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Protective Effect of Rosa Canina Extract Against Doxorubicin-induced Testicular Toxicity in Mice

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HIGHLIGHTS

- Doxorubicin is able to target germ cells, and resulting in aspermia and infertility.
- *R. canina* extract treatment improve testicular and sperm parameters.
- *R. canina* extract has protective effects against doxorubicin-induced testicular toxicity.

Abstract: The present study was aimed to investigate the in vivo effects of Rosa canina extract on doxorubicin-induced testicular toxicity in mice for the first time. Male NMRI mice were randomly divided into six treatment groups (10=per group) as follows: (I) vehicles, (II) doxorubicin alone (3 mg/kg, i.p. on days 7, 14 and 21), (III and IV): Rosa canina extract alone (100 mg/kg and 200 mg/kg per day, i.p. for 28 days), (V and VI) Rosa canina extract plus doxorubicin (each dose given 1 h post Rosa canina). Doxorubicin-treated mice displayed smaller body and testicular weights, decreased serum levels of testosterone, loss in the number of germ cells and Sertoli cells, and reduced sperm count, viability, morphology

and motility. Doxorubicin treatment significantly decreased the mean testis diameter, seminiferous tubular diameter and seminiferous epithelial height and increased seminiferous luminal diameter. However, *Rosa canina* pretreatment could effectively improve all of these abnormalities in doxorubicin- treated mice. The treatment with a higher dose of the extract (200 mg/kg) was more effective compared to doxorubicin and the lower dose of the extract. These findings suggested that the *Rosa canina* extract has protective effects against doxorubicin-induced reproductive toxicity.

Keywords: *Rosa canina* extract, Doxorubicin, Testes, Sperm, Mice.

INTRODUCTION

Doxorubicin, one of the most well-known chemotherapeutic agents, has long been widely used for its potent efficiency to treat both solid and non-solid malignant tumors [1,2]. However, its clinical use has been accompanied by adverse effects on the male reproductive system [3]. The exact mechanism of doxorubicin-induced testicular toxicity is little known, but previous studies have provided evidences that doxorubicin retards testicular growth, impedes the process of spermatogenesis, which eventually leads to male infertility through imposing oxidative stress and cellular apoptosis [4]. The drug intercalates into DNA strands and results in double-strand DNA breaks and cell death [1,2,5] in meiotically dividing spermatocytes and spermatogonia [6-10]. Moreover, doxorubicin-induced oxidative stress [2] in testicular tissue may also cause DNA damage and cell death [4].

The high susceptibility of testes and sperm to doxorubicin -induced testicular oxidative stress may be due to a weak anti-oxidant defense system in testicular tissue and semen [4,11-13]. Besides, earlier studies demonstrated that antioxidant supplementation improved the quality of the semen profile in infertile men [14]. Based on this concept, a variety of antioxidants have been employed to protect the testes from doxorubicin injury [15,16,4].

Rosa canina (*R. canina*), a member of the *Rosaceae* family [17] have been studied as antioxidant and anti-inflammatory agents [18]. High antioxidant power of *R. canina* is mainly due to its strong free radical scavenging and hydrogen peroxide scavenging activities [19]. Such activities can be attributed to the presence of the active constituents [20] such as phenolic acids, proanthocyanidins, flavonoids, unsaturated and polyunsaturated fatty acids, phospholipids, minerals, carotenoids and highest vitamin C content (ascorbic acid) and other vitamins (vitamins B1(thiamin), B2, K, PP [21,17]. In addition, *R. canina* has been considered as a protective agent against DNA damage [22]. However, despite its clinical effectiveness as diuretic, arthritis, laxative, kidney function disturbances, urinary tract disorders, colds, gout, fever, and gastroprotective agents [21,23]; the impact of this extract on spermatogenesis and male infertility has not been reported yet. Considering the widespread use clinically of doxorubicin against malignant tumors and its consequences to male fertility [2,3], and the protective effect of *R. canina* extract over some other tissues, we decided to study the in vivo protective effects of *R. canina* against doxorubicin-induced testicular toxicity in mice.

MATERIAL AND METHODS

Plant material and preparation of the aqueous extract

The fruits of *Rosa canina* were collected from Parav Mountain in Kermanshah province (west of Iran). A dried voucher specimen (No, 578) was deposited in the Herbarium of Biology Department, Razi University (HBRU). Fruits were washed and dried with a clean cloth. Pulps of the fruits were separated and divided into small pieces. The crushed pulps were dried in an oven at 60 °C for 24 hours. Dried pulps were milled and stored in a closed container at -20 °C until extraction. Fifty grams of dried powder of *R. canina* pulp was extracted on the stirrer with 1000 ml of distilled water for 24h at room temperature. After filtration, the aqueous extract was evaporated to dryness on a rotary evaporator under reduced pressure below 40°C.

Animals and pharmacological treatments

Male NMRI mice were obtained from the Experimental Animal Facility of the Pasteur Institute (Tehran, Iran), housed under standard conditions of controlled humidity, temperature (25 ± 2°C) and light (12 h light/12 h dark), and fed with commercial pellet and water during the study period. Animal handling and care were performed in accordance with the policies of the Razi University Animal Care and Use Committee, and the Guide for the Care and Use of Laboratory Animals (B/195). Male NMRI mice were divided randomly into six treatment groups (10 mice per group) and caged separately as follows: treatment I: mice served as control group and received none but a normal saline injection; treatment II: mice were treated with doxorubicin (Milan, Italy) at the dose of 3mg/kg on day 7, 14 and 21 for totally three shots and accumulated dose of 9 mg/kg; treatment III and IV: mice were given *R. canina* extract 100 and 200 mg/kg for 28 days, respectively; treatment V and VI: mice were received *R. canina* extract 100 and 200 mg/kg followed by doxorubicin injection (3 mg/kg, each dose given 1 h after *R. canina*), respectively. All the treatments were given by intraperitoneal route.

Harvest of serum and testicular samples

Experimental and control animals were killed by cervical dislocation, on day 28 after the first treatment, and the body and testicular weights were measured in all treatment groups. For to do histopathologic and morphometric examinations, their testis fixed in 10% buffered formalin for 24 hours, dehydrated by gradually increasing concentrations of ethanol and embedded in paraffin. Five micrometer cross sections were stained with hematoxylin and eosin (H&E). The epididymal cauda were obtained for the analysis of sperm concentration, viability, motility and morphology. Blood samples were harvested and centrifuged at 2000×g for 10 min to obtain serum for evaluating serum testosterone concentrations.

Epididymal sperm count and viability

The epididymal cauda of each mouse were excised and cut into small pieces in a petri dishes containing Ham's F-10 culture medium (nutrient mixture-Ham-X1, Gibco, UK) supplemented with 10 % fetal bovine serum (FBS; Gibco). The samples incubated for 30 minutes at 37 °C to let the sperms swim up [24]. To assess the epididymal sperm count and

viability, 10 µl of 0.4% trypan blue dye (v/v; Gibco) was added to 40 µl of the sperm sample and incubated at room temperature for 5 min. Then, the specimen was transferred to Neubauer's counting chamber of hemacytometer. The total number of viable (colorless) and dead (colored) sperms were counted with using a light microscope at 40 x magnifications. The relative viability of sperms was calculated by dividing the total viable sperms (unstained) by the total sperms (stained and unstained) $\times 100$ [25].

Sperm motility

One drop of the sperm homogenate was placed on a pre-warmed microscope slide at 37 °C, and cover with a clean cover slip. Photographs were taken in 30 frames per second in each sample and recorded by a digital camera (Olympus DP72 Digital Camera; Tokyo, Japan) attached to inverted fluorescence microscope (Olympus IX-71, Japan). At least 400 sperm were counted for sperm motility analysis per sample with $\times 400$ magnification. Sperm progression was classified as follows: rapid progressive, slow progressive, non-progressive and immotile [26,27].

Sperm morphology

For sperm morphology assessment, a drop of sperm suspension was drawn on clean glass slide. The sperms were fixed in ethanol alcohol and allowed to dry in air overnight. The slides were stained with papanicolaou staining technique and examined at $\times 400$ magnification to detect the morphological abnormalities as follows: pin head, round head, giant head, double head, bent neck, coiled tail, bent tail, short tail, double tail and cytoplasmic droplet. For each sperm sample, the percentage of abnormal sperms was scored from at least 10 fields and 200 sperms [26,27].

Serum testosterone concentration

Serum testosterone concentrations were determined by using AccBind ELISA Microwells (testosterone ELISA kit) (Monobind Inc., USA) [28].

Histopathological analysis

To assess histopathological alterations, two hundred round or nearly round seminiferous tubule cross-sections per animal were examined. The cross-sections of tubular profiles were categorized into four grades, 1 = 0–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%. These alterations were categorized according to the level of damage observed in the seminiferous epithelium of tubular profiles and the frequency of occurrence in each treatment group. The determinant histopathological alterations were considered: a) the germinal lineage cells detached from the epithelium into the tubular lumen, b) intraepithelial vacuolation formation, c) the cellular debris d) the depletion of epithelial (depletion level 1: Sertoli cell-only; depletion level 2: Sertoli cell + spermatogonia; depletion level 3: At least two layers of cells). The images were captured using an inverted fluorescence microscope (Olympus IX-71, Japan) [29].

Morphometrical analysis

Testis sections used for morphometrical analysis were examined and photographed by an inverted fluorescence microscope (Olympus IX-71, Japan). Testicular diameter; seminiferous tubule diameter; luminal diameter and germinal epithelium height (μm) were measured in each treatment group. From the spermatogenic cells on the inner surface of the basement membrane lining the lumen of the tubules considered for measurement of diameter and germinal epithelial height [30]. For this, two hundred round or nearly round cross-sections of tubular profiles per animal were examined at $\times 100$ magnification.

Tubular frequency according to the different germ cell types

To determine alterations in the kinetics of spermatogenesis, the frequencies of tubular sections containing each germ cell type (spermatogonia, spermatocytes, round spermatids and elongated spermatids) and Sertoli cells at the stage VII of the spermatogenic cycle were assessed. For this, two hundred random tubular sections from 20 random testicular cross-sections per animal were examined at $\times 100$ magnifications. The identification of germ cell types and stage VII of the seminiferous epithelium cycle was performed according to Nakata and co-workers [30,31].

Statistical analysis

All data were analyzed by using SPSS (version 20; SPSS Inc) and were expressed as mean and standard errors of the means (SEM). The statistical significance was tested by one-Way ANOVA and Duncan test. A value of $p < 0.05$ was considered to determine statistically significant.

RESULTS

The body and testicular weights

The body weight, absolute and relative weight of testis in treatment II showed a significantly decrease in comparison to all the other treatment groups ($p < 0.05$) (fig. 1 A, B and C). However, these parameters were not significantly affected by any of treatment III and IV when compared with treatment I. In addition, no significant differences were observed regarding the body weight in the treatment V and VI compared with treatment I. Absolute and relative weight of testis in the treatment V and VI significantly restored than treatment II, respectively ($p < 0.05$).

Figure 1. Body weight (A), absolute weight of testis (B) relative weight (per BW, %) (C) in mice treated with doxorubicin and *R. canina*. Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Sperm count and viability

The results of the sperm count and the percentages of sperm viability were significantly lower in the treatment II in comparison to all the other treatment groups ($p < 0.05$) (Tab. 1). In contrast, there was a significant increase in these parameters in the treatment III and IV compared with all the other treatment groups, respectively ($p < 0.05$). In addition, the mean sperm count and the percentages of sperm viability in the treatment V and VI were significantly higher than treatment II, respectively ($p < 0.05$). Changes in these parameters were dose-dependent manner in response to *R. canina* extract (treatment III and IV, and V and VI, respectively).

Table 1. Frequency of mouse sperm count and viability after treatment with doxorubicin and *R. canina* extract.

Groups	Sperm count ($\times 10^6$)	Sperm viability (%)
Treatment I	16.40 $\pm$ 0.22 ^{b,c,d,e,f}	77.32 $\pm$ 0.76 ^{b,c,d,e,f}
Treatment II	8.52 $\pm$ 0.23 ^{a,c,d,e,f}	47.36 $\pm$ 1.22 ^{b,c,d,e,f}
Treatment III	17.80 $\pm$ 0.20 ^{a,b,d,e,f}	81.47 $\pm$ 0.90 ^{a,b,e,f}
Treatment IV	19.08 $\pm$ 0.24 ^{a,b,c,e,f}	82.41 $\pm$ 1.05 ^{a,b,e,f}
Treatment V	11.88 $\pm$ 0.27 ^{a,b,c,d,f}	70.38 $\pm$ 1.26 ^{a,b,c,d}
Treatment VI	14.52 $\pm$ 0.25 ^{a,b,c,d,e}	73.07 $\pm$ 1.75 ^{a,b,c,d}

Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Sperm motility

Based on the results, there was a significant decrease in the mean percentage of sperm progressivity and non-progressivity and a significant increase in the mean percentage of immotile sperm in the treatment II compared with all the other the treatment groups,

respectively ($p < 0.05$) (Tab. 2). Reversibly, the percentage of progressive motility was significantly higher in the treatment III and IV than treatment I as well as in the treatment V and VI than the treatment II, respectively ($p < 0.05$). The mean percentage of slow progressive sperm was noted when the treatment V and VI compared with all the treatment groups ($p < 0.05$). The mean percentage of immotile sperm was significantly lower in the treatment III and IV than treatment I as well as the treatment V and VI than treatment II, respectively ($p < 0.05$). However, no significant differences were observed regarding the mean percentage of immotile sperm in the treatment V and VI compared with treatment I. In addition, sperm non-progressivity in the treatment IV was significantly lower than treatment I ($p < 0.05$).

Table 2. Frequency of mouse sperm motility after treatment with doxorubicin and *R. canina* extract.

Groups	Fast progressive (%)	Slow progressive (%)	Non-progressive (%)	Immotile (%)
Treatment I	40.80±1.65 ^{b,c,d}	16.60±0.93 ^{e,f}	18.60±1.20 ^{b,d}	24.00±0.89 ^{b,c,d}
Treatment II	6.00±1.04 ^{a,c,d,e,f}	15.40±1.02 ^{e,f}	6.60±0.87 ^{a,c,d,e,f}	72.20±2.03 ^{a,c,d,e,f}
Treatment III	51.20±1.39 ^{a,b,e,f}	16.20±0.86 ^{e,f}	15.60±0.92 ^b	18.00±1.26 ^{a,b,e,f}
Treatment IV	52.80±2.70 ^{a,b,e,f}	15.60±1.02 ^{e,f}	14.20±0.86 ^{a,b}	17.00±0.70 ^{a,b,e,f}
Treatment V	38.80±1.24 ^{b,c,d}	20.40±1.43 ^{a,b,c,d}	17.20±1.28 ^b	22.80±0.86 ^{b,c,d}
Treatment VI	40.40±1.50 ^{b,c,d}	21.00±1.22 ^{a,b,c,d}	16.20±1.01 ^b	22.40±0.93 ^{b,c,d}

Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Sperm morphology

Level of sperm abnormality was significantly higher in the treatment II when compared with all the other groups ($p < 0.05$) (Tab. 3; fig. 2). However, no significant differences were observed in the treatment III and IV compared with treatment I. In addition, level of sperm abnormality in the treatment V and VI were significantly lower than treatment II, respectively ($p < 0.05$).

Figure 2. Effect of doxorubicin and *Rosa canina* on sperm morphology, Pap staining, Magnification of 40X. Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin.

Table 3. Frequency of abnormal morphology of mouse sperm after treatment with doxorubicin and *R. canina* extract.

Groups	Treatment I	Treatment II	Treatment III	Treatment IV	Treatment V	Treatment VI
abnormal (%)	29.60±1.96 ^{bef}	74.40±1.63 ^{acdef}	27.80±1.28 ^{bef}	27.00±1.70 ^{bef}	60.60±1.95 ^{abcd}	55.80±1.43 ^{abcd}

Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Serum testosterone level

Serum testosterone levels were significantly lower in the treatment II compared to other treatment groups ($p < 0.05$). In contrast, the levels were higher in the treatment III and IV compared to treatment I, but the effect was significant only in IV than treatment I ($p < 0.05$). Serum testosterone levels in the treatment V and VI were also significantly higher than treatment II, respectively ($p < 0.05$) (fig. 3).

Figure 3. Comparison of the means of serum testosterone concentration after treatment with doxorubicin and *R. canina* extract. Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III; ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Histopathology

The affected seminiferous tubules of treatment II exhibited a high frequency of tubules with germ cell depletion and degenerated appearance of germ cells, sometimes the seminiferous epithelium lined only with Sertoli cells (depletion level 1) (Tab. 4; fig. 4) ($p < 0.05$). In addition, the germinal lineage cells detached from the epithelium into the tubular lumen, intraepithelial vacuolization and cellular debris was noticed in the tubular lumen of many seminiferous tubules in the treatment II ($p < 0.05$). Conversely, there was a normal preserved seminiferous epithelium in the treatment III and IV, similar to those observed in treatment I, respectively. In addition, tubular sections in the treatment V and VI displayed a lower frequency of tubular sections with germ cell depletion (severe and moderate) than treatment II, respectively ($p < 0.05$). The lower presence of intraepithelial vacuoles and sloughed germ cell into the lumen were observed in these treatment groups, respectively ($p < 0.05$).

Figure 4. Photomicrographs of cross sections of seminiferous tubules of Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin. H&E method. I, III, IV; showing organized seminiferous epithelium (Ep) with normally

different germ cell layers containing various cell types, tubular lumen (L); interstitial tissue (IT). Observe some tubular sections in II showing the cellular debris, intraepithelial vacuolation formation (thick arrow), the germinal lineage cells detached from the epithelium into the tubular lumen and depletion of epithelial such as Sertoli cell-only (thin arrows). V and VI; in both figures, note damaged seminiferous epithelium in seminiferous tubule (asterisks) neighboring other seminiferous epithelium containing normally different germ cell layers (thick arrow).

Table 4. Percentage of mice presenting specific histopathological alterations and score of the frequencies (1–4)¹ of the tubular alterations after treatment with doxorubicin and *R. canina* extract.

Types of histological alterations	Percentual (%) score (1–4)	Treatment groups					
		I	II	III	IV	V	VI
Sloughed germinal cell into the lumen.	Percentage of mice with this alteration	10 ^{be}	100 ^{acdef}	10 ^{be}	10 ^{be}	20 ^{abcd}	10 ^{be}
	Score of frequency of the tubular alteration	1	4	1	1	1	1
Intraepithelial vacuolization.	Percentage of mice with this alteration	10 ^{be}	100 ^{acdef}	10 ^{be}	10 ^{be}	20 ^{abcd}	10 ^{be}
	Score of frequency of the tubular alteration	1	4	1	1	1	1
Sertoli cell-only (depletion level 1).	Percentage of mice with this alteration	0 ^{bef}	100 ^{acdef}	0 ^{bef}	0 ^{bef}	20 ^{abf}	10 ^{abcde}
	Score of frequency of the tubular alteration	1	4	1	1	2	1
Sertoli cell + spermatogonia (depletion level 2).	Percentage of mice with this alteration	0 ^{bef}	100 ^{acdef}	0 ^{bef}	0 ^{bef}	60 ^{abcd}	30 ^{abcde}
	Score of frequency of the tubular alteration	1	4	1	1	2	1
At least two layers of cells (depletion level 3).	Percentage of mice with this alteration	0 ^{bcef}	100 ^{acdef}	10 ^{abdef}	0 ^{bcef}	40 ^{abcd}	30 ^{abcde}
	Score of frequency of the tubular alteration	1	4	1	1	2	2

¹Scores 1–4: 1 = 0–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%. Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; P < 0.05). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Morphometric testicular analysis

Based on the results, there was a significant decrease in the mean testicular diameter, seminiferous tubular diameter and height of the seminiferous tubule epithelium in the treatment II (p<0.05). Conversely, luminal diameter in this treatment had a significant increase compared to the values observed in treatment I (p<0.05). An increase in the mean seminiferous tubular diameter and seminiferous epithelial height was seen in the treatment III and IV than treatment I, respectively. The increase was significant only in treatment IV (p<0.05). These values significantly restored in the treatment V and VI compared to treatment II, respectively (p<0.05). Despite decrease in the mean seminiferous luminal diameter in two treatment groups compared to treatment II, the difference was significant only in treatment VI (p<0.05) (Tab. 5; fig. 4).

Table 5. Values of testicular histomorphometrical parameters after treatment with doxorubicin and *R. canina* extract.

Groups	Testicular diameter (μm)	Seminiferous tubular diameter (μm)	Seminiferous epithelial height (μm)	Seminiferous luminal diameter (μm)
Treatment I	3410.36 $\pm$ 21.2 ^{bef}	229.23 $\pm$ 3.12 ^{bdef}	73.22 $\pm$ 1.77 ^{bdef}	80.75 $\pm$ 2.91 ^{be}
Treatment II	2795.25 $\pm$ 53.2 ^{acdef}	176.92 $\pm$ 2.22 ^{acdef}	35.99 $\pm$ 1.70 ^{acdef}	101.40 $\pm$ 2.66 ^{acdf}
Treatment III	3342.14 $\pm$ 63.3 ^{bdef}	234.46 $\pm$ 2.70 ^{bdef}	76.73 $\pm$ 1.99 ^{bef}	83.19 $\pm$ 3.06 ^{be}
Treatment IV	3492.54 $\pm$ 27.9 ^{bcef}	250.56 $\pm$ 2.94 ^{abcef}	81.28 $\pm$ 1.87 ^{abef}	86.30 $\pm$ 3.35 ^{be}
Treatment V	3117.85 $\pm$ 39.2 ^{abcd}	201.97 $\pm$ 3.65 ^{abcdf}	51.84 $\pm$ 2.30 ^{abcdf}	96.65 $\pm$ 4.52 ^{acdf}
Treatment VI	3193.17 $\pm$ 64.16 ^{abcd}	214.37 $\pm$ 3.83 ^{abcde}	64.59 $\pm$ 3.89 ^{abcde}	82.17 $\pm$ 4.01 ^{be}

Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg; Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III; ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

Tubular frequency according to the different germ cell types

The frequency of seminiferous tubule sections containing of Sertoli cells and different spermatogenic cell types (types A spermatogonial, preleptotene spermatocytes, pachytene spermatocytes, round spermatids and elongated spermatids) in the treatment II was significantly lower compared to those from other treatment groups at the same stage (VII) ($p < 0.05$) (Tab. 6; fig. 4) Conversely, these parameters were significantly higher in the treatment V and VI when compared to treatment II at the same stage (VII), respectively ($p < 0.05$). In addition, there was a significant increase in preleptotene spermatocytes, pachytene spermatocytes and round spermatids in treatment IV compared to the treatment I ($p < 0.05$).

Table 6. Frequencies of the different types of germinal lineage cells per 100 seminiferous tubule examined in mice testis after treatment with doxorubicin and *R. canina* extract in stage VII seminiferous.

Groups	Sertoli	Spermatogonia (type A)	Spermatocytes		Spermatids	
			PI	P	Round spermatids	Elongated spermatids
Treatment I	17.73 $\pm$ 0.43 ^{bef}	2.73 $\pm$ 0.208 ^{bef}	41.28 $\pm$ 1.52 ^{bdef}	45.20 $\pm$ 1.63 ^{bde}	120.06 $\pm$ 4.50 ^{bdef}	128.26 $\pm$ 5.89 ^{bef}
Treatment II	14.26 $\pm$ 0.28 ^{acdef}	0.40 $\pm$ 0.13 ^{acdef}	1.66 $\pm$ 0.37 ^{acdef}	5.46 $\pm$ 1.12 ^{acdef}	12.5 $\pm$ 1.71 ^{acdef}	69.9 $\pm$ 4.34 ^{acdef}
Treatment III	17.33 $\pm$ 0.25 ^{be}	2.66 $\pm$ 0.18 ^{bef}	39.93 $\pm$ 2.35 ^{bdef}	48.26 $\pm$ 1.48 ^{be}	122.26 $\pm$ 4.66 ^{bef}	126.46 $\pm$ 4.64 ^{bef}
Treatment IV	17.40 $\pm$ 0.41 ^{be}	2.80 $\pm$ 0.22 ^{bef}	48.40 $\pm$ 1.12 ^{abcef}	56.40 $\pm$ 1.15 ^{abdef}	132.46 $\pm$ 4.40 ^{abef}	127.93 $\pm$ 5.45 ^{bef}
Treatment V	15.53 $\pm$ 0.35 ^{abcd}	1.12 $\pm$ 0.15 ^{abcdf}	14.06 $\pm$ 2.68 ^{abcdf}	29.33 $\pm$ 4.62 ^{abcdf}	56.26 $\pm$ 5.54 ^{abcdf}	88.00 $\pm$ 4.62 ^{abcd}
Treatment VI	16.46 $\pm$ 0.33 ^{ab}	1.95 $\pm$ 0.18 ^{abcde}	21.80 $\pm$ 1.34 ^{abcde}	43.26 $\pm$ 2.66 ^{bde}	91.73 $\pm$ 3.00 ^{abcde}	101.06 $\pm$ 6.77 ^{abcd}

PI, preleptotene spermatocytes; P, pachytene spermatocytes. Treatment I: control; Treatment II: doxorubicin; Treatment III: *R. canina* extract 100 mg/kg; Treatment IV: *R. canina* extract 200 mg/kg;

Treatment V: *R. canina* 100 mg/kg /doxorubicin; Treatment VI: *R. canina* 200 mg/kg /doxorubicin (mean + SEM; $P < 0.05$). ^a Significant compared with I; ^b Significant compared with II; ^c Significant compared with III, ^d Significant compared with IV; ^e Significant compared with V; ^f Significant compared with VI.

DISCUSSION

Considering the widespread use clinically of anticancer drugs such as doxorubicin against malignant tumors and their consequences to male fertility [15,2,3], fertility preservation strategies for patients undergoing chemotherapy treatment becomes an important issue [32]. Previous studies provide evidences that doxorubicin caused testicular toxicity through eliciting oxidative stress and apoptosis [4].

It demonstrated that oxidative stress involved in low sperm quality of male infertility [33]. On the other hand, despite the enzymatic and non-enzymatic antioxidants in the testes and seminal plasma, ample evidence has implied that there was a weak anti-oxidant defense system in testicular tissue and semen [11-13,34,35]. Based on this concept, a variety of antioxidants have been employed to protect the testes from anticancer drugs [15,16].

Several studies revising a high antioxidant and anti-inflammatory properties of *R. canina* extract [18] showed that these properties are responsible for the protective effects against oxidative stress induced by renal reperfusion injury [36]. Moreover, it demonstrated that *R. canina* extract displayed an anti-inflammatory effect in an in vivo animal model of ulcerogenic gastric damage induced by ethanol as necrotizing [23]. The protective effect of *R. canina* extract over some other tissues has already been proven [36,23], whereas, there is no report on its the protective effect regarding reproductive system.

Doxorubicin is administered to mice at doses up to 9 mg/kg [37]. The previous reports clearly indicated that the administration of doxorubicin intraperitoneally at the dose of 3mg/kg for totally three shots (accumulated dose of 9 mg/kg) caused retardation of testicular growth and reduction in spermatogenic function through eliciting oxidative stress and cell apoptosis [4]. On the same way, the two different doses of the *R. canina* extract were selected due to its high antioxidant and anti-inflammatory activity. It mentioned the clinical effect of *R. canina* extract 200 mg/kg was more effective [23].

Considering the long-term side effects of doxorubicin administration [37,4] and the potential of *R. canina* extract to improve treatment efficacy, the aim of this study was to verify if 100 and 200 mg/kg of *R. canina* extract [23] could reduce male infertility caused by doxorubicin in mice.

In the current study, a significantly lower body and testicular weights was observed in doxorubicin-treated mice, in comparison to the other groups as described in other experimental studies that administered doxorubicin alone [4]. Because testicular weight depends on the mass of the differentiated germ cells [38], a sharp decline of testicular weight can be explained by a high percentage of affected seminiferous tubules in doxorubicin –treated mice which are lined by only a layer of Sertoli cells and rare germ cells or tubules contained cells with degenerated degenerative characteristics [9]. Morphometrical parameters and germ cell frequencies provide more information about the

germ cell degeneration result from the testicular damage [38,10]. In agreement with these reports, our results revealed that the marked reduction in morphometrical parameters and consequent decrease of testicular weight in doxorubicin treated- mice have occurred due to a massive germ cell loss [4,39,38,10,9].

Earlier studies have been shown that a low dose administration of doxorubicin is able to target germ cells, mainly type A spermatogonia and can also decrease spermatogonia (intermediate spermatogonia and type B spermatogonia), primary spermatocytes and round spermatid, Sertoli cell and resulting in consequent depletion of seminiferous epithelium [7-10,40], as described in present study that administered doxorubicin alone. Doxorubicin also decreased the number of Sertoli cells [40] and caused suppression of spermatogenesis by acting through Sertoli cells [41].

In contrast, the present results showed that administration of both doses of *R. canina* extract not only no adverse effects on morphometrical parameters and the different spermatogenic cell types in the testes, but also the higher dose of *R. canina* extract (200 mg/kg) was more effective against this anticancer drug compared to the lower dose of the *R. canina* extract.

Considering that, in mice, a complete wave of spermatogenesis takes around 35 days, we can suppose that, pachytene spermatocytes at 14 days; late round spermatids of step 7-8 at 28 days generated from the target spermatogonia. Therefore, according to the elapsed time between treatment and the killing time of the animals applied in the present study (28 days), pachytene spermatocytes and round spermatids [31,42] were affected by doxorubicin or *R. canina* treatment. The frequency of tubular sections containing different spermatogenic cell types showed that *R. canina* treatment increased the number of preleptotene spermatocytes, pachytene spermatocytes and round spermatids. Conversely, antispermatogenic plants suppressed spermatogenesis and decreased seminiferous epithelium height by marked reductions in the number of pachytene spermatocytes and round spermatids which reflected in the number of spermatozoa in the epididymal fluid [43]. Therefore, it can be concluded that *R. canina* extract with progression of meiotic process would increase spermatogenic cells, which were reflected in the epididymal fluid parameters. In addition, it was noted improvement of the histopathological properties, and a distinctive reduction of the histomorphometrical damage and germ cell loss in the mice treated with *R. canina* extract/doxorubicin, in comparison to those solely doxorubicin-treated. These results are suggestive of the protective effect of the *R. canina* extract against doxorubicin gonadotoxicity.

The impact of the nutritional environment as an important aspect has been demonstrated on male reproduction in mammals [44]. Non-enzymatic antioxidant system is mainly including proanthocyanidins, flavonoids, vitamin E, A, C [45-49]. As previously referred, *R. canina* are known to have some these active constituents and particularly vitamin C [21,50]. The importance of vitamin C in the support of spermatogenesis may explain at least in part through its ability to reduce vitamin E and maintain this antioxidant in an active state [47]. Vitamin E is a powerful antioxidant and a vital factor for the maintenance spermatogenesis in mammalian. Animals with deficiencies of vitamin C and E exhibit disruption of both spermatogenesis process and testosterone production [48]. In contrast,

administration of vitamin C improved sperm concentration, sperm motility, testosterone production and fertility in normal animals and animal's exposure to stress [51,52,48]. Consistent with these reports, *R. canina* extract not only increased testosterone level in normal mice, but also it improved the reduction of testosterone level resulted from doxorubicin treatment. Despite reduction in count, viability, motility and morphology in doxorubicin treated mice [4,9,10,16], *R. canina* pretreatment improved these parameters that were compromised by doxorubicin treatment in accordance with histomorphometrical findings. The significantly higher sperm count, viability, motility and morphology obtained in *R. canina* extract alone than control corroborate these findings and leads us to believe that *R. canina* extract has protective effect against doxorubicin-induced testes gonadotoxicity.

On the other hand, it demonstrated that other free radical scavengers and antioxidants such as proanthocyanidins may cause the restoration of antioxidant defense system. Proanthocyanidins as a promising chemopreventive agent, have a protective effect in the reduce of doxorubicin-induced mutagenesis and cell proliferation changes in somatic and germ cells and as well as improved sperm parameters of mice since it serves as free radical scavengers and antioxidants [15,45]. Similar results have also been described in *Crataegus monogyna* fruit aqueous extract, since this extract is rich in proanthocyanidins and flavonoids [53,54] and many of phenolic compounds with cytoprotective properties by scavenging radicals [55]. *Crataegus monogyna* co-treatment would effectively offset the oxidative impact imposed by doxorubicin on the testicular and sperm parameters through restoration of antioxidant defense system [16]. Therefore, the present study corroborates the critical role of this agent in improving the quality of the semen profile through restoration of antioxidant defense system.

CONCLUSION

In conclusion, our results indicate that *R. canina* extract is able to improve testicular and sperm parameters in mice treated with doxorubicin. The present study is the first study to investigate the protective effect of *R. canina* extract against doxorubicin-induced testicular toxicity. Further studies maybe needed to further investigate the potential role of *R. canina* extract as antioxidant agent on the male reproductive system.

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