Cisplatin induced testicular damage through mitochondria mediated apoptosis, inflammation and oxidative stress in rats: impact of resveratrol

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Abstract. The target of this study was to explore the role of mitochondria mediated apoptosis and inflammation in cisplatin-induced testicular damage and to evaluate the ameliorative effect of resveratrol. Adult male Wistar rats were randomly allocated to 4 groups. Group I (Control) received normal saline, Group II (Resveratrol) received resveratrol (50 mg/kg/day), Group III (Cisplatin) received cisplatin (7.5 mg/kg/week, i.p.) and Group IV (Resveratrol + Cisplatin) received resveratrol and cisplatin in the same regimen of treatment. Treatment with resveratrol in Groups II and IV started 48h before cisplatin injection and continued for further 4 successive weeks. Cisplatin-treated rats showed reduced body weight, absolute testes weight and sperm count, motility and viability. On the other hand, cisplatin treatment increased the percentage of sperm abnormalities. It also decreased serum testosterone level, mitochondrial membrane potential while, increased cytochrome C liberation from the mitochondria into the cytosol. The activities of caspase-3 & -9 were increased. The level of TNF-α, IL-6 and Bax were increased whereas Bcl-2 was decreased. Oxidative stress markers were found to increase with a concomitant reduction in the antioxidant enzymes and GSH levels. These results were confirmed by immunohistochemical and histopathological analysis. Contrary to all these results, there were improvements in cisplatin induced testicular damage through attenuation of mitochondria mediated apoptosis, inflammation, and oxidative stress owing to resveratrol pretreatment. Thus, resveratrol, as a potential therapeutic agent, may hold promise in preventing mitochondria mediated apoptosis and inflammation in cisplatin-induced testicular damage in rats.

Key words: Cisplatin, Resveratrol, Mitochondria, Apoptosis, Testis

CISPLATIN is one of the most potent and widely used antineoplastic drugs [1]. Regardless of well-legalized effectiveness of cisplatin in curing a diversity of tumors, its use is restricted because of the dose- and duration-dependent cell resistance [2]. Further, its nonspecific action leads to deleterious effects in the kidneys [3], testes [4] and liver [5]. The testicular toxicity caused by cisplatin is severe and irreversible [6]. It may lead to azoospermia and reduced testosterone level [7].

Apoptosis is a substantial process in the development and maintenance of homeostasis [9]. However, redundant or inconvenient apoptosis in the testis will lead to abnormal spermatogenesis or testicular neoplasms [10, 11]. Mitochondria-mediated apoptosis is induced by intracellular stimuli, such as oxidative stress and nutritive privation, which causes an imbalance in the expression of Bcl-2 family, up-regulate pro-apoptotic (Bax) and downregulate anti-apoptotic (Bcl-2), resulting in increasing mitochondrial membrane permeability and liberation of cytochrome C from the mitochondria into the cytosol [12]. Cytochrome C enhances association of caspase-9 and apoptosome. Caspase-9 elicits the caspase cascade, resulting in activation of caspase-3 and finally apoptosis [13].

Phytochemicals having potent antioxidant activity, when given along with chemotherapeutic agents, provide preferable efficacy of chemotherapeutic drugs and attenuate vital tissue toxicity [14]. Resveratrol is one of such phytochemicals, a polyphenolic phytoalexin that is found in several different plants in nature, particularly in grapes [15]. Resveratrol inhibits reactive oxygen species (ROS) formation and preserve normal cells from
DNA injury and apoptosis by modulating antiapoptotic mediator (Bcl-2) and inhibiting proapoptotic (Bax, cytochrome C and caspase 3/9) mediators [16]. The efficiency of resveratrol has been widely studied against cisplatin induced cardiotoxicity [17], ototoxicity [18] and nephrotoxicity [19]. A large body of evidence point that resveratrol perhaps useful to numerous aspects of human reproduction [20]. However, there are no numerous reports on how resveratrol manages the testicular dysfunction caused by cisplatin. In addition, there is a rareness of input in the literature regarding the role of mitochondria, apoptosis and inflammation in testicular damage induced by cisplatin in rats. The target of the current study was to explore the role of mitochondria mediated apoptosis and inflammation in cisplatin-induced testicular damage and to evaluate the ameliorative effect of resveratrol.

**Materials and Methods**

**Reagents**
Cisplatin, resveratrol (3,5,4-hydroxystilbene), and other reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA.

**Animals and treatments**
Adult male Wistar rats (200 ± 10 g) were placed in polypropylene cages and kept on a 12 h light:dark cycle with a temperature of 20–25°C and free access to food and water. The animals were assigned in random to 4 equal-sized groups (n = 6). Group I (Control) received normal saline intraperitoneally (i.p.) as vehicle. Group II (Resveratrol) received resveratrol (50 mg/kg/day diluted in dimethyl sulfoxide) once daily by i.p. injection. Group III (Cisplatin) treated with 7.5 mg/kg/week by i.p. injection diluted in normal saline. Group IV (Resveratrol + Cisplatin) received resveratrol and cisplatin by the same regimen of treatment. Treatment with resveratrol in Groups II and IV started 48 h before starting the experiment and treatment in all Groups continued for further 4 successive weeks. The doses of resveratrol [21, 22] and cisplatin [23-25] were selected as per previously published. Protein content were specified using protein assay kit. All experimental procedures were approved by the research ethical committee of the Faculty of Pharmacy (boys), Al-Azhar University, Cairo, Egypt, and comply with the Guide for the Care and Use of Laboratory Animals [26].

**Necropsy**
Blood was collected from the retro-orbital venous plexus of overnight-fasted rats after 24 hours. The serum was procured by incubating the collected blood at 37°C for 30 min followed by centrifugation for 10 min at 3,000 g. The obtained serum was held at –80°C while testosterone inspection. Rats were euthanized under ether anesthesia and the testes were isolated, cleaned of excess tissues, weighed and washed with ice-cold phosphate-buffered saline (PBS, pH 7.4). Sperm count, motility and viability were determined in the epididymides of each rat. Sperm suspension was also used for determination of sperm morphology.

Homogenization of the right testis from each rat was carried out in 10% (w/v) ice-cold Tris–HCl buffer (150 mM, pH 7.4). This was followed by centrifugation of the homogenate at 2,000 g for 10 min at 4°C to remove the pellet. The supernatant was further centrifuged at 10,000 g for 30 min at 4°C to separate the cytosolic (in the supernatant) and the mitochondrial (in the pellets) fractions of the testis [27]. The cytosol was used for all biochemical studies except for the mitochondrial membrane potential was measured in the mitochondrial fraction. The cytochrome C was measured in both fractions. The left testis from each rat was fixed in Bouin’s solution and manipulated for immunohistochemistry for cytochrome C and histopathological examination.

**Sperm count and motility**
Sperm count and motility were determined in the epididymides as previously mentioned and the percentage motility was calculated [28].

**Sperm viability and morphology**
Sperm viability was specified using eosin/nigrosine stain as previously mentioned [29] and the percentage viability was calculated [30]. The total abnormality of sperm head and tail was expressed as percentage [31].

**Serum testosterone**
Testosterone was inspected using Testosterone ELISA kit [32].

**Mitochondrial membrane potential (Δψm)**
The mitochondrial membrane potential (Δψm) was deliberated, in the mitochondrial fraction of the testis, using the JC-1 assay kit [33].

**Bax and Bcl-2**
The concentrations of Bax and Bcl-2 were deliberated using commercially rat Bax and Bcl-2 ELISA kits according to the protocols approved by the manufacturers.

**Cytochrome C release**
Cytochrome C (Cyt C) release from the mitochondria into the cytosol was determined by measuring its concen-
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tration in both the mitochondria and the cytosol using Cyt C ELISA kit.

**Caspase-3 & -9**

The activities of caspase-3 and -9 were inspected by the colorimetric assay kit using specific chromogen. Enzyme activity was presented as μmol pN/A/mg protein.

**TNF-α and IL-6**

The TNF-α and IL-6 levels were inspected in the testis cytosol using sandwich enzyme-linked immunosorbent assay using primary rabbit anti-TNF-α antibody and anti-IL-6 antibody and secondary goat anti-rabbit IgG-peroxidase antibody according to the instructions approved by the manufacturer.

**Oxidative status**

**Hydrogen peroxide production**

Generation of hydrogen peroxide (H₂O₂) was inspected as previously mentioned [34].

**Lipid peroxidation**

The colored product resulting from interaction between malondialdehyde (MDA), an endproduct of lipid peroxidation, and thiobarbituric acid can be measured at 532 nm [35].

**Protein carbonyl content**

Protein carbonyl content in the testis cytosol was determined as previously described [36].

**Antioxidant status**

**Superoxide dismutase**

Superoxide dismutase (SOD) was deliberated as previously mentioned [37]. The activity of SOD was expressed as nmol pyrogallol oxidized/min/mg protein.

**Catalase**

Catalase (CAT) activity was measured as previously described [38]. The activity of CAT was expressed in μmol of hydrogen peroxide consumed/min/mg protein.

**Glutathione peroxidase**

Glutathione peroxidase (GPx) activity was determined as previously mentioned [39]. The activity of GPx was expressed as nmol of NADPH oxidized/min/mg protein.

**Total reduced glutathione**

Total reduced glutathione (GSH) content was estimated as previously described [40]. The quantity of GSH was given in μg/mg protein.

**Immunohistochemical assessment of cytochrome C**

It was carried out in the left testis using Cyt C polyclonal antibody. Cyt C immunoreactivity; a mitochondrial pattern was seen as a punctate pattern of discrete spots within the cytoplasm, while a cytoplasmic pattern was diffuse cellular immunostaining without distinct spots [41]. The staining intensity was scored as previously described [42] from 0 to 3; 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = strong staining.

**Histopathology of the testis**

Hematoxylin and eosin (H&E) was used to stain the testis tissue sections prior to light microscope histopathological examination [43].

**Statistical analysis**

One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used to compare the differences between obtained values (mean ± SD, n = 6). P < 0.05 indicated a statistically significant difference. Statistical analysis was performed using GraphPad Instat 3.

**Results**

**Body weight, testes weight and sperm parameters**

Table 1 demonstrates the changes in body weight, testes weight and sperm parameters. Cisplatin treatment of rats resulted in a significant diminution in body weight and absolute testes weight (Group III) (p < 0.01 & p < 0.05 respectively) as compared to the corresponding control. Sperm count, motility and viability showed significant diminution (p < 0.001, p < 0.05 & p < 0.01 respectively) in response to cisplatin treatment (Group III) as compared to the corresponding control. Cisplatin treatment showed significant increase (p < 0.01) in the percentage of sperm abnormality (Group III). The values of body weight, absolute testes weight and sperm count, motility and viability in Group IV (rats treated with resveratrol and cisplatin) did not differ significantly from their corresponding controls (Group I).

**Serum testosterone**

Serum testosterone level was diminished (p < 0.05, 14.59%) after cisplatin treatment (Group III) while in Group IV did not significantly differ when compared to the corresponding control (Group I) in response to resveratrol pretreatment (Fig. 1).

**Mitochondrial membrane potential (Δψm)**

Mitochondrial membrane potential (Δψm) was significantly diminished (p < 0.05, 18.33%) in the cisplatin treated animals (Group III). Pretreatment of rats with resveratrol (Group IV) maintained the Δψm near the normal value (Fig. 2).

**Bax and Bcl-2**

Bax and Bcl-2 levels were presented in Fig. 3A & B
respectively. The concentration of Bax was significantly increased \( p < 0.01, 14.22\% \), while Bcl-2 showed a significant reduction \( p < 0.05, 15.96\% \) in the testis of cisplatin treated rats (Group III) relative to the corresponding control. The Bax and Bcl-2 levels in Group IV (rats treated with resveratrol and cisplatin) did not differ significantly from the corresponding control. Treatment of animals with cisplatin (Group III) increased the Bax/Bcl-2 ratio into 2.74 folds as compared to 2.02 folds in the control group. This ratio was decreased into 2.16 folds by pretreatment with resveratrol (Group IV).

**Cytochrome C release**

Cisplatin treatment showed significant increase in Cyt C liberation from the mitochondria into the cytosol (Fig. 4A & B respectively). The concentration of Cyt C in the testicular mitochondria was diminished significantly \( p < 0.001, 73.43\% \) in the cisplatin treated rats (Group III), while its concentration in the cytosol was increased significantly \( p < 0.001, 62.79\% \) in the cisplatin treated rats (Group III). The concentration of Cyt C in both the testicular mitochondria and the cytosol of Group IV did not differ significantly from the corresponding control.

**Caspases-3 & -9**

Caspases-3 & -9 activities were markedly higher \( p < 0.001 & p < 0.05 \) respectively) after cisplatin treatment (Group III). The activities of these enzymes in Group IV did not differ significantly relative to their corresponding controls (Fig. 5A & B respectively).

**Tumor necrosis factor alpha (TNF-α) and Interleukin-6 (IL-6)**

The concentrations of TNF-α and IL-6 rose significantly \( p < 0.05 \) (26.73% & 31.18% respectively) after treatment with cisplatin (Group III) relative to their corresponding controls. The values of these inflammatory mediators did not show any significant difference in animals pretreated with resveratrol (Group IV) as compared to the respective controls (Fig. 6A & B).

**Oxidative and antioxidant status**

Table 2 presents the oxidative and the antioxidant status. There was a significant increase in lipid peroxidation (LPO) and the production of hydrogen peroxide \( \text{H}_2\text{O}_2 \) \( p < 0.01 \) (23.3% & 22.06% respectively) after treatment with cisplatin (Group III) relative to the corresponding controls. There was a significant increase in the protein carbonyl content \( p < 0.05, 19.53\% \) after cisplatin treatment (Group III) relative to the corresponding control. These values in Group IV (rats treated with resveratrol and cisplatin) did not change significantly from the corresponding controls. Cisplatin treatment (Group III) resulted in reduction in the activities of superoxide dismutase (SOD) \( p < 0.05, 17.44\% \), catalase (CAT) \( p < 0.001, 23.82\% \) and glutathione peroxi-

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**Table 1** Effect of cisplatin and resveratrol on body weight, testes weight and sperm parameters

| Parameter                  | Group I (control) | Group II (resveratrol) | Group III (cisplatin) | Group IV (resveratrol + cisplatin) |
|----------------------------|-------------------|------------------------|-----------------------|-----------------------------------|
| Body weight (g)            | 201.17 ± 7.63     | 202.83 ± 5.27          | 187.67 ± 7.76**      | 200.33 ± 4.23**                   |
| Absolute testes weight (g) | 2.62 ± 0.29       | 2.6 ± 0.21             | 2.23 ± 0.13**        | 2.57 ± 0.13**                     |
| Cauda sperm count (×10⁶/rat)| 61.33 ± 3.83      | 61.67 ± 4.41           | 51.5 ± 2.74****      | 57.83 ± 3.19**                    |
| Sperm motility (%)         | 82.33 ± 4.97      | 83.17 ± 4.83           | 72.5 ± 4.09**        | 80.67 ± 5.61**                    |
| Sperm viability (%)        | 79.33 ± 4.03      | 80.67 ± 3.33           | 71 ± 3.46**          | 77.83 ± 2.04***                   |
| Sperm abnormalities (%)    | 7.33 ± 1.37       | 7.17 ± 1.17            | 10.5 ± 1.87***       | 8 ± 1.26***                       |

Data are shown as mean ± S.D. (n = 6). ★Group I and Groups II–IV; ★★Group III and Group IV were compared by ANOVA to detect statistical differences. The symbols signify a statistical significance relative to corresponding control where * \( p < 0.05; ** p < 0.01; *** p < 0.001.\)

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**Fig. 1** Effect of cisplatin and resveratrol on serum testosterone

Group I: Control, Group II: Resveratrol, Group III: Cisplatin, Group IV: Cisplatin + Resveratrol.

Data are expressed as mean ± S.D. (n = 6). Serum testosterone is expressed as ng/mL. ★Group I and Groups II–IV; ★★Group III and Group IV were compared by ANOVA to detect statistical differences. The symbols signify a statistical significance relative to corresponding control where * \( p < 0.05; ** p < 0.01; *** p < 0.001.\)
Fig. 2 Effect of cisplatin and resveratrol on mitochondrial membrane potential
Group I: Control, Group II: Resveratrol, Group III: Cisplatin, Group IV: Cisplatin + Resveratrol.
Data are given as mean ± S.D. (n = 6). The Δψm is expressed as ratio of J-aggregates:J-monomers. *Group I and Groups II–IV; **Group III and Group IV were compared by ANOVA to detect statistical differences. The symbols signify a statistical significance relative to corresponding control where * p < 0.05; ** p < 0.01; *** p < 0.001.

Fig. 3 Effect of cisplatin and resveratrol on Bax and Bcl-2 levels
Group I: Control, Group II: Resveratrol, Group III: Cisplatin, Group IV: Cisplatin + Resveratrol.
Data are given as mean ± S.D. (n = 6). The concentration of BAX and Bcl-2 are expressed as pg/mg protein. *Group I and Groups II–IV; **Group III and Group IV were compared by ANOVA to detect statistical differences. The symbols signify a statistical significance relative to corresponding control where * p < 0.05; ** p < 0.01; *** p < 0.001.

dase (GPx) (p < 0.01, 15.69%) and total reduced glutathione (GSH) (p < 0.05, 27.14%) when compared with corresponding controls. No significant differences were found in the antioxidant enzymatic activities and GSH level (Group IV) relative to the corresponding controls (Table 2).

Immunohistochemistry of cytochrome C
The immunohistochemical examination of Cyt C is illustrated in Fig. 7. It revealed negative (0) reactivity for cytochrome C in germinal epithelial lining (black arrow) with mild (1) punctate reactivity in interstitial cells (red arrow) in the testis of the control (Group I, Fig. 7a). Resveratrol-treated animals (Group II, Fig. 7b) showed negative (0) reactivity for cytochrome C in germinal epithelial lining (black arrow). Testes of cisplatin treated rats (Group III, Fig. 7c) showed strong (3) diffuse cytoplasmic reactivity for Cyt C in germinal epithelial lining (black arrow) and in interstitial cells (red arrow). The liberation of Cyt C into the cytoplasm was decreased in the resveratrol pretreated animals (Group IV, Fig. 7d). Testicular tissue of group IV showed negative (0) reactivity for Cyt C in germinal epithelial lining (black arrow) with
mild (1) diffuse cytoplasmic reactivity in interstitial cells (red arrow). Cyt C immunoreactivity; a mitochondrial pattern was seen as a punctate pattern of discrete spots within the cytoplasm, while a cytoplasmic pattern was diffuse cellular immunostaining without distinct spots.

**Histopathological examination**

Histopathological examination was presented in Fig. 8. Normal histological construction was seen in control animal’s testes (Group I, Fig. 8a). Group I showed seminiferous tubules with average basement membrane (BM) (black arrow), Sertoli cells (blue arrow), spermagonia (red arrow), primary spermatocytes (yellow arrow), spermatids (green arrow), many spermatozoa (orange arrow), and average interstitium with Leydig cells (white arrow). Resveratrol-treated animals (Group II, Fig. 8b) had normal testicular histology and normal spermatogenesis. Group II demonstrates seminiferous tubules with average BM (black arrow), spermatagonia (blue arrow), primary spermatocytes (red arrow), spermatids (green arrow), many spermatozoa (yellow arrow), and average interstitium with Leydig cells (orange arrow). The pretreatment of resveratrol (Group IV) improved the histological pictures of the cisplatin-treated rats (Group III).

**Discussion**

Testicular dysfunction caused by cisplatin is notified to be a major cause of infertility in males subjected to chemotherapy [44]. In this study, the body weight and the absolute testes weight were significantly diminished in response to cisplatin treatment. The decrease in body weight may be caused by cisplatin-induced gastrointestin-
Table 2  Effect of cisplatin and resveratrol on oxidative and antioxidant status

| Parameter               | Group I (control) | Group II (resveratrol) | Group III (cisplatin) | Group IV (resveratrol + cisplatin) |
|-------------------------|-------------------|------------------------|-----------------------|-----------------------------------|
| H$_2$O$_2$ production   | 43.83 ± 3.92      | 41.83 ± 3.31           | 53.5 ± 4.72**         | 44.33 ± 4.08***                   |
| LPO                     | 5.58 ± 0.4        | 5.42 ± 0.37            | 6.88 ± 0.59***        | 5.77 ± 0.58**                     |
| Protein carbonyl content| 2.97 ± 0.39       | 2.85 ± 0.31            | 3.55 ± 0.34**         | 3.02 ± 0.25**                     |
| SOD                     | 35.33 ± 3.88      | 35.83 ± 2.48           | 29.17 ± 4.12**        | 34.67 ± 2.42**                    |
| CAT                     | 6.17 ± 0.5        | 6.18 ± 0.52            | 4.7 ± 0.48***         | 5.72 ± 0.57**                     |
| GPx                     | 42.5 ± 2.35       | 42.83 ± 2.14           | 35.83 ± 2.93***       | 41.5 ± 4.37*                      |
| GSH                     | 15.33 ± 2.66      | 16 ± 2.37              | 11.17 ± 2.23**        | 15.15 ± 1.52**                    |

H$_2$O$_2$ production: nmol of H$_2$O$_2$ generated/min/mg protein; LPO: µmol of MDA equivalent formed/min/mg protein; Protein carbonyl content: nmol/mg protein; SOD: nmol pyrogallol oxidized/min/mg protein; CAT: µmol of hydrogen peroxide consumed/min/mg protein; GPx: nmol of NADPH oxidized/min/mg protein; GSH: Total reduced glutathione, µg/mg protein. Data are expressed as mean ± S.D. (n = 6). * Group I and Groups II–IV; ** Group III and Group IV were compared by ANOVA to detect statistical differences. The symbols signify a statistical significance relative to corresponding control where * p < 0.05; ** p < 0.01; *** p < 0.001.

Fig. 7  Representative illustrations of immunohistochemical staining of cytochrome C (Cyt C) of the rat testes treated with cisplatin and resveratrol.

Group I Control (a), Group II Resveratrol (b), Group III Cisplatin (c), Group IV Cisplatin + Resveratrol (d).

Testes from control (Group I, 7a) revealed negative (0) reactivity for cytochrome C in germinal epithelial lining (black arrow) with mild (1) punctate reactivity in interstitial cells (red arrow). Testes of animals treated with resveratrol (Group II, 7b) showed negative (0) reactivity for cytochrome C in germinal epithelial lining (black arrow). Testes of cisplatin treated rats (Group III, 7c) showed strong (3) diffuse cytoplasmic reactivity for cytochrome C in germinal epithelial lining (black arrow) and in interstitial cells (red arrow). There was decrease in the release of cytochrome C into the cytoplasm of the testis of resveratrol pretreated animals (Group IV, 7d). Group IV showed negative (0) reactivity for cytochrome C in germinal epithelial lining (black arrow) with mild (1) diffuse cytoplasmic reactivity in interstitial cells (red arrow). Cytochrome C immunoreactivity; a punctate pattern of discrete spots within the cytoplasm was a mitochondrial pattern, while diffuse cellular immunostaining without distinct spots was considered a cytoplasmic pattern (Cytochrome C immunostain ×400).
nal toxicity [45]. Cisplatin is highly emetogenic and causes profound gastrointestinal alterations as nausea, anorexia, diarrhoea and malabsorption which may result in weight loss [46, 47]. Diminished the absolute weight of testes and produced change in sperm features. The weight of testis particularly depends on the mass of differentiated spermatogenic cells; thus, depression in testicular weight suggests germ cell injury [48]. It is reported that the testis demands persistent testosterone stimulation for its normal growth and function [48]. The depression in the weight of testes could be due to decrease in testosterone biosynthesis [49]. However, administration of resveratrol markedly mitigated the effect of cisplatin on body and testes weight. Cisplatin suppressed sperm count, motility and viability and increased abnormal sperm morphology. The diminished sperm concentration after cisplatin treatment probably due to depressed testosterone level [48]. Moreover, the diminution in sperm count comes in accordance with the reduction in testes weight. Further, the decreased sperm count and motility direct attention to lipid peroxidation and reactive oxygen species (ROS) produced by cisplatin in the testis [50-53]. Treatment with resveratrol prohibited the deteriorating effects of cisplatin on sperm count, sperm motility, and viability and decreased abnormal sperm morphology. Testosterone is demanded for normal development and maturation of germ cells in the testis and then diminished level of testosterone may direct germ cells toward apoptosis [54]. The diminished serum testosterone in cisplatin-treated rats perhaps due to the deteriorating effect of ROS on Leydig cells [55]. Maintenance of mitochondrial membrane potential (ΔΨm) is fundamental for cellular endurance and its damage might lead to cellular apoptosis [56]. The diminution in ΔΨm

Fig. 8  Representative illustrations of histological morphology of rat testes treated with cisplatin and resveratrol
Group I: Control (a), Group II: Resveratrol (b), Group III: Cisplatin (c), Group IV: Cisplatin + Resveratrol (d).
Testes from control (Group I, 8a) showed seminiferous tubules with BM (black arrow), Sertoli cells (blue arrow), spermatogonia (red arrow), primary spermatocytes (yellow arrow), spermatids (green arrow), many spermatozoa (orange arrow), and average interstitium with Leydig cells (white arrow).
Testes of animals treated with resveratrol (Group II, 8b) exhibited seminiferous tubules with average BM (black arrow), spermatogonia (blue arrow), primary spermatocytes (red arrow), spermatids (green arrow), many spermatozoa (yellow arrow), and average interstitium with Leydig cells (orange arrow).
Testes of cisplatin treated rats (Group III, 8c) showed scattered distorted empty seminiferous tubules (loss of spermatogenesis) with thick basement membrane (black arrow), marked reduction of germinal lining with markedly degenerated cells (blue arrow).
Histopathological morphology of testes was improved in animals pretreated with resveratrol (Group IV, 8d). The findings in Group IV showed normal histological structure in the seminiferous tubules and the interstitial tissues with complete spermatogenesis series in the lumen. These sections showed seminiferous tubules with average BM (black arrow), spermatogonia (blue arrow), primary spermatocytes (red arrow), spermatids (green arrow), many spermatozoa (yellow arrow), and average interstitium with Leydig cells (orange arrow). Hematoxylin and eosin (H & E ×40) was used for cross-section staining.
leads to liberation of cytochrome C (Cyt C) into the cytosol with activation of apoptosis [57]. Moreover, the alteration in the mitochondria of spermatogenic cells will impair the process of oxidative phosphorylation, and influence spermatogenesis and sperm motility [58]. Results of this study elucidated that the ΔΨm decreased following cisplatin treatment and resveratrol ameliorated it, indicating the anti-apoptotic and improvement effect of resveratrol. In the intrinsic pathway, the crucial point in the apoptotic signaling series is the liberation of Cyt C from mitochondria, after breakdown of ΔΨm, into the cytosol [59, 60]. Cytosolic Cyt C joins with Apaf-1 forming apoptosome, and then activates caspase-9 and caspase-3, the key factor in execution of cell apoptosis. In the present study cisplatin induced ΔΨm collapse. The collapse of ΔΨm can be controlled by the anti-apoptotic Bcl-2 [61] which inhibits its collapse and pro-apoptotic Bax which promote the collapse. Bcl-2 is in the mitochondrial wall controls apoptosis by preserving mitochondrial membrane integrity, regulating mitochondrial permeability and suppressing Cyt C liberation [62]. The pro-apoptotic Bax promotes liberation of Cyt C to the mitochondria from the cytosol. Bax increases in cases of oxidative stress and disrupts the membrane permeability and liberates Cyt C in the cytosol [63]. The results of this study showed that cisplatin decreased ΔΨm, declined the anti-apoptotic protein Bcl-2, and increased the pro-apoptotic Bax, thereby eventually increasing the Bax/Bcl-2 ratio and elevating the concentration of Cyt C in the cytosol. Meantime, the caspase-9 and caspase-3 were activated, manifesting mitochondria-associated apoptosis in the testes. Moreover, the induced apoptosis of germ cells may cause atrophy of the testes [64]. The increased ROS induces mitochondrial-dependent apoptosis by increasing the mitochondrial membrane permeability and further through activation of caspases [65]. In the present study resveratrol inhibited apoptosis by reducing ROS production, decreasing cytosolic Bax while increasing Bcl-2 concentrations and hence decreasing Bax/Bcl-2 ratio, inhibiting Cyt C liberation from the mitochondria into the cytosol and inhibiting caspase-9 &-3 activities in the testes of cisplatin-treated rats.

Inflammatory cytokines, including TNF-α and IL-6, play a substantial role in spermatogenesis and testicular steroidogenesis under normal conditions [66]. However, these cytokines can be upregulated in the testis in inflammatory status and deteriorate normal spermatogenesis [67]. Enhanced level of these biomarkers in the testis of cisplatin treated rats in this study clearly indicates an inflammatory event. Excess ROS generation induces inflammatory cascades with increased production of TNF-α and IL-6. This magnifies gonadotoxicity and spermiotoxicity leading to testicular dysfunction. These abnormalities were recovered almost to baseline level in the resveratrol pretreated animals. Cisplatin was reported to generate excessive ROS [68]. Data from this study manifested a significant increase in LPO and H₂O₂ production in the testis of rats treated with cisplatin that was correlated with a significant mitigation in the activity of the antioxidant enzymes SOD, CAT and GPx and GSH level. The decreased activities of the antioxidant enzymes and GSH level perhaps due to their exhaustion during the reduction of ROS. SOD catalyzes the conversion of superoxide anions to H₂O₂ and molecular oxygen, while CAT detoxifies H₂O₂ [69]. The decreased CAT activity may indicate less capability of testicular mitochondria to remove H₂O₂ generated in response to cisplatin [70]. The decreased activity of GPx perhaps due to the observed deficiency of the substrate GSH [71]. CAT and GPx conserve SOD against inactivation by H₂O₂. Mutually, the SOD keeps CAT and GPx against suppression by superoxide anion [72]. GSH plays a substantial role in the antioxidant defenses to remove ROS [73, 74]. Treatment with resveratrol decreased ROS, prevented oxidative stress, protected the testis against lipid peroxidation, decreased the production of inflammatory cytokines caused by cisplatin administration and increased antioxidant enzymes and GSH level.

The examination of testicular tissue stained with anti-Cyt C antibody revealed more immunopositive cells in the cytosol than in the mitochondria of the cisplatin-treated rats. The cisplatin group revealed a higher degree of immunopositivity in the germinal epithelial lining. There were fewer immunopositive cells in the cisplatin group pretreated with resveratrol than in the cisplatin group alone.

Histopathological sections of testicular tissue showed scattered distorted empty somniferous tubules with degeneration and loss of spermatogenesis in the cisplatin treated group. Such deterioration in testicular architecture is mainly referred to severe apoptosis and oxidative injury produced by cisplatin.

Treatment with resveratrol effectively mitigated the architectural and functional testicular perturbations induced by cisplatin together with restoring the body and testes weights. Therefore, resveratrol might be believed as a novel curative target for preserving testis function under cisplatin treatment and any pathological conditions of the testis. The main limitation of this study is that serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were not measured. FSH regulates spermatogenesis, whereas the LH controls Leydig cell function and testosterone secretion.

Based on the biochemical, histopathological and immunohistochemical analyses, it could be concluded that cisplatin induced testicular dysfunction, mitochon-
drial mediated apoptosis, inflammation and oxidative stress and resveratrol ameliorated the testicular dysfunction caused by cisplatin in the rat testis. Thus, resveratrol, as a potential therapeutic agent, may hold promise in preventing mitochondria mediated apoptosis and inflammation in cisplatin-induced testicular damage in rats.

No Conflict of Interest

Acknowledgment

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