

# Resveratrol treatment reduces apoptosis and morphological alterations in cisplatin induced testis damage

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Received: 09 January 2019 / Revised: 26 March 2019 / Accepted: 28 March 2019

**ABSTRACT:** Cisplatin commonly used as a chemotheropotic agent however, it is associated with numerous side effects such as reproductive cytotoxicity. It causes spermatogenic cell death and DNA damage in spermatozoa via the formation of reactive oxygene species. Resveratrol (3,5,4'-*trans*-trihydroxystilbene), a natural phytoalexin, is a potent antioxidant agent, present in a wide variety of dietary sources including grapes, plums and peanuts. The aim of present study to evaluate the beneficial effects of resveratrol on cisplatin induced testis damage. Male Sprague Dawley rats were used in the study and four experimental groups were formed as: 1- saline applied control, 2- resveratrol applied control, 3- cisplatin and 4- cisplatin+resveratrol groups. Following a single dose of cisplatin (7 mg/kg i.p.), either saline or resveratrol (10 mg/kg, orally) was administered for 5 days. Testis samples were prepared for histopathological and ultrastructural evaluations, cell proliferation and apoptosis. Tissue malondialdehyde (MDA), glutathione (GSH) levels and myeloperoxidase (MPO) activity were determined biochemically. Degenerated and atrophic tubules of tissue, apoptotic cells, MDA level and MPO activity were increased although proliferation index and GSH level were decreased in cisplatin group. Degenerated tight junctions between the Sertoli cells and vacuole formation in germinal epithelial cells were also revealed at this group. However, resveratrol treatment reduced degenerated and atrophic tubules, apoptotic cells, vacuole formation in germinal epithelial cells, MDA level and MPO activity and increased proliferation index and GSH level in testis. These results showed that resveratrol ameliorates cisplatin induced testis injury by the impairment of oxidative stress and apoptosis.

**KEYWORDS:** Cisplatin; resveratrol; testis; apoptosis; cell proliferation; ultrastructure.

## 1. INTRODUCTION

Cisplatin (Cis) is a highly active antineoplastic agent commonly used in the treatment of several types of human cancer such as, testicular [1] ovarian [2], prostate [3] and bladder carcinoma [4], neuroblastoma [5] and head-neck carcinoma [6]. However, the clinical use of Cis is often restricted because of its many side effects, such as nephrotoxicity [7], ototoxicity [8], neurotoxicity [9], cardiotoxicity [10] and gonadal toxicity [11]. Also an increase of apoptosis in germ cells is seen because of the side effects of Cis and the other chemoterapeutics on testis [12, 13]. It was observed that Cis treatment altered oxidative markers such as increase of malondialdehyde (MDA), and decrease of endogenous glutathione (GSH) which cause disturb the organs' pathophysiology in many systems including urogenital system [14].

Exogenous many antioxidants such as melatonin [15] and quercetin [16] have been investigated as protecting against oxidative damage and combined with chemotherapeutics. Resveratrol (3,4,5-*trans*-trihydroxystilbene, RVT), a potent antioxidant, is a phytoalexin and present in plants such as grapes, peanuts berries and pines. RVT reduces lipid peroxidation by its free radical scavenger, improves the activities of a variety of antioxidant enzymes and prevents DNA damage [17]. RVT increases endogenous antioxidant levels such as GSH, superoxide dismutase (SOD), catalase (CAT), glutathion reductase by alleviating oxidative damage [18]. RVT also has antimutagenic, antiproliferative and anticarcinogenic effects besides its antioxidant effects [19]. According to these findings, antioxidants proposed to evaluate protective and therapeutic effects on tissue damage caused by chemotherapeutics.

Excessive generation of reactive oxygen speices (ROS) can cause testicular damage. Cisplatin treatment also increases formation of ROS in testis which causes the seminiferous tubule damage with germ cell

**How to cite this article:** Özyılmaz Yay N, Şener G, Ercan F. Resveratrol treatment reduces apoptosis and morphological alterations in cisplatin induced testis damage. J Res Pharm. 2019; 23(4): 621-631.

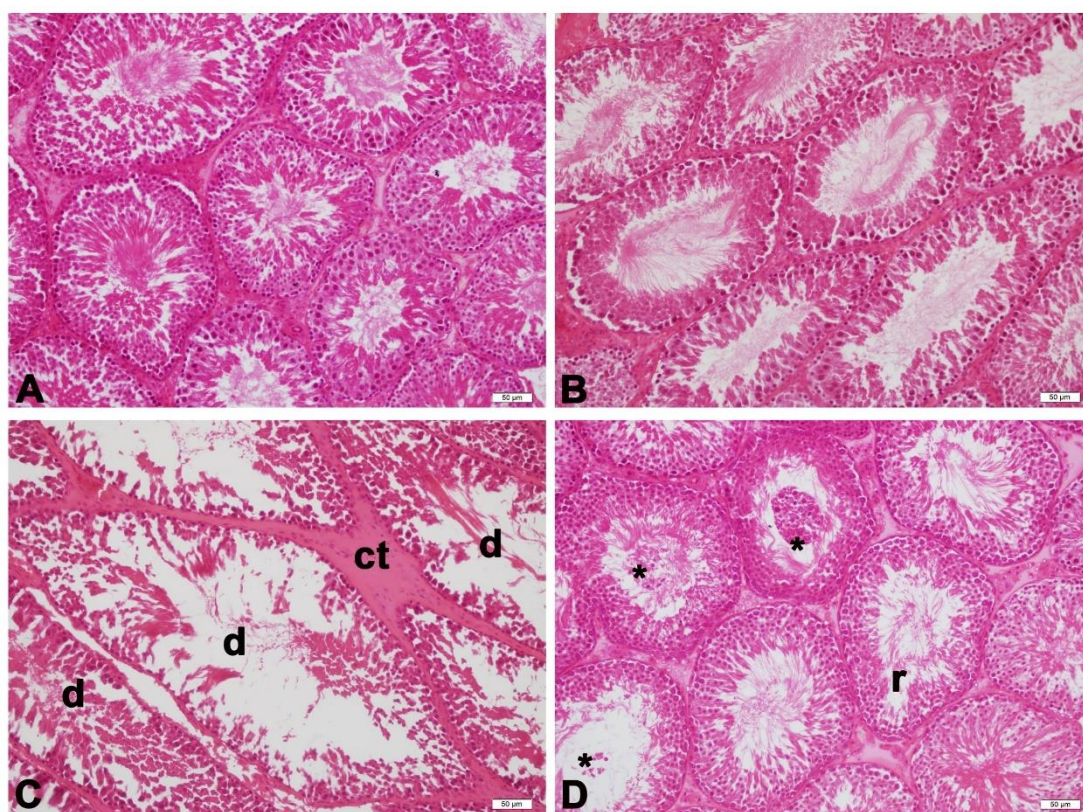
apoptosis, disfunction of Leydig cells and disorder of testicular stereodogenesis and culminating infertility [20]. For protecting the testicular damage from the side effects of cisplatin many antioxidant substances such as lycopene and ellagic acid [13], acetyl L-carnitine [21] and apocynin [22] were investigated in rats.

In this study we aimed to investigate cisplatin's gonodotoxic side effects in the rat testis and the possible beneficial antioxidant and antiapoptotic effects of RVT by histochemical, ultrastructural and biochemical methods.

## 2. RESULTS

### 2.1. Histopathological evaluation

Normal seminiferous tubules and spermatogenic cells in testis were observed in the control and RVT groups. In the Cis group degenerative, regressive and atrophic tubules were increased. Also, many seminiferous tubules with cellular debris in the lumen, increase of basement membrane thickness and connective tissue elements in the interstitial area were observed in this group. In the Cis+RVT group, the number of atrophic and degenerative seminiferous tubules decreased significantly. Most of the seminiferous tubules were in normal morphology (Figure 1).

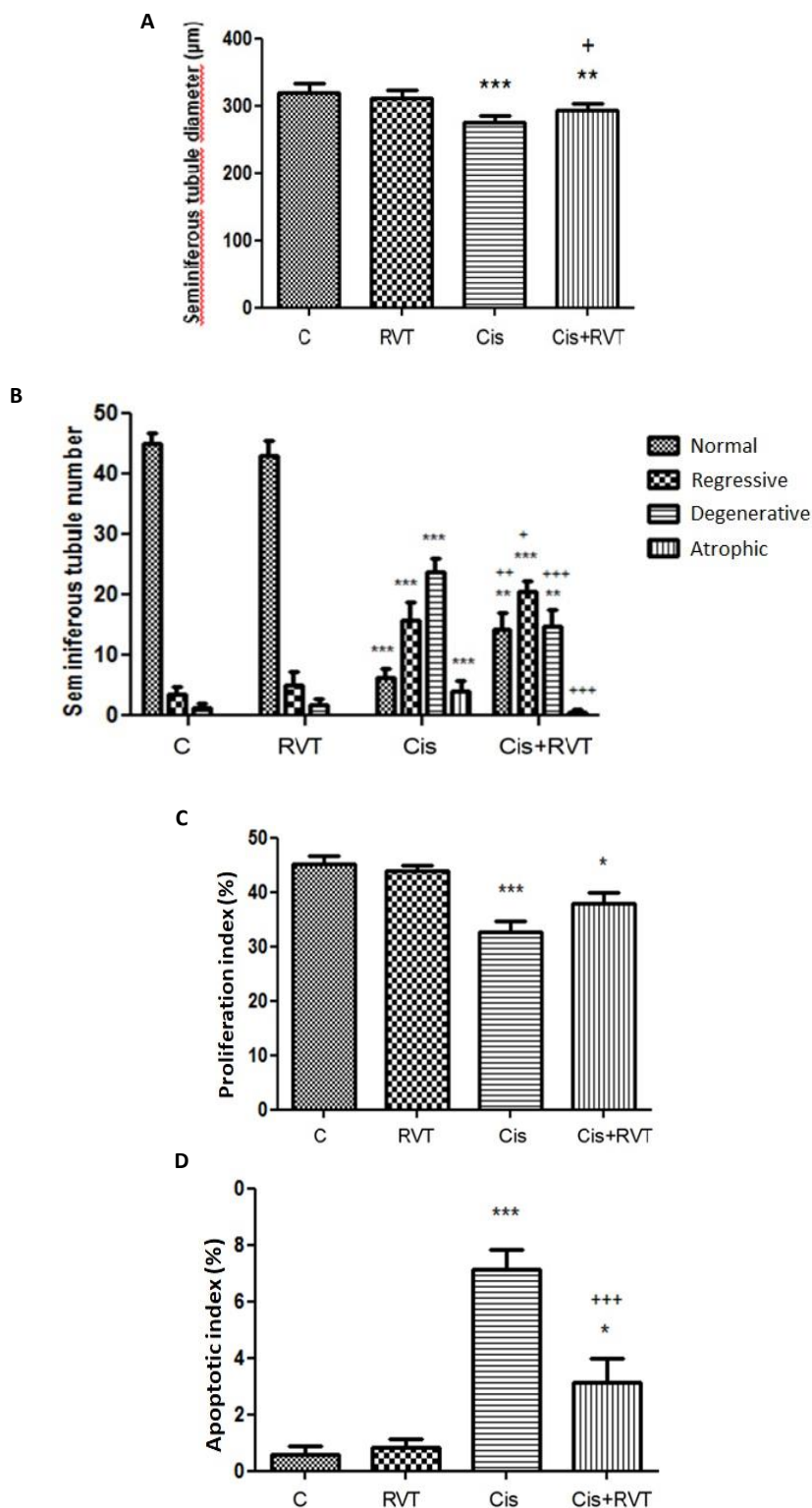


**Figure 1. Representative light micrographs of testicular tissue in experimental groups. A:** Regular seminiferous tubules with germinal epithelium are seen in control (A) and RVT applied control (B) groups. Degenerated seminiferous tubules (d) with dilatation between the germinal epithelial cells, increase of connective tissue elements (ct) in interstitial area are seen in Cis group (C). Cellular debris (\*) in lumen seminiferous tubules and regressive seminiferous tubule (r) are seen in Cis+RVT group (D). H&E staining.

The diameter of seminiferous tubules was  $319,4 \pm 13,3 \mu\text{m}$  in control group and  $310,6 \pm 11,8 \mu\text{m}$  in RVT group. There were significant decrease in seminiferous tubule diameter in Cis group ( $275,2 \pm 10,7 \mu\text{m}$ ) compare with the control group. However, the seminiferous tubule diameter was significantly increased in Cis+RVT group ( $292,8 \pm 9,7 \mu\text{m}$ ) compared with Cis group (Figure 2A).

Histopathological scoring results are shown in Figure 2B. In the Cis group, atrophic ( $p < 0.001$ ), degenerative ( $p < 0.001$ ) and regressive ( $p < 0.001$ ) seminiferous tubules were significantly increased compared with the control group. In the Cis+RVT group, the number of normal tubules ( $P < 0.01$ ) and regressive tubules

( $p < 0.05$ ) were significantly increased and degenerative ( $p < 0.001$ ) and atrophic tubules ( $p < 0.001$ ) were significantly decreased compared with the Cis group (Figure 2B).



**Figure 2.** Seminiferous tubule diameter (A) and histopathology scores (B), proliferative index (C) and apoptotic index (D) in experimental groups. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$  versus control group. +:  $p < 0.05$ , ++:  $p < 0.01$ , +++:  $p < 0.001$  versus Cis group.

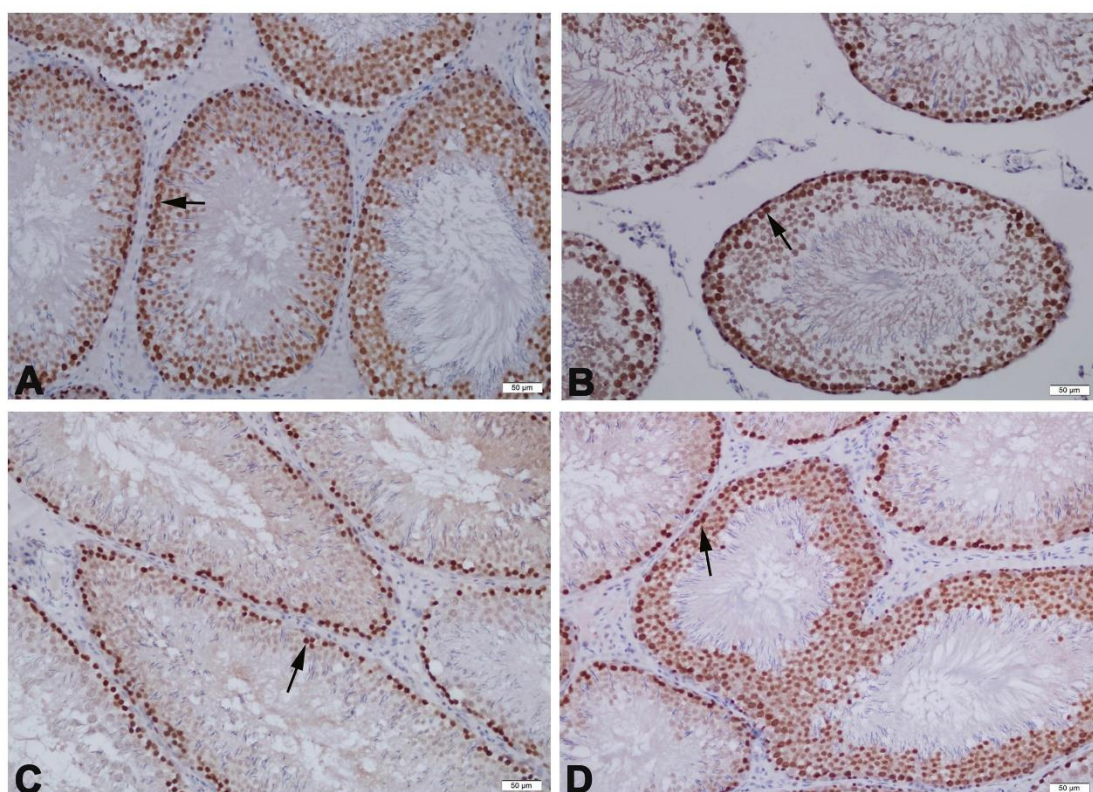


## 2.2. Evaluation of PCNA assay

Brown-coloured PCNA + cells were observed in each seminiferous tubules in all experimental groups (Figure 3). Proliferation index was significantly decreased at Cis ( $p < 0.001$ ) group compared to control group. However increase of proliferation index was observed at Cis+RVT group ( $p < 0.001$ ) compared with Cis group (Figure 2C).

## 2.3. Evaluation of TUNEL assay

Brown-coloured TUNEL+ cells in seminiferous tubules were observed in all groups (Figure 4). The number of seminiferous tubules with three or more TUNEL+ cells was significantly increased at Cis ( $p < 0.001$ ) group. However seminiferous tubules with three or more TUNEL+ cells was significantly decreased at Cis+RVT group ( $p < 0.001$ ) compared with Cis group. (Figure 2D).



**Figure 3.** Representative light micrographs of PCNA stained testicular tissue in experimental groups. Regular seminiferous tubules with PCNA positive spermatogenic cells (arrow) in control (A) and RVT applied control (B) groups; decrease number of PCNA positive cells (arrow) in seminiferous tubules in Cis group (C); regular seminiferous tubules with many PCNA positive cells (arrow) in Cis+RVT group (D) are seen.

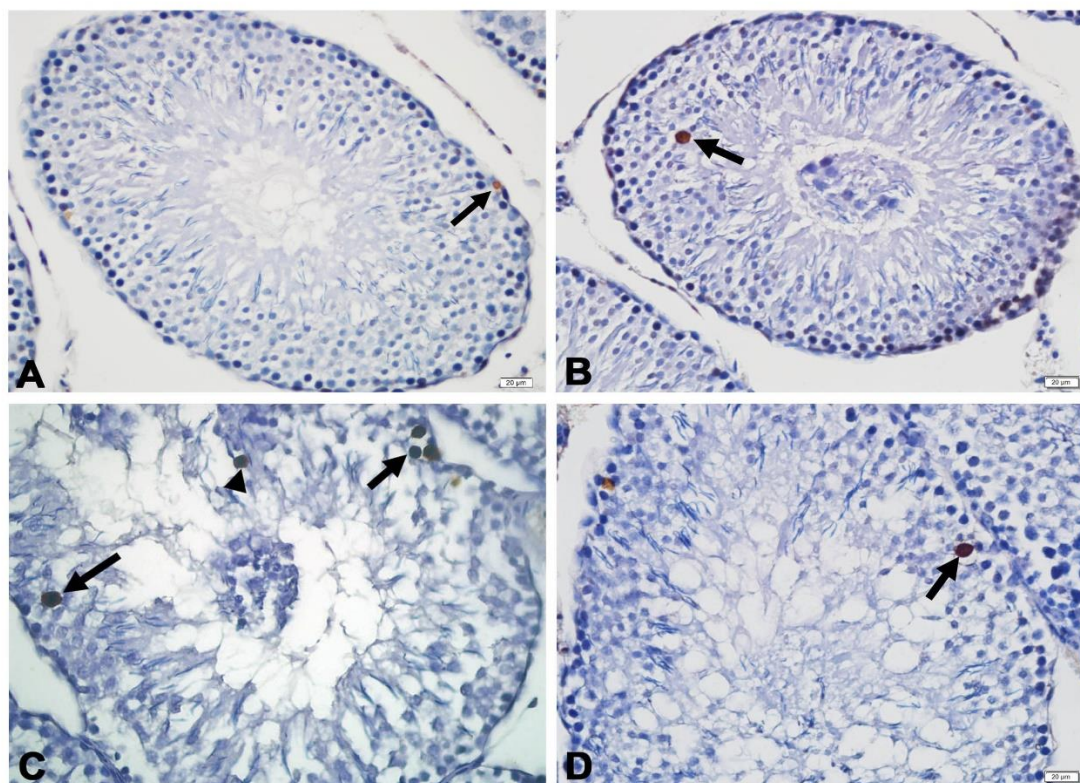
## 2.4. Electron microscopic evaluation

Normal seminiferous tubules with regular tight junctions between the Sertoli cells were seen in the control and RVT groups. Dilatation in the intercellular space near the basal lamina and severe large vacuoles in the cytoplasm of spermatogonia and Sertoli cells were observed in the Cis group. Regular ultrastructure of spermatogonia and Sertoli cells with tight junctions were observed in most regions of the Cis+RVT group. Even though most of the tubules showed regular ultrastructure with tight junctions of Sertoli cells, some tubules consisted of vacuole formation in the cytoplasm of Sertoli cells in this group (Figure 5).

## 2.5. Evaluation of MDA and GSH levels and MPO activity

The MDA levels in testis were significantly higher in the Cis group ( $p < 0.001$ ) than in the control group, while treatment with RVT significantly reduced MDA level ( $P < 0.05$ ) comparing to Cis group (Figure 6A). Cis caused substantial depletion of tissue GSH level ( $p < 0.001$ ). RVT treatment increased GSH level, but this

increase was not found significant (Figure 6B). The MPO activity was significantly increased in Cis group ( $p < 0.001$ ) than the control group ( $p < 0.001$ ) while treatment with RVT ( $p < 0.001$ ) significantly reduced MPO activity (Figure 6C).



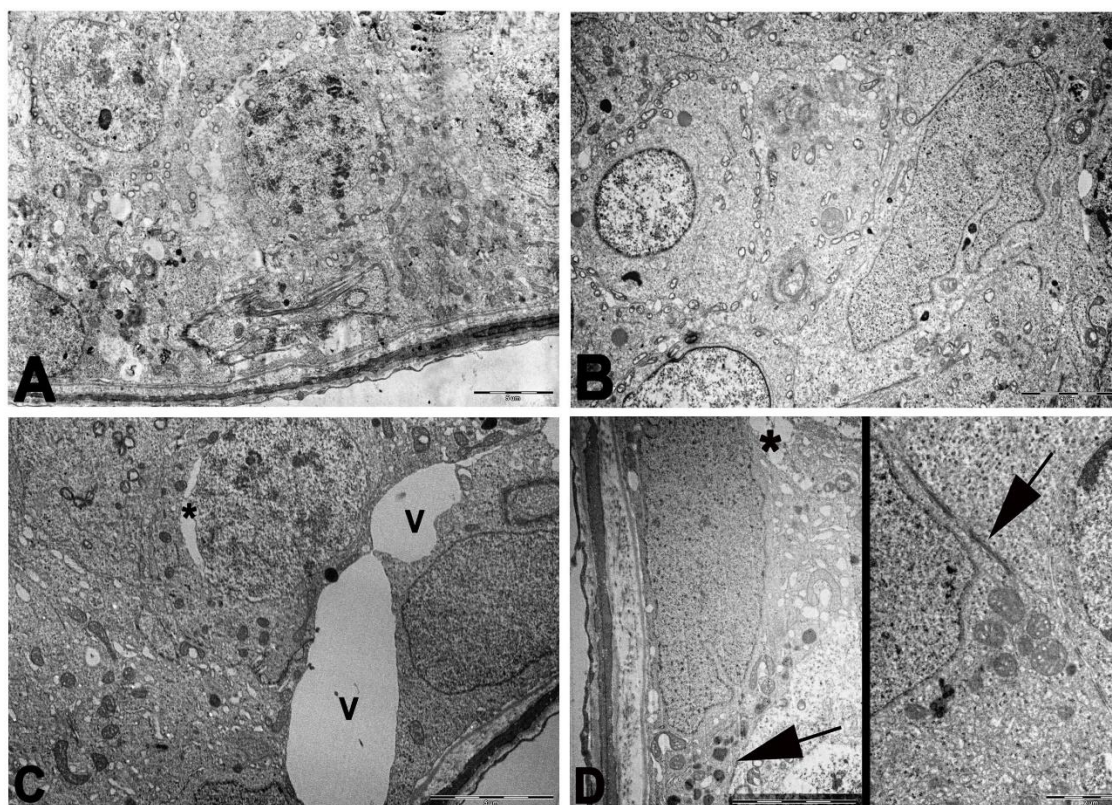
**Figure 4.** Representative light micrographs of TUNEL stained testis tissues in experimental groups. A few number of TUNEL positive cells (arrow) in control (A) and RVT applied control (B) groups; increase number of TUNEL positive cells (arrow) in germinal epithelium and luminal region (arrowhead) of Cis group (C) and decrease number of TUNEL positive cells (arrow) in the Cis+RVT group (D) are seen.

### 3. DISCUSSION

The present study showed that Cis induces histological alterations with a decrease of testicular diameter, an increase of regressive, degenerative and atrophic seminiferous tubules, decrease of spermatogenic cell proliferation and increase of apoptosis. Moreover, Cis induces oxidative injury and inflammatory activity with an increase of MDA level and MPO activity and depletion of antioxidant GSH level. RVT treatment ameliorates all these histological and biochemical alterations and protects against Cis-induced testicular damage.

Cisplatin is one of the most effective antineoplastic agent quite used in the treatment of several types of cancers including urogenital system carcinoma. However, Cis has side effects such as nephrotoxicity, hepatotoxicity, cardiotoxicity, ototoxicity and gonadal toxicity. Cis induced tissue injury mechanisms has not been well-explained. Cis treatment causes cytotoxicity including formation of ROS, DNA damage, induction of apoptosis and inflammation, reduction of DNA synthesis and mitosis [23, 24]. Also, studies demonstrated that Cis promotes histopathological changes in testicular tissue, decrease of seminiferous tubule diameter, spermatogenic cell proliferation and increase of apoptosis in seminiferous tubules [21, 25]. In this present study a single dose (7 mg/kg) administration of Cis were significantly decreased seminiferous tubule diameter and PCNA positive cells in Cis group. Degenerated and atrophic tubules, and apoptotic cells in germinal epithelium were significantly increased in Cis group. RVT treatment enhanced the seminiferous tubule diameter with decrease of atrophic tubules by inhibition of apoptosis.





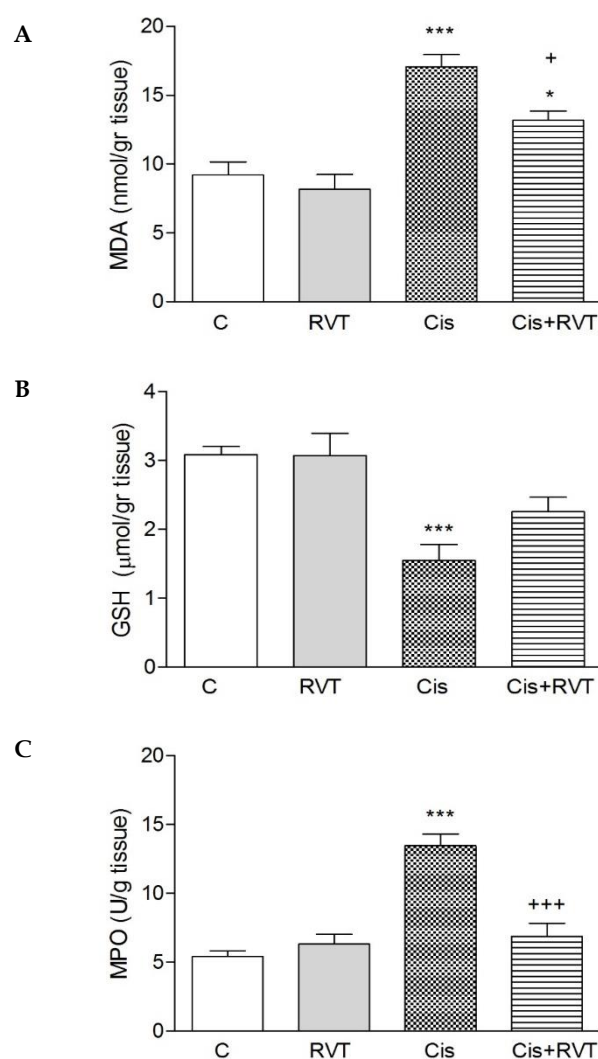
**Figure 5.** Representative electron micrographs of testicular tissue in experimental groups. Regular basal lamina, germinal cells and Sertoli cells with tight junctions in control (A) and RVT applied control (B) groups; large vacuole formation (v) around tight junctions in the intercellular spaces and intracytoplasmic vacuolar structures (\*) in the Cis group (C); regular morphology of basal lamina, tight junctions (→) and some intracellular vacuoles (\*) in the RVT+ Cis group (D) are seen.

It is known that Cis treatment promotes oxidative stress by increasing ROS. Cis treatment increased the formation of MDA, an index of lipid peroxidation, and decreased of endogenous antioxidant enzyme as GSH in the testes. Moreover, Cis treatment causes the inflammation by the accumulation of leukocytes in kidney and liver tissues [26]. Activated leukocytes such as neutrophils release reactive oxygen metabolites and MPO into the extracellular fluid. Neutrophil infiltration occurs in the acute inflammation and increases the MPO activity. Cis application may cause testis damage by increase of MDA level, inflammation and the MPO activity [27]. It was shown that treatment with different antioxidants such as rutin [28] and melatonin [29] reduces ROS generation as well as MPO activity in different oxidative injury models. In our study increase of MDA level and MPO activity and a decrease of GSH level were observed in the Cis group. RVT treatment ameliorated MDA production and MPO activity and replenished GSH level in Cis induced oxidative damage in rat testis.

Previous studies demonstrated that Cis inhibits tumor growth by inducing apoptosis in patients [30] and Cis induces apoptosis in rat testis and spermatozoa [31-33]. Several studies showed that Cis treatment increases the formation of oxidative stress, DNA fragmentation and induces apoptosis in testis, and different antioxidants and antioxidant rich extracts such as rutin [34], Ginkgo biloba extract [27], and Eugenia jambolana extract [35] inhibits apoptosis in Cis induced testis damage. It was shown that RVT inhibits apoptosis in Cis induced kidney injury [36], ischemia reperfusion induced cardiac injury [37] and acrylamide- induced oxidative stress in many tissues [38]. In this study, we observed that RVT inhibits the formation of apoptosis in Cis induced testis injury by the regulation of oxidant/antioxidant balance.

RVT's antioxidant, antiinflammatory and antiapoptotic roles were studied in different experimental testis injury models such as ischemia/perfusion [39], cadmium chloride treatment [40] and polyvinyl chloride-treatment [41]. It was shown that RVT protects the Cis induced testis damage in mice by the inhibition of oxidative stress [42]. Furthermore, it was observed that 45 days RVT (20mg/kg body weight) treatment ameliorates oxidant, steroidogenic and spermatogenic activity which was deteriorated by the injection of 3 mg/kg Cis for 3 days [43]. Parallel to this study's results, we observed that RVT treatment enhanced

antioxidant activity, testis morphology, inhibited apoptosis and inflammation from side effects of a Cis. Additionally our ultrastructural results showed that RVT ameliorated the germinal epithelial cells, Sertoli cells and tight junctions from a destructive effects of Cis.



**Figure 6.** MDA (A) and GSH (B) levels, and MPO (C) activity in experimental groups. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$  versus control group. +:  $p < 0.05$ , +++:  $p < 0.001$  versus Cis group.

#### 4. CONCLUSION

Cis treatment caused decrease of spermatogenic cell line, endogenous GSH level, ultrastructural degeneration of both spermatogenic and Sertoli cells, increase of oxidative, inflammatory and apoptotic activity and RVT ameliorates all these parameters in testis. This study showed that RVT ameliorates Cis induced testis damage by the antioxidant and antiapoptotic effects. It is concluded that, using of RVT might prevent Cis induced testis damage by the inhibition of ROS formation and apoptosis.

#### 5. MATERIALS AND METHODS

##### 5.1. Animals

Adult male Sprague-Dawley rats were housed individually in a light- and temperature-controlled room on a 12-h/12-h light-dark cycle and fed a standard pellet lab chow. All experimental protocols were approved by the Animal Care and Use Committee of the Marmara University (21.11.2013-106.2013.mar).

## 5.2. Experimental groups

Twenty-eight rats were divided into four groups: 1) Control (n=7) , 2) RVT (n=7), 3) Cis (n=7) , 4) Cis+RVT (n=7). We applied either saline or 10 mg/kg RVT orally for 5 days after a single dose of 7 mg/kg i.p. cisplatin to the Cis groups. Cis [22] and RVT [19] doses were decided according to our previous studies. Only saline or RVT was applied to the control groups. After 5 days, rats were decapitated under ether anesthesia and testis tissues were removed for histochemical, ultrastructural and biochemical evaluation.

## 5.3. Light microscopic preparation and histopathological scoring

The right testes of rats were removed, and fixed in Bouin's solution for 48 h and then processed routinely for paraffin embedding. Approximately 4 µm thick paraffin sections stained with hematoxylin and eosin (H&E) for histopathological analysis. Fifty seminiferous tubules from a section of mid-testicular region for each animal were photographed and evaluated for tubule diameter and histopathological scoring by the NIH Image Analysis (IMAGE J, National Institutes of Health, Bethesda, MD, USA) program. Modified Hess's histopathological scoring was evaluated as normal, regressive, degenerative or atrophic tubules [44].

## 5.4. Proliferating Cell Nuclear Antigen Method

The samples from left testes of all groups were fixed with 10% neutral formaldehyde, processed routinely for paraffin embedding. Approximately 5µm thick sections were deparaffinized, rehydrated in a graded series of ethanol, and immersed in 3% hydrogen peroxide for 5 min twice. Then boiled in citrate buffer (10mM; pH 6.0) for 20 minutes for proliferating cell nuclear antigen (PCNA) retrieval. Following washing in PBS 2X5 min slides were incubated with blocking solution (invitrogen PCNA staining kit, cat. No.93-1143) for 10 min. Sections were incubated in a humidified chamber with mouse anti-PCNA primary antibody (ready to use, Invitrogen PCNA Staining Kit cat. no.93-1143) overnight at 4 to 8 °C. Following washing with PBS, slides were incubated with biotinlated secondary antibody for 10 min, washed with PBS, incubated with streptavidin peroxidase for 10 min. After washing with then washing with PBS, the slides were incubated with 3, 3-diaminobenzidine tetrahydrochloride dihydrate (DAB) for 5 min. Slides were stained with hematoxylin and mounted with entellan (Merck, Darmstadt, Germany). For evaluation of proliferative index randomly selected 20 seminiferous tubules were counted both PCNA+ and PCNA- germ cells. Proliferative index were calculated as PCNA + cells/total cells in each seminiferous tubules.

## 5.5. Terminal transferase-mediated dUTP-biotin Nick end Labeling Method

The apoptotic cells were evaluated by terminal transferase-mediated dUTP-biotin Nick end Labeling (TUNEL) accordaning with the manufacturer's directive (Apoptag Plus Peroxidase in situ Apoptosis kit, Chemicon International, S7101, Temecula, CA, USA). The procedure was as follows: every fifth parafine sections (a total of five sections from each animal) was incubated with proteinase K washed with distilled water and incubated with 3% hydrogen peroxide in PBS. The sections were then washed with PBS, put in the balance buffer and incubated with recombinant terminal transferase TdT enzyme at 37 °C. The sections were rinsed in washing buffer, washed in PBS, incubated with anti-digoxigenin conjugate, and then washed with PBS. The slides were incubated in 3, 3-diaminobenzidine tetrahydrochloride dihydrate (DAB) and washed with distilled water, stained with Mayer's haematoxylin. The slides were dehydrated with alcohol series, cleared with toluene and mounted with entellan (Merck, Darmstadt, Germany). In each section, randomly selected 20 seminiferous tubules were counted for TUNEL-positive cells. Seminiferous tubules contains three or more TUNEL + cells were evaluted and calculated to total seminiferous tubules.

All stained sections were evaluated and photographed with the digital camera (Olympus C-5060, Tokyo, Japan) of a photomicroscope (Olympus BX51, Tokyo, Japan).

## 5.6. Transmission electron microscopic (TEM) preparation

Approximately 3 mm<sup>3</sup> tissue samples from the left testis were fixed into 2.5% glutaraldehyde in PBS (0.1 M, pH 7.2), postfixated in 1% osmium tetroxide in PBS (0.1 M, pH 7.2), dehydrated in an increased series of ethanol and embedded in Epon 812 resin (Fluka, Sigma-Aldrich Chemica, Steinheim, Switzerland). Ultrathin sections were contrasted with uranyl acetate and lead citrate. The ultrathin sections were examined using a transmission electron microscope (JEOL 1200 EXII, Tokyo, Japan) and photographed with digital camera (Morada Soft Imaging System, Olympus, USA).



## 5.7. Biochemical Analysis

### 5.7.1. Malondyaldehyde and glutathione H assays

The half of the left testes samples were homogenized in ice cold 150 mM KCl for assignment of MDA and GSH levels.

MDA levels were analysed as lipid peroxidation products [45]. Results are expressed as nmol MDA/g tissue. GSH was determined by the spectrophotometric method using the Ellman's reagent [46]. Results are expressed  $\mu\text{mol}$  GSH/g tissue.

### 5.7.2. Myeloperoxidase activity

Tissue associated myeloperoxidase (MPO) activity was evaluated according to Hillegas *et al.* [47] method. Testis samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41,400 g (10 min), pellets were suspended in 50 mM PBS containing 0.5% hexadecyltrimethylammonium bromide. MPO activity was evaluated by measuring the  $\text{H}_2\text{O}_2$ -dependent oxidation of o-dianizidine 2 HCl. One unit of enzyme activity was described as the amount of MPO that causes alteration in absorbance of 1.0 unit/min at 460 nm and was expressed as unit/g tissue.

### 5.7.3. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA). Differences between groups were determined with Tukey's multiple comparisons test, and the data were expressed as mean  $\pm$  standard error of the mean (SEM). Significance of differences was taken at the level of  $p < 0.05$ . Calculations were performed using Instant statistical analysis package (Prism 5.0 GraphPad Software, San Diego, CA, USA).

**Acknowledgement:** The authors thank the Marmara University Scientific Research Project Commission for their financial support (SAG-C-YLP-090414-0076).

**Author contributions:** Conception - N.Ö.Y., G.Ş., F.E.; Design - N.Ö.Y., G.Ş., F.E.; Supervision - G.Ş., F.E.; Resources - N.Ö.Y., F.E.; Materials - N.Ö.Y., G.Ş., F.E.; Data Collection and/or Processing - N.Ö.Y., F.E.; Analysis and/or Interpretation - N.Ö.Y., G.Ş., F.E.; Literature Search - N.Ö.Y., F.E.; Writing Manuscript - N.Ö.Y., F.E.; Critical Review - N.Ö.Y., G.Ş., F.E.

**Conflict of interest statement:** The authors declared no conflict of interest.

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