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Original Article

Protective effect of Damask rose (*Rosa damascena*) essential oil against testicular injury and alterations in epididymal sperm parameters in rats exposed to high-dose glucocorticoid

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Abstract

Background: Glucocorticoid (GC) excess adversely affects male fertility. **Aims:** This study examined the protective effects of *Rosa damascena* essential oil (RD oil) on testicular structure and function as well as sperm parameters in a rat model of GC-induced testicular injury. **Methods:** Forty-nine adult rats were divided into seven groups: negative control (NC): normal rats; positive control (PC): received dexamethasone at 7 mg/kg/day (intraperitoneally for 7 days); vehicle control (VC) and RD oil-treated groups (T1-T4): received olive oil as vehicle or treated with 40, 80, 160 and 320 mg/kg/day of RD oil by oral gavages concurrently with dexamethasone. **Results:** Dexamethasone decreased body weight and serum testosterone of PC rats, accompanied by germ cell depletion, epithelial thinning, and a marked increase in apoptotic cells in the testes compared to NC rats ($P < 0.05$). In PC rats, reductions were observed in epididymal total sperm count, the number of morphologically normal and viable spermatozoa, sperm DNA integrity, and the percentage of acrosome-intact spermatozoa. In contrast, sperm kinematic parameters remained largely unchanged. RD oil, particularly at higher doses, positively affected testosterone levels, spermatogenesis, and seminiferous epithelial thickness, and reduced testicular apoptosis. In addition, RD oil improved certain sperm parameters, including the percentage of acrosome-intact sperms and DNA integrity. **Conclusion:** RD oil shows a protective effect against GC-induced injury to spermatogenesis and testicular tissue, as well as derangement in serum testosterone. These effects are associated with anti-apoptotic effects, especially at higher doses. Certain impaired sperm parameters are also improved by RD oil. These findings set the stage for future investigations on RD oil in GC-related infertility.

Key words: Apoptosis, Glucocorticoid, Rat, *Rosa damascena*, Testis

Introduction

Glucocorticoids (GCs) are commonly prescribed for their profound anti-inflammatory and immunosuppressive effects. According to Pofi *et al.* (2023), currently, between 1% and 3% of the global population receives GC treatment, and there has been more than a 30% increase in long-term prescriptions over the last two decades. On the other hand, emotional stress has steadily increased globally over the past few decades, resulting in a rise in endogenous GC levels (Piao *et al.*, 2024). Unfortunately, excess GC level is associated with a long list of immediate and long-term complications, among them is suppression of male fertility (Gensler, 2013;

Whirlledge and Cidlowski, 2017).

Historically, approximately 50 years ago, Evain *et al.* (1976) demonstrated that testicular interstitial cells and seminiferous tubules of rats contain specific cytoplasmic and nuclear GC-binding sites. These researchers also reported that administration of dexamethasone for three days reduces testosterone production stimulated by human chorionic gonadotropin in rats (Evain *et al.*, 1976). Following that, Schultz *et al.* (1993) described GC receptor-like immunoreactivity in primary spermatocytes and Leydig cells of adult rat testicles. They suggested that GCs can directly affect spermatogenesis and steroidogenesis in the testis.

Today, the detrimental impact of GC excess, whether

exogenous or stress-induced, on testicular function and consequently male fertility has been demonstrated through clinical experience and findings from numerous experimental studies (Whirledge and Cidlowski, 2017; Hampf and Stárka, 2020). Various researchers have used animal models, primarily mice and rats, and found that administering GCs at elevated doses for a relatively short period leads to harmful effects on testicular structure and function. In some studies, a few parameters concerning epididymal sperm have also been assessed; however, information regarding the effects of GCs on epididymal sperm characteristics in subacute studies is generally limited (Yazawa *et al.*, 2000; Orazizadeh *et al.*, 2010; Khorsandi *et al.*, 2013; Azimi Zangabad *et al.*, 2023).

The initiation of apoptosis in testicular cells, including germ cells and Leydig cells, has been identified by various researchers as a significant mechanism through which GCs induce harmful effects on the reproductive system of male rats, along with a reduction in serum testosterone levels (Yazawa *et al.*, 2000; Gao *et al.*, 2002; Orazizadeh *et al.*, 2010; Kheirabad *et al.*, 2016).

Although drugs, such as glucose-lowering or antihypertensive medications, are prescribed to reduce comorbidities like diabetes or hypertension associated with GC excess (Fardet and Fève, 2014), currently, there is no specific treatment available to effectively assist in reducing potential testicular injury in male patients experiencing stress or undergoing GC therapy.

Herbal remedies have attracted interest in this regard, leading to research on the potential benefits of herbal preparations or natural products against GC-related infertility. To the best of our knowledge, various extracts have been examined in this context, including grape seed extract (Hasona, 2018), aqueous extract of *Allium cepa* L. (onion) (Nassan *et al.*, 2021), Egyptian *Portulaca oleracea* L. (Purslane) and *Cichorium intybus* L. (Chicory) extracts (Saleh *et al.*, 2022), as well as *Nigella sativa* essential oil (Abouzaripour *et al.*, 2024), among others.

Damask rose (*Rosa damascena* Mill. L.), known by the name “Gole Mohammadi” in Iran, is a member of the Rosaceae family with a mesmerizing scent and a long history of cultivation, mostly to exploit its essential oil (RD oil) in the perfume industry (Boskabady *et al.*, 2011). Aromatherapy with rose oil has shown protective effects against the detrimental effects of formaldehyde inhalation on the testicular tissue of rats (Köse *et al.*, 2012). Interestingly, Hamed *et al.* (2018) reported that oral administration of RD oil increases sperm counts and the number of spermatogonia, primary spermatocytes, Sertoli cells, and Leydig cells in diabetic rats. In double-blind, randomized, placebo-controlled clinical trials by Farnia *et al.* (2015, 2017), orally administered RD oil has improved sexual function in male patients with selective serotonin-reuptake inhibitors-induced sexual dysfunction and also in those with opium use disorder under methadone maintenance therapy. In the latter patients, administration of the RD oil has also increased serum testosterone levels (Farnia *et al.*, 2015, 2017). Some

components of RD oil have also shown anti-apoptotic effects *in vivo* (Wang *et al.*, 2016; Jayaraj *et al.*, 2022).

Despite these promising results, the plausible protective effects of RD oil on GC-induced testicular injury have not been evaluated yet. This motivated us to conduct a study to clarify the effects of RD oil on the testicular structure and function of rats with GC-induced testicular injury. To achieve this goal, serum testosterone level, histopathological and histomorphometric parameters of testicular tissue, Johnsen scoring, as well as sperm parameters were determined in rats with GC-induced testicular injury that were treated with different doses of RD oil. Terminal nucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) assay was also performed for the determination of anti-apoptotic effects.

Materials and Methods

The RD oil

The RD oil (Batch No. 63014078) was provided by Barij Essence Pharmaceutical Co., Tehran, Iran, and was prepared by hydrodistillation. According to the manufacturer's analysis report, the RD oil contained citronellol (26.4%), geraniol (5.9%), nerol (2.9%), and 2-phenyl ethanol (1.4%) (w/w) as the major constituents determined by the gas chromatography method (the analysis sheet is available as Supplementary Figure 1 (SF1)).

Animals and study design

An experimental study with parallel control groups was designed. Forty-nine adult male Sprague-Dawley rats with a body weight of 200 ± 20 g were kept in transparent polycarbonate cages with wood-shaving bedding under standard environmental conditions (temperature $21 \pm 2^\circ\text{C}$ and 12/12 h light/dark cycles) with free access to commercial feed and tap water. Visual checking was performed twice daily to assess the health status of the animals.

After one week of the adaptation period, animals were randomly divided into seven equal groups ($n=7$ each) and treated as follows: negative control group (NC): normal rats that received no special treatment during the experiment; positive control group (PC): these rats received dexamethasone phosphate (Dexacoid®, 0.4%, Nasr, Tehran, Iran) at the dose level of 7 mg/kg/day by intraperitoneal injections for 7 consecutive days for induction of testicular injury (the dose level selection was based on the previous study (Yazawa *et al.*, 2000); vehicle control group (VC): testicular injury was induced in these rats as the PC group and also they received olive oil by oral gavages as the vehicle of RD oil; RD oil treated groups (T1, T2, T3, and T4): in addition to dexamethasone, these rats were concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. The RD oil was dissolved in olive oil as the vehicle. The dose levels for RD oil were chosen based on a previous study by Dadkhah *et al.* (2020) with

modifications.

At the end of the treatment period and after an overnight fast, rats were weighed and anesthetized with an intraperitoneal injection of ketamine (90 mg/kg; ketamine HCl 10%, Alfasan) and xylazine (10 mg/kg; Xylamax® 2%, Rooyan Darou) cocktail. Cardiocentesis was performed to collect blood samples. The blood samples were taken between 10:00 and 12:00 a.m. All rats were euthanized under deep CO₂ anesthesia. The right testicle was removed immediately, weighed, and then transferred to neutral buffered formalin (10%) for histological examination. The right cauda epididymis was weighed and used for evaluating sperm parameters.

All methods employed in this research received approval from the Shiraz University institutional ethics committee (code No.: IR.US.PSYEDU.REC.1402.077) and were in accordance with Directive 2010/63/EU regarding the protection of animals utilized for scientific purposes.

Determination of serum testosterone level

Blood samples were centrifuged at 1500 g for 10 min. Harvested sera were stored at -70°C until use. An ELISA kit (Monobind Inc., CA, USA, Cat. No. 3725-300A) was used to determine serum testosterone based on competitive enzyme immunoassay. Within and between-assay CVs of the kit were both <10%. The assay was performed according to the manufacturer's protocol.

Johnsen Scoring, histopathological, and histomorphometric evaluation

Five-micrometer-thick cross-sections from testicular samples were prepared and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope for plausible lesions in seminiferous tubules and interstitial tissue cells by an expert unaware of the grouping system. To evaluate spermatogenesis, Johnsen scoring was performed under a light microscope with a ×10 objective magnification for 10 tubules in each field. In case of doubt, the presence of spermatozoa was checked by higher magnification. Each tubule was scored from 1 to 10 based on criteria previously described by Johnsen (1970), and the mean score was calculated for each sample.

For histomorphometric examinations, we also used five-micrometer-thick cross-sections from testicular samples, stained with hematoxylin and eosin (H&E), which were investigated by light microscopy. Three slides were prepared from each sample. In each slide, 3 or 4 indiscriminately chosen seminiferous tubules from stage VII were used for analysis. The diameter of the seminiferous tubule and epithelial thickness of circular to nearly circular tubules were determined in 10 tubules/sample by using Digimizer image analysis software. The tubules were chosen indiscriminately, and the criteria to find circular or nearly circular tubules were based on the study by Ogedengbe *et al.* (2018).

TUNEL assay

After deparaffinization and rehydration, the slides

were washed with phosphate-buffered saline (PBS). Then, samples were exposed to hydrogen peroxide and methanol (1:9) for 10 min, after which they were washed 3 times with PBS (five min each time). Proteinase K (IHC Select®, Cat. No. 21627-M, Merck Millipore) diluted at 1:1000 with PBS was used for 30 min at 37°C, followed by 3 times washing with PBS. Then, 0.3% Triton™ X-100 (Cat. No. T8787, Sigma-Aldrich) for 10 min was used to increase the permeability of the nucleus. Samples were then washed 3 times with PBS. An In Situ Cell Death Detection Kit, POD (Cat. No. 11684817910, Roche) was used. Samples were exposed to terminal deoxynucleotidyl transferase (TdT) enzyme (50 µL with 450 µL of label solution [fluorescein-dUTP]) for 2 h at 37°C, followed by being washed 3 times with PBS. Finally, the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Cat. No. D9542, Sigma-Aldrich), and slides were examined by an Olympus fluorescence microscope. Three tubules were analyzed per slide, with five fields examined for each tubule, and then the average for each slide was calculated. ImageJ 1.52n software was used to calculate the TUNEL fluorescence intensity.

Sperm analysis

Collection of epididymal sperms

Immediately following sacrifice, the testicles were exposed, and the cauda epididymides were carefully excised. The epididymal tails were cut into several small fragments and placed in multi-well sterile culture plates containing 3 ml of pre-warmed (37°C) sperm analysis medium. The medium consisted of Tris (3.025 g), citrate (1.7 g), glucose (1.25 g), crystalline penicillin G (150,000 U), crystalline streptomycin sulfate (150 mg), and sterile double-distilled water to a final volume of 100 ml, adjusted to pH 6.76. Plates were maintained on a warm stage at 38.5°C for 15 min to allow spermatozoa to disperse into the medium before analysis.

Total epididymal sperm count

Sperm counts were determined utilizing a Neubauer hemocytometer (Brand™, Germany) following the dilution and devitalization of sperm cells in distilled water at a ratio of 1:9. Fragments of epididymal tissue were meticulously collected and weighed on a laboratory digital precision scale (Sartorius™, Japan) with a sensitivity of 0.0001 g. Ultimately, the total quantity of epididymal sperm cells per mg of epididymal tissue was determined for each sample.

Spermatozoa viability and morphology

The Eosin-Nigrosin supravital staining technique was employed to assess the viability of spermatozoa. In summary, a 5 µL sample of sperm was combined with an equal volume of commercial staining solution (Minitube™, Germany) on a pre-warmed microscopic slide at 37°C, followed by smearing after 5 s. Spermatozoa exhibiting reddish-pink heads were classified as non-viable, while those with colorless heads were deemed viable. Additionally, the prepared smears

facilitated the examination of morphological abnormalities in the sperm cells. Spermatozoa were categorized as normal or abnormal based on observation of morphological abnormalities in different anatomic regions. A minimum of 200 cells was analyzed for each sample.

Spermatozoa kinematic parameters

Epididymal spermatozoa motility parameters were assessed subjectively within 10 min following the dissection of the epididymis tails into the analysis medium. This evaluation was conducted using a computer-assisted semen analysis system, specifically the HFT™ CASA System (HFT Co., Tehran, Iran) (Table 1), in conjunction with an Olympus bright-field microscope (Olympus, Tokyo, Japan) at a magnification of 100. A 5 µL sample of the analysis medium containing sperm cells was collected and introduced into a pre-warmed (37°C) counting chamber (Sperm360 Labs, 10-micron depth, India) for immediate assessment. A minimum of 400 cells in 4 random microscopic fields (×100 magnification) were counted. Frame rate, particle size thresholds, velocity cut-offs, and definitions of progressive motility were standardized across all groups. Given the elongated shape of rat sperm and the minor variation in diameter between the head and midpiece, special care was taken with the particle size and tracking thresholds to reduce the risk of misidentification. Even with meticulous tuning of CASA parameters, it was often not possible to completely eliminate segment over-detection in the analysis of rodent sperm. The kinematic parameters of the sperm included the total motile spermatozoa percentage (TM), the percentage of spermatozoa exhibiting rapid progressive motility (PM), as well as the velocities measured along the curved line (VCL, µm/s), the average path (VAP, µm/s), and the straight line (VSL, µm/s). Spermatozoa velocity ratios, including straightness, linearity, and wobble, were also calculated. A spermatozoon was classified as immotile when the VAP value was less than 10 µm/second, and the CASA frame rate was set to 50 Hz.

Table 1: HFT CASA technical settings

Parameters function	Parameter	Values
Sperm detection	Min area of sperm	8 Pixels
	Max area of sperm	40 Pixels
	Mean area of sperm	20 Pixels
Activity classification	Motile min speed 1	9.9 µm/s
	Motile min speed 2	12 µm/s
	Motile max speed	250 µm/s
	VSL low	5 µm/s
	VSL high	10 µm/s
	LIN low	15%
	LIN high	30%

VSL: Linear velocity, and LIN: Linearity

Sperm DNA integrity (sperm chromatin dispersion assay)

The sperm chromatin dispersion (SCD) assay, specifically focusing on double-strand breaks, was

employed, as previously described (Fernández *et al.*, 2003). In summary, sperm cells were introduced into a low-melting-point agarose gel placed on microscope slides and subsequently treated with 0.08 N HCl along with lysing solutions that included 1% sodium dodecyl sulfate, 2 M NaCl, and 0.8 M dithiothreitol. The slides were then stained using Diff-Quick reagent. Following acid denaturation and the removal of nuclear proteins, spermatozoa with intact DNA exhibited a prominent halo of dispersed DNA surrounding the head (DNAL). In contrast, sperm cells exhibiting partial and severe DNA fragmentation displayed smaller halos (DNAS) or no halo (DNAN) at all, respectively. A total of 100 sperm cells were analyzed to calculate the mean percentages of spermatozoa with partially fragmented and intact DNA.

Sperm DNA integrity (acridine orange staining)

Before staining, the smear was treated with an acid-detergent solution at pH 1.2, composed of 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton-X 100. Following this, the smear was stained for 10 min using freshly prepared AO at a concentration of 0.19 mg/ml in a citrate phosphate buffer, which consisted of 80 ml of 0.1 M citric acid and 5 ml of 0.3 M NaH₂PO₄, adjusted to pH 2.5. A minimum of 200 sperm cells were analyzed for each sample by an epi-fluorescent microscope (ZEISS Primo Star iLED, Carl Zeiss Microimaging GmbH, Germany) under ×1000 magnification. Sperm heads that displayed a green fluorescence were classified as having normal DNA (double-stranded), whereas those exhibiting red or orange fluorescence were identified as having damaged DNA (single-stranded).

Integrity of sperm acrosome

The Coomassie Brilliant Blue G-250 staining protocol was employed. Initially, the sperm samples were fixed in a 4% paraformaldehyde solution (comprising 2.5 mM NaH₂PO₄, 110 mM Na₂HPO₄, and 4% paraformaldehyde at pH 7.4) at 24°C for 10 min. Following centrifugation, sperms were subjected to two washes with 1.5 ml of 100 mM ammonium acetate (pH 9.0). The resultant sperm pellet was subsequently resuspended in 1 ml of the same ammonium acetate solution. A 50 µL aliquot of the sperm suspension was then spread onto glass microscope slides using another glass slide, and the preparations were allowed to air dry. The sperm on the slides were then incubated for 2 min in a freshly formulated Coomassie stain, which consisted of 0.22% Coomassie Brilliant Blue G-250 (Sigma-Aldrich, USA), 50% methanol, 10% glacial acetic acid, and 40% water. After staining, the slides were rinsed with distilled water to eliminate any excess dye. The stained spermatozoa were examined under a bright-field light microscope at a magnification of ×400. A distinct blue line along the head of the spermatozoon indicated intact acrosome, with a minimum of 100 analyzed spermatozoa.

Data analysis

All data were presented as mean and SD. The

normality of data was analyzed by the Shapiro-Wilk test. Data analysis was performed by One-way ANOVA followed by Tukey's multiple-comparison test. A paired t-test was used to compare the body weight of rats in each group between days 1 and 8 of the experiment. $P < 0.05$ was considered the level of significance for all comparisons. The outliers were identified by the ROUT (Robust Regression and Outlier removal) method with the ROUT coefficient (Q) of 5%. The effect size, which quantifies the strength of a treatment response between groups, is indicated to show practical significance (Maher *et al.*, 2013). Based on GraphPad Prism 6 software indications, since there's only one factor, R squared for the model equals eta squared for that effect.

Data analysis and preparation of graphs were performed with GraphPad Prism 6 software.

Results

Body weight change and testicular weight/body weight ratio

Rats in different groups showed statistically the same body weight at the beginning of the experiment (day 1) (data not shown). As shown in Fig. 1, at the end of the experiment (day 8), the body weight of rats in the NC group increased compared to day 1 ($P = 0.0002$), while other groups showed a slight decrease in body weight during this period ($P > 0.05$). One-way ANOVA showed a significant difference in body weight change among groups during the experiment [$P < 0.0001$, R square=0.597]. Rats that received dexamethasone (VC, PC, T1-T4) showed a significant negative change in body weight compared to the NC group ($P < 0.0001$ for all comparisons). Regarding testicular weight/body weight ratio, one-way ANOVA revealed a significant difference among groups in this parameter [$P = 0.0068$, R square=0.427]. Administration of dexamethasone was not associated with a significant change in the testicular weight/body weight ratio of rats in the VC and PC groups compared to NC rats ($P > 0.05$). Moreover, rats that were treated with RD oil (T1-T4) had a statistically similar testicular weight/body weight ratio compared to

PC/VC rats; although a higher testicular weight/body weight ratio of T1-T4 rats was observed compared to the NC group ($P < 0.05$ for all comparisons).

Serum testosterone level

One-way ANOVA showed a significant difference in serum testosterone levels among groups [$P = 0.0005$, R square=0.568]. As shown in Fig. 2, the administration of dexamethasone to rats in PC and VC groups was associated with a significant decrease in serum testosterone levels compared to that of the NC rats ($P < 0.05$ for both comparisons). Treating rats with RD oil at dose levels of 40, 80, and 160 mg/kg (T1-T3) did not result in a significant change in serum testosterone levels compared to the PC group ($P > 0.05$). However, rats in the T4 group that received the highest dose level of RD oil showed a significant increase in serum testosterone level compared to PC rats ($P < 0.05$). Administration of RD oil at 320 mg/kg reversed the serum testosterone level of rats to that of the NC group ($P > 0.05$).

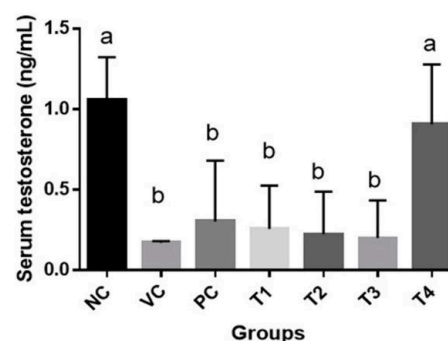


Fig. 2: Serum testosterone levels (mean and SD) in different groups. NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, and T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P < 0.05$.

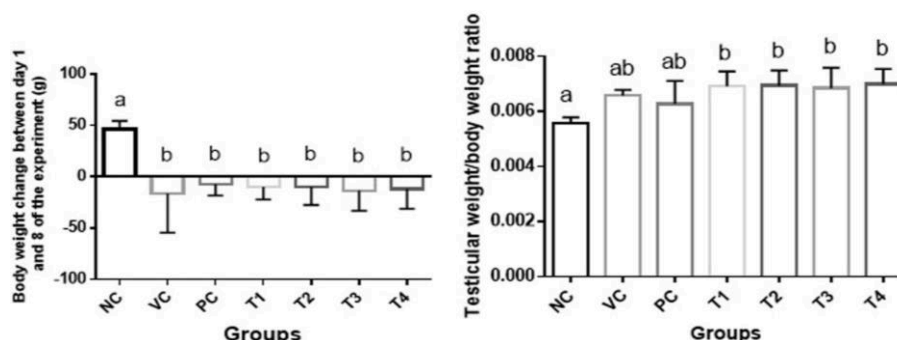


Fig. 1: Body weight change and testicular weight/body weight ratio (mean and SD) in different groups. NC: Normal rats; VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil; PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days; T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P < 0.05$.

Descriptive histopathological changes

Testicular tissue sections of the NC group revealed the normal structure of the seminiferous tubules and spermatogenesis, while decreased spermatogenesis, accompanied by epithelial thinning, germ cell depletion, and epithelial vacuolization, were observed in some tubules of the PC group. Moreover, a decreased

abundance of Leydig cells in the interstitial tissue was observed in this group. Administration of RD oil, except for the T1 group, was associated with better spermatogenesis. Especially in the T4 group, the tissue lesions were decreased compared to that of the PC group, and the structure of the tubules was much more similar to the NC group (Figs. 3A-H).

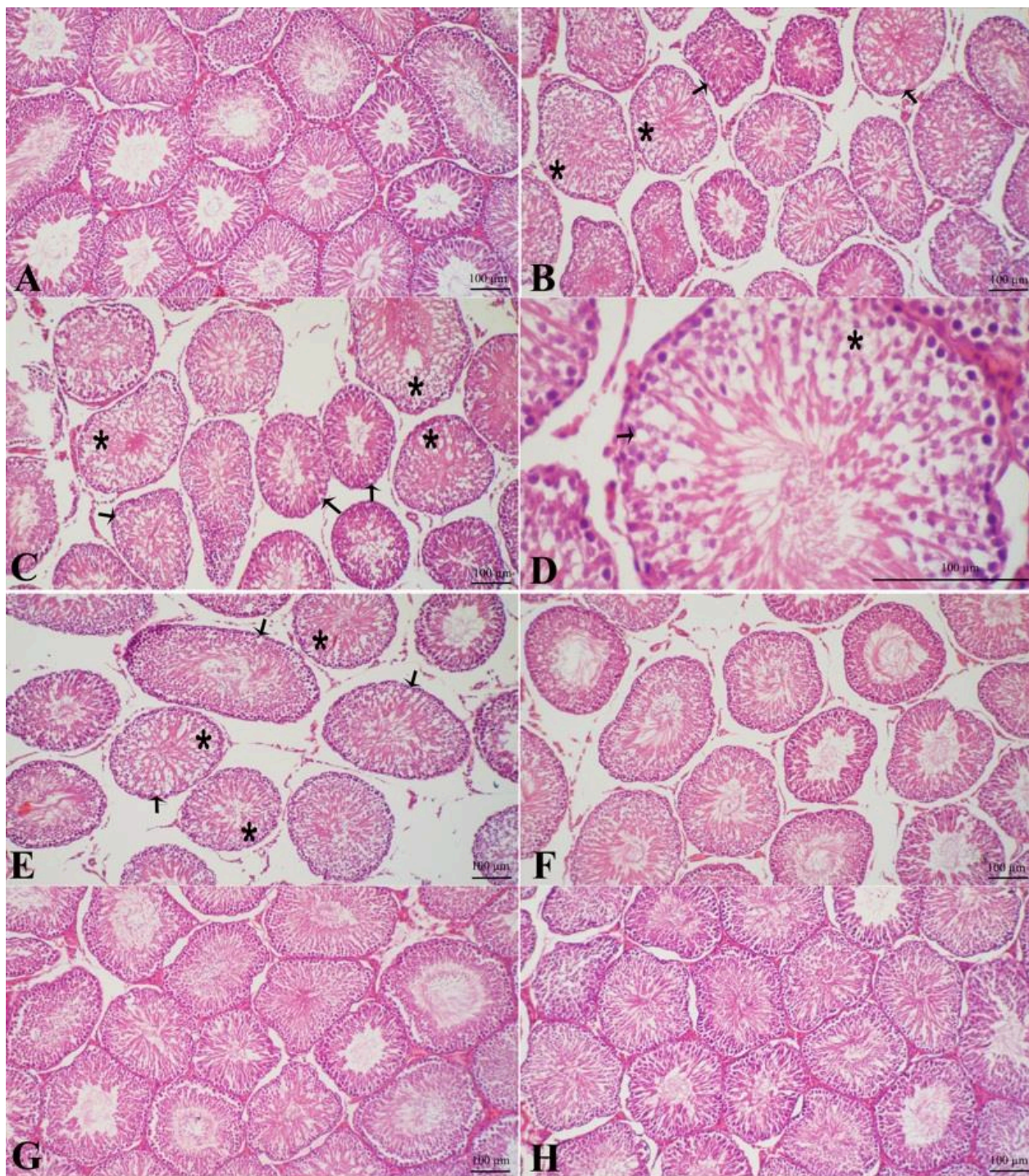


Fig. 3: Representative histopathological photomicrographs of testicular tissue in different groups. (A) Negative control group: normal structure of the seminiferous tubules, (H&E, scale bar, 100 µm); (B and C) Vehicle and positive control groups: decreased abundance and depletion of epithelial germ cells (*) with vacuolization (arrows) in some tubules, (H&E, scale bar, 100 µm); (D) Higher magnification from positive control group: decreased germ cells (*) with vacuolization (arrow), (H&E, scale bar, 100 µm); (E) T1 group: decreased germ cells (*) with vacuolization (arrows), (H&E, scale bar, 100 µm); (F-H) T2-T4 groups. Improved lesions so that the T4 group was almost similar to the negative control group, (H&E, scale bar, 100 µm)

Histomorphometric parameters

Although the seminiferous tubules' diameter was statistically the same among different groups [$P=0.1651$, $R\text{ square}=0.242$], a significant difference was observed in epithelial thickness of the tubules [$P<0.0001$, $R\text{ square}=0.750$]. According to the results of the *post-hoc* test, the administration of dexamethasone to rats of the VC and PC groups was associated with a significant decrease in epithelial thickness of seminiferous tubules compared to that of the NC rats ($P<0.0001$ for both comparisons). The epithelial thickness of tubules in rats of the T3 and T4 groups was appreciably higher than that of the PC rats ($P<0.05$ and $P<0.01$, respectively). Administration of RD oil to T3 and T4 rats could not increase the epithelial thickness of tubules to the level of NC rats ($P<0.001$ and $P<0.05$, respectively) (Fig. 4).

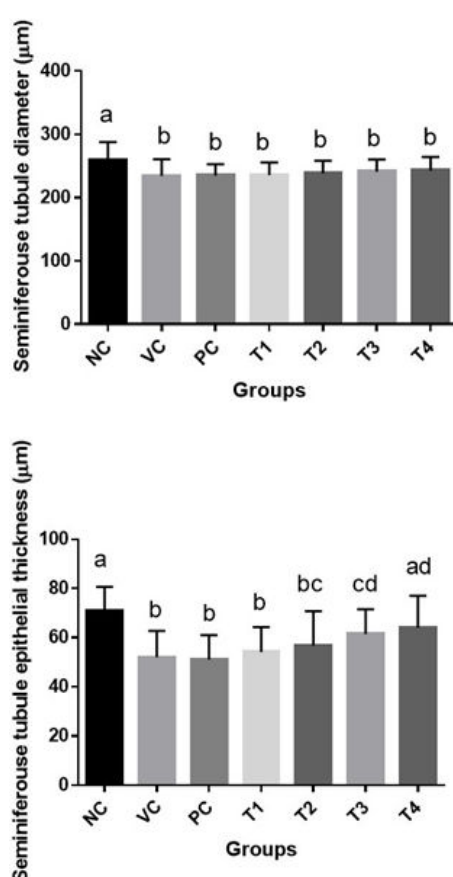


Fig. 4: Histomorphometric parameters of testicular tissue (mean and SD) in different groups. NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, and T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P<0.05$

Johnsen scores

In one-way ANOVA, a significant difference was

observed in Johnsen scores among groups [$P<0.0001$, $R\text{ square}=0.962$]. VC and PC Groups had significantly lower Johnsen scores than that of the NC rats ($P<0.0001$ for all comparisons). Administration of RD oil at two higher dose levels (T3 and T4) resulted in a significant increase in Johnsen score than that of the PC rats ($P<0.001$ and $P<0.0001$, respectively). Rats in the T4 group showed significantly higher scores than T3 rats ($P<0.001$) (Fig. 5).

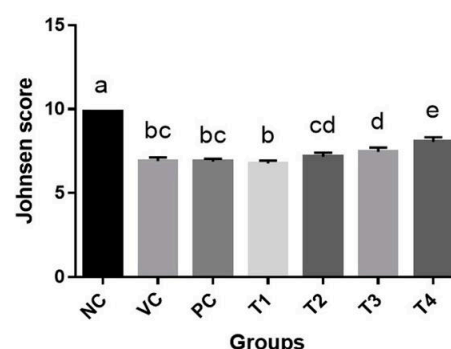


Fig. 5: Johnsen scores (mean and SD) of different groups. NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, and T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P<0.05$

TUNEL assay

Figure 6A shows photomicrographs of testicular tissue after fluorescent TUNEL staining. One-way ANOVA revealed a significant difference in the percentage of TUNEL-positive cells among groups [$P<0.0001$, $R\text{ square}=0.991$]. Dexamethasone administration to rats of the PC group significantly increased the percentage of TUNEL-positive cells compared to that of the NC group ($P<0.0001$). Treated rats with RD oil showed a significantly decreased percentage of TUNEL-positive cells compared to PC rats ($P<0.0001$ for all comparisons). Rats in the T4 group had a significantly lower percentage of TUNEL-positive cells than other RD oil-treated rats ($P<0.001$ for all comparisons). Administration of RD oil could not reverse the percentage of TUNEL-positive cells to the NC group levels ($P<0.0001$ for all comparisons) (Fig. 6B).

Sperm count, viability, and morphology

One-way ANOVA revealed a significant change in total epididymal sperm count as well as total sperm count/mg of cauda epididymis among groups [$P<0.0001$, $R\text{ square}=0.772$ and $P<0.0001$, $R\text{ square}=0.712$, respectively]. Post hoc test showed that administration of dexamethasone was associated with a significant decrease in total epididymal spermatozoa count and total

spermatozoa count/mg of cauda epididymis of rats of VC and PC groups compared to those of the NC rats ($P<0.001$ for all comparisons). Treatment with RD oil did not significantly alter the above-mentioned parameters in T1-T4 groups compared to VC or PC rats ($P>0.05$).

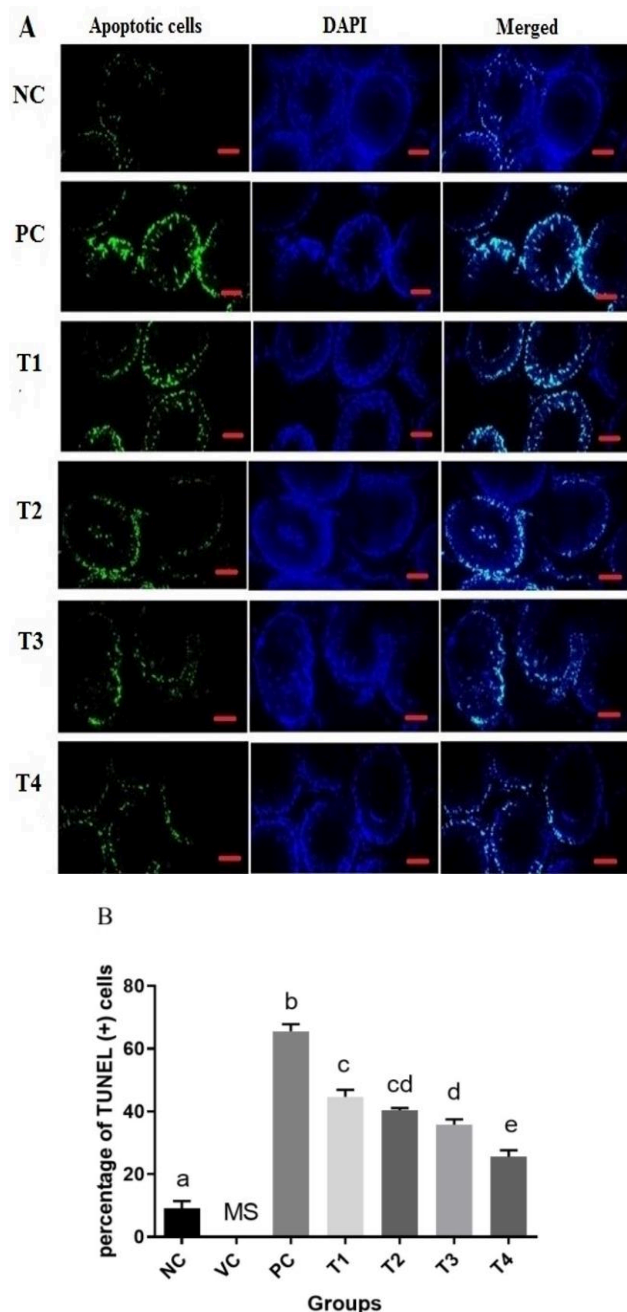


Fig. 6: (A) Representative photomicrographs of testicular tissue after fluorescent TUNEL staining (scale bar 200 μ m) showed increased apoptotic cells in dexamethasone-treated groups compared to NC rats, and (B) Effect of RD oil on the percentage of TUNEL positive cells (mean and SD) in different groups ($n=3$). NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the

dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days, and MS: Missed samples. Samples were missed during processing and preparation, and could not be replaced. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P<0.05$.

A significant difference was also observed among groups in the percentage of viable sperm [$P<0.0001$, R square=0.622]. The percentage of viable spermatozoa significantly decreased in the VC and PC groups than that of the NC rats ($P<0.05$). Although all groups of rats that were treated with RD oil showed statistically similar percentages of viable spermatozoa compared to that of the NC rats ($P>0.05$), rats that were treated with RD oil at the T2 and T3 groups showed significantly higher percentages of sperm viability compared to that of the PC group ($P<0.001$ and $P<0.05$, respectively).

The percentage of sperm with normal morphology was also significantly different among groups [$P=0.0001$, R square=0.574]. This parameter was significantly lower in the VC and PC groups compared to NC rats ($P<0.01$ for both comparisons). Rats in the T2 group had a significantly higher percentage of sperm with normal morphology than VC and PC rats ($P<0.05$ for both comparisons). Figures 7A-F show the morphology of sperm. Data related to the above-mentioned parameters in different groups are summarized in Fig. 8.

Sperm kinematic parameters

Administration of dexamethasone was associated with only insignificant changes in sperm kinematic parameters of the PC group compared to NC rats. Rats in T4 groups had significantly higher percentages of progressive motile sperm and velocity of curved line than those of the PC rats ($P<0.05$ for both comparisons). Aside from this, rats in the T1-T4 groups showed statistically the same sperm kinematic parameters compared to those of the NC, VC, or PC groups ($P>0.05$) (Table 2).

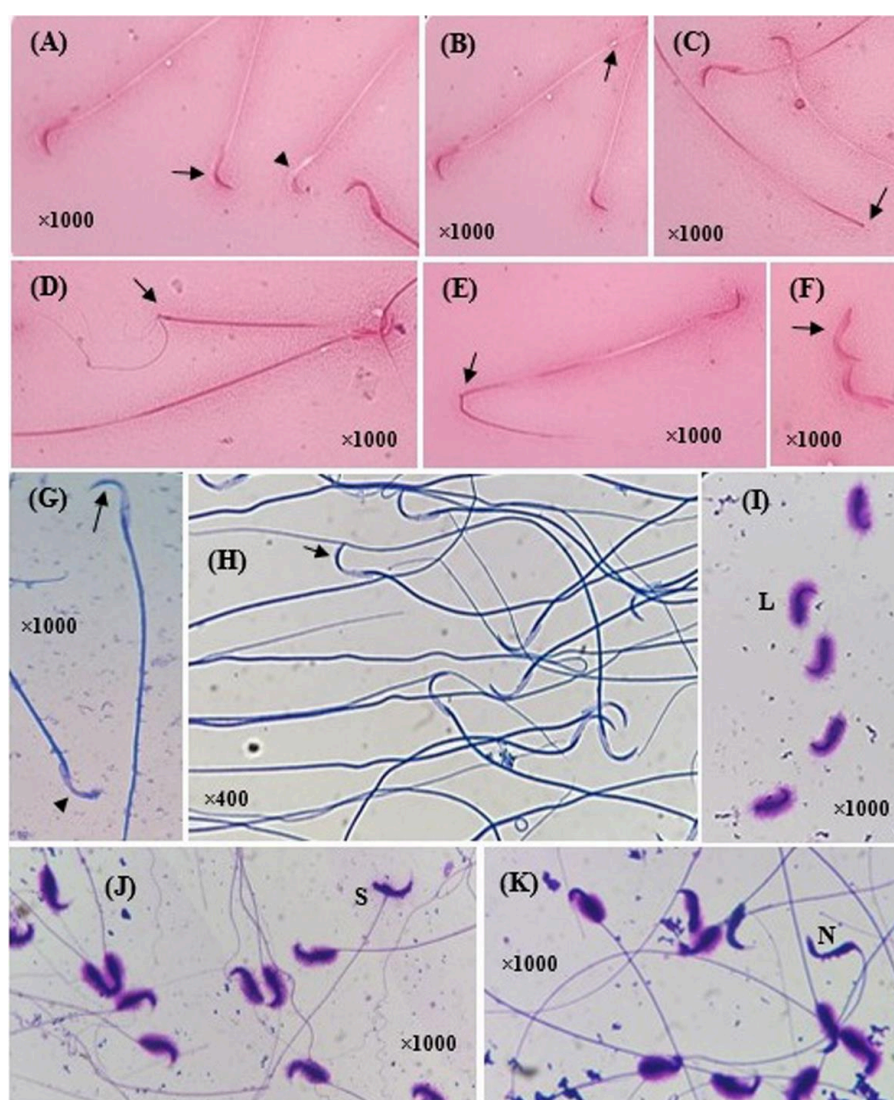
Sperm DNA integrity and the integrity of the sperm acrosome

The percentage of sperm with acrosome integrity was lower in rats of the PC group compared to NC ($P<0.05$) rats. RD oil-treated rats showed significantly higher spermatozoa with intact acrosome than that of the PC group ($P<0.01$ for comparison with T1 and T3, $P<0.001$ for comparison with T2 and T4). This parameter was statistically the same among NC and RD oil-treated groups ($P>0.05$). Parameters related to sperm DNA integrity were statistically different in rats of PC or VC groups compared to NC rats ($P<0.01$ for all comparisons). Administration of RD oil to rats in T1 to T4 groups was associated with an appreciable change in these parameters compared to PC rats ($P<0.01$ for all comparisons). Data are summarized in Table 3. Figures 7G-K shows images related to DNA integrity as determined by the two above-mentioned methods in rats.

Table 2: Sperm kinematic parameters (mean and SD) of rats in different groups. The parameters were not affected by dexamethasone administration

Parameters	Groups							Effect size	ANOVA P-value
	NC	VC	PC	T1	T2	T3	T4		
Total motile spermatozoa (%)	96.8	99.2	93.1	99.5	97.81	98.5	99.6	R ² =0.292	P=0.0777
Progressive motile spermatozoa (%)	±2.91	±0.292	±6.69	±0.660	±3.85	±1.66	±0.492	R ² =0.291	P=0.0180
	39.3	41.2	39.1	42.8	45.6	45	47.4		
Velocity of curved line (µm/s)	±7.38	±0.827	±6.40	±3.19	±6.01	±3.52	±4.74*	R ² =0.250	P=0.0180
	172	186	153	183	187	177	194		
Velocity along the average path (µm/s)	±20.4	±8.11	±39.2	±11.5	±24.7	±27.8	±10.11*	R ² =0.314	P=0.0534
	79.7	89.8	66.9	86	89.2	85	92.4		
Straight line velocity (µm/s)	±12.6	±8.36	±22.6	±6.23	±12.6	±13.6	±5.01	R ² =0.288	P=0.0826
	53.6	63.5	43.6	56.2	60.4	56.2	63.1		
Straightness (%)	±11.6	±11.8	±17.3	±4.92	±10.4	±10.4	±5.41	R ² =0.179	P=0.3677
	30.9	33.8	29.8	30.6	32.2	31.6	32.4		
Linearity (%)	±3.45	±4.75	±3.71	±0.978	±2.31	±1.31	±1.43	R ² =0.207	P=0.2651
	66.8	70.3	64.1	65.3	67.5	65.9	68.2		
Wobble (%)	±4.17	±6.6	±4.92	±1.39	±2.94	±2.43	±3.48	R ² =0.187	P=0.3572
	46.1	48	47.3	46.8	47.6	48	47.6		
	±2.44	±2.39	±0.823	±0.763	±1.58	±0.563	±0.812		

NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, and T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. No significant difference was observed among groups ($P>0.05$), except for significant difference between T4 and PC group for percent of progressive motile sperms and velocity of curved line (µm/s). * The significant difference is shown by asterisk sign ($P<0.05$, for both cases)

**Fig. 7:** Images of eosin-nigrosin supravital-stained spermatozoa (A-F). Live (arrow) versus dead (triangle) spermatozoa are shown in

panel A, while Panels B-F show normal and abnormal morphologic forms. In panel (B) Distal protoplasmic droplet, (C) A decapitated spermatozoa, (D) and (E) Sperm cells with bent tails, and (F) Free spermatozoa head are shown by arrows. Panels G and H show sperm acrosome integrity as assessed by the modified Coomassie Blue staining method; Spermatozoa with intact acrosome indicate a sharp blue line along their heads (arrow), and spermatozoa with damaged/reacted acrosome do not show the sharp blue line (triangle). Panels I-K show the results of the sperm chromatin dispersion (SCD) test. Nuclei with a large size halo (L) were considered non-fragmented DNA; nuclei with no halo (N) were considered to have totally fragmented DNA; nuclei with small size halo (S) were considered to have partially fragmented DNA. All images were taken under bright field compound microscope at given magnifications written on each image

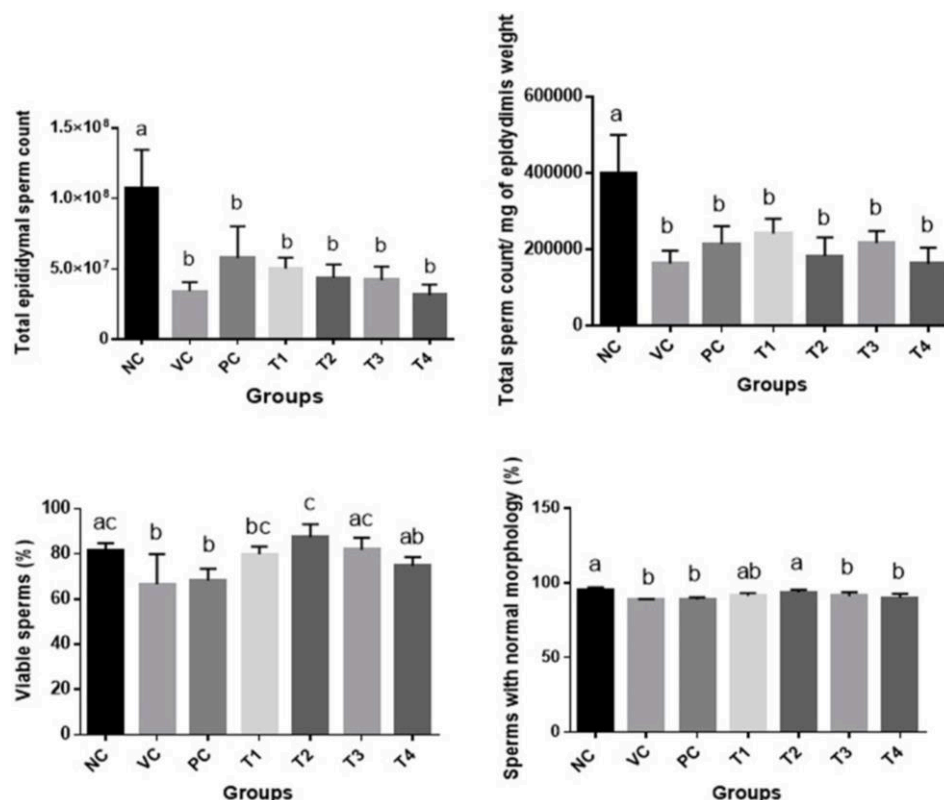


Fig. 8: Epididymal sperm count, viability and morphology parameters (mean and SD) in different groups. NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days, and T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days. Columns marked with at least one similar letter are not significantly different, while when no common letter is present, the values are significantly different at $P < 0.05$

Table 3: Spermatozoa acrosome and DNA integrity parameters (mean and SD) of rats in different groups

Parameters	Groups							Effect size	ANOVA P-value
	NC	VC	PC	T1	T2	T3	T4		
Acrosome intact spermatozoa (%)	98.4 ±1.14 ^a	MS	96.4 ±1.39 ^b	98.8 ±0.836 ^a	99.3 ±0.816 ^a	98.7 ±1.11 ^a	99.4 ±0.894 ^a	$R^2=0.539$	$P=0.0003$
Spermatozoa with large halo (DNAL) (%)	94.4 ±1.51 ^a	62.5 ±3.53 ^b	51.4 ±11.4 ^b	91.2 ±2.98 ^a	88.3 ±4.92 ^a	89.3 ±3.32 ^a	90.4 ±4.56 ^a	$R^2=0.894$	$P<0.0001$
Spermatozoa with small halo (DNAS) (%)	5.60 ±1.51 ^a	27.5 ±3.53 ^b	39.4 ±10.1 ^b	7.75 ±2.06 ^a	11.3 ±5.12 ^a	9.66 ±3.14 ^a	9.20 ±4.02 ^a	$R^2=0.859$	$P<0.0001$
Spermatozoa with no halo (DNAN) (%)	0 ±0 ^a	10.0 ±0 ^b	10.4 ±4.89 ^b	1.00 ±1.15 ^a	0.333 ±0.816 ^a	1.00 ±1.09 ^a	0.400 ±0.894 ^a	$R^2=0.801$	$P<0.0001$
Spermatozoa DNA integrity (%)	96.0 ±1.41 ^a	MS	92.8 ±1.09 ^b	98.8 ±1.09 ^{ac}	97.6 ±1.63 ^{ac}	98.8 ±1.06 ^c	97.0 ±1.22 ^{ac}	$R^2=0.684$	$P<0.0001$

NC: Normal rats, VC: Vehicle control group that received dexamethasone and olive oil by oral gavages as the vehicle of RD oil, PC: Positive control group that received dexamethasone at the dose level of 7 mg/kg/day by intraperitoneal injection for 7 days; T1-T4 groups that received dexamethasone and concomitantly treated with RD oil at the dose levels of 40, 80, 160 and 320 mg/kg/day by oral gavage for 7 days, and MS: Missed samples. Samples were missed during processing and preparation and could not be replaced. Administration of RD oil at different doses positively affected sperm acrosome and DNA integrity parameters. The values in a row with no common superscript letter are statistically different ($P < 0.05$)

Discussion

Consistent with previous studies, we observed that dexamethasone reduces the body weight of rats without affecting the testicular weight to body weight ratio (Yazawa *et al.*, 2000; Hasona, 2018). As anticipated, the negative impacts of dexamethasone were also evident at the testicular level, as shown by unfavorable histopathological changes in testicular tissue and reduced serum testosterone levels.

Information regarding the impact of GCs on epididymal sperm characteristics in subacute studies, like the one we conducted, is scarce. Based on our findings, GC administration in this model decreases epididymal sperm count and viability and also adversely affects sperm morphology. In rats, it takes 52 days from the initiation of stem cell division to the maturation of spermatozoa plus 7-10 days to reach the cauda epididymis (Robb *et al.*, 1978; Clouthier *et al.*, 1996). Since the duration of dexamethasone administration in the present study was limited to 7 days (subacute exposure), the destructive changes induced by dexamethasone at the testicular level (for instance, reduced spermatogenesis) are possibly not yet reflected in the sperms obtained from the cauda epididymis.

It is demonstrated that changes in cauda epididymal sperm parameters observed within 7-10 days post-exposure to harmful factors might be attributable to toxic effects on the epididymis (De Grava Kempinas and Klinefelter, 2014). Regarding the fact that our study period conforms to this time interval, it seems that dexamethasone might have toxic effects on sperm at the epididymal level. Research shows that GC receptors can be found in the nucleus and cytoplasm of various epididymal cells of rats, including epithelial, smooth muscle, and interstitial cells, as well as in nerve fibers, indicating that the epididymis is regulated by GCs (Silva *et al.*, 2010). Also, excess levels of GCs may be detrimental to normal epididymal function (Gladstones, 2009). Hence, the effects of dexamethasone on epididymal tissue might be related to its adverse effects on the above-mentioned parameters of sperm, as observed in this study. However, this needs to be more precisely evaluated in future studies.

As previously stated, although the kinematic parameters of sperm remained unaffected in rats exposed to GC, the percentage of acrosome-intact sperm and the DNA integrity of sperm diminished due to GC administration in the PC group. Consistently, it has been shown that treating human normozoospermic and varicocele samples with dexamethasone results in an augmented acrosome reaction (Rago *et al.*, 2024). When spermatozoa are missing acrosomal enzymes or when they lose these enzymes too early due to a spontaneous acrosome reaction, fertilization failure increases (Chan *et al.*, 1999). The integrity of the sperm genome is also crucial for the development of healthy offspring and can serve as a valuable diagnostic indicator of sperm reproductive capability (López-Fernández *et al.*, 2008). DNA damage can occur during spermatozoa

development in the testis; however, as the sperm cells transit the epididymis, the chromatin becomes more compacted by the formation of disulfide cross-links and turns into a toroid shape. This chromatin shape provides greater DNA protection from mechanical disruption than somatic cells (Robertson *et al.*, 2024). Since 1988, it has been observed that GCs are capable of inducing DNA fragmentation in cells, as described by Distelhorst in human lymphoid leukemia cells. This effect has also been reported in breast cancer cells (Flaherty *et al.*, 2017) and human oral keratinocytes (Valente *et al.*, 2021).

In the present study, administration of RD oil positively affected certain sperm parameters as well as testicular microstructure (histopathological and histomorphometric parameters), spermatogenesis (Johnsen scores), and serum testosterone levels. These effects were associated with appreciably reduced levels of apoptosis in testicular cells, including tubular epithelial and interstitial (Leydig) cells.

Induction of apoptosis in testicular cells (germ cells and Leydig cells) has been described by different researchers as an important mechanism for GC-induced damage to male rats' reproductive system, as well as decreased serum testosterone levels (Yazawa *et al.*, 2000; Gao *et al.*, 2002; Kheirabad *et al.*, 2016).

In the present study, the different doses of RD oil showed appreciable anti-apoptotic effects, which were most prominent at the highest dose (320 mg/kg). We could not find another study that has evaluated the effect of RD oil on apoptosis in an *in vivo* model. However, pre-medication with the hydro-alcoholic extract of *R. damascena* significantly reduced the TUNEL-positive neurons in CA1, CA3, and dentate gyrus areas of the hippocampus in a rat model of pentylene-tetrazole-induced seizure (Homayoun *et al.*, 2020). Moreover, citronellol, as the major constituent of RD oil, has shown anti-apoptotic effects in a rat model of Parkinson's disease (Jayaraj *et al.*, 2022). The anti-apoptotic effect has also been reported for geraniol, as the other important constituent of RD oil, in rats with traumatic injury of the spinal cord (Wang *et al.*, 2016).

The beneficial impacts of RD oil on testicular characteristics and serum testosterone levels may be at least in part due to its protective ability against dexamethasone-triggered cell death in germ cells and Leydig cells. The observation that the protective and anti-apoptotic effects of RD oil were more appreciable at elevated dose levels reinforces this notion.

A notable limitation of this research appears to be that, despite administering high doses of GC, the duration of the study did not encompass an entire spermatogenic cycle. It is important to highlight that we performed histopathological evaluations and scorings on testicular tissue, which are typically employed to forecast the future condition of spermatogenesis. This method enabled us to assess possible alterations in spermatogenesis, even with the limited exposure duration. Another limitation of the research is the insufficient exploration of the impacts of GC and/or RD

oil at the epididymal level and on diverse hypothalamic/hypophyseal hormones. Furthermore, while certain beneficial effects of RD oil may be associated with its anti-apoptotic properties on testicular cells, the potential mechanisms by which this essential oil protects spermatozoa against, for example, DNA damage still require clarification in future investigations, especially at the molecular level.

In conclusion, while earlier studies have highlighted the beneficial impacts of RD oil on specific male reproductive system disorders, our research demonstrates that RD oil treatment also provides a protective benefit against the detrimental effects on spermatogenesis, serum testosterone levels, and testicular tissue that are caused by high-dose GC administration in an animal model. Moreover, the findings of this study add to our understanding of the detrimental effects of GC on epididymal sperm in models when a high dose of GC is administered for a period shorter than the time needed for a full spermatogenic cycle. Positive effects of RD oil on certain deranged sperm parameters, as were observed in the present study, were also promising. These findings set the stage for future investigations on the effects of this herbal treatment in human male patients facing infertility associated with GC excess.

Conflict of interest

The authors declare that they have no competing interests.

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Use of generative AI and AI-assisted technologies statement

We have not utilized any AI tools or technologies in the preparation of this manuscript. Some sentences are paraphrased by Grammarly.

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