First International Journal of Andrology
ANDROLOGIA

ORIGINAL ARTICLE

Protective role of *Diospyros lotus* on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats

S. Saral[1], E. Ozcelik[1], A. Cetin[2], O. Saral[1], N. Basak[3], M. Aydın[4] & O. Ciftci[5]

1 Department of Nutrition and Dietetics, School of Healthy Sciences, Artvin Coruh University, Artvin, Turkey;
2 Department of Histology and Embryology, Faculty of Medicine, Inonu University, Malatya, Turkey;
3 Department of Pharmacology, Faculty of Medicine, Inonu University, Malatya, Turkey;
4 Department of Obstetric and Gynecology, Faculty of Veterinary Medicine, Firat University, Elazıg, Turkey;
5 Department of Medical Pharmacology, Faculty of Medicine, Inonu University, Malatya, Turkey

Keywords
Cisplatin—*Diospyros lotus*—oxidative stress—testicular damage

Correspondence
Dr. Eda Ozcelik, Artvin Coruh University, School of Healthy Sciences, Department of Nutrition and Dietetics, Artvin/Turkey.
Tel.: +466 2121301/2144;
E-mail: eda_ozcelik@yahoo.com

Accepted: May 18, 2015

doi: 10.1111/and.12448

Summary

The aim of this study was to investigate the protective effect of *Diospyros lotus* (DL) on cisplatin (CP)-induced testicular damage in male rats. Twenty-eight male rats were randomly divided into four groups: group 1 – control, given isotonic saline solution; group 2 – CP 7 mg kg^{-1} given intraperitoneally as single dose; group 3 – DL 1000 mg kg^{-1} per day given orally for 10 days; group 4 – CP and DL given together at the same doses. CP caused a significant increase in thiobarbituric acid-reactive substances (TBARS) level and a significant decrease in superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione (GSH) levels in rats testis tissues compared to the control group. CP caused a significant increase in lipid peroxidation in testis tissues compared to the control group, whereas DL led to a significant increase in SOD and GSH levels. However, there were no statistically significant changes in GPx and CAT levels. In addition, serum testosterone levels, sperm concentration and sperm motility were significantly decreased, but abnormal sperm rate and histological changes were increased with CP. However, these effects of CP on sperm parameters, histological changes and the tissue weights were eliminated by DL treatment. In conclusion, our study showed that the reproductive toxicity caused by CP may be prevented by DL treatment.

Introduction

Cisplatin (CP) is one of the most widely used and effective agent for the treatment of a variety of cancers, including bladder, ovarian, head and neck, cervical, lung and colorectal cancer. However, CP has many side effects, specifically testicular toxicity affecting spermatogenesis, chromosomal abnormalities in spermatozoa and fertility, so the clinical use of CP is often limited (Köberle *et al.*, 2010; Salem *et al.*, 2012). Pathogenesis of testicular damage after CP exposure is usually attributed to oxidative stress (Silici *et al.*, 2009). It is an important factor in the aetiology of low sperm activity, inducing morphological changes (Aziz *et al.*, 2004) and oxidative damage to DNA, membranes and proteins (Aitken & De Iuliis, 2007; Moretti *et al.*, 2012). Both short-term and long-term effects of CP inducing testicular oxidative stress have been previously documented in studies (Cherry *et al.*, 2004; Ahmed *et al.*, 2011). CP was reported to induce long-term failure of spermatogenesis and to inhibit testosterone secretion. Additionally, CP has been shown to stimulate lipid peroxidation and reduction in the activity of enzymes that protect against oxidative damage in testicular tissues (Amin & Hamza, 2006; Ateşşahin *et al.*, 2006).

Protection from oxidative stress is intervened by an effective free radical defence system, including antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and antioxidant such as glutathione (GSH) (Bhat *et al.*, 1999). Free radicals and other reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide, are an entire class of highly reactive molecules derived from the normal metabolism of oxygen or from exogenous factors and agents. ROS are not only strongly associated with lipid

peroxidation resulting in disruption of food materials, but are also related to the development of pathology of various diseases, including cancer (Esmaeili *et al.*, 2010). However, CP treatment leads to a significant decrease in reproductive organ weights, sperm quality and inhibition of testosterone secretion (Beytur *et al.*, 2012).

Caspases are a family of important signalling molecules with diverse duties depending on the subtype and organ involved. The activation of caspases also is a marker for cellular damage in diseases. Their involvement as an indicator alone and as a potential leverage point for drug research makes them widely researched molecules. Caspase-3 serves as a junction point for different signalling pathways. It is well appropriated as a read-out in an apoptosis assay (Lavrik *et al.*, 2005).

During the last two decades, the increased attention in new approaches to the immunotherapy of cancer and an important request for therapeutic agents that can modulate the immunodeficiency have encouraged studies on the immunomodulatory mechanism of natural substances (Silici *et al.*, 2009). Several studies have accumulated in documenting the beneficial effects of a variety of natural substances in CP-induced toxicity (Ahmed *et al.*, 2011). *Diospyros lotus* (DL) is a deciduous tree, which is a traditional nutrient grown particularly in the Black Sea Region of Turkey. It has been cultivated in several countries for its edible fruits which are considered for its medicinal importance. In folk medicine, DL was used as a sedative, nutritive, antiseptic, antidiabetic, antitumour and for the treatment of constipation. Chemical research of the fruits led to the recognition of some fatty acids, sugars and nonvolatile acids (Loizzo *et al.*, 2009; Uddin *et al.*, 2011).

The aims of this study were to investigate the effects of CP on sperm characteristics, plasma testosterone levels, histopathological and biochemical changes related to oxidative stress in testes and to examine the protective effect of DL on these parameters.

Materials and methods

Chemicals

Cisplatin (10 mg/10 ml, Code 1876A) was obtained from Faulding Pharmaceuticals Plc (Warwickshire, UK). DL fruits were provided by Kumludere village (Trabzon, Turkey). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or of the highest grade available.

Preparation of extract

Diospyros lotus fruits were oven dried at 38 °C for 5 days. Dried fruits of DL were coarsely ground (2–3 mm) before extraction. The extraction process was made according to the method developed by Moghaddam *et al.* (2012).

Animals and treatment

A total of 28 healthy adult male Spraque Dawley rats (between 2–3 months old and 250–300 g. in weight) obtained from Experimental Animal Institute, Malatya–Turkey for this experiment. Animals were housed in sterilised polypropylene rat cages, in 12-h light–dark cycle, at an ambient temperature of 21 °C. Diet and water for them were given *ad libitum*. Experiments were performed based on animal ethics guidelines of Institutional Animals Ethics Committee.

Rats were randomly divided into four equal groups ($n = 7$ in each group). CP was intraperitoneally (i.p.) administered at the dose of 7 mg kg^{-1} single injection. Our previous studies (Beytur *et al.*, 2012; Ciftci *et al.*, 2014) indicate that single injection CP caused testicular damage in rats at the dose of 7 mg kg^{-1} (i.p.). DL, suspended in tap water, was given at the doses of 1000 mg kg^{-1} per day for 10 consecutive days. At the dose of 1000 mg kg^{-1} is the most effective dose for assessing the antioxidant potential of fruits extract of DL in CP-induced oxidative damage on animals. This dose was guided by previous studies (Rashed *et al.*, 2013). Group 1 (control) served as negative control and was given isotonic saline (i.p.) and tap water (orally) as vehicles. In group 2 (CP group), CP was given a single injection, and then, tap water was given to rats for 10 days. Rats in the group 3 (DL group) were treated with DL for 10 days without CP. In the group 4, rats were treated with CP and DL (CP+DL group) together. Tissue and blood samples were collected on day 10 of CP treatment. The animals were sacrificed under anaesthesia, and testis tissues were immediately removed and dissected over ice-cold glass. Blood samples were collected from the left ventricle with an injector under anaesthesia. Serums were obtained after whole-blood centrifugation (3000 ***g***, 20 min, at 4 °C). Tissue and serum samples were stored at −45 °C in a deep freeze until analysis (Beytur *et al.*, 2012; Ciftci *et al.*, 2014).

Biochemical assay

The homogenisation of tissues was carried out in Teflon-glass homogeniser with 150 mM KCl (pH 7.4) to obtain 1 : 10 (w/v) dilution of the whole homogenate. The homogenates were centrifuged at 18 000 ***g*** (4 °C) for 30 min. to determine thiobarbituric acid-reactive substances (TBARS), reduced GSH levels, and CAT activities and at 25 000 × ***g*** for 50 min. to determine GPx and CuZn-SOD activities (Ciftci *et al.*, 2014). The levels of homogenised tissue TBARS, as an index of lipid peroxi-

dation, were determined by thiobarbituric acid reaction using the method of Yagi (1998). The product was evaluated spectrophotometrically at 532 nm, and the results are expressed as nmol g^{-1} tissue.

The GSH content of the testis homogenate was measured at 412 nm using the method of Sedlak & Lindsay (1968). The GSH level was expressed as nmol ml^{-1}. SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O_2^- generated by the xanthine/xanthine oxidase system (Sun *et al.*, 1988). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The product was evaluated spectrophotometrically at 560 nm. Results are expressed as IU mg^{-1} protein. CAT activity of tissues was determined according to the method of Aebi (1974). The enzymatic decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. The enzyme activities are given in k mg^{-1} protein. GPx activity levels were measured using the method of Paglia & Valentine (1967), in which GPx activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was followed spectrophotometrically at 340 nm and 37 °C. GPx activity is expressed as IU mg^{-1} protein. Tissue protein content was determined according to the method developed by Lowry *et al.*, (1951) using bovine serum albumin as standard.

Evaluation of sperm parameters

The epididymal sperm concentration was determined with a haemocytometer using a modified method briefly described by Ciftci *et al.* (2012a). The right epididymis was finely minced by anatomical scissors in 1 ml of isotonic saline in a Petri dish and allowed to incubate at room temperature. Then, the supernatant fluid containing all epididymal sperm cells was counted with the help of a light microscope at 200× magnifications. The percentage of forward progressive sperm motility was evaluated using a light microscope with heated (37 °C) stage as described by Ciftci *et al.* (2012b). The percentage of forward progressive sperm motility was evaluated visually at 400× magnification. Motility estimates were performed from three different fields on each sample. The mean of the three successive estimations was used as the final motility score. To determine the percentage of morphological abnormal spermatozoa, the slides stained with eosin–nigrosin were prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 sperm cells were examined on each slide (2100 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as a percentage (Ciftci *et al.*, 2012a,b).

Histological evaluation

For light microscopic evaluation, testis samples were fixed in 10% formalin and were embedded in paraffin. Paraffin-embedded specimens were cut into 5-μm-thick sections, mounted on slides and stained with haematoxylin–eosin (H-E). Tissue samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

For immunohistochemical analysis, thick sections were mounted on polylysine-coated slides. After rehydrating, samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min at room temperature, and the sections were washed with phosphate-buffered saline (PBS). Then, the sections were kept in 0.3% H_2O_2 for 7 min and afterwards washed with PBS. Sections were incubated with primary rabbit-polyclonal Caspase-3 antibody (Ab4061, Abcam Ltd, Cambridge, UK) for 2 h. They then were rinsed in PBS and incubated with biotinylated goat antipolyvalent for 10 min at room temperature. Staining was completed with chromogen+substrate for 15 min, and slides were counterstained with Mayer's haematoxylin for 1 min, rinsed in tap water and dehydrated. The Caspase-3 kit was used according to the manufacturer's instructions.

Measurement of seminiferous tubule diameter and germinal epithelial cell thickness

In each section, the diameters of 20 separate circular seminiferous tubules were randomly measured using a 20× objective. We totally measure 100 tubule diameter in each group. The mean seminiferous tubular diameter (MSTD) of each testis was determined in micrometres (μm). We measured MSTD and germinal epithelial cell thickness (GECT) using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd.).

Hormonal analysis

Serum testosterone level was determined by enzyme-linked immunosorbent assay (ELISA) using anti-rat ELISA commercial kits from Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer's instructions. The plates were read at 405 nm using the CA-2000 ELISA microplate reader and washer (CIOM Medical Co., Ltd., Changchun, China). Testosterone quantities

in the samples were calculated from standard curves of testosterone using a linear regression method.

Statistical analysis

Biochemical, spermatological and hormonal values were presented as mean ± SD. Significant differences (P value) were accepted as $P \leq 0.01$. A computer program SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For biochemical, hormonal and spermatological values, statistical analyses were performed using one-way ANOVA and *post hoc* Duncan significant difference test. Histological results were compared with Kruskal–Wallis variance analysis and chi-square.

Results

Biochemical results

Rat testis SOD, GPx, CAT, GSH and TBARS levels are given in Table 1. The results indicated that CP led to significant increase in TBARS levels, contrariwise SOD, GPx, CAT and GSH levels were significantly decreased with CP treatment compared to control and other groups. Additionally, only DL treatment did not significantly change in TBARS, SOD, GPx, CAT and GSH levels compared to control group (Table 1). On the other hand, in combined treatment group (CP+DL), increased TBARS levels caused by CP were nearly control value treatment DL, and it was determined that testis TBARS levels were significantly decreased and this value closely the control value. Similarly, lower GSH and SOD levels were significantly increased and were nearly control values. However, GPx and CAT levels did not positively affect with CP+DL treatment.

Hormonal results

Serum testosterone levels are presented in Fig. 1. It was determined that serum testosterone levels with CP treatment were significantly decreased ($P < 0.01$) compared to control and other groups. DL treatment did not significantly change serum testosterone levels compared to control. Additionally, DL treatment together with CP not positively affected serum testosterone levels against CP toxicity in terms of testosterone levels.

Histological results

In this study, all the figures demonstrate the histological findings of each group in testis. In the control (Fig. 2a) and DL (Fig. 2b) groups, testis tissue showed a normal testicular architecture of the seminiferous tubule morphology and interstitium and had intact germinal epithelium. In CP group (Fig. 3), some degenerative changes were observed such as loss of maturation in the germinal cells (Fig. 3a, b), tubular degeneration (Fig. 3b), oedema (stars) and vacuolisation (arrows) (Fig. 3c), arrested spermatocytes in different stage in division (Fig. 3d), eosinophilic cytoplasm, multinucleated giant cells (Fig. 3e, f). In CP+DL group (Fig. 4a, b), we detected an improved histological appearance in testis.

Immunohistochemically Caspase-3-stained cells were not observed in control (Fig. 5a) and DL (Fig. 5b) groups. The percentages of Caspase-3-positive cells were high in CP group (Fig. 5c). The density of immunohistochemically Caspase-3-stained cells was decreased in CP+DL group (Fig. 5d). The value of MSTD and GECT was given in Table 4. The results indicated that the MSTD and GECT were significantly decreased in CP groups compared with control and the others. On the other hand, the decrease in the levels of MSTD and GECT were normally against CP group. Besides, they were no significant differences between control and only given DL group.

Spermatological results

The effects of CP and DL on reproductive organ weights and sperm characteristics were given in Table 2 and Table 3 respectively. There was no significant effect on organ weights except testis weights with CP or DL treatments. Testis weights both right and left were significantly decreased with CP treatment. But DL treatment together with CP was significantly prevented this decreases caused

Table 1 The levels of SOD, GPx, CAT, GSH and TBARS in rat testis tissue

	Control	CP	DL	CP+DL
TBARS (nmol g^{-1} tissue)	2.58 ± 0.83^{a}	4.89 ± 0.71^{b}	2.48 ± 0.12^{a}	3.13 ± 0.53^{c}
Reduced GSH (nmol ml^{-1})	194.3 ± 4.09^{a}	143.1 ± 3.76^{b}	188.6 ± 4.92^{a}	166.8 ± 4.08^{c}
CAT (k mg^{-1} protein)	0.024 ± 0.005^{a}	0.017 ± 0.003^{b}	0.023 ± 0.002^{a}	0.019 ± 0.003^{b}
SOD (U mg^{-1} protein)	29.53 ± 0.46^{a}	21.44 ± 0.93^{b}	30.73 ± 0.62^{a}	26.12 ± 1.68^{c}
GPx (U mg^{-1} protein)	2.85 ± 0.068^{a}	1.91 ± 0.090^{b}	2.79 ± 0.107^{a}	2.17 ± 0.109^{b}

Means bearing different superscripts within the same row were significantly ($P \leq 0.01$) different.SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; GSH, glutathione; TBARS, thiobarbituric acid-reactive substances; CP, cisplatin; DL, *Diospyros lotus*.

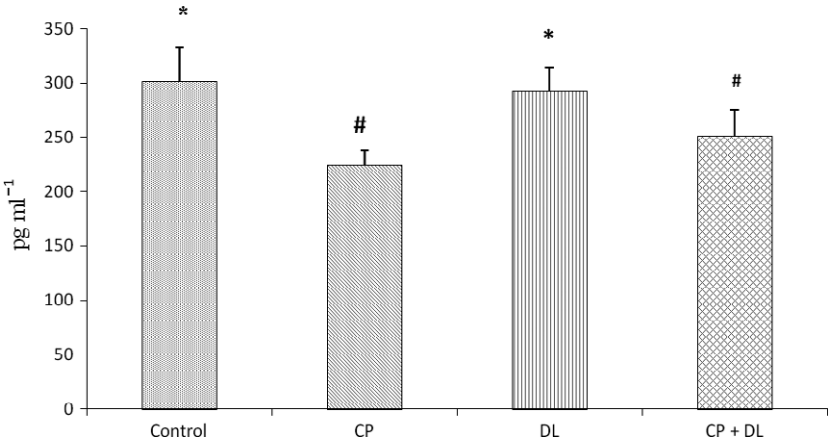

Fig. 1 Serum testosterone levels in rats (pg ml^{-1}±SEM). *,# showed significant ($P \leq 0.01$) differences between groups.

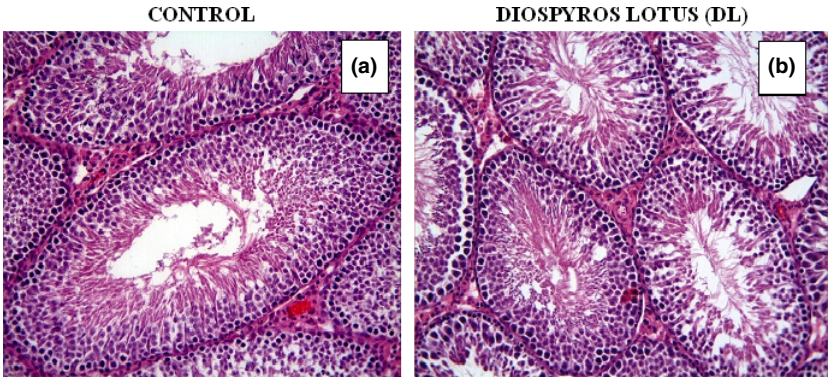

Fig. 2 Control (a) and *Diospyros lotus* (b) groups: Seminiferous tubules showed normal histological appearance. Arrangement of germinal epithelium, seminiferous tubules and interstitial cells was normal. a,b: H-E; ×20.

by CP. Additionally, CP treatment significantly ($P < 0.01$) decreased epididymal sperm concentration and sperm motility and increased abnormal sperm rate in rats. On the other hand, in CP+DL group, epididymal sperm concentration and sperm motility increased and abnormal sperm rate was decreased compared with CP group. We showed that these values were nearly similar to the control group (Table 3).

Discussion

Cisplatin is a highly effective antitumour drug used to treat many types of different tumours testicular, ovary, bladder, cervix, endometrium, lung, head and neck. Nonetheless, its usage is restricted by its toxical effects on the reproductive system (Cherry *et al.*, 2004; Howell & Shalet, 2005; Ilbey *et al.*, 2009a). For that, the prevention of side effects of CP is very important in terms of treatment protocol, benefits in quality of life and extending the limits of dose (Kamisli *et al.*, 2013). In the present study, testicular damage induced by CP treatment was characterised by significant increases lipid peroxidation, decreased antioxidant status, changed sperm characteristics and serum testosterone level in rats. Nonetheless, we determined that these toxic effects of CP on the reproductive system can be avoided by DL when used together with CP. These results were confirmed by histopathological and immunohistochemical examinations.

Oxidative stress occurs in overproduction of oxygen free radical precursors and/or decreased antioxidant defence system. The oxygen free radical generation is associated with impaired GSH metabolism, changes in the antioxidant enzymes and lipid peroxidation. The enzymes such as SOD, GSH-Px and CAT are endogenous defence mechanisms, whose activities eliminate superoxide, hydrogen peroxide and hydroxyl radicals (Ilbey *et al.*, 2009b; Yildirim *et al.*, 2013). These enzymes are the first line of defence against oxidative injury. The inhibition of antioxidant system may cause the accumulation of H_2O_2 or products of its decomposition (Yildirim *et al.*, 2013). In the present study, CP significantly induced lipid peroxidation in rat testis tissue via increased TBARS levels. The studies indicate that CP treatment caused significant lipid peroxidation in rat testis tissue (Amin & Hamza, 2006; Beytur *et al.*, 2012; Sajjad, 2012), and these studies confirmed our findings. The increased lipid peroxidation is one of the toxic case of CP administration in testis. CP treatment causes excessive ROS accumulation with an

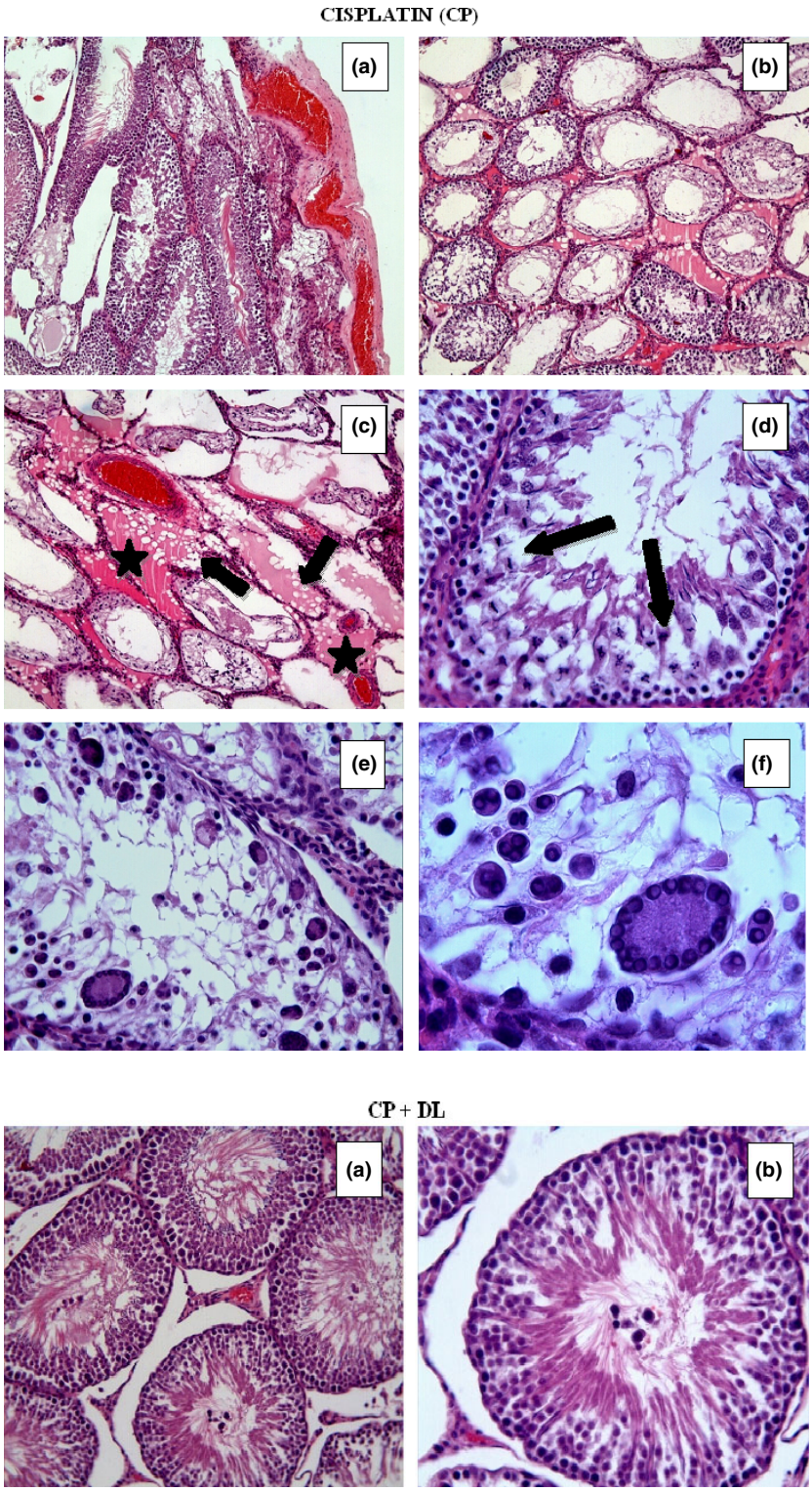

Fig. 3 Cisplatin (CP) group: Distortion of germinal cells (a,b), tubule degeneration (b), oedema (stars) and vacuolisation (arrows) (c), arrested spermatocytes in different stage in division (d), eosinophilic cytoplasm, multinucleated giant cells (e,f) were observed in CP group. a, b: H-E; ×10, c: H-E; ×20, d, e: H-E; ×40, f: H-E; ×100.

Fig. 4 Cisplatin (CP)+*Diospyros lotus* (DL) group: In CP+DL group was observed improved histological appearance in testis tissue. A few cells observed in lumen (a,b). a: H-E; ×20, b: H-E; ×40.

antioxidant status unbalance, leading to lipid peroxidation and GSH depletion (Howell & Shalet, 2005; Kim *et al.*, 2006). As a result of our study, CP caused to decrease levels of GSH, SOD, GPx and CAT. It was observed that CP induced negative effects in antioxidant enzymes. Our study results correlate with previous findings of CP treatment in animals (Cherry *et al.*, 2004; Atessahin *et al.*, 1999).

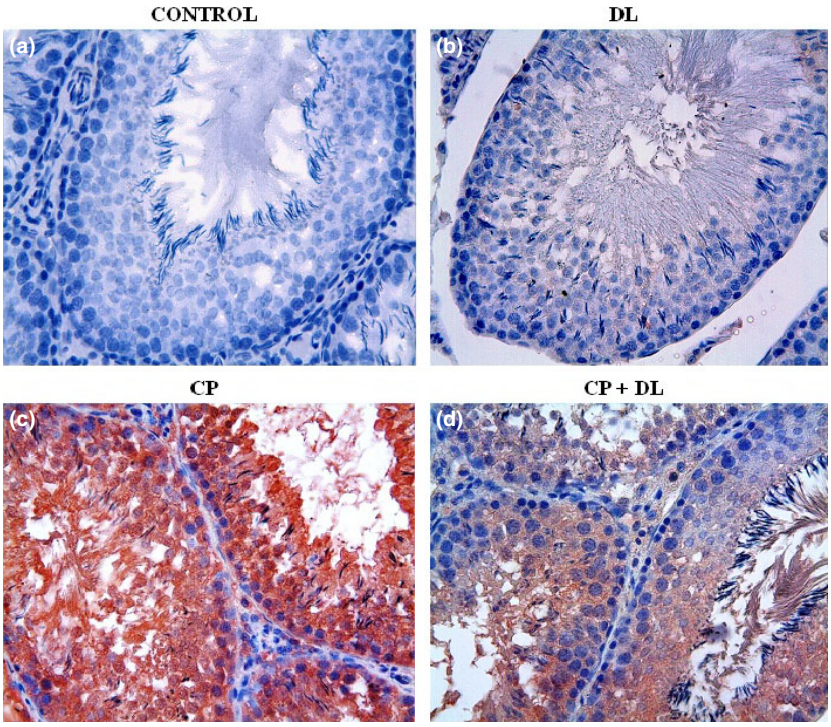

Fig. 5 Immunohistochemical expression of caspase-3 in control (a), *Diospyros lotus* (DL) (b), cisplatin (CP) (c), CP+DL (d) groups. The number of stained (positive) cells decreased in CP+DL group. Caspase-3; ×40.

Table 2 Testis, epididymis, seminal vesicle and prostate weights in CP-, DL- or CP+DL-administered rats. (n = 7)

	Testes weight (g)		Epididymis weight (g)			
Groups	Right	Left	Right	Left	Seminal vesicle weight (g)	Prostate weight (g)
Control	1.239 ± 0.02^{a}	1.221 ± 0.04^{a}	0.434 ± 0.02	0.491 ± 0.02	1.203 ± 0.08	0.511 ± 0.05
CP	0.843 ± 0.05^{b}	0.868 ± 0.06^{b}	0.316 ± 0.02	0.316 ± 0.02	1.210 ± 0.05	0.483 ± 0.04
DL	1.286 ± 0.03^{a}	1.316 ± 0.03^{a}	0.517 ± 0.03	0.493 ± 0.01	1.328 ± 0.04	0.527 ± 0.03
CP+DL	1.084 ± 0.10^{c}	1.091 ± 0.07^{c}	0.390 ± 0.02	0.376 ± 0.02	1.040 ± 0.10	0.548 ± 0.04

Data expressed as mean ± SEM. Different letters a, b and c within same line showed significant ($P < 0.01$) differences between control and treatments groups.CP, cisplatin; DL, *Diospyros lotus*.

Table 3 Epididymal sperm concentration, sperm motility, abnormal sperm rate in CP-, DL- or CP+DL-administered rats. (n = 7)

	Parameters				
Groups	Sperm motility (%)	Epididymal sperm concentration (million g^{-1} tissue)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	92.07 ± 0.95^{a}	299.28 ± 13. 24^{a}	4.57 ± 0.35^{a}	4.71 ± 0.35^{a}	9.28 ± 0.52^{a}
CP	61.92 ± 1.75^{b}	157.28.±2.15^{b}	10.42 ± 0.56^{b}	9.14 ± 0.63^{b}	19.28 ± 0.47^{b}
DL	93.04 ± 0.93^{a}	307.14 ± 11.17^{a}	4.85 ± 0.34^{a}	4.42 ± 0.48^{a}	9.28 ± 0.68^{a}
CP+DL	71.42 ± 1.42^{c}	211.85 ± 4.78^{a}	8.14 ± 0.69^{c}	7.85 ± 0.50^{c}	16.00 ± 0.61^{c}

Data expressed as mean ± SEM. Different letters a, b and c within same line showed significant ($P < 0.01$) differences between control and treatments groups. CP, cisplatin group; DL, *Diospyros lotus* group; CP+DL, Cisplatin+Diospyros lotus group.

The cytotoxic effects of CP on germ cells led the researchers to supplemental process that could protect fertility in men applied traditional anticancer therapies. Plant extracts have been used for the treatment of various diseases in traditional system of medicine throughout the world. Traditional medicine will serve as an important

Table 4 The effect of CP and DL on MSTD and GECT. Mean ± SD

Groups	MSTD	GECT
Control	306.27 ± 1.97[a]	92.99 ± 1.20[a]
CP	220.09 ± 2.59[b]	51.97 ± 0.91[b]
CP+DL	273.74 ± 1.77[c]	73.22 ± 0.91[c]
DL	297.80 ± 2.82[a]	82.60 ± 1.09[a]

The mean differences of the values bearing different superscript letters within the same column are statistically significant. ($P \leq 0.01$).
SD, Standard deviation; CP, cisplatin; DL, *Diospyros lotus;* MSTD, mean seminiferous tubular diameter.

search engine and safe natural products research to rediscover sources of new drugs (Peesa, 2013). Researchers showed that the best dosage of DL fruits extract for improvement of morphologic changes in the organs was found to be 1000 mg kg^{-1} (Azadbakhta *et al.*, 2010). In our study, the same dose prevented lipid peroxidation through reduced TBARS levels in testis tissue. Thus, DL supplementation may play a protective role against CP-induced oxidative stress. Rashed *et al.* (Rashed *et al.*, 2013) demonstrated that DL extract has different bioactive components and good antioxidant effect, and it prevents lipid peroxidation. These results are in agreement with our findings.

Recently, much attention has been focused on the protective effects of antioxidants and naturally occurring substances against CP-induced testicular toxicity, but little is known about herbal plants as protective agents (Amin & Hamza, 2006). Our data indicate that DL has a protective action against CP-induced testicular toxicity as evidenced by the elevated levels of the GSH and SOD. However, GPx and CAT levels did not significantly change in CP+DL group compared with CP group. Treatment of DL clearly restored the testicular damage and caused to increase GSH levels. The depleted level of GSH in CP toxicity may due to scavenging of toxic radicals and inhibition of the synthesis and increased rates of turnover. Depletion of cellular GSH, which may act as a radical scavenger, potentiates the CP-induced cytotoxicity (Yildirim *et al.*, 2013).

Our histopathological data indicate that CP caused significant alterations in testis tissue structure such as germ cell degeneration, epithelial sloughing, vacuoles, thinning of germinal cell layers and tubular shrinkage. In addition, our findings indicate that histopathological damages decreased when DL combined with CP. These results correlate with previous findings of CP treatment in animals (Lirdi *et al.*, 2008; Ilbey *et al.*,2009b). Decrease in elevated oxidative stress in testis tissue with DL caused significant recovery, and DL may contribute to male infertility.

Caspase-3 is believed to be a major member of protease family. The convenient regulation of the caspase cascade plays an important role in sperm differentiation and testicular maturity. However, caspases have been implicated in the pathogenesis of plural andrological pathologies such as impaired spermatogenesis, decreased sperm motility and increased levels of sperm DNA fragmentation, testicular torsion, varicocele and immunological infertility (Said *et al.*, 2004). In our study, caspase-3-positive cells were high in CP group. DL combination with CP treatment caused decrease in caspase-3-stained cells. In particular, our results were able to show that failure of activation of caspase-3 in CP induced testicular damage.

Leydig cells produce the testosterone of primary male steroid hormone, which promotes spermatogenesis and fertility in puberty and throughout adulthood. Decreased serum testosterone levels also occurred in male mice following exposure to CP (Amin & Hamza, 2006; Ilbey *et al.*, 2009a; Silici *et al.*, 2009). In our study, serum testosterone level has significantly decreased with CP treatment in rats. But, DL treatment did not change serum testosterone levels compared with the control group. There was no study about the effects of DL treatment on serum testosterone levels. This effect of CP may occur through interference with LH receptor expression, breaking of the cholesterol mobilisation to mitochondrial cytochrome P450scc, or decreases of the activity of this enzyme, thus interfering with the initial steps in testosterone production (Silici *et al.*, 2009; Beytur *et al.*, 2012).

The studies have been reported that male rats receiving CP exhibit decreases in reproductive organ weights and impaired fertility along with alterations in the growth and development of next generations (Cherry *et al.*, 2004; Ilbey *et al.*, 2009b). In our study, CP treatment caused significant decrease in sperm concentration and sperm motility with significant effect on the reproductive organ weights (testis, seminal vesicles, prostate). CP could significantly decrease organ weights. Additionally, in this study, we showed that DL significantly increased sperm motility with sperm concentration and prevented toxic effect of CP. The increase in sperm motility after DL administration may be explained by the decreased free radical production, increased antioxidant enzymes and decreased lipid peroxidation. DL treatment can be beneficial due to its antioxidant properties and may be positively effect infertility caused by CP.

Diospyros lotus has been considered to be mediated via its beneficial effects on the antioxidant defence system, the scavenging of free radicals and/or via preventing lipid peroxidation. Results from this study indicate that the novel natural antioxidant DL might have protective effect against CP-induced testicular damage and oxidative stress in rat.

References

Aebi H (1974) Catalase. In: Methods of Enzymatic Analysis. Bergmeyer HU (ed). Academic Press, New York, pp. 673–677.

Ahmed EA, Omar HM, elghaffar S, Ragb SM, Nasser AY (2011) The antioxidant activity of Vitamin C, DPPD and L-cysteine against Cisplatin-induced testicular oxidative damage in rats. *Food Chem Toxicol* 49:1115–1121.

Aitken RJ, De Iuliis GN (2007) Value of DNA integrity assays for fertility evaluation. *Soc Reprod Fertil Suppl* 65:81–92.

Amin A, Hamza AA (2006) Effects of ginger and roselle on cisplatin-induced reproductive toxicity in rats. *Asian J Androl* 8:607–612.

Atessahin A, Karahan I, Turk G, Gur S, Yilmaz S, Ceribasi AO (2006) Protective role of lycopene on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats. *Reprod Toxicol* 21:42–47.

Azadbakhta M, Safapour S, Ahmadi A, Ghasemi M, Shokrzadeh M (2010) Anti-diabetic effects of aqueous fruits extract of *Diospyros lotus* L. on streptozotocin-induced diabetic rats and the possible morphologic changes in the liver, kidney and heart. *J Pharmacognosy Phytother* 2:10–16.

Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ Jr, Agarwal A (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* 81:349–354.

Beytur A, Ciftci O, Oguz F, Oguzturk H, Yılmaz F (2012) Montelukast attenuates side efects of cisplatin including testicular, spermatological, and hormonal damage in male rats. *Cancer Chemother Pharmacol* 69:207–213.

Bhat SG, Nie Z, Ramkumar V (1999) Cisplatin up-regulates adenosine A receptors in rat testes. *Eur J Pharmacol* 382:35–43.

Cherry SM, Hunt PA, Hassold TJ (2004) Cisplatin disrupts mammalian spermatogenesis But does not affect recombination or chromosome segregation. *Mutat Res* 564:115–128.

Ciftci O, Aydin M, Ozdemir I, Vardi N (2012a) Quercetin prevents 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced testicular damage in rats. *Andrologia* 44:164–173.

Ciftci O, Ozdemir Ã, Aydin M, Beytur A (2012b) Beneficial effects of chrysin on the reproductive system of adult male rats. *Andrologia* 44:181–186.

Ciftci O, Cetin A, Aydin M, Kaya K, Oguz F (2014) Fish oil, contained in eicosapentaenoic acid and docosahexaenoic acid, attenuates testicular and spermatological damage induced by cisplatin in rats. *Andrologia* 46:1161–1168.

Esmaeili MA, Kanani MR, Sonboli A (2010) Salvia reuterana extract prevents formation of advanced glycation end products: an *in vitro* study. *IJPS* 6:33–50.

Howell SJ, Shalet SM (2005) Spermatogenesis after cancer treatment: damage and recovery. *J Natl Cancer Inst Monogr* 34:12–17.

Ilbey YO, Ozbek E, Cekmen M, Simsek A, Otunctemur A, Somay A (2009a) Protective effect of curcumin in cisplatin-induced oxidative injury in rat testis: mitogen-activated protein kinase and nuclear factor-kappa B signaling pathways. *Hum Reprod* 24:1717–1725.

Ilbey YO, Ozbek E, Cekmen M, Simsek A, Cekmen M, Otunctemur A, Somay A (2009b) Chemoprotective effect of a nuclear factor-kB inhibitor, pyrrolidine dithiocarbamate, against cisplatin-induced testicular damage in rats. *J Androl* 30:505–514.

Kamisli S, Ciftci O, Kaya K, Cetin A, Kamisli O, Ozcan C (2013) Hesperidin protects brain and sciatic nerve tissues against cisplatin-induced oxidative, histological and electromyographical side effects in rats. *Toxicol Ind Health* 3:1–11.

Kim YH, Kim YW, Oh YJ, Back NI, Chung SA, Chung HG, Jeong TS, Choi MS, Lee KT (2006) Protective effect of the ethanolic extract of the roots Brassica rapa on cisplatin-induced nephrotoxicity in LLC-PKl cells and rats. *Biol Pharm Bull* 29:2436–2441.

Köberle B, Tomicic MT, Usanova S, Kaina B (2010) Cisplatin resistance: preclinical findings and clinical implications. *Biochim Biophys Acta* 1806:172–182.

Lavrik IN, Golks A, Krammer PH (2005) Caspases: pharmacological manipulation of cell death. *J Clin Invest* 115:2665–2672.

Lirdi LC, Stumpp T, Sasso-Cerri E, Miraglia SM (2008) Amifostine protective effect on cisplatin-treated rat testis. *Anat Rec (Hoboken)* 291:797–808.

Loizzo MR, Said A, Tundis R, Hawas UW, Rashed K, Menichini F, Frega NG, Menichini F (2009) Antioxidant and antiproliferative activity of *Diospyros lotus* extract and isolated compounds. *Plant Foods Hum Nutr* 64:264–270.

Lowry OH, Rosebrough NJ, Farr AL, Randall RI (1951) Protein measurement with folin phenol reagent. *J Biol Chem* 193:265–275.

Moghaddam AH, Nabavi SM, Nabavi SF, Bigdellou R, Mohammadzadeh S, Ebrahimzadeh MA (2012) Antioxidant, antihemolytic and nephroprotective activity of aqueous extract of *Diospyros lotus* seeds. *Acta Pol Pharm* 69:687–692.

Moretti E, Mazzi L, Terzuoli G, Bonechi C, Iacoponi F, Martini S, Rossi C, Collodel G (2012) Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. *Reprod Toxicol* 34:651–657.

Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 70:158–169.

Peesa JP (2013) Nephroprotective potential of herbal medicines: a review. *Asian J Pharm Tech* 3:115–118.

Rashed KN, Chang CW, Wu LY, Peng WH (2013) Hepatoprotective activity of *Diospyros lotus* fruits on acute liver injury induced by carbon tetrachloride and phytochemical analysis. *Topcls J Herb Med* 2:75–83.

Said TM, Paasch U, Glander HJ, Agarwal A (2004) Role of caspases in male infertility. *Hum Reprod Update* 10:39–51.

Sajjad H (2012) Toxicity effect of cisplatin–treatment on rat testis tissue. *Ann Biol Res* 3:2297–2303.

Salem EA, Salem NA, Maarouf AE, Serefoglu EC, Hellstrom WJG (2012) Selenium and lycopene attenuate cisplatin-induced testicular toxicity associated with oxidative stress in Wistar rats. *Urology* 79:1184–e1–6.

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25:192–205.

Silici S, Ekmekcioglu O, Eraslan G, Demirtas A (2009) Antioxidative effect of royal jelly in cisplatin-induced testes damage. *Urology* 74:545–551.

Sun Y, Oberley LW, Li YA (1988) Simple method for clinical assay of superoxide dismutase. *Clin Chem* 34:497–500.

Uddin G, Rauf A, Siddiqui BS, Shah SQ (2011) Preliminary comparative phyto-chemical screening of *Diospyros lotus* Stewart. *Middle-East J Sci Res* 10:78–81.

Yagi K (1998) Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol Biol* 108:101–106.

Yildirim NC, Kandemir FM, Ceribasi S, Ozkaraca M, Benzer F (2013) Pomegranate seed extract attenuates chemotherapy-induced liver damage in an experimental model of rabbits. *Cell Mol Biol (Noisy-le-grand)* 59(Suppl):OL1842–1847.