—Original Article—

Resveratrol Reverses Cadmium Chloride-induced Testicular Damage and Subfertility by Downregulating p53 and Bax and Upregulating Gonadotropins and Bcl-2 gene Expression

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Abstract. This study was performed to investigate the protective and therapeutic effects of resveratrol (RES) against $CdCl_2$-induced toxicity in rat testes. Seven experimental groups of adult male rats were formulated as follows: A) controls+NS, B) control+vehicle (saline solution of hydroxypropyl cyclodextrin), C) RES treated, D) $CdCl_2$+NS, E) $CdCl_2$+vehicle, F) RES followed by $CdCl_2$ and M) $CdCl_2$ followed by RES. At the end of the protocol, serum levels of FSH, LH and testosterone were measured in all groups, and testicular levels of TBARS and superoxide dismutase (SOD) activity were measured. Epididymal semen analysis was performed, and testicular expression of Bcl-2, p53 and Bax was assessed by RT-PCR. Also, histopathological changes of the testes were examined microscopically. Administration of RES before or after cadmium chloride in rats improved semen parameters including count, motility, daily sperm production and morphology, increased serum concentrations of gonadotropins and testosterone, decreased testicular lipid peroxidation and increased SOD activity. RES not only attenuated cadmium chloride-induced testicular histopathology but was also able to protect against the onset of cadmium chloride testicular toxicity. Cadmium chloride downregulated the anti-apoptotic gene Bcl2 and upregulated the expression of pro-apoptotic genes p53 and Bax. Resveratrol protected against and partially reversed cadmium chloride testicular toxicity via upregulation of Bcl2 and downregulation of p53 and Bax gene expression. The antioxidant activity of RES protects against cadmium chloride testicular toxicity and partially reverses its effect via upregulation of BCl2 and downregulation of p53 and Bax expression.
Key words: Cadmium, Infertility, Resveratrol, Sperm, Testis

The adverse effects of greatest concern in the field of toxicology are those of chronic toxicity, cancer and reproductive dysfunction [1]. It has long been suggested that at least half of the cases of human male infertility of unknown etiology may be attributable to various environmental and occupational exposures [2]. The possibility that exposures to multiple environmental agents are associated with reproductive and developmental disorders in human populations has recently generated much public interest [3].

Cadmium (Cd) is one of the environmental pollutants arising from electroplating, fertilizers, pigments, smoking and plastics, both in manufacturing and the environment [4]. Therefore, humans and animals can easily be exposed to Cd by consuming plants, water and air [4]. It is accumulated in the human body, has a half-life exceeding 10 years and has been linked with a number of health problems [5] including marked damage to the liver and kidneys [6], red blood cells [7], the heart [8] and the skeletal muscle [9]. Epidemiological studies provided equivocal results concerning the effects of Cd on sex hormone concentration, sperm parameters and male infertility [10]. These studies reported that the testes are included among the most targeted organs for Cd intoxication [10]. Recent evidence shows that rodent testes are more susceptible to Cd toxicity, as manifested by obvious testicular damage without pathological changes to other organs [11], although the detected levels of Cd accumulated in the testis are relatively low compared with those in many other tissues such as the liver after Cd administration [3,4]. Exposure to Cd can negatively affect the male reproductive system via degenerative changes in the testes, epididymis, and seminal vesicles [12]. Recently, azoospermic persons were found to have higher serum

and seminal plasma Cd levels compared with oligospermic ones [13]. Also, a positive relationship was found between Cd exposure and asthenozoospermia in a rat model [14].

In general, cadmium toxicity in the testes is multifaceted. This is likely because Cd has the capacity to induce oxidative stress [15, 16] as well as apoptosis of germ cells in humans and animals [17, 18]. In these studies, Cd was shown to increase the expression of the pro-apoptotic genes p53 and Bax while reducing the expression of Bcl-2, an anti-apoptotic gene. Furthermore, Cd is involved in disruption of the blood-testis barrier via specific signal transduction pathway and signalling molecules, such as p38 mitogen-activated protein kinase [15, 16]. Recently, at the molecular level, a single subcutaneous injection of Cd at doses of 10, 15 or 20 µmol/kg to mature rats decreased the expression levels of the genes encoding the follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), testis-specific histone 2B, and transition proteins 1 and 2, which are preferentially expressed in Sertoli cells, Leydig cells, spermatocytes, and spermatids, respectively, 96 h after injection [16].

In regard to maintaining normal male reproductive function and semen parameters, studies have shown that antioxidants could protect spermatozoa from reactive oxygen species (ROS), prevent DNA fragmentation, improve semen quality, reduce damage to spermatozoa, block premature sperm maturation and provide an overall stimulation to sperm cells under various toxic conditions [19]. The majority of these studies lacked appropriate controls, focused on healthy individuals or had indirect end-points of success. Several other studies were noted for their quality and design, and demonstrated compelling evidence regarding the efficacy of antioxidants in improving semen parameters [19].

Resveratrol (*trans*-3,5,4'-trihydroxystilbene; RES) is a naturally occurring polyphone synthesized by a variety of plant species in response to injury, UV irradiation and fungal attack. It is present in grapes, berries, peanuts and in red wine [20]. Besides its known cardioprotective effects, RES exhibits anticancer properties: it suppresses cell proliferation, has a growth inhibitory effect and potentiates apoptotic effects of cytokines, chemotherapeutic agents and ionizing radiation as reviewed by Aggarwal *et al.* [21]. In addition to being an antioxidant and a vasorelaxing agent, it modulates lipoprotein metabolism, inhibits platelet aggregation and exerts a therapeutic activity. Given the structural similarities of RES to diethylstilbestrol (DES) and estradiol and its activity as a modulator of the estrogen-response systems, it has been classified as a phytoestrogen [21].

Regarding male fertility, recent *in vivo* studies in animal models demonstrated that RES administration enhances sperm production in rats by stimulating the hypothalamic-pituitary-gonadal axis without inducing adverse effects [22]. RES has a positive effect by triggering penile erection and by enhancing blood testosterone levels, testicular sperm count and epididymal sperm motility, as demonstrated in rabbits [23]. A protective effect of RES against oxidative damage but not against the loss of motility induced by the cryopreservation of human semen has recently been observed as well [24]. To date, the protective effect of RES against Cd-induced testicular toxicity has not been investigated. It was of interest, therefore, to investigate potential preventive or therapeutic effects of RES against cadmium-induced testicular toxicity in rats. Thus, in the current study, we investigated the antioxidant potential of RES as well as its effect on the levels of testicular mRNA expression of Bcl-2, p53 and Bax in the testes of male rats intoxicated with cadmium chloride ($CdCl_2$) in an attempt to understand the molecular mechanistic action of this drug.

Materials and Methods

Drugs and chemicals

Resveratrol is only commercially available as the trans-isomer (trans-Resveratrol), and the stable and pharmacologically active form of RES was purchased from Sigma-Aldrich (St. Louis, MO, USA). RES was prepared by dissolving in a saline solution (0.9% NaCl) of 20% hydroxypropyl cyclodextrin (American Maize-Products, Hammond, IN, USA) to the desired final volume used in the experimental procedure. Cadmium chloride ($CdCl_2$) in crystalline form was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline to the desired final volume used in the experimental procedure. Quantitative ELISA kits for detecting rat serum total testosterone (Cat. No. 582701) and follicular stimulating hormone (FSH, Cat. No. 500710) were purchased from Chemical (Ann Arbor, MI, USA). An ELISA kit for detecting rat serum luteinizing hormone (LH, Cat. No. KT-21064) was obtained from Kamiya Biomedical Company (Seattle, WA, USA). Assay kits for determination of malondialdehyde (MDA, Cat No. NWK-MDA01) were purchased from NWLSS (Vancouver, WA, USA). An assay kit for determination of superoxide dismutase (SOD, Cat No. 706002) activity was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Animals

Adult male Wistar that were 10 weeks of age and weighed 250 ± 10 g were used for the experiments. The animals were obtained from the animal house of the College of Medicine, where they were fed standard rat pellets and allowed free access to water before the experiment. They were housed at a controlled ambient temperature of 25 ± 2 C and 50 ± 10% relative humidity, with 12-h light/12-h dark cycles. Experiments were performed with the approval of the Research Ethics Committee at the College of Medicine, King Khalid University, Abha, Saudi Arabia (Rec. No. 2013-02-11), and all procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental design

After an adaptation period of one week, the rats were randomly divided into seven groups of 10 rats each based the drugs used in the intervention: The rats in group A (control untreated rats) were the normal control animals and received1 ml of normal saline (0.9% NaCl), while the animals in group B (sham group) received 1 ml of saline solution containing of 20% hydroxypropyl cyclodextrin. The rats in group C received RES at a dose of 20 mg/kg body weight (bwt) in a total volume of 1 ml [25]. Testicular Cd toxicity was initiated in all other animals by intraperitoneal injection of a single dose of 1 mg/kg bwt $CdCl_2$ dissolved in 0.9% saline intraperitoneally [26]. The $CdCl_2$-treated rats were then randomly divided into three groups based on the treatments: a model group ($CdCl_2$ treated, group D) that received 1 ml normal saline, a control group that received $CdCl_2$ plus 1 ml of saline solution containing 20% hydroxypropyl

cyclodextrin (group E) and a RES-treated group (CdCl$_2$+RES group, group F) that received 20 mg/kg bwt RES in a total volume of 1 ml (26). In the control or CdCl$_2$-treated groups, treatment with the vehicle, hydroxypropyl cyclodextrin or RES continued for 15 days on a daily basis basis and was administered orally using a special gavage needle. In an additional group RES-pretreated group, group M), rats were first pretreatedwith 1 ml RES (50 mg/kg bwt) for 15 days orally, were then injected with a single dose of 1 mg/kg bwt CdCl$_2$ intraperitoneally 6 hours after the last RES treatment on day 15 and then continued on the normal saline treatment for another 15 days. The dose selected for CdCl$_2$ was based on previous dose-response studies that showed the maximum testicular damage and poorest semen quality occur at this dose [26]. Similarly, the dose selected for RES was based on previous studies that showed beneficial effects of RES on semen parameters at this dose and the safety of this dose [21–25].

Tissue collection and biochemical analysis

Six hours after the last treatment on day 15 of the protocol for groups A-F or day 30 in group M, all the rats from all groups were anesthetized with light diethyl ether, and 3 ml blood samples were collected using a 3 ml syringe directly from the heart using the ventricular puncture method into plain 5 ml untreated glass tubes, where they were allowed to clot for 15 min at room temperature. Samples were centrifuged at 4000 rpm for 10 min to obtain the serum, which was used to determine the levels of testosterone, FSH and LH, as per the manufacturer's instructions in the assay kits. Further, all the animals in all groups were sacrificed by decapitation, and both testes were removed and transferred into Petri dishes. The adipose tissues, connective tissues and blood vessels were removed from them. The epididymis was then removed and used for the fresh sperm count and analysis. The right testis from each rat in all groups was then frozen at –80 C for the determination of daily sperm production. At the same time, the left testis was divided into three fractions. One small fraction was used for histopathological evaluation and the two other fractions were frozen in liquid nitrogen and stored at 80 C. Subsequently, one of the fractions was stored and used for determination the levels of malanodialdehyde (MDA) and the activity of superoxide dismutase (SOD) as per the manufacturer's instructions in the assay kits, while the other fraction was used for the determination of testicular Bcl-2, p53 and Bax mRNA expression levels using RT-PCR.

Semen analysis: sperm count and motility

The right *cauda epididymis* from each rat was weighed, diluted in 1:20 physiological saline solution (0.9% NaCl) in a Petri dish and minced with a scalpel blade in the mid-to-distal region of the epididymis. The suspension was kept at 37 C for 5 min to allow for the sperms to disperse in the medium. The sperm suspension was gently mixed 20 times and placed in a hemocytometer, and total numbers of the sperms were counted under a Nikon microscope (Nikon Eclipse E600) at a final magnification of × 400. Sperms were counted in 5 small squares of the main large central square, with each square consisting of 16 smaller squares. Therefore, a correction factor of 50 was applied to calculate the total number of sperms per millilitre and converted to 0.1 g weight. Two samples were counted

per epididymis, and one epididymis was collected from each of the 10 rats in each experimental group. Further sample analyses included counting motile and immotile sperms in a total of 400 sperm sample, and the results were expressed in percent.

Semen analysis: sperm morphology

A drop of Eosin stain was added to the sperm suspension, which was kept for 5 min, at 37 C. Then, a drop of sperm suspension was placed on a clean slide and was gently spread to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology according to the method of Feustan *et al.* [27]. The following sperm abnormalities were counted in two separate fields in each of the sperm samples described above: absence of head, absence of tail, tail bending, tail coiling, mid-piece curving and mid-piece bending.

Semen analysis: estimation of daily sperm production

Daily sperm production was estimated using the protocol described by Fernandes *et al.* [28], in which resistant sperms were counted following homogenization of the testis sample. Each frozen right decapsulated testis was homogenized in 5 ml 0.9% (w/v) NaCl and Triton X-100 (0.05%, v/v) using a Waring blender. The preparation was diluted 10-fold, and 4 samples were transferred to a Neubauer chamber, and late spermatids were counted. The variation between duplicate testicular sperm counts was less than 10%. Daily sperm production (DSP) values were obtained using a transit time factor of 6.1 days, which is the number of days a rats' spermatids are typically present in the seminiferous epithelium.

Preparation of testis homogenate

Parts of the frozen left testes from all groups were washed with phosphate buffered saline (PBS), pH 7.4, containing 0.16 mg/ml of heparin to remove any red blood cells (erythrocytes) and clots. Then they were homogenized with an ultrasonic homogenizer in cold phosphate buffer, pH 7.0, containing ethylenediaminetetraacetic acid (EDTA) for measurement of thiobarbituric acid reactive substances (TBARS) and with 20 mM of cold N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, containing 1 mM ethylene glycol-bis(2-aminoethoxy)-tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose for measurement of SOD [29]. The supernatant was put in separate tubes and stored at –70 C.

Measurement of lipid peroxidation level

Lipid peroxidation levels in testicular homogenates were measured by the thiobarbituric acid (TBA) reaction using a commercial kit based on the method of Ohkawa *et al.* [30]. This method was used to measure spectrophotometrically the color produced by the reaction of TBA with malondialdehyde (MDA) at 532 nm. For this purpose, TBARS levels were measured using a commercial assay, the malondialdehyde Assay, according to the manufacturer's instructions. Tissue supernatants (50 µl) were added to test tubes containing 2 µl of butylated hydroxytoluene (BHT) in methanol. Next, 50 µl of acid reagent (1 M phosphoric acid) was added, and finally, 50 µl of TBA solution was added. The tubes were mixed vigorously and incubated for 60 min at 60 C. The mixture was centrifuged at 10,000 × *g* for 3 min. The supernatants were put into wells on a microplate in aliquots

of 75 μl, and absorbance was measured with a plate reader at 532 nm. TBARS (MDA) levels were expressed as nmol/mg protein.

Measurement of superoxide dismutase (SOD) activity

SOD activity in the testicular tissue homogenates supernatants was measured as previously described by Sun *et al.* [31]. For this purpose, SOD activity was measured using a commercial assay kit according to the manufacturer's instructions. The SOD assay consisted of a combination of the following reagents: 0.3 mM xanthine oxidase, 0.6 mM diethylenetriaminepentaacetic acid (DETAPAC), 150 μM nitroblue tetrazolium (NBT), 400 mM sodium carbonate (Na_2CO_3), and bovine serum albumin (1 g/l). The principle of the method is based on the inhibition of NBT reduction by superoxide radicals produced by the xanthine/xanthine oxidase system. For the assay, standard SOD solutions and tissue supernatant (10 μl) were added to wells containing 200 μl of NBT solution that was diluted by adding 19.95 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM DETAPAC solution and 0.1 mM hypoxanthine. Finally, 20 μl of xanthine oxidase was added to the wells at an interval of 20 sec. After incubation at 25 C for 20 min, the reaction was terminated by the addition of 1 ml of 0.8 mM cupric chloride. The level of formazan was measured spectrophotometrically by reading the absorbance at 560 nm with the help of a plate reader. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/mg protein.

RNA extraction and RT-PCR

The procedure was optimized for semiquantitative detection using the primer pairs and conditions described in Table 1. Published sequences of PCR primers used for the detection of Bcl-2, Bax, p53 and β-actin [32, 33] were used. Total RNA was extracted from frozen parts of left testicle tissue (30 mg) using an RNeasy Mini Kit (Qiagen Pty, Victoria, Australia) according to manufacturer's directions. The concentration of total RNA was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Kyoto, Japan). The purity was estimated by the 260/280nm absorbance ratio. Single-strand cDNA synthesis was performed as follows: 30 μl of reverse transcription mixture contained 1 μg of DNase I pretreated total RNA, 0.75 μg of oligo d (T) primer, 6 μl of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor, and 240 U of reverse transcriptase (Invitrogen). The RT reaction was carried out at 40 C for 70 min followed by heat inactivation at 95 C for 3 min. The tested genes and the internal control (β-actin) were amplified by PCR using 2 μl RT products from each sample in a 20 μl reaction containing *Taq* polymerase (0.01 U/ml), dNTPs (100 mM), $MgCl_2$ (1.5 mM) and buffer (50 mM Tris-HCl). PCR reactions consisted of a first denaturing cycle at 97 C for 5 min, followed by a variable number of cycles of amplification, consisting of denaturation at 96 C for 30 sec, annealing for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 15 min was included. Annealing temperature was adjusted for each target: 60 C for P53 and 55 C for BCl-2, Bax and β-actin. A control reaction without reverse transcriptase was included for every sample of RNA isolated to verify the absence of contamination. PCR products (10 μl) were electrophoresed on 2% agarose gels containing 100 ng/ml ethidium bromide, and photographed with a Polaroid camera under

Table 1. Primers and conditions used in PCR reactions

Target	Primer sequence (5' to 3')	AT (C)	Size (bp)
p53	5 - CTACTAAGGTCGTGAGACGCTGCC-3[c] 5 - TCAGCATACAGGTTTCCTTCCACC-3[d]	60	106
Bax	5- 5_-GGTTGCCCTCTTCTACTTT-3 [c] 5- AGCCACCCTGGTCTTG-3[d]	55	143
BCl-2	5-ACTTTGCAGAGATGTCCAGT-3[c] 5_-CGGTTCAGGTACTCAGCAT-3[d]	55	217
β-actin	5-CGTTGACATCCGTAAAGAC-3[c] 5-TAGGAGCCAGGGCAGTA-3 [d]	55	110

AT: Annealing temperature. [c] Sense. [d] Antisense.

ultraviolet illumination. Gel images were scanned, and the bands for Bcl-2, Bax, p53 and β-actin were quantified by densitometry using the NIH Image software. Bcl-2, Bax and p53 intensities intensities were normalized to those of the corresponding β-actin band intensity for each sample.

Histopathological studies

Specimens from testes of all experimental groups were fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol (70–100%) and then prepared using standard procedures for hematoxylin and eosin staining.

Statistical analysis

Statistical analyses were performed by using the GraphPad Prism statistical software package (version 6). Data are presented as means with their standard deviations (mean ± SD). Normality and homogeneity of the data were confirmed before ANOVA, and differences among the experimental groups were assessed by one-way ANOVA followed by Tukey's *t* test.

Results

Semen parameters and morphology

The results of epididymal sperm counts, motility, daily sperm production and sperm morphology are shown in Fig. 1 and Fig. 2 and Table 2. There was no significant difference in any semen parameters measured between the control group (group A) and the sham group that received NS solution containing hydroxypropyl cyclodextrin. The epididymal sperm count, motility, testicular resistant sperm and DSP were significantly greater (36.5, 27.4, 25 and 29.1%, respectively) in RES-treated rats (group C) than in controls (P< 0.0001) (Figs, 1 and 2). Sperm abnormalities, including absence of head, absence of tail, tail bending, tail coiling and mid-piece bending, were not more frequent in RES-treated rats (Table 2). Consequently, the percentage of total abnormality was not affected by the treatment, indicating that the overall sperm quality was not impaired by RES. On the other hand, a single $Cdcl_2$ injection along with NS (group D) or with the vehicle (group E) resulted in significant decreases (P<0.0001) in the number (58.7 and 61.7%, respectively) and motility (41.9 and 40.5%, respectively) of epididymal sperms (per 0.1 g of epididymis) as well as in testicular homogenization-resistant sperms (47.3 and 48.3%, respectively). Daily sperm production was also reduced (DSP/
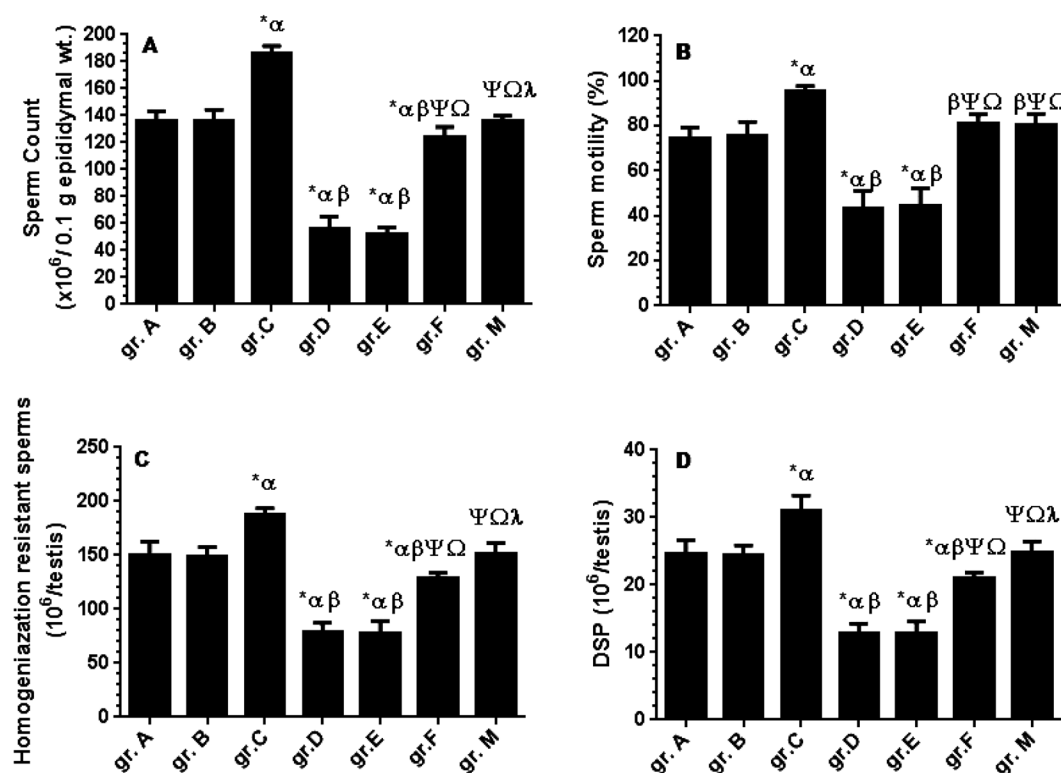
Fig. 1. Epididymal sperm count (A), motility (B), testicular homogenization-resistant sperm (C) and daily sperm production (DSP, D) levels in the control and all other experimental groups of rats. Data are expressed as the mean ± SD for 10 samples in each group. Values were considered significantly different at P<0.05. * Significantly different when compared with group A (control group+NS). α: Significantly different when compared with group B (Control+NS containing 20% hydroxypropyl cyclodextrin). β: Significantly different when compared with group C (control+RES). Ψ: Significantly different when compared with group D (CdCl$_2$+NS). Ω: Significantly different when compared to group E (CdCl$_2$+NS containing 20% hydroxypropyl cyclodextrin). λ: Significantly different when compared with group F (CdCl$_2$ then RES). Group M: RES then CdCl$_2$ treatment in rats.

testis) (47.68 and 48.3%, respectively) as compared with control rats. Results obtained from the morphological assessment of sperms (Table 2) indicated that the total percentages of abnormal sperms were significantly higher in these groups of rats (59.39 ± 8.26 and 62.57 ± 10.25%, respectively) as compared with their levels in the control group (10.47 ± 1.043). The majority of the significant abnormalities included increased percentages absence of tail, absence of head and tail coiling (Fig. 2). The ANOVA analysis showed no significant differences in any measured semen parameters between groups D and E. However the groups post-treated (group F) or pre-treated (group M) with RES showed similar significant improvements (P<0.0001) in sperm count, motility and DSP as well as normal percentages of sperm morphological phenotypes as compared with groups E and D, rats and their numbers were not significantly different from those of the control group.

Serum hormones

At the end of the study, the serum concentrations of follicular stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were significantly greater (31.2, 48.2 and 48.6%, respectively) in the RES-treated rats than in the control (group A) or sham (group B) groups

(Fig. 3) (P< 0.0001). In the groups intoxicated with CdCl$_2$ (groups D and E), the average levels of serum FSH, LH and testosterone were significantly decreased (P<0.05) in comparison with those of the control group. The percentage of decreases in the levels of these hormones in these groups were 62.8 and 65.7% for FSH, 59.8 and 60.9% for LH and 35.8 and 38.9% for testosterone, respectively. However, rats post-treated or pre-treated with RES (groups F and M, respectively) showed significant improvements (P<0.05) in the levels of these hormones as compared with CdCl$_2$ treated rats (groups D and E). However, the ANOVA analysis revealed that the levels of these hormones in group F, which was post-treated with RES, remained significantly lower than those of the control rats (11.3, 16.03 and 26.4%, respectively). Interestingly, group M, which was pre-treated with RES, showed the highest recovery of all changed hormonal levels examined, and its levels were not significantly different from those of the control group.

TBARS levels and SOD activity

In the testis, there were no significant differences in TBARS levels or SDO activity between the sham and control group (P= 0.9999 and P=0.9644, respectively). However, the SOD activity was
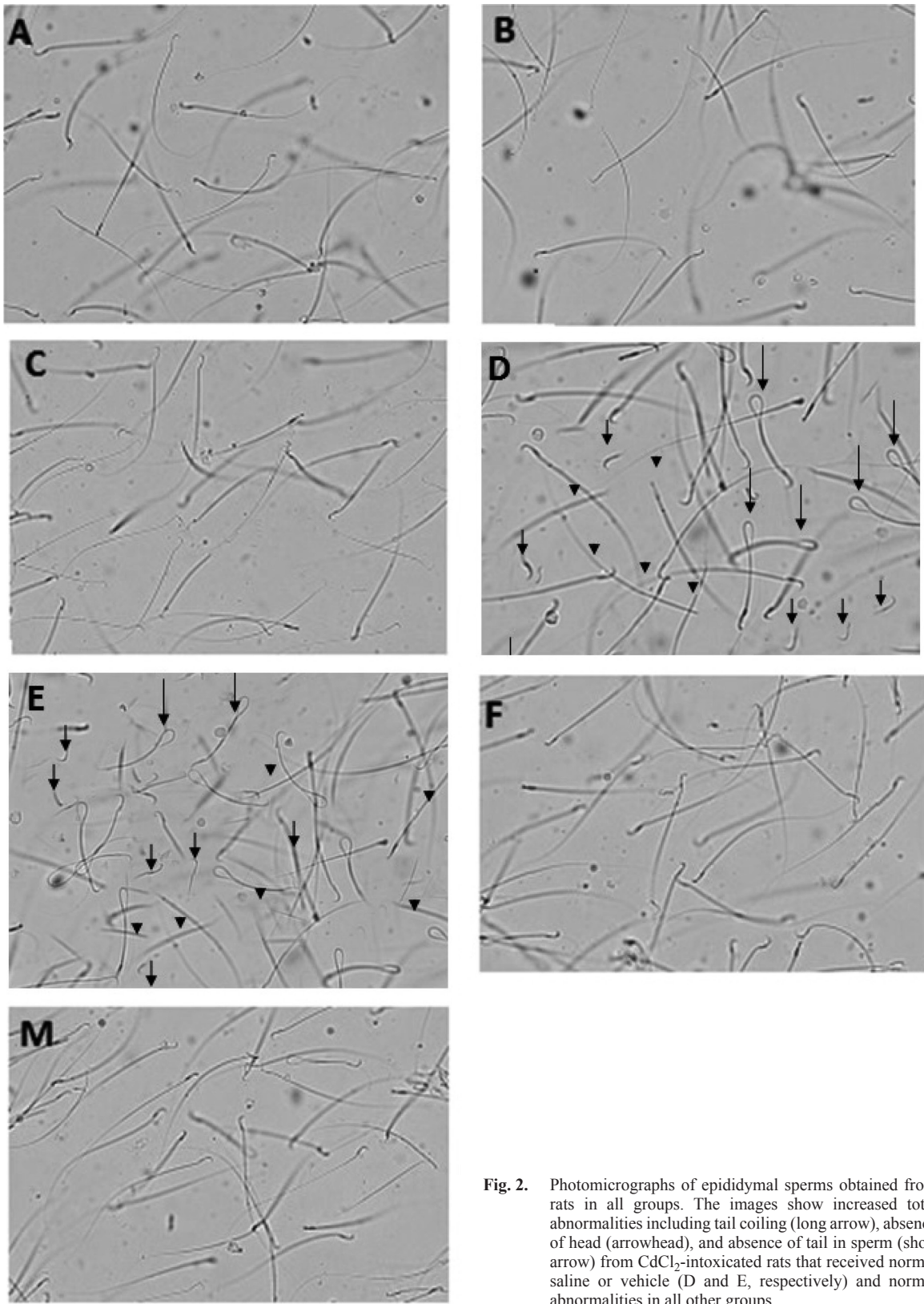
Fig. 2. Photomicrographs of epididymal sperms obtained from rats in all groups. The images show increased total abnormalities including tail coiling (long arrow), absence of head (arrowhead), and absence of tail in sperm (short arrow) from CdCl$_2$-intoxicated rats that received normal saline or vehicle (D and E, respectively) and normal abnormalities in all other groups.
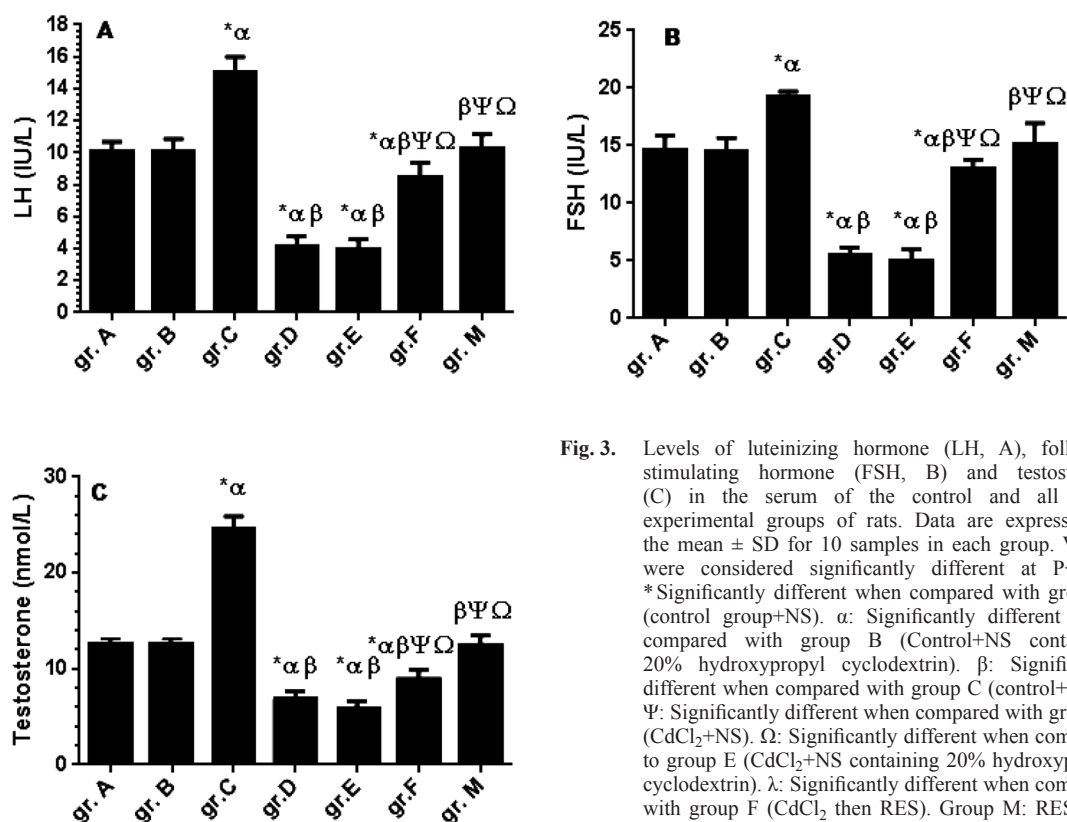
Fig. 3. Levels of luteinizing hormone (LH, A), follicular stimulating hormone (FSH, B) and testosterone (C) in the serum of the control and all other experimental groups of rats. Data are expressed as the mean ± SD for 10 samples in each group. Values were considered significantly different at P<0.05. *Significantly different when compared with group A (control group+NS). α: Significantly different when compared with group B (Control+NS containing 20% hydroxypropyl cyclodextrin). β: Significantly different when compared with group C (control+RES). Ψ: Significantly different when compared with group D (CdCl₂+NS). Ω: Significantly different when compared to group E (CdCl₂+NS containing 20% hydroxypropyl cyclodextrin). λ: Significantly different when compared with group F (CdCl₂ then RES). Group M: RES then CdCl₂ treatment in rats.

Table 2. Characterization of epididymal sperm morphology in the control and all other experimental groups

Group	Absence of tail	Absence of head	Tail bending	Mid-piece bending	Tail coiling	Total abnormality
Group A	0.91 ± 0.043	1.98 ± 0.13	2.11 ± 0.2	2.30 ± 0.56	3.19 ± 0.11	10.47 ± 1.043
Group B	1.01 ± 0.096	2.11 ± 0.34	1.98 ± 0.365	2.16 ± 0.367	3.11 ± 0.34	10.37 ± 1.51
Group C	0.89 ± 0.11	2.16 ± 0.24*	1.96 ± 0.14	1.98 ± 0.23	3.25 ± 0.45	10.24 ± 1.17
Group D	12.45 ± 2.34[abc]	17.45 ± 3.45[abc]	1.89 ± 0.13	1.97 ± 0.23	25.63 ± 2.11[abc]	59.39 ± 8.26
Group E	13.26 ± 3.15[abc]	18.25 ± 3.46[abc]	2.04 ± 0.21	2.14 ± 0.25	26.88 ± 3.18[abc]	62.57 ± 10.25
Group F	1.03 ± 0.034[cde]	2.01 ± 0.23[cde]	2.17 ± 0.31	1.91 ± 0.17	3.56 ± 0.27[cde]	10.68 ± 1.01
Group M	1.12 ± 0.14[cde]	1.89 ± 0.16[cde]	1.96 ± 0.31	2.01 ± 0.23	3.45 ± 0.35[cde]	10.25 ± 0.12[cde]

Data are expressed as the mean ± SD for 10 samples in each group. Values were considered significantly different at P<0.05. 10 sample [a] Significantly different when compared with group A (control group+NS). [b] Significantly different when compared with group B (Control+NS containing 20% hydroxypropyl cyclodextrin). [c] Significantly different when compared with group C (Control+RES). [d] Significantly different when compared with group D (CdCl₂+NS). [e] Significantly different when compared with group E (CdCl₂+NS containing 20% hydroxypropyl cyclodextrin). Group F treated with CdCl₂ and then RES. Group M: RES then CdCl₂ treatment in rats. NS: Normal saline.

significantly greater (44.7%) and the TBARS levels were significantly lower (36.8%) in the RES-treated group (group C) as compared to with the levels in the control group. On the other hand, the levels of testicular TBARS were significantly higher (P<0.0001) (98.1 and 104.9%) and SOD activities were significantly (P<0.0001) lower (55.1 and 56.7%) in Cdcl₂-intoxicated rats administered NS or vehicle (groups D and E respectively) as compared with the levels in the control rats (28.9% and 14.13%, respectively) (Fig. 4). No significant differences were found in the levels of these parameters between groups D and E. Similar normal levels of these parameters were found in groups F and M, which were post-treated or pre-treated with RES, respectively, and their levels were significantly lower than those of the CdCl₂-treated rats but not significantly different when compared with the levels of the control group.

Histopathlogical findings

The seminiferous tubules of the control and sham groups (groups A and B) were completely differentiated. Sections of testis from these

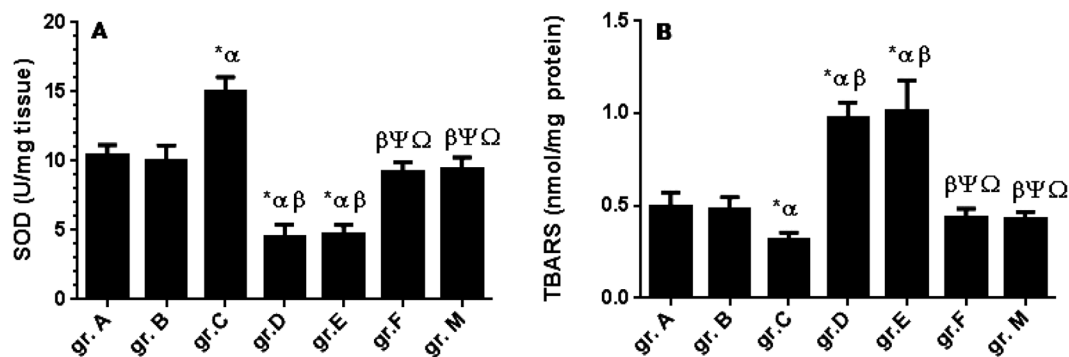**Fig. 4.** Superoxide dismutase activity (A) and lipid peroxidation content (TBARS, B) in the testis homogenates of all groups of rats. Data are expressed as the mean ± SD for 10 samples in each group. Values were considered significantly different at P<0.05. *Significantly different when compared with groupA (control group+NS). α: Significantly different when compared with group B (Control+NS containing 20% hydroxypropyl cyclodextrin). β: Significantly different when compared with group C (control+RES). Ψ: Significantly different when compared with group D (CdCl$_2$+NS). Ω: Significantly different when compared to group E (CdCl$_2$+NS containing 20% hydroxypropyl cyclodextrin). λ: Significantly different when compared with group F (CdCl$_2$ then RES). Group M: RES then CdCl$_2$ treatment in rats.

control and sham groups revealed that seminiferous tubule had each a definite membrane and a small lumen densely filled with sperm tails. Spermatogenic cells including spermatogonia, primary spermatocytes, early spermatids, late spermatids and Sertoli cells were seen to be abundant and healthy (Fig. 5A and B). Similar morphology to the control and sham groups was seen in the RES-treated group (Fig. 5C). On the other hand, examination of the testes of rats treated with CdCl$_2$ and NS (group D) revealed degeneration of spermatogonial cells lining the seminiferous tubules and that lumens of tubules were filled with degenerated germ cells. Also, vacuolization of the seminiferous epithelium and partial to complete absence of germ cells associated with intestinal edema, damaged Sertoli cells, interstitial hemorrhage and necrosis of Leydig cells were also noticed (Fig. 5D). The testes of rats treated with CdCl$_2$ and vehicle (group E) revealed similar histopathological changes to those observed in group D (Fig. 5E). However, Morphological examination of the testes in groups F and M (post-treated or pre-treated with RES, respectively) showed normal testicular morphology and spermatogenesis with normal seminiferous tubule architectures, disappearance of vacuolation in all stages of spermatogenesis and well-preserved Sertoli cells. Spermatogonia, primary spermatocytes, spermatids, and mature sperm were clearly seen in the seminiferous, with increased dense packing of mature sperm in the lumen and a decreased number of sperm heads. Decreased intestinal edema and interstitial hemorrhage were observed in these groups (Fig. 5F and M).

In the statistical analysis for comparison of the diameter and epithelium thickness of the seminiferous duct, the histomorphologic changes included a significant increase in seminiferous tubule diameter but not in epithelium thickness in the RES-treated group as compared with the control groups administered normal saline or vehicle (Table 3). On the other hand, CdCl$_2$-intoxicated rats that received normal saline or vehicle showed significant decreases in both diameter and epithelium thickness in their seminiferous tubules as compared with their corresponding control groups. Seminiferous tubules from groups

pre-treated or post-treated with RES showed significant amelioration in both diameter and thickness of their seminiferous tubules as compared with CdCl$_2$-intoxicated rats. The ANOVA analysis showed that the diameters of the seminiferous tubules in these groups of rats were not significantly different when compared to each other but were significantly higher than those obtained in the control group and significantly lower those obtained in the RES-treated group. The thicknesses of the tubule epithelium in these groups of rats were not significantly different from those measured in the control group or RES-treated group (Table 3).

mRNA Levels of p53, Bax, and Bcl-2

Figure 6A shows the transcriptional changes of p53, Bax, and Bcl-2 in the testes from all groups of rats. All tested transcripts were detected, and RT-PCR resulted in fragments similar in size to those expected. The levels of the β-actin transcript remained relatively constant in the testes of all groups. In the control or sham groups, p53 transcripts were barely detectable as compared with Bax and Bcl-2, with the Bcl-2 band being the most prominent. In comparison with control and sham groups, RES administration to control rats resulted in upregulation of mRNAs of Bcl-2 (1.690 ± 0.036 *vs.* 0.8977 ± 0.033 and 0.9267 ± 0.016) and downregulation of both p53 (0.1387 ± 0.003 *vs.* 0.260 ± 0.01 and 0.251 ± 0.15) and Bax (0.113 ± 0.005 *vs.* 0.382 ± 0.022 and 0.381 ± 0.018), respectively. In comparison of both groups intoxicated with CdCl$_2$ and receiving NS or the vehicle (groups D and E) and with the control group, mRNA expression of p53 and Bax was significantly induced (2.202 ± 0.054 and 2.167 ± 0.043 *vs.* 0.26 ± 0.01) and (0.892 ± 0.02 and 0.849 ± 0.015 *vs.* 0.382 ± 0.022), respectively, while Bcl-2 expression was suppressed (0.408 ± 0.0165 and 0.347 ± 0.0187 *vs.* 0.8977 ± 0.033). On the other hand, in comparison with the CdCl$_2$-treated group, post- (group F) and pretreatment (group M) with RES selectively increased Bcl$_2$ expression (1.15 ± 0.058 and 1.167 *vs.* 0.408 ± 0.0165) and downregulated p53 (0.325 ± 0.01 and 0.317 ± 0.005
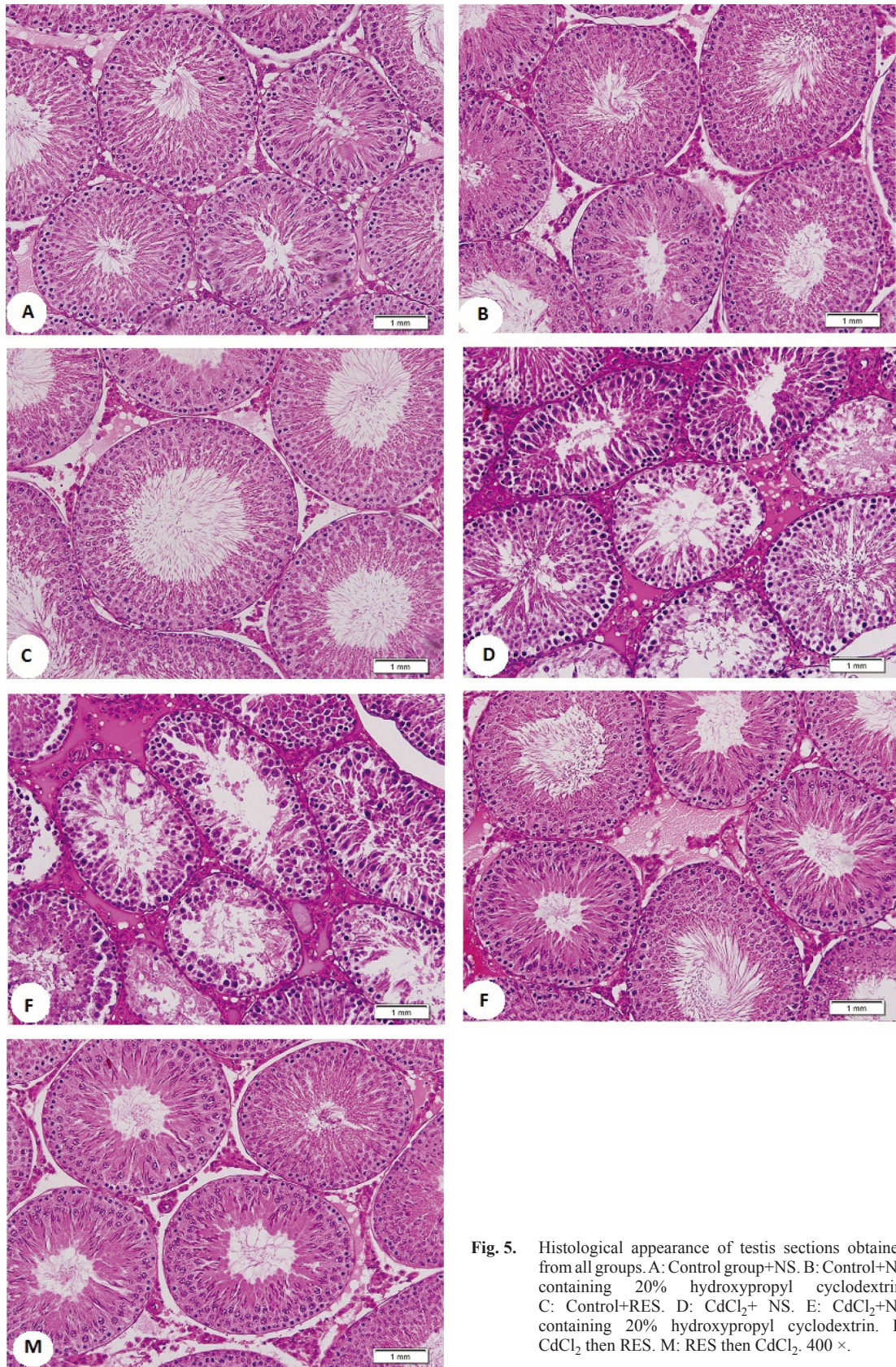
Fig. 5. Histological appearance of testis sections obtained from all groups. A: Control group+NS. B: Control+NS containing 20% hydroxypropyl cyclodextrin. C: Control+RES. D: $CdCl_2$+ NS. E: $CdCl_2$+NS containing 20% hydroxypropyl cyclodextrin. F: $CdCl_2$ then RES. M: RES then $CdCl_2$. 400 ×.

Table 3. The histomorphometric changes of testicular tissue in rats in the control group and all other experimental groups

| Groups	Seminiferous tubule diameter (μm)	Thickness of seminiferous duct epithelium (μm)
Group A	326 ± 20.1	83.45 ± 5.67
Group B	329 ± 11.56	85.34 ± 4.69
Group C	354 ± 19.87[ab]	85.44 ± 3.34
Group D	276 ± 12.67[abc]	61.04 ± 3.13[abc]
Group E	277 ± 14.17[abc]	57.9 ± 2.78[abc]
Group F	337 ± 19.5[abcde]	81.56 ± 3.23[de]
Group M	338 ± 11.79 [abcde]	84.12 ± 3.41[de]

Data are expressed as the mean ± SD for 10 samples in each group. Values were considered significantly different at P<0.05. 10 sample [a] Significantly different when compared with group A (control group+NS). [b] Significantly different when compared with group B (Control+NS containing 20% hydroxypropyl cyclodextrin). [c] Significantly different when compared with group C (Control+RES). [d] Significantly different when compared with group D (CdCl₂+NS). [e] Significantly different when compared with group E (CdCl₂+NS containing 20% hydroxypropyl cyclodextrin). Group F treated with CdCl₂ and then RES. Group M: RES then CdCl₂ treatment in rats. NS: Normal saline.

vs. 2.202 ± 0.054) and Bax (0.0114 ± 0.0045 and 0.0111 ± 0.003 *vs.* 0.892 ± 0.02) gene expression, respectively. ANOVA revealed that the levels of p53, BCl-2 and Bax in groups F and M were not statistically different compared with those expressed in the control and sham groups (groups A and B).

Discussion

The present study is the first to highlight the possible putative mechanisms behind the therapeutic and protective role of RES in ameliorating testicular toxicity and poor semen quality in CdCl₂-intoxicated rats. The current study is the first in the literature to show the novel anti-apoptotic role of RES in such mechanisms. Although the testicular damage induced by CdCl₂ is well recognized, the precise mechanisms underlying its toxicity in the testes have remained unclear. In general, CdCl₂ testicular toxicity in the testis is probably as a result of interactions of a complex network of causes. In the current study, CdCl₂ administration increased lipid peroxidation and oxidative stress in the testes of treated rats, which was associated with the observed testicular damage. These finding are in a agreement with previous research showing similar finding in rats tests intoxicated with CdCl₂ [15, 16, 34–36]. The toxic effect of CdCl₂ on the testes is known to deplete glutathione and protein protein-bound sulfhydryl groups, which results in enhanced production of reactive oxygen species (ROS) such as superoxide ion, hydroxyl radicals and hydrogen peroxide [34]. In this study, rats exposed to CdCl₂ showed a significant reduction in the activity of antioxidant enzyme (SOD) and a concomitant enhancement in lipid peroxidation (MDA), in accordance with earlier reports of CdCl₂ intoxication in rats [34, 36].

Similar to previous findings, CdCl₂ intoxication has been shown to increase the expression of pro-apoptotic proteins p53 and Bax, while reducing the expression of Bcl-2, an anti-apoptotic protein
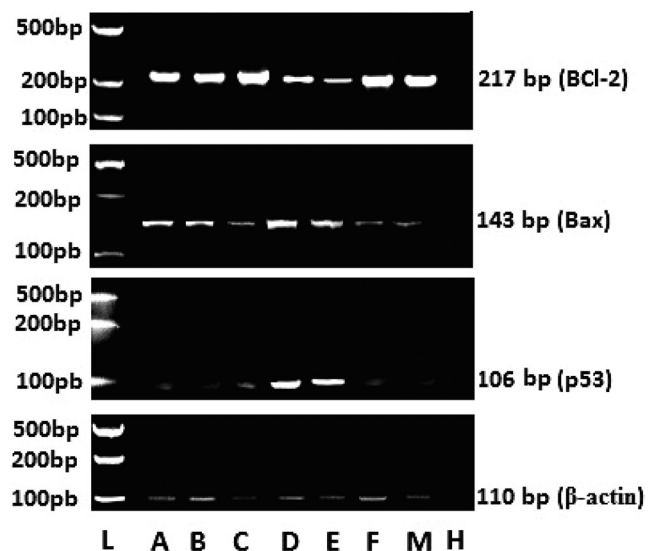
Fig. 6. Semiquantitative reverse transcription PCR products and relative expression of testicular mRNA of Bcl-2, Bax and p53 in reference to β-actin mRNA (housekeeping gene). The RT-PCR products obtained from all groups were separated by 2% agarose gel electrophoresis with 100 ng/ml ethidium bromide. L: Ladder. A: Control group+NS. B: Control+NS containing 20% hydroxypropyl cyclodextrin. C: Control+RES. D: CdCl₂+NS. E: CdCl₂+NS containing 20% hydroxypropyl cyclodextrin. F: CdCl₂ then RES. M: RES then CdCl₂. H: Negative control in which reverse transcriptase was omitted.

[17, 18]. Under normal conditions, p53 levels are maintained at a low state by virtue of the extremely short half-life of the polypeptide but are induced in response to cellular stress, functioning as a transcriptional transactivator in DNA repair, apoptosis and tumor suppression pathways [37]. Induction of p53 is associated with a rapid increase in its levels and with an increased ability to bind DNA and mediate transcriptional activation [37]. The products encoded by p53 can regulate the expression of Bax, which may mediate p53-dependent cell apoptosis [38]. It was reported that p53 might lead to apoptosis indirectly by downregulating expression of Bcl-2 [38]. Bax can promote apoptosis by homodimerizing or heterodimerizing with Bcl-2. Therefore, the alteration of the Bax to Bcl-2 ratio appears to determine whether some cells live or die [39]. Similar to these reported findings, in our current study, CdCl₂ induced concomitant increases in expression of P53 and Bax but led to decreased expression of Bcl-2, suggesting their important roles in testis damage, apoptotic cell death and decreased semen parameters elicited by CdCl₂.

The present study showed a decrease in sperm count and sperm motility and increase abnormality (absence of head, absence of tail and tail coiling) in male rats treated with CdCl₂. These reductions and abnormalities in sperm quality and number could be multifactorial but are at least due to reduced spermatogenesis due to increased oxidative stress and and the apoptotic mechanism observed in the testes of the treated rats. Zemjanis [40] reported that spermatozoa abnormalities such as absence of tail, absence of head, tail coiling

and mid-piece bending are considered to reflect disturbances in spermatogenesis, whereas secondary abnormalities such as abnormal acrosome are believed to arise after spermatogenesis is completed due to epididymal dysfunction. It has been reported that oxidative stress affects the sperm cell via interference with the membrane fluidity, which is the main factor for sperm motility and fusion with the oocyte [41]. Similar results were reported by Neveen et al. [42], who found that exposure of adult male miceto $CdCl_2$ significantly decreased sperm counts, total number of sperm per mg of testis and daily sperm production efficiency. In addition, Bench et al. [5], reported that $CdCl_2$ has a detrimental effect on testicular function (stages of spermatogenesis) that could result in reduced sperm production leading to reduced male fertility. Moreover, $CdCl_2$ was found to be known as a competitor of calcium, which is essential for sperm motility regulation [43, 44]. Further evidence was derived from a study for Benoff et al. [14] in which aberrant sperm motility was correlated with altered expression of L-type voltage-dependen channel isoforms found on the sperm tail, which regulate calcium and Cd influx.

The present study showed a decrease in the hormonal levels of testosterone, LH and FSH in rats intoxicated with $CdCl_2$. These finding are in the same line with the perviously mentioned effect of $CdCl_2$ on the pituitary gonadal axis. Our results are in accordance with those obtained by Lafuente et al., [45] Kuo et al. [46], Pillai et al. [47] and Watanabe et al. [48], who reported decreased levels of LH, FSH and testosterone in mice and rats intoxicated with $CdCl_2$. The decreased levels of FSH and LH could be explained by the study of Yang et al., who suggested that $CdCl_2$ induces apoptosis of the anterior pituitary both in vivo and in vitro in a dose-dependent manner [49]. Also, nitric oxide (NO) production after $CdCl_2$ administration has been implicated to play a role in such decrease [50]. Concerning LH and steroidogenesis and Fatma et al. [51] reported that LH acts upon the Leydig cells of the testis and is responsible for the production of testosterone, an androgen that exerts both endocrine activity and intratesticular activity in spermatogenesis. It was reported that $CdCl_2$ administration significantly increased NO production [50], leading to a decrease in testosterone synthesis in the Leydig cells by acting centrally on the pituitary gland and inhibiting LH secretion [52]. Our findings are in accordance with previous reports from Piasek and Laskey [53], who demonstrated lowering in steroidogenesis in $CdCl_2$-treated female rats. Further evidence was derived from a study for Murugesan et al. [54], in which poor pituitary LH secretion with reduced Leydig cell steroidogenesis was reported in a highly contaminated environment.

The histopathological changes observed in the present study are in agreement with the findings of El-Ashmawy and Youssef [55], who demonstrated that a single dose of $CdCl_2$ induced severe necrosis and degeneration of seminiferous tubules with complete loss of spermatogenic cell layers and absence of centrally located spermatozoa. The present results are also in accordance with a report by El-Missiry and Shalaby [56], whoillustrated that $CdCl_2$ can induce lipid peroxidation and damage in testicular tissue, including necrosis and apoptosis, in rats. Considering the high sensitivity of the testicular tissue to $CdCl_2$ insult, preventive intervention is of major concern. Previous studies have shown that antioxidants protect spermatozoa from reactive oxygen species (ROS), prevent DNA fragmentation,

improve semen quality, reduce damage to spermatozoa, block premature sperm maturation and provide an overall stimulation to sperm cells under various toxic conditions [19]. Our study is the first to describe a novel effect of trans-resveratrol (RES), namely, an increase in spermatozoa production and motility and improved histological changes in both healthy and $CdCl_2$-intoxicated rats with concomitant regulation of apoptosis by boosting the levels of an anti-apoptotic protein, BCl-2, and decreasing the levels of pro-apoptotic proteins, p53 and Bax. The data in the current study showed that the therapeutic effect of RES on $CdCl_2$-induced testicular damage and hormonal disturbance is more profound than its protective effect, as shown by the significant improvement in sex hormones, semen parameters and histological changes.

Our results show that the main two functions of the testes, synthesis of steroid hormones and production of spermatozoa, which are controlled by gonadotrophins and testosterone, were enhanced in healthy and $CdCl_2$-intoxicated male rats receiving daily RES. These changes were verified to be RES specific and not vehicle generated. This lack of toxicity in the control group was not surprising because it was previously demonstrated that the oral administration of trans-resveratrol at a dose of 20 mg/kg bwt/day for 28 days was not harmful to male rats [25]. Moreover, our results are supported by a recent toxicological study reporting that exposure of male and female rats to a dose of 300 mg/kg bwt/day of trans-resveratrol for 28 days did not have adverse effects [57].

Evidently, it seems that resveratrol improves semen parameters in healthy and in $CdCl_2$-intoxicated rats by acting through different mechanisms. The most likely mechanism of its action is its potent antioxidant effect, endocrine function and its direct or indirect effect on apoptosis. In our current study, pre-and post treatment with RES normalized $CdCl_2$-induced testicular oxidative stress as indicated by the significant increase in SOD activity and significant decrease in MDA levels. It was shown previously that during spermatogenesis stages VI–VIII in rats, there is a significant increase in superoxide dismutase mRNA expression coinciding with the presence in the tubules of elongated spermatids with excess cytoplasmatic retention. The cytoplasm was shown to produce high levels of ROS [58]. One of the biological activities of RES is its antioxidant potential, since RES is able to reach peroxidized rigid membranes and increase membrane fluidity in order to interact more efficiently with radicals in the altered lipid bilayer [59]. Therefore, RES exhibits a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS [59]. Therefore, trans-resveratrol could be acting by decreasing the steady-state or $CdCl_2$-induced high levels of ROS and lipidperoxidation factors in the seminiferous tubules, thus increasing sperm and androgen production. Together, these activities could also account for the increase in sperm output observed in healthy rats or $CdCl_2$-intoxicated rats. Also, Lagouge et al [60] proved that the effects of small concentrations of RES were associated with induction of genes for oxidative phosphorylation and mitochondrial biogenesis and thus stimulating mitochondrial functions of the cell, from which we may conclude that RES, apart from being an antioxidant, could mobilize the spermatozoa energetic metabolism and therefore improve spermatozoa viability and motility.

Also, the protective or therapeutic effects of RES could also be mediated by its promising effect on apoptosis. Our study is the first

in the literature to show that RES increased or improved the mRNA expression of Bcl-2 levels and decreased expression of both p53 and Bax genes in both healthy and CdCl$_2$-intoxicated rats suggesting a possible mechanism of action of this drug in protecting germ cells and testicular tissue from damage. This is the first report of a stimulatory effect of RES on the secretion of gonadotrophins, the major endocrine regulators of spermatogenesis. The concentrations of FSH, which acts within the tubules to stimulate spermatogenesis, and LH, which signals the production of testosterone in Leydig cells, were elevated in the RES group compared with the control rats. Testosterone, which is essential for promoting spermatogenesis, was also enhanced. Regarding increases in these hormones levels in both control and Cd-intoxicated rats, it has been suggested that the effect of RES on sperm count may be caused by the hypophisary stimulation of testicular function, and a possible explanation of that for this was suggested by Juan *et al.* [22], who attributed it to the binding of RES to the estrogen receptor (ER) as a mixed weak agonist/antagonist without estrogenic properties [61]. Also, RES may increased increase NO production [50], leading to an increase in testosterone synthesis in the Leydig cells by acting centrally on the pituitary gland and stimulating LH and FSH secretions. Moreover, as RES was shown to have an anti-apoptotic effect in the study, it may prevent enhancement of hormonal secretion due to cadmium-induced apoptosis of the anterior pituitary, resulting in normal levels of LH and FSH [49].

In conclusion, we have demonstrated the therapeutic and protective efficacy of RES against CdCl$_2$-induced testicular damage and disturbed hormonal levels and semen parameters. Even though the exact mechanism of action of CdCl$_2$ at the molecular level cannot be completely deduced from the results presented above, it could possibly be due to a combination of its antioxidant and anti-apoptotic mechanisms. Further studies are required to examine the molecular pathways responsible for its anti-apoptotic effect.

Acknowledgments

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