard morphology and lumen consisting of germ cells; b) DOX group exhibiting sloughed epithelium and lumen having less number of germ cells; c) DOX + Isorhamnetin group displaying less sloughed epithelium and lumen containing superfluous germ cells in comparison to DOX exposed group; d) Isorhamnetin-treated group exhibiting compact epithelium and lumen full of germs cells as in control group (H &E).

Discussion

The purpose of this study was to evaluate the protective role of Isorhamnetin on DOX-induced testicular damage in male rats. A single dose of DOX caused reproductive toxicity by damaging the biochemical, spermatogenic, hormonal, and histopathological profiles in testicular tissues; however, cotreatment with Isorhamnetin recovered these injuries. This is probably due to the stimulating effects of Isorhamnetin on testosterone concentration and antioxidant enzyme activities in the testes.

DOX treatment significantly decreased the activities of ACP, LDH, and γ-GT in the testes. Whereas Isorhamnetin was found to prevent the decrease in the activities of ACP, LDH, and γ-GT in the testes of DOX-treated rats. Activities of testicular marker enzymes, such as ACP, LDH, and γ-GT, are considered as the functional indicators of spermatogenesis[31], and the decreased activity of these enzymes in DOX-administered animals represents a defect in spermatogenesis and testicular maturation. Similar patterns of testicular LDH and ACP activities have been reported with several chemicals causing testicular toxicity.[32] LDH is closely associated with the maturation of spermatogenic epithelium and ACP is involved in protein synthesis in the testes, while γ-GT promotes sperm maturation.[33] Coadministration of rats with DOX + Isorhamnetin resulted in a significant increase in testicular LDH, ACP, and γ-GT when compared with the DOX-alone-treated group, which indicates the testicular protective activity of isorhamnetin.

There is a complex system of antioxidant enzymes and free radical scavengers in the testes, which safeguards the spermatogenic and steroidogenic processes from the impacts of oxidative stress (OS). The OS increases when excessive free radicals overwhelm the antioxidant defense of the male reproductive tract, thereby damaging cells, tissues, and organs.[34] At physiological levels, ROS plays a dynamic role in stimulating the activation, capacitation, and acrosome reaction.[35] ROS overproduction disrupts the sperm antioxidant system, which leads to a condition known as OS.[36] ROS include hydroxyl radicals (•OH), superoxide anion ($\bullet O_2^-$), and H_2O_2.[37] Overproduction of ROS might be a contributing factor for 30–80% of all infertility cases.[38] Spermatozoa are particularly susceptible to oxidative damage due to their limited intracellular defense system and the high content of polyunsaturated fatty acids (PUFAs) on their plasma membranes.[39] The oxidative damage in PUFAs is substantially destructive because it proceeds self-perpetuating chain reaction and leads to the formation of hazardous substances, such as thiobarbituric acid reactive substances and MDA, which are considered as indicators of lipid peroxidation (LP).[40] In the present study, DOX administration resulted in a significant decrease in the CAT, SOD, GPx, and GSR activities, while it elevated the level of MDA, confirming the previous report that DOX decreases the activities of antioxidant enzymes[41] and elevates the MDA level.[42] As reported, earlier enzymatic antioxidants are the first line of defense that removes free radicals.[40] SOD is 1 of the various influential cellular antioxidant enzymes, which catalyze the dismutation of O_2^- into H_2O_2 and molecular oxygen[43]; on the other hand, H_2O_2 is converted to H_2O by CAT and GPx.[44] The concentration of glutathione is retained by GSR, which restores the reduced glutathione peroxidase (GSH) from glutathione disulfide (oxidized form) for the continuous activity of GPx.[45] MDA levels in seminal plasma and spermatozoa are negatively correlated with sperm parameters.[36] However, DOX-induced OS was remarkably prevented by Isorhamnetin treatment, as displayed by the reduced MDA level and increased activities of SOD, CAT, GPx, and GSR due to its free radical scavenging or antioxidant potential. Xu et al.[46] also reported that Isorhamnetin holds real potential in the mitigation of myocardial ischemia–reperfusion injury by lowering the level of MDA and increasing the cellular CAT, SOD, and GSH-Px.

DOX commenced profound damage in epididymal sperm count, progressive motility, and viability in addition to a reduction in the number of HOS coiled-tail sperm, whereas a more distinguished level of abnormality was observed in the head, midpiece, and tail of sperm. This may be due to the redox dysregulation as well as cell cycle arrest and apoptosis prompted by DOX treatment.[47,48] LP also triggers the loss of membrane integrity and fluidity, causing increased cell permeability, inactivation or decrease in the membrane enzyme

activity, structural damage, and cell death.[36] This may lead to a decrease in sperm counts in the epididymal fluid.[40] But Isorhamnetin treatment significantly prevented all these spermatogenic impairments, which might be due to its potent ROS scavenging activity.

DOX exposure reduced the steroidogenic enzymes (3β-HSD, 17β-HSD, and StAR) expression, which is compatible with the earlier investigation in which DOX administration impairs steroidogenesis.[49] The principle enzymes, 3β-HSD and 17β-HSD, regulate steroidogenic activities and play a primary role in androgenesis.[50] Additionally, StAR is a rate-limiting enzyme that mediates cholesterol (testosterone precursor) transportation into the internal membrane of mitochondria to ensure testosterone biosynthesis.[51,52] Lower expression of these androgenic enzymes due to DOX-prompted OS resulted in a decreased testosterone concentration. Conversely, Isorhamnetin significantly prevented the suppression of testosterone by upregulating the expression of steroidogenic enzymes, which may be due to its androgenic nature potential.

Spermatogenesis is a continuous, highly regulated process of male germ cell proliferation and differentiation. This process takes place in the seminiferous tubules of the testes for the generation of sperm throughout the lifetime. Differentiation of spermatogonia occurs through a linear process, including mitotic expansions, meiotic divisions, and morphological transformations. The commitment of spermatogonia to the differentiation pathway happens when undifferentiated spermatogonia undergo an irreversible transition (in mouse) or division (in primate) to produce differentiating spermatogonia. The commitment of undifferentiated spermatogonia to differentiating spermatogonia and normal spermatogenesis requires the action of gonadotropins. Secretion of gonadotropins, including LH and FSH, by the pituitary gland is under the control of the hypothalamic gonadotropin-releasing hormone.[53] In the testes, 2 specific transmembrane receptors, FSHR and LHR, mediate the actions of gonadotropins, FSH and LH, respectively. Within the testes, FSHR is selectively expressed by Sertoli cells (SCs) in the seminiferous tubules, whereas LHR is expressed by Leydig cells (LCs) in the interstitial space. Therefore, FSH directly, but LH indirectly, via androgen receptor acts on spermatogenesis by regulating SC factors. In response to LH signaling, testosterone is produced by the LCs in a pulsatile fashion, and in response to FSH, a nonsteroidal hormone is produced by the SCs in a nonpulsatile manner.[54] The major role of gonadotropins FSH and LH is to establish normal spermatogenesis and sperm production during puberty and adulthood. However, in the present study, DOX administration reduced the production of gonadotropins (LH and FSH) and testosterone, possibly due to the disturbed hypothalamus-pituitary-gonadal axis.[55] Conversely, Isorhamnetin treatment significantly improved hormonal concentration. Isorhamnetin may prevent these toxic alterations in hormonal levels by conferring protection to the hypothalamic-pituitary-testicular axis, which consequently protected these spermatogenic damages.

In the present study, our results showed that DOX administration elevated the expression of apoptotic proteins, Bax, and caspase-3, while it lowered the expression of Bcl-2. Kun-Peng et al.[56] revealed that DOX-instigates imbalance in apoptotic proteins. Proapoptotic and antiapoptotic proteins carry out apoptosis via mitochondrial-dependent and -independent pathways.[57] The apoptotic markers, Bcl-2 and Bax, are related to the Bcl-2 family.[58] The relative imbalance between these proteins results in apoptotic cell death. Bax is an apoptotic marker, which encourages cell death, while Bcl-2 is an antiapoptotic protein that provokes the repression of apoptosis.[59] Reduction in Bcl-2 and elevation in Bax adversely alter the permeability of the mitochondrial membrane, resulting in a rise in the liberation of cytochrome c within the cytosol.[60] This augmented cytochrome c in cytosol ultimately activates the expression of caspase-3, which leads to apoptosis and cell death.[61] However, Isorhamnetin protected these testicle damages via downregulating the expression of Bax and caspase-3 and escalating the expression of Bcl-2. This study also highlights the antiapoptotic potential of Isorhamnetin in testes of DOX-treated rats. Isorhamnetin elevated the antiapoptotic proteins, Bcl-2 expression, while lowering the expression of Bax and caspase-3, which authenticates the previous study in which isorhamnetin promoted the expression of antiapoptotic proteins, Bcl-2, while decreasing the expression of Bax and caspase-3 in cardiomyocytes.[46]

According to previous studies, DOX has consistently stimulated testicular-cell death due to the upsurge of ROS in the male reproductive system.[62] In the current work, DOX administration elicited severe histopathological damages in testicular tissues by inducing a diminution in height and diameter of seminiferous tubules' epithelium, as well as tunica propria thickness, whereas the diameter of the tubular lumen and interstitial spaces were increased. Moreover, DOX downregulated the number of spermatogonia, primary and secondary spermatocytes in addition to spermatids. DOX treatment resulted in testicular damage and spermatogenic death[63] due to the production of OS and LP in testicles.[64] However, isorhamnetin treatment remarkably protected the architectural degeneration of testis and the number of germ cells in the male reproductive system, owing to its antioxidant and androgenic potential. Moreover, findings of Ganbold et al.[65] proposed that isorhamnetin treatment alleviated histopathologic anomalies of nonalcoholic steatohepatitis-induced liver injury by reducing steatosis and apoptosis. These findings proved the cytoprotective potential of isorhamnetin and also endorsed the idea of its usage along with DOX to protect DOX-generated fertility issues in male cancer patients.

Conclusion

DOX exposure induced remarkable damages in the biochemical, spermatogenic hormonal, and histopathological profiles in testicular tissues. Additionally, the activity of antioxidant enzymes, the expression of apoptotic/antiapoptotic proteins and steroidogenic enzymes, as well as testicular marker enzymes, exhibited a state of imbalance due to DOX exposure, and thus resulted in the deteriorated performance of the entire male reproductive system. However, Isorhamnetin restored all these damages due to its antioxidant, androgenic, and antiapoptotic potential.

List of abbreviations

3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; Bax: BCL2-associated X protein; Bcl-2: B-cell lymphoma 2; CAT: catalase; DOX: doxorubicin; DNA: deoxyribonucleic acid; FSH: follicle-stimulating hormone; GPx: glutathione peroxidase; GSH: reduced glutathione peroxidase; GSR: glutathione reductase; H_2O: water; H_2O_2: hydrogen peroxide; HOS test: hypo-osmotic swelling test; LC: Leydig cell; LDH: lactate dehydrogenase; LH: luteinizing hormone; LP: lipid peroxidation; MDA: malondialdehyde; PUFAs: polyunsaturated fatty acids; OS: oxidative stress; RNA: ribonucleic acid; ROS: reactive oxygen species; SCs: Sertoli cells; SOD: superoxide dismutase; StAR: steroidogenic acute regulatory protein.

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Authors' contributions

SM, MUI, and QuA designed the study, conceived the study, and analyzed the results. SM and MUI conceived an initial part of the study, performed the experiment and histology, and helped in compiling the results. SR, QuA, SR, MUI, and TA helped in writing the results. SM, SR, MUI, QuA, TA, HS, and AA made a substantial contribution in the interpretation of data and revising the manuscript for intellectual content. All authors read and approved the final manuscript.

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Conflict of interest statement

None declared.

Declarations

Ethics approval

Animals were treated in compliance with the European Union of Animal Care and Experimentation (CEE Council 86/609) approved protocol.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data are contained in the manuscript.

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