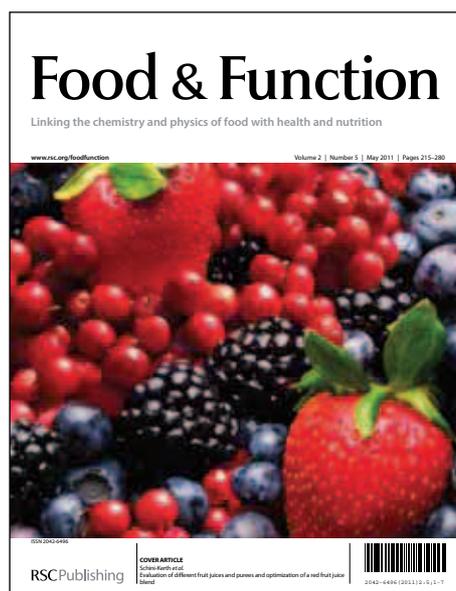


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ARTICLE

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

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Abstract

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) on DDP-induced spermiotoxicity. GSPE at 200 mg/kg/day and 400 mg/kg/day, respectively, was orally treated for 15 consecutive days, starting 10 days before a single intraperitoneal dose of DDP (7 mg/kg). 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/day. In conclusion, GSPE has the dose dependent protective effects against DDP-induced rat testicular toxicity.

Key words: cisplatin, grape seed proanthocyanidin extracts, testicular toxicity, oxidative stress

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ARTICLE

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 (Colpi et al., 2004; Howell and Shalet, 2005). Also, DDP is widely used for the treatment of testicular cancer, and it has a >90% (Wang and Lippard, 2005) cure rate. The success of DDP for the treatment of cancer is limited by its undesirable side effects on reproductive system (Cherry et al., 2004; Ishikawa et al., 2004).

The reproductive toxicity and nephrotoxicity induced by DDP is generally attributed to oxidative stress (Ahmed et al., 2011; Pérez-Rojas et al., 2011; Chirino and Pedraza-Chaverri, 2009). The pathogenesis by which DDP cause testicular injury are 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) (Ahmed et al., 2011; Salem et al., 2012; Ilbey et al., 2009; Turk et al., 2008). ROS like hydrogen peroxide (H₂O₂), hydroxyl radical (•OH), superoxide anion (O₂⁻) and singlet molecular oxygen (1O₂) are normally generated in subcellular compartments of testis, 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 antioxidant system, leading to cellular dysfunction (Salem et al., 2012; Ilbey et al., 2009; Turk et al., 2008). 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 polyunsaturated fatty acids and low antioxidant capacity (Vernet et al., 2004).

Grape seed proanthocyanidin extracts (GSPE) are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics. GSPE has been reported to have therapeutic potentials due to their antioxidant, anti-inflammatory, radical scavenging, renal protecting and antitumor properties (Houde et al., 2006; Shao et al., 2003; Vayalil et al., 2004). GSPE exhibited free radical scavenging abilities towards biologically generated free radicals such as superoxide anion, hydroxyl radicals and peroxy radicals, and exhibited superior performance as compared to vitamin C, vitamin E, and β-carotene (Bagchi et al., 1997; Sato et al., 1999). GSPE may prevent cytotoxicity mediated by free radical and lipid peroxidation, and protect low density lipoproteins from oxidation if absorbed and biologically active in vivo (Bagchi et al., 1998). GSPE is marketed as a dietary supplement in the United States due to its powerful antioxidant activity, low toxicity and no genotoxic potential (Ray et al., 2001).

Considering the antioxidant property of GSPE, the present study is, therefore, designed to examine the impairment of DDP treatment and test the potential protective ability of GSPE to 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 Factory; Grape seed procyanidins (Purity is more than 95% analysed by UV, in which procyanidolic oligomers and procyanidin B2 is more than 60% and 1.8% respectively analysed by HPLC) was purchased from Tianjin Peak Natural Product Research Development Co., Ltd; Take an examination of Coomassie brilliant blue protein, reduce glutathione (GSH), Glutathione peroxidase (GSH-Px), Superoxide dismutase (SOD), malondialdehyde (MDA) kits are 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 average weight (140–160g) 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 bw, a dose that is well known to induce testicular toxicity in rats (Ilbey et al., 2009). GSPE were suspended in distilled water and administered to the animals by gavage at the 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 (Yousef et al., 2009; Abir et al., 2009). Group 1 was used as control; groups 3, 4 and 5 were orally treated with GSPE (400 mg/kg bw, 200 mg/kg bw and 400 mg/kg bw respectively) for 15 consecutive days. Groups 2, 4 and 5 received a single intraperitoneal dose of DDP 7mg/kg 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 and assayed immediately. Cleared of adhering connective tissue and weighed. 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 deep-freezer and weighed. They were then transferred to cold glass tubes and diluted with a nine-fold volume of phosphate buffer (pH7.4). For the enzymatic analysis, testicular tissues were minced and then homogenized using a Teflon-glass homogeniser at $16,000\times\text{g}$ for 3 min in cold physiological saline on ice. The homogenates were centrifuged at 3000 rpm for 15min 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. (Yokoi et al., 2003). 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 as 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. Total sperm number was determined with a hemocytometer. Approximately 10 μl 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 $200\times$ 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 light microscope with heater table, an aliquot of this solution was placed on the slide, and percentage motility was evaluated visually at a magnification of $400\times$. 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. (Atessahin et al., 2006) was used for 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 light microscope at $400\times$ magnification.

2.5 Biochemical assays

Concentrations of malondialdehyde (MDA), as proceeding lipid peroxidation (LPO), and reduce 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 according to the kits that were purchased from Nanjing built biological engineering research institute. SOD and GSH-Px activities were measured by 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 \pm 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, a 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 (400mg/kg) significantly attenuated DDP-induced changes. However GSPE(200mg/kg) 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 GSPE alone group was close to that in the control group, and there was a significant difference between the GSPE group and the DDP group ($p < 0.01$).

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 sperm compared with the control group, the administration of GSPE (400mg/kg) 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 (400mg/kg) treatment provided a marked reduction ($P < 0.05$) in the increased MDA levels. However DDP + GSPE (400mg/kg) treatment resulted in insignificant increases ($P > 0.05$) in MDA levels of the testicular tissue compared with the DDP-alone group.

DDP treatment caused the 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 ((200mg/kg and 400mg/kg) to DDP -treated rats prevented the DDP -induced decreases in these antioxidants. Moreover, the administration of GSPE (400mg/kg) alone caused significantly increase in GSH levels ($P < 0.01$), GSH-Px ($P < 0.01$) and SOD activities ($P < 0.05$) and decrease in MDA levels when compared with both control group and DDP group.

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

(400mg/kg) treatment provided a marked ($P < 0.01$) amelioration in these parameters (**Table 4**). When the structure of testis was histopathologically examined; degeneration, necrosis and interstitial oedema were detected in testis of DDP-

Table 1

Testis and epididymis weights in all groups

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

Data given are the mean±standard deviations (n = 10).

^a $P < 0.01$ compared with control group.

^b $P < 0.01$. compared with DDP group.

Table 2

Epididymal sperm concentration, sperm motility, abnormal sperms and testicular sperm concentration in all groups

Parameters	Epididymal sperm concentration (105 sperms/ml)	sperm motility(%)	abnormal sperms(%)	testicular sperm concentration (105 sperms/ml)
Control	22.83±4.21	73.3±4.7	2.1±0.69	18.77±2.62
DDP	13.92±3.06 ^a	51.4±7.0 ^a	3.6±0.42 ^a	12.08±1.47 ^a
GSPE(400mg/kg)	24.71±3.00 ^b	75.0±7.6 ^b	2.1±0.35 ^b	19.69±2.31 ^b
DDP+ GSPE(200mg/kg)	15.68±2.59	58.7±7.9 ^c	3.2±0.36	16.88±2.63
DDP+ GSPE(400mg/kg)	19.69±3.44 ^b	64.4±7.4 ^b	2.5±0.21 ^b	18.00±1.47 ^b

Data given are the mean±standard deviations (n = 10).

^a $P < 0.01$ compared with control group.

^b $P < 0.01$. compared with DDP group.

^c $P < 0.05$. compared with DDP group

Table 3

The GSH and MDA levels, GSH-Px and SOD activity in testis tissue of all groups

Parameters	GSH ($\mu\text{mol/g prot}$)	GSH-Px (U/g prot)	SOD (U/g prot)	MDA (nmol/g prot)
Control	495.74±56.30	630.62±18.16	153.80±12.31	2.04±0.48
DDP	427.89±65.76 ^a	593.71±16.62 ^a	139.78±11.79 ^b	2.89±0.76 ^a
GSPE(400mg/kg)	569.76±39.18 ^{ac}	668.85±23.99 ^{ac}	174.00±9.51 ^{ac}	2.00±0.59 ^c
DDP+ GSPE(200mg/kg)	507.09±29.09 ^c	629.00±16.58 ^c	156.15±9.91 ^c	2.41±0.69
DDP+ GSPE(400mg/kg)	523.91±43.70 ^c	623.90±17.21 ^d	170.31±8.51 ^c	2.11±0.56 ^d

Data given are the mean±standard deviations (n = 10).

^a $P < 0.01$ compared with control group.

^b $P < 0.05$ compared with control group.

^c $P < 0.01$. compared with DDP group.

^d $P < 0.05$. compared with DDP group

treated group when compared to the control group (**Fig. 2**). The administration of GSPE(400mg/kg) to DDP-treated rats

improved nearly all the DDP -induced damages in the structure of testis (**Fig. 5**).

Table 4

The diameter of seminiferous tubules and germinal cell thickness in testis tissue of all groups

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

Data given are the mean ± standard deviations (n = 10).

^a P < 0.01 compared with control group.

^b P < 0.01 compared with DDP group.

^c P < 0.05 compared with DDP group

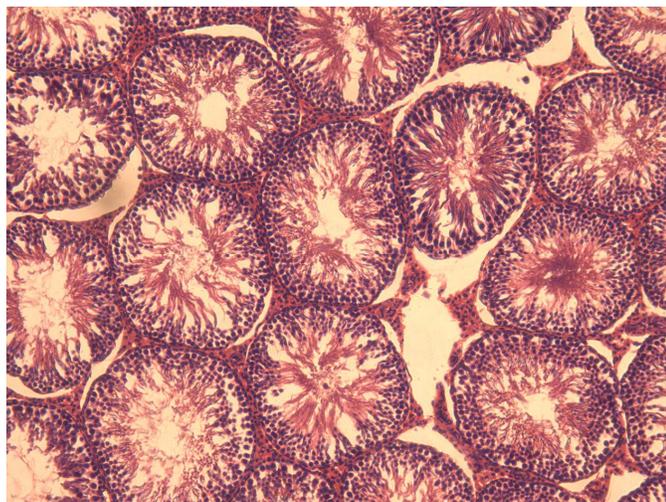


Fig. 1. The normal testis tissue of rat. (H&E, 200 ×)

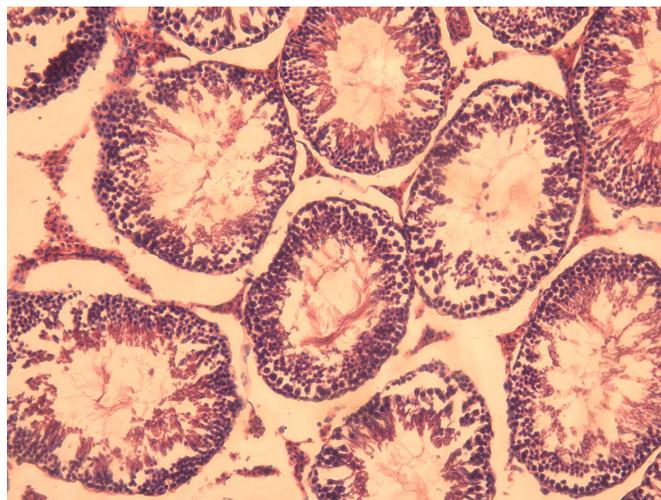


Fig. 2. The effect of DDP on testis tissue of rat. Seminiferous tubules show degeneration, necrosis, and interstitial oedema (H&E, 200 ×).

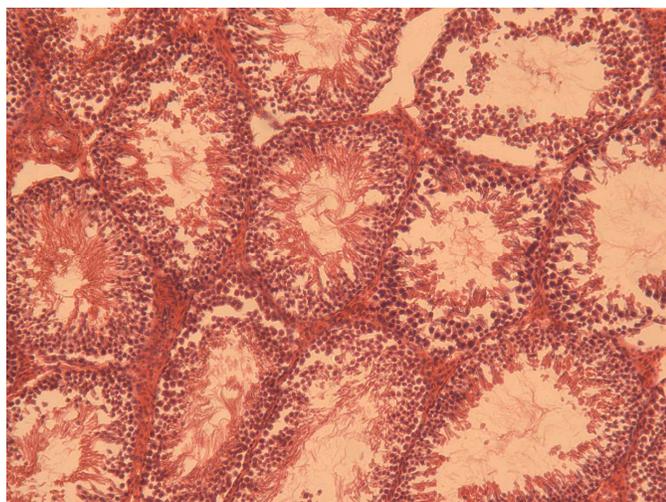


Fig. 3. The effect of GSPE(400mg/kg) on testis tissue of rat. (H&E, 200 ×).



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

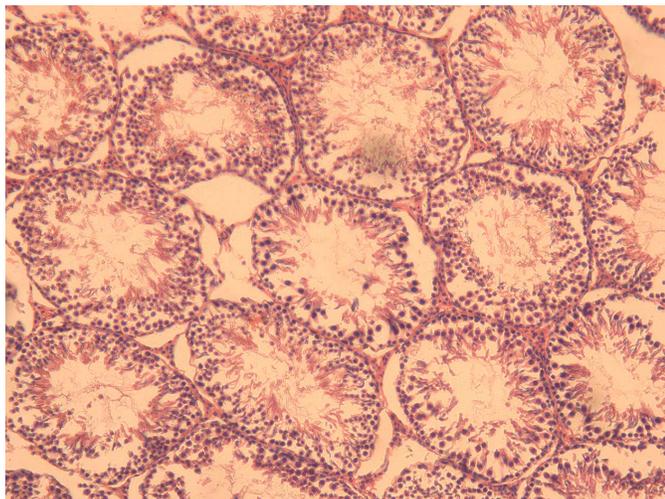


Fig. 5. The effect of GSPE(400mg/kg) on testis tissue of rat treated with DDP. Seminiferous tubules show nearly normal structure(H&E, 200×).

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 (Howell and Shalet, 2001). Many drugs used for chemotherapy, especially alkylating agents, have gonadotoxic effects, and their reproductive toxicity is associated with variables such as antineoplastic agent group, number of chemotherapeutic agents used, their total doses, treatment duration, and individual sensitivity (Howell and Shalet, 2001; Martin et al., 1999). DDP is an effective alkylating chemotherapeutic agent using for the treatment of testicular, ovary, head, neck, and cervix cancer types. Recently, it has attracted more attention owing to impairment in testicular function following the chemotherapy (Cherry et al., 2004; Ishikawa et al., 2004). Some investigators have reported that DDP administration caused temporary or permanent azoospermia or oligospermia. (Salem et al., 2012; Ilbey et al., 2009; Turk et al., 2008) According to Boekelheide, (Boekelheide, 2005) 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 the dose of 7 mg/kg resulted in both a significantly decrease in sperm concentration, sperm motility and increase in all of 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 (Cherry et al., 2004). Chemotherapeutic regimen-induced testicular damage is drug specific and dose related. In the present study, administration of DDP reduced testes 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 report of some workers (Salem et al., 2012; Ilbey et al., 2009; Turk et al., 2008) and confirm the spermiotoxic effects of DDP in rat testes.

The cellular/biochemical mechanisms by which DDP causes reproductive toxicity is poorly understood; Several *in vitro* and *in vivo* studies (Ahmed et al., 2011; Salem et al., 2012; Ilbey et al., 2009; Turk et al., 2008) 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 (Baker and Aitken, 2005).

In the present study, the DDP treatment caused significant increase in MDA level, and decrease in 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 in the compensation of free radicals generated when 7 mg/kg 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 anion. 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., (Turk et al., 2008) DDP has been reported to cause an increase in plasma, sperm, and testicular tissue MDA levels. Furthermore, Salem et al 21 reported a significant increase in the MDA levels of the testis tissue. The decrease in activity of the antioxidant enzymes may predispose the sperms to increased free radical damage.

GSPE, a combination of biologically active bioflavonoids including oligomeric proanthocyanidins, have been shown to exert a novel spectrum of biological, therapeutic, and chemopreventive properties. (Bagchi et al., 2002). 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 (Gemma et al., 2009). The GSPE used in the present study contain more than 60% oligomers as described in 2.1 chemicals. As a result, we found 200 mg /kg GSPE significantly improved sperm quality in animals treated with DDP. Pretreatment with GSPE (200 mg /kg and 400 mg /kg) significantly inhibited the increase 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 GSPE more significantly remediated testicular function than pre-treatment with 200 mg /kg 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 rescue of these parameters with GSPE, especially in the 400 mg /kg 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 time to use 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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