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Grape seed proanthocyanidin extract prevents DDP-induced testicular toxicity in rats

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Oxidative stress has been proven to be involved in cisplatin (DDP)-induced toxicity. The aim of the present study was to investigate a possible protective role of grape seed proanthocyanidin extract (GSPE) in DDP-induced spermiotoxicity. GSPE at 200 mg kg^{-1} d^{-1} and 400 mg kg^{-1} d^{-1} was orally administered for 15 consecutive days, starting 10 days before a single intraperitoneal dose of DDP (7 mg kg^{-1}). Results revealed that testicular and epididymal weight, epididymal sperm count, motility and morphology, the activities of GSH-Px and SOD, and GSH levels were significantly decreased whereas the level of MDA was significantly increased in the DDP group rats. GSPE treatment significantly attenuated the harmful effects of DDP-induced lipid peroxidation, oxidative stress, loss of genital organ weight, as well as function of reproductive organs. These changes were restored to near normal levels by GSPE at 400 mg kg^{-1} d^{-1}. In conclusion, GSPE has dose dependent protective effects against DDP-induced rat testicular toxicity.

1. Introduction

Chemotherapy has improved the quality of life of cancer patients and given hope for remission. Despite successes, even the most effective anti-cancer drugs may cause unwanted lesions. DDP is a highly effective antineoplastic DNA alkylating agent in treatment of various solid tumors including cancers of the bladder, ovary, cervix, endometrium, head, neck, and lung.[1,2] Also, DDP is widely used for the treatment of testicular cancer, and it has a >90% (ref. 3) cure rate. The success of DDP for the treatment of cancer is limited by its undesirable side effects on the reproductive system.[4,5]

The reproductive toxicity and nephrotoxicity induced by DDP are generally attributed to oxidative stress.[6–8] The pathogenesis by which DDP causes testicular injury is poorly understood; however, it has been demonstrated in numerous studies that DDP treatment is related to induction of oxidative stress by generation of free radicals and reactive oxygen species (ROS).[6,9–11] ROS like hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), superoxide anions (O_2^{-}) and singlet molecular oxygen ($^1O_2$) are normally generated in subcellular compartments of testes, particularly mitochondria, which are subsequently scavenged by antioxidant defense systems of the corresponding cellular compartments. However, this balance can easily be broken by chemicals such as DDP, which impair the balance of the antioxidant system, leading to cellular dysfunction.[9–11] When produced in excessive amounts, the ROS stimulate DNA fragmentation and a loss of sperm function associated with peroxidative damage to the mitochondria and sperm membrane. Furthermore, spermatozoa are more susceptible to peroxidative damage because of a high concentration of poly-unsaturated fatty acids and low antioxidant capacity.[12]

Grape seed proanthocyanidin extracts (GSPEs) are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics. GSPE has been reported to have therapeutic potentials due to its antioxidant, anti-inflammatory, radical scavenging, renal protecting and antitumor properties.[13–15] GSPE exhibited free radical scavenging abilities towards biologically generated free radicals such as superoxide anions, hydroxyl radicals and peroxyl radicals, and exhibited superior performance to vitamin C, vitamin E, and β-carotene.[16,17] GSPE may prevent cytotoxicity mediated by free radicals and lipid peroxidation, and protect low density lipoproteins from oxidation if absorbed and biologically active *in vivo*.[18] GSPE is marketed as a dietary supplement in the United States due to its powerful antioxidant activity, low toxicity and no genotoxic potential.[19]

Considering the antioxidant properties of GSPE, the present study is, therefore, designed to examine the impairment of DDP treatment and test the potential protective ability of GSPE in rats.

2. Materials and methods

2.1 Chemicals

DDP (type of freeze-dried powder for injection, with saline water dissolved) was obtained from Shandong Qilu Pharmaceutical

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Factory; grape seed procyanidins (purity is more than 95% as analysed by UV, in which procyanidolic oligomers and procyanidin B2 are more than 60% and 1.8% respectively as analysed by HPLC) were purchased from Tianjin Peak Natural Product Research Development Co., Ltd. For Coomassie brilliant blue protein assay, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA) kits were obtained from Nanjing Jiancheng Bioengineering Institute.

2.2 Animals and experimental design

This study was conducted in accordance with our institutional guidelines on the use of live animals for research, and the experimental protocol was approved by the Experimental Animal Ethical Committee of Function Test Center for Functional Food, College of Arts and Science, Beijing Union University. Fifty male Sprague Dawley rats of average weight (140–160 g) were used. Animals in the present study were obtained from the Laboratory of Animal Center of Academy of Military Medical Sciences of China. They were kept under standard laboratory conditions (12 h light:12 h dark and 24 ± 3 °C) and fed standard commercial laboratory chow (pellet form, in the sack). Feed and water were provided *ad libitum*.

The rats were randomly divided into five groups, each group containing ten rats. DDP was suspended in physiological saline and injected intraperitoneally (i.p.) at 7 mg kg^{-1} bw, a dose that is well known to induce testicular toxicity in rats.[10] GSPE was suspended in distilled water and administered to the animals by gavage at a dose of 200 or 400 mg kg^{-1} bw. The dose of GSPE used in this study was selected on the basis of the previous studies.[20,21] Group 1 was used as control; groups 3, 4 and 5 were orally treated with GSPE (400 mg kg^{-1} bw, 200 mg kg^{-1} bw and 400 mg kg^{-1} bw respectively) for 15 consecutive days. Groups 2, 4 and 5 received a single intraperitoneal dose of DDP 7 mg kg^{-1} bw on the 10th day of the experiment.

2.3 Sample collection

The rats were sacrificed on the fifth day after DDP injection. Blood samples were collected into tubes and centrifuged at 3000 rpm for 10 min. The testes and epididymis were quickly removed, cleared of adhering connective tissue, weighed and assayed immediately. One of the testes was fixed in neutral-formalin solution for histopathological examinations. The other testis samples were also stored at −80 °C until biochemical analyses. Testis tissues were taken from the deep-freezer and weighed. They were then transferred to cold glass tubes and diluted with a nine-fold volume of phosphate buffer (pH 7.4). For the enzymatic analysis, testicular tissues were minced and then homogenized using a Teflon-glass homogenizer at 16 000 × *g* for 3 min in cold physiological saline on ice. The homogenates were centrifuged at 3000 rpm for 15 min at 4 °C and the supernatant cytosols were kept frozen at −20 °C for the subsequent biochemical assays.

2.4 Epididymal sperm concentration and motility

Spermatozoa in the epididymis were counted by a modified method of Yokoi *et al.*[22] Briefly, the epididymis was minced with anatomical scissors in 5 ml of physiological saline, placed in a rocker for 10 min, and incubated at room temperature for 2 min. The supernatant fluid was diluted to 1 : 100 with a solution containing 5 g of sodium bicarbonate, 1 ml formalin (35%) and 25 mg eosin per 100 ml of distilled water. The total sperm number was determined using a hemocytometer. Approximately 10 dl of the diluted sperm suspension was transferred to each counting chamber and was allowed to stand for 5 min for counting under a light microscope at 200× magnification.

The progressive motility was evaluated. The fluid obtained from the left caudal epididymis with a pipette was diluted to 0.5 ml with tris buffer solution. A slide was placed on the light microscope with a heating table, an aliquot of this solution was placed on the slide, and percentage motility was evaluated visually at a magnification of 400×. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score. Samples for motility evaluation were kept at 35 °C.

The described method by Atessahin *et al.*[23] was used for the determination of the percentage of morphologically abnormal spermatozoa. According to the method, the slides were prepared with India ink. A total of 300 sperm cells were counted on each slide under the light microscope at 400× magnification.

2.5 Biochemical assays

Concentrations of malondialdehyde (MDA), as proceeding lipid peroxidation (LPO), and reduced glutathione (GSH) levels were measured in the supernatant cytosols that were kept frozen at −20 °C. Also the supernatant cytosols were used to determine superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. MDA and GSH concentrations were assayed using the kits that were purchased from Nanjing built biological engineering research institute. SOD and GSH-Px activities were measured using the kits that were obtained from Nanjing Jiancheng Bioengineering Institute.

2.6 Histopathological examination

Testicle samples were taken and fixed in 10% neutral-buffered formalin, processed routinely, and stained with haematoxylin–eosin (H & E). Light microscopy was used for the evaluations. The diameter and germinal cell thickness of the seminiferous tubules (ST) from five different areas of each testicle were measured using an ocular micrometre in a light microscope, and the average size of ST and germinal cell thickness were calculated.

2.7 Statistical analysis

The SPSS program (version 12.0) was used for the statistical analysis. Data are presented as mean ± standard error, and a value of $P < 0.05$ was considered as significant. Values were compared by one-way analysis of variance (ANOVA) and *post hoc* Duncan (D) test to determine the differences among all the groups.

3. Results

3.1 Organ weights

The values of testis and epididymis weights are shown in Table 1. At the end of the study, significant decreases in weights of testes ($P < 0.01$) and epididymides ($P < 0.01$) were observed as a result of DDP administration as compared to the control group. GSPE (400 mg kg^{-1}) significantly attenuated DDP-induced changes. However GSPE (200 mg kg^{-1}) resulted in insignificant increases ($P > 0.05$) in weights of testes ($P < 0.01$) and epididymides ($P < 0.05$) compared with the DDP group. Besides, the weights of testes and epididymides in the GSPE alone group were close to those in the control group, and there was a significant difference between the GSPE group and the DDP group ($P < 0.01$).

Table 1 Testis and epididymis weights in all groups[a]

Parameters	Testis weight (g)	Epididymis weight (g)
Control	2.533 ± 0.1424	0.5318 ± 0.0687
DDP	2.1900 ± 0.2022[b]	0.3956 ± 0.0543[b]
GSPE (400 mg kg^{-1})	2.4918 ± 0.0748[c]	0.5117 ± 0.0833[c]
DDP + GSPE (200 mg kg^{-1})	2.2483 ± 0.1650	0.4383 ± 0.0879
DDP + GSPE (400 mg kg^{-1})	2.3785 ± 0.1342[c]	0.4769 ± 0.0572[c]

[a] Data given are the mean ± standard deviations ($n = 10$). [b] $P < 0.01$ compared with the control group. [c] $P < 0.01$ compared with the DDP group.

3.2 Sperm characteristics

Epididymal sperm concentration, sperm motility, abnormal sperm rates and testicular sperm concentration are shown in Table 2. Although DDP treatment significantly decreased sperm concentration ($P < 0.01$) and sperm motility ($P < 0.01$) and increased the percentage of abnormality ($P < 0.01$) of sperms compared with the control group, the administration of GSPE (400 mg kg^{-1}) significantly prevented the DDP-induced side effects in sperm quality, including concentration ($P < 0.01$), motility ($P < 0.01$) and abnormality ($P < 0.01$), compared with the DDP group.

3.3 Levels of MDA and GSH and activity of GSH-Px and SOD in testis tissue

The MDA and GSH levels and GSH-Px and SOD activities of all the treatment groups are shown in Table 3. DDP administration caused significant ($P < 0.01$) increases in MDA levels of the testicular tissue compared with the control group. DDP + GSPE (400 mg kg^{-1}) treatment provided a marked reduction ($P < 0.05$) in the increased MDA levels. However DDP + GSPE (400 mg kg^{-1}) treatment resulted in insignificant increases ($P > 0.05$) in MDA levels of the testicular tissue compared with the DDP-alone group.

DDP treatment caused significant decreases in GSH levels ($P < 0.01$), GSH-Px ($P < 0.01$) and SOD activities ($P < 0.05$) of testicular tissue when compared with the control group. However, administration of GSPE (200 mg kg^{-1} and 400 mg kg^{-1}) to DDP-treated rats prevented the DDP-induced decreases in these antioxidants. Moreover, the administration of GSPE (400 mg kg^{-1}) alone caused a significant increase in GSH levels ($P < 0.01$), GSH-Px ($P < 0.01$) and SOD activities ($P < 0.05$) and a decrease in MDA levels when compared with both control and DDP groups (Fig. 1).

3.4 Histopathological observations

While DDP treatment caused significant decreases in the diameter of ST and germinal cell thickness of the testis compared to the control group ($P < 0.01$), DDP plus GSPE (400 mg kg^{-1}) treatment provided a marked ($P < 0.01$) amelioration in these parameters (Table 4). When the structure of the testes was histopathologically examined; degeneration, necrosis and interstitial oedema were detected in the testes of the DDP-treated group (Fig. 2) when compared to the control group (Fig. 1). The administration of GSPE (400 mg kg^{-1}) to DDP-treated rats (Fig. 3 and 4) improved nearly all the DDP-induced damage in the structure of testes (Fig. 5).

4. Discussion

Cytotoxic chemotherapy has improved the survival rates in many conditions, particularly testicular malignancies. Treatment is, however, associated with significant morbidity, and testicular dysfunction is among the most common long-term side effects of this therapy.[24] Many drugs used for chemotherapy, especially alkylating agents, have gonadotoxic effects, and their reproductive toxicity is associated with variables such as the antineoplastic agent group, the number of chemotherapeutic agents used, their total doses, treatment duration, and individual sensitivity.[24,25] DDP is an effective alkylating chemotherapeutic agent using for the treatment of testicular, ovary,

Table 2 Epididymal sperm concentration, sperm motility, abnormal sperms and testicular sperm concentration in all groups[a]

Parameters	Epididymal sperm concentration (10^5 sperms per ml)	Sperm motility (%)	Abnormal sperms (%)	Testicular sperm concentration (10^5 sperms per ml)
Control	22.83 ± 4.21	73.3 ± 4.7	2.1 ± 0.69	18.77 ± 2.62
DDP	13.92 ± 3.06[b]	51.4 ± 7.0[b]	3.6 ± 0.42[b]	12.08 ± 1.47[b]
GSPE (400 mg kg^{-1})	24.71 ± 3.00[c]	75.0 ± 7.6[c]	2.1 ± 0.35[c]	19.69 ± 2.31[c]
DDP + GSPE (200 mg kg^{-1})	15.68 ± 2.59	58.7 ± 7.9[d]	3.2 ± 0.36	16.88 ± 2.63
DDP + GSPE (400 mg kg^{-1})	19.69 ± 3.44[c]	64.4 ± 7.4[c]	2.5 ± 0.21[c]	18.00 ± 1.47[c]

[a] Data given are the mean ± standard deviations ($n = 10$). [b] $P < 0.01$ compared with the control group. [c] $P < 0.01$ compared with the DDP group. [d] $P < 0.05$ compared with the DDP group.

Table 3 The GSH and MDA levels, GSH-Px and SOD activity in testis tissue of all groups[a]

Parameters	GSH (μmol per g protein)	GSH-Px (U per g protein)	SOD (U per g protein)	MDA (nmol per g protein)
Control	495.74 ± 56.30	630.62 ± 18.16	153.80 ± 12.31	2.04 ± 0.48
DDP	427.89 ± 65.76[b]	593.71 ± 16.62[b]	139.78 ± 11.79[c]	2.89 ± 0.76[b]
GSPE (400 mg kg^{-1})	569.76 ± 39.18[b,d]	668.85 ± 23.99[b,d]	174.00 ± 9.51[b,d]	2.00 ± 0.59[d]
DDP + GSPE (200 mg kg^{-1})	507.09 ± 29.09[d]	629.00 ± 16.58[d]	156.15 ± 9.91[d]	2.41 ± 0.69
DDP + GSPE (400 mg kg^{-1})	523.91 ± 43.70[d]	623.90 ± 17.21[e]	170.31 ± 8.51[d]	2.11 ± 0.56[e]

[a] Data given are the mean ± standard deviations ($n = 10$). [b] $P < 0.01$ compared with the control group. [c] $P < 0.05$ compared with the control group. [d] $P < 0.01$ compared with the DDP group. [e] $P < 0.05$ compared with the DDP group.

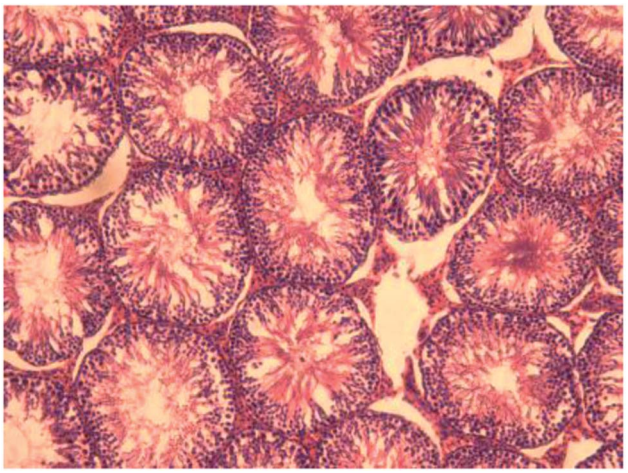

Fig. 1 The normal testis tissue of rat (H&E, 200×).

Table 4 The diameter of seminiferous tubules and germinal cell thickness in testis tissue of all groups[a]

Parameters	Diameter of ST (μm)	Germinal cell thickness (μm)
Control	313.83 ± 16.52	103.16 ± 10.42
DDP	284.39 ± 18.21[b]	81.49 ± 8.35[b]
GSPE (400 mg kg^{-1})	300.30 ± 10.93[d]	99.89 ± 17.86[c]
DDP + GSPE (200 mg kg^{-1})	285.80 ± 11.19	88.49 ± 7.84
DDP + GSPE (400 mg kg^{-1})	301.65 ± 16.83[d]	95.01 ± 6.17[d]

[a] Data given are the mean ± standard deviations ($n = 10$). [b] $P < 0.01$ compared with the control group. [c] $P < 0.01$ compared with the DDP group. [d] $P < 0.05$ compared with the DDP group.

head, neck, and cervix cancer types. Recently, it has attracted more attention owing to impairment in testicular function following the chemotherapy.[4,5] Some investigators have reported that DDP administration caused temporary or permanent azoospermia or oligospermia.[9–11] According to Boekelheide,[26] although DDP exposure in men can produce long-lasting azoospermia and testicular atrophy, animal studies of potential cellular targets and mechanisms of toxicity within the testis indicate that DDP has broad activity, targeting Leydig cells, Sertoli cells, and germ cells. The results of the present study indicated that DDP administration at a dose of 7 mg kg^{-1} resulted in both a significant decrease in sperm concentration, sperm motility and increase in all sperm abnormality rates in the rats.

Germinal epithelial damage, resulting in oligospermia or azoospermia, has long been a recognized consequence of treatment with chemotherapeutic agents.[4] Chemotherapeutic

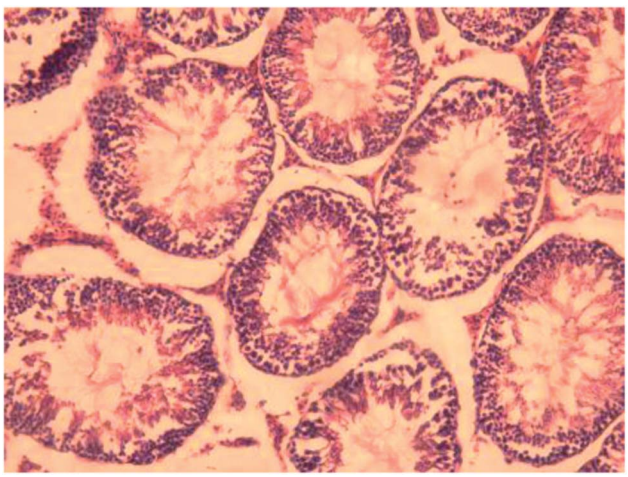

Fig. 2 The effect of DDP on testis tissue of rat. Seminiferous tubules show degeneration, necrosis, and interstitial oedema (H&E, 200×).

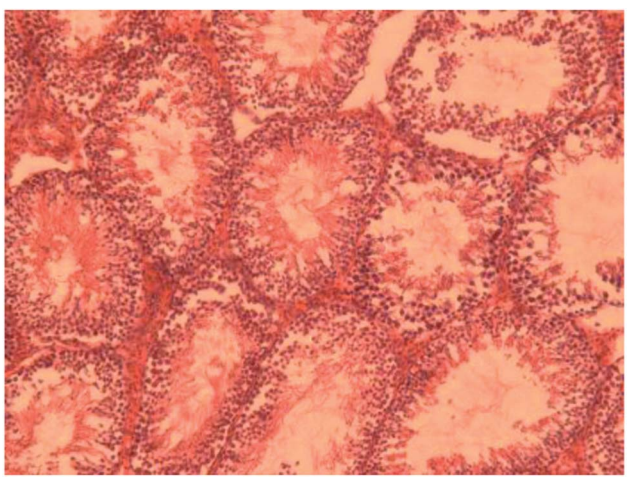

Fig. 3 The effect of GSPE (400 mg kg^{-1}) on testis tissue of rat (H&E, 200×).

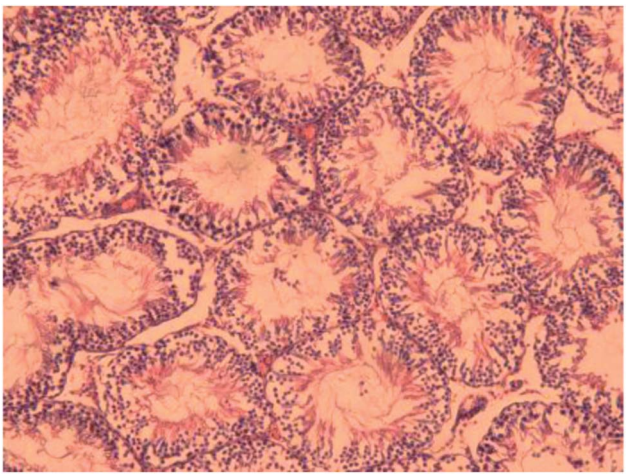

Fig. 4 The effect of GSPE (200 mg kg^{-1}) on testis tissue of rat treated with DDP. Seminiferous tubules show lightly degeneration, necrosis, and interstitial oedema (H&E, 200×).

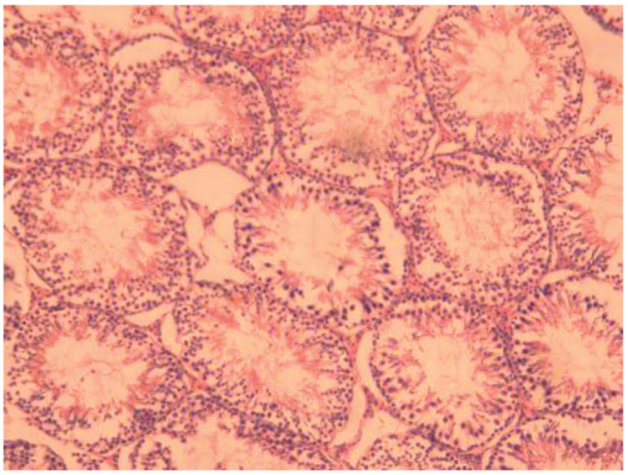

Fig. 5 The effect of GSPE (400 mg kg^{-1}) on testis tissue of rat treated with DDP. Seminiferous tubules show nearly normal structure (H&E, 200×).

regimen-induced testicular damage is drug specific and dose related. In the present study, administration of DDP reduced testis weight when compared with control, and histopathologic examination showed severe degeneration, necrosis, and reductions in seminiferous tubule and germinal cell thickness in the testes of rats treated with DDP alone. Our findings, especially impairment in sperm characteristics and in histopathological findings, are compatible with reports of some workers[9–11] and confirm the spermiotoxic effects of DDP in rat testes.

The cellular/biochemical mechanisms by which DDP causes reproductive toxicity are poorly understood. Several *in vitro* and *in vivo* studies[6,10,11] have suggested that exposure of experimental animals to DDP is accompanied by the induction of oxidative stress. Oxidative stress is a condition that is associated with an imbalance between the production and removal of reactive oxygen species (ROS) and free radicals, characterized by an increase in lipid peroxidation and a decrease in antioxidant enzymes. Excess ROS and free radical generation has been identified in the seminal plasma and sperm of infertile and subfertile males.[27]

In the present study, the DDP treatment caused a significant increase in the MDA level, and decrease in the GSH level, GSH-Px and SOD activities of testicular tissue compared to the control. The changes in the indicated parameters suggest the activities of antioxidant enzymes to be insufficient for the compensation of free radicals generated when 7 mg kg^{-1} DDP was administered at the indicated dose and for the indicated period. Also, the increase in the MDA level also confirms this situation. A reduction in the activity of SOD causes an increase in the level of superoxide anions. In addition, the decrease in the activities of the enzymes can be explained either with their consumption and induction during the conversion of free radicals into less harmful or harmless metabolites or secondarily with the direct inhibitory or stimulatory effect of DDP on enzyme activity. Among relevant studies that have been conducted over rats, and in a study carried out by Turk *et al.*,[11] DDP has been reported to cause an increase in plasma, sperm, and testicular tissue MDA levels. Furthermore, Salem *et al.*[9] reported a significant increase in the MDA levels of the testis tissue. The decrease in the activity of the antioxidant enzymes may predispose the sperms to increased free radical damage.

GSPE, a combination of biologically active bioflavonoids including oligomeric proanthocyanidins, has been shown to exert a novel spectrum of biological, therapeutic, and chemopreventive properties.[28] A study of an *in vitro* model has also determined that dimeric and trimeric oligomers are the most powerful PC molecules that mimic the complete GSPE.[29] The GSPE used in the present study contains more than 60% oligomers as described in section 2.1 chemicals. As a result, we found 200 mg kg^{-1} GSPE significantly improved the sperm quality in animals treated with DDP. Pretreatment with GSPE (200 mg kg^{-1} and 400 mg kg^{-1}) significantly inhibited the increase in MDA and GSH depletion and increase the activity of SOD and GSH-Px in the testis induced by DDP exposure. Pretreatment with 400 mg kg^{-1} GSPE more significantly remediated testicular function than pre-treatment with 200 mg kg^{-1} GSPE. The possible explanation for the protective effects of GSPE against DDP-induced increase in lipid peroxidation is its ability to react with the oxygen metabolites. Our study showed a significant decrease in sperm concentration, sperm motility and increase in abnormal sperm rates in DDP treated rats and recovery of these parameters with GSPE, especially in the 400 mg kg^{-1} group. We suggest that the protective effect of GSPE against DDP-induced abnormal sperm rates is attributed to the antioxidant properties of GSPE. These observations might also indicate that GSPE has protective and therapeutic effects on DDP induced oxidative stress. To our knowledge, this is the first use of GSPE in a similar protocol. Future research should focus on the effect of specific compounds in GSPE, such as dimers and trimers.

In conclusion, this study apparently suggests that GSPE has a protective effect against testicular toxicity caused by DDP. This protective effect of GSPE seems to be closely involved with the

suppression of oxidative stress. Therefore, GSPE may be used combined with DDP in chemotherapeutic treatments to improve DDP-induced injuries in sperm quality and oxidative stress parameters.

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