First International Journal of Andrology
ANDROLOGIA

ORIGINAL ARTICLE

Effectiveness of cinnamon (*Cinnamomum zeylanicum*) bark oil in the prevention of carbon tetrachloride-induced damages on the male reproductive system

A. Yüce[1], G. Türk[2], S. Çeribaşı[3], M. Güvenç[1], M. Çiftçi[4], M. Sönmez[2], Ş. Özer Kaya[2], M. Çay[1] & M. Aksakal[1]

1 Faculty of Veterinary Medicine, Department of Physiology, Firat University, Elazığ, Turkey;
2 Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Firat University, Elazığ, Turkey;
3 Faculty of Veterinary Medicine, Department of Pathology, Firat University, Elazığ, Turkey;
4 Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Firat University, Elazığ, Turkey

Keywords
Apoptosis—carbon tetrachloride—cinnamon bark oil—lipid peroxidation—sperm—testis

Correspondence
Assoc. Prof. Dr Gaffari Türk, PhD,
Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination,
Fırat University, 23119, Elazığ, Turkey.
Tel: +90 424 237 00 00/3892;
Fax: +90 424 238 81 73;
E-mails: gturk@firat.edu.tr;
gaffariturk@hotmail.com

Accepted: December 23, 2012

doi: 10.1111/and.12072

Summary

In this study, it was aimed to investigate the likelihood of detrimental effects of carbon tetrachloride (CCl_4) on male reproductive system through oxidative stress mechanism and also protective effects of cinnamon bark oil (CBO). For this purpose, 28 healthy male Wistar rats were divided into four groups, seven rats in each. Group 1 received only olive oil daily; group 2 was treated with 100 mg kg^{-1} CBO daily; group 3 was treated with only 0.25 ml kg^{-1} CCl_4 weekly; and group 4 received weekly CCl_4 + daily CBO. All administrations were made by intragastric catheter and maintained for 10 weeks. Body and reproductive organ weights, sperm characteristics, testicular oxidative stress markers and testicular apoptosis were examined. CCl_4 administration caused significant decreases in body and reproductive organ weights, testicular catalase (CAT) activity, sperm motility and concentration, and significant increases in lipid peroxidation (LPO) level, abnormal sperm rate and apoptotic index along with some histopathological damages compared with the control group. However, significant improvements were observed in absolute weights of testis and epididymis, all sperm quality parameters, LPO level, apoptotic index and testicular histopathological structure following the administration of CCl_4 together with CBO when compared to group given CCl_4 only. The findings of this study clearly suggest that CBO has protective effect against damages in male reproductive organs and cells induced by CCl_4.

Introduction

Carbon tetrachloride (CCl_4) is a colourless toxic substance and has been used as a dry-cleaning agent, fabric spotting fluid and solvent, reagent in chemical synthesis, fire extinguisher fluid and grain fumigant. It is released into the environment predominantly through direct emissions to air, with lower amounts discharged to soil and water. CCl_4 is rapidly absorbed by any route of exposure in humans and animals. Once absorbed, it is widely distributed among tissues, especially those with high lipid content, reaching peak concentrations in <1–6 h, depending on exposure concentration or dose (U.S. EPA. IRIS, 2010). Therefore, nontarget humans and animals are extensively exposed to CCl_4 due to its common use and releasing to the environment.

Carbon tetrachloride is also known as a potent hepatotoxic and cirrhotic agent because it is mainly metabolised by the liver. Therefore, the mechanism of CCl_4-induced liver injury is well studied in the rat model (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008; Xu *et al.*, 2010; Karakus *et al.*, 2011; El Denshary *et al.*, 2012). Possible mechanism for the CCl_4-induced hepatotoxicity is that cytochrome P450 (CYP) activates CCl_4 into its active metabolite, trichloromethyl radical. The metabolic bioactivation of CCl_4 thereby leads to overproduction of

reactive free radicals, which cause increases in lipid peroxidation (LPO) level and protein oxidation in the liver (Sheweita *et al.*, 2001).

The plasma membrane of spermatozoa contains a high amount of polyunsaturated fatty acids (PUFAs). Therefore, it is particularly susceptible to peroxidative damage. The LPO destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and the defects of membrane integrity (Turner & Lysiak, 2008). On the other hand, CYP genes in the male reproductive system such as CYP2E1 in the rat prostate and testis (Jiang *et al.*, 1998), CYP17, which is involved in steroidogenesis (Nebert & Russell, 2002), in testicular germ cells of mice (Liu *et al.*, 2007) and CYP1A2 and CYP1B1 in hamster epididymal tissue (Hudson *et al.*, 2001) reported to be identified. Based on the existence of some CYP genes in the male reproductive organs, CCl_4 causes usually oxidative damage to the lipids and proteins of the reproductive tissues (Abraham *et al.*, 1999). It has been reported that acute or chronic CCl_4 administration to adult male rats causes increments in testicular tissue LPO level (Abraham *et al.*, 1999; Fadhel & Amran, 2002; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012), sperm abnormalities and testicular tissue DNA fragmentation (Abdou *et al.*, 2012; Khan, 2012); reductions in weights of body and testes (Castilla-Cortazar *et al.*, 2004; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009), sperm count and motility (Khan, 2012) and antioxidant enzymes (Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012); degeneration in testicular histologic structure (Kalla & Bansal, 1975; Castilla-Cortazar *et al.*, 2004; Horn *et al.*, 2006; Khan & Ahmed, 2009); and disturbances in steroid and gonadotropin hormones (Castilla-Cortazar *et al.*, 2004; Khan & Ahmed, 2009; Khan, 2012).

Antioxidants are compounds that scavenge and suppress the formation of ROS and LPO. Hence, the application of ROS scavengers is likely to improve the stress-induced damages in testis and sperm function (Vernet *et al.*, 2004). For this purpose, some herbal antioxidants were used to prevent testicular oxidative stress (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012), hormonal disturbances (Khan & Ahmed, 2009; Khan, 2012), sperm abnormalities (Abdou *et al.*, 2012), reduced sperm count, motility and testicular tissue DNA fragmentation (Khan, 2012) and some testicular histopathological lesions (Manjrekar *et al.*, 2008; Khan & Ahmed, 2009) exerted by CCl_4 in rats. Cinnamon has also been used as a spice and as traditional herbal medicine for centuries. The most important volatile oils derived from cinnamon are *C. zeylanicum* bark and leaf oils, *C. cassia* (cassia oil) and *C. camphora* (Jayaprakasha & Rao, 2011). Different Cinnamomum extracts have been reported to have free radical scavenger and potent antioxidant activity (Jayaprakasha *et al.*, 2006; Prasad *et al.*, 2009; Ciftci *et al.*, 2010; Azab *et al.*, 2011; Yüce *et al.*, 2013). In addition, *C. zeylanicum* consumption provides marked improvements in sperm quality and reproductive organ weights of healthy (Yüce *et al.*, 2013) and diabetic (Hafez, 2010; Shalaby & Mouneir, 2010) rats. However, there is no evidence on the effect of cinnamon bark oil (CBO) on CCl_4-induced reproductive dysfunction in male rats. Therefore, this study was conducted to investigate whether CBO has any preventive effect on CCl_4-induced adverse changes in sperm quality, testicular apoptosis and histopathological lesions associated with the oxidative stress.

Materials and methods

Cinnamon bark oil and chemicals

Cinnamon bark oil was purchased from a local store (Altınterim Co., Elazığ, Turkey). According to the manufacturer's procedure; *C. zeylanicum* barks were transported in polypropylene bags and were dried to constant weight in room temperature. CBO was obtained by hydrodistillation method. The plant materials (about 100 g) were then ground into small pieces and were placed in a flask (2 l) together with double-distilled water (1.5 l). The mixture was boiled for 4 h. The extract was condensed in cooling vapour to collect the essential oil. The extracted oil was dried over anhydrous sodium sulphate. CBO was kept at 4 °C until being used. The other chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

Animals and treatment protocol

The experimental protocols were approved by the local Committees for using Animals of Firat University (Elazig, Turkey). Animal care and experimental protocols complied with the NIH Guide for the Care and Use of Laboratory Animals. Twenty-eight healthy adult male Wistar albino rats, aged 5 months, were obtained from Firat University Experimental Research Centre (Elazig, Turkey) and maintained therein during the study. The animals were housed in polycarbonate cages in a room with a 12-h day–night cycle, at a temperature of 24 ± 3 °C and humidity of 45% to 65%. During the whole experimental period, animals were fed with a balanced commercial diet (Elazig Food Company, Elazig, Turkey), and fresh distilled drinking water was given *ad libitum*.

The rats were randomly divided into four groups; each containing seven rats. One milliliter pure olive oil was daily administered by gavage to rats in the first group,

and they served as *control*. One milliliter olive oil containing 100 mg kg^{-1} CBO was daily given by gavage to rats in the second group (Group CBO). Rats in third group were weekly treated with 1 ml olive oil containing 0.25 ml kg^{-1} CCl_4 (Group CCl_4). Animals in fourth group received weekly CCl_4 and daily CBO (Group CCl_4 + CBO). All administrations were maintained for 10 weeks. Olive oil was used as vehicle because CCl_4 is an oil-dissolved chemical. The doses of CCl_4 (Horn *et al.*, 2006) and CBO (Shah *et al.*, 1998; Yüce *et al.*, 2013) given to rats in this study, generally used for long-term studies, were selected based on the previous reports. Because the spermatogenic cycle, including spermatocytogenesis, meiosis and spermiogenesis, is 48–52 days (Bennett & Vickery, 1970) and epididymal transit of spermatozoa is approximately 1 week (Kempinas *et al.*, 1998) in rats, the treatment period used herein was set at 10 weeks to achieve a maximum effect. Each rat was weighed weekly, and the dose levels of CCl_4 and CBO in oil suspension were adjusted for changes in body weights during the experimental period.

Sample collection and homogenate preparation

The rats were sacrificed using ether anaesthesia at the end of 10th week. Testes, epididymides, seminal vesicles and ventral prostate were removed, cleared from adhering connective tissue and weighed. One of the testis samples was fixed in Bouin's solution for histopathological examination. The other testis samples were stored at −20 °C for biochemical analyses. Testes were taken from a −20 °C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a ninefold volume of PBS (pH 7.4). For the enzymatic analyses, testes were minced in a glass and homogenised by a Teflon-glass homogenisator for 3 min in cold physiological saline on ice (Türk *et al.*, 2011).

Determination of testicular oxidative stress markers

All analyses were performed with the aid of a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). LPO level was measured according to the concentration of thiobarbituric acid reactive substances, and the amount of malondialdehyde (MDA) produced was used as an index of LPO. MDA level at 532 nm was expressed as nmol g $protein^{-1}$ (Placer *et al.*, 1966).

Reduced glutathione (rGSH) level was measured using the method described by Sedlak & Lindsay (1968). The level of rGSH at 412 nm was expressed as nmol g $protein^{-1}$. Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence & Burk (1976). The GSH-Px activity at 340 nm was expressed as IU g $protein^{-1}$. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as k g $protein^{-1}$, where k is the first-order rate constant (Aebi, 1983). Protein concentration was determined using the method of Lowry *et al.* (1951).

Sperm analyses

All sperm analyses were made by using the methods reported in the study of Türk *et al.* (2008). The sperm concentration in the right cauda epididymal tissue was determined with a haemocytometer. Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. The percentage of sperm motility was evaluated using a light microscope with a heated stage. To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M of sodium citrate) were prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide (2100 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage.

Histopathological examination

Testis tissues were fixed in Bouin's solution for 48 h, and they were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5 μm thicknesses and stained with Mayer's haematoxylin and eosin. Twenty-five seminiferous tubules (ST) were randomly examined per section, their diameters and germinal cell layer thickness (GCLT; from the basal membrane towards the lumen of the tubule) were measured using an ocular micrometre in a light microscope, and the mean size of ST and GCLT were calculated. Johnsen's testicular scoring (Johnsen, 1970) was performed for control and treated-groups. Twenty-five ST from each section were evaluated, and a score between 1 (very poor) and 10 (excellent) was given to each tubule according to Johnsen's criteria. The degree of damages was graded as follows: mild (+), moderate (++) and severe (+++).

Determination of testicular apoptotic cell index

The apoptotic germ cells were defined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay with the ApopTag Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Briefly, the fixed testicular tissues in Bouin's solution were embedded in paraffin and sectioned at 4 μm

thickness. The paraffin sections were deparaffinised in xylene, dehydrated through graded alcohol and washed in PBS. The sections were treated with 20 mg ml^{-1} proteinase K for 5 min, followed by treatment with 3% H_2O_2 for 5 min to inhibit endogenous peroxidase. After rewashing with PBS, sections were then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-11-dUTP at 37 °C for 1 h in humidified chamber, and then, stop–wash buffer was applied for 30 min at 37 °C. Sections were visualised with 3-amino-9-ethylcarbazole (AEC) substrate. Negative controls were performed using distilled water in the place of TdT enzyme. Finally, sections were counterstained with Mayer's haematoxylin, rinsed in tap water and mounted with glycerol. TUNEL-positive apoptotic index was calculated as follow:

$$\text{TUNEL-positive apoptotic index}(\%) = \frac{\text{Total apoptotic cell count in 25 ST}}{\text{Total germinal cell count in 25 ST}} \times 100$$

Statistical analysis

Data are presented as mean ± SEM. The degree of significance was set at $P < 0.05$. It was determined that raw data showed normal distribution according to Shapiro–Wilk normality test. Based on the normality test, one-way analyses of variance and *post hoc* Tukey's HSD test were used to determine the differences between the groups with respect to all parameters studied. All the analyses were carried out using the SPSS/PC software programme (Version 15.0; SPSS, Chicago, IL, USA).

Results

Changes in body and reproductive organ weights

Carbon tetrachloride administration caused a significant ($P < 0.05$) decrease in the final body weight when compared to control group. This decreased value in CCl_4 group was brought to a value near to control group, although not significant, by CBO administration to CCl_4-treated rats (Fig. 1). Absolute and relative reproductive organ weights are presented in Figs 2 and 3 respectively. Only CBO administration significantly increased the absolute ($P < 0.001$) and relative ($P < 0.01$) weights of the right cauda epididymis and also the relative testis weight ($P < 0.05$) in comparison with the control group. Significant reductions ($P < 0.001$) were observed in all absolute reproductive organ weights and relative weights of epididymis, seminal vesicles and prostate following CCl_4 administration only. However, significant improvements ($P < 0.001$) were determined in absolute weights of testis and epididymis in CCl_4 + CBO group when compared to CCl_4 group only.

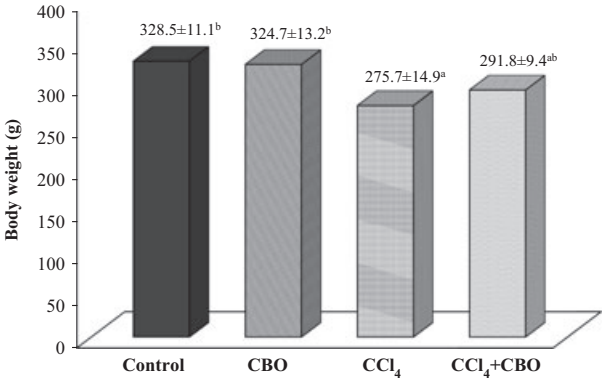

Fig. 1 Mean ± SEM values of body weight in different treatment groups (CBO, cinnamon bark oil; CCl_4, carbon tetrachloride). Mean values having different superscripts (a and b; $P < 0.05$) in each group significantly differ from each other.

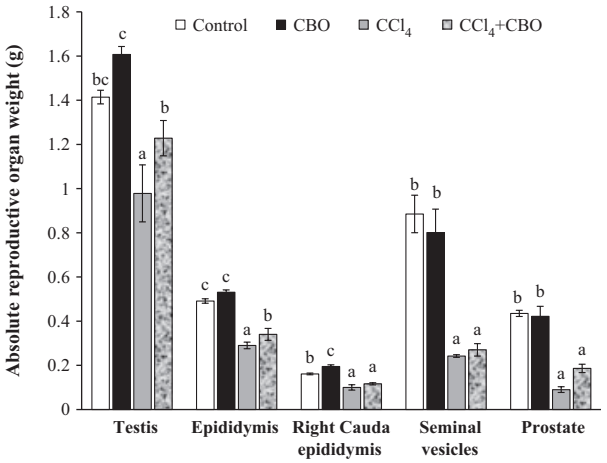

Fig. 2 Mean ± SEM values of absolute reproductive organ weight in different treatment groups (CBO, cinnamon bark oil; CCl_4, carbon tetrachloride). The mean values having different superscripts (a–c; $P < 0.001$) in each group significantly differ from each other.

Changes in testicular oxidative stress markers

The LPO level (MDA) and antioxidant enzyme activities of all the groups are given in Table 1. Although CBO administration alone provided improvements in all oxidative stress markers, the increase in rGSH level reached the statistically significant level ($P < 0.01$) when compared to the control group. Only CCl_4 administration resulted in significantly increased MDA level ($P < 0.001$) and significantly decreased CAT activity ($P < 0.01$) when compared to the control group. However, CBO administration to

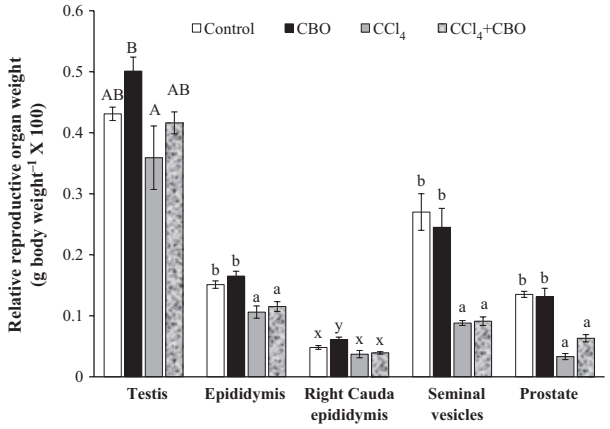

Fig. 3 Mean ± SEM values of relative reproductive organ weight in different treatment groups (CBO, cinnamon bark oil; CCl_4, carbon tetrachloride). Mean values having different superscripts (A and B; $P < 0.05$, x and y; $P < 0.01$, a and b; $P < 0.001$) in each group significantly differ from each other.

CCl_4-treated rats led to a significant decrease ($P < 0.001$) in MDA level, but not in CAT activity, in comparison with the CCl_4 group only.

Changes in sperm parameters

The effects of CBO on epididymal sperm concentration, sperm motility and abnormal sperm rate are presented in Table 2. Only CBO administration significantly increased the sperm concentration ($P < 0.001$) in comparison with the control group. Significant decreases ($P < 0.001$) in sperm motility and concentration as well as significant increases ($P < 0.001$) in head, tail and total abnormal sperm rates were observed in CCl_4 group when compared to control group. However, significant improvements ($P < 0.001$) were determined in all sperm parameters of CCl_4 + CBO group as compared to the CCl_4 group only.

Changes in testicular histologic structure and apoptotic cell index

No histopathological lesions (Table 3) were observed in testicular tissues of control (Fig. 4a) and CBO (Fig. 4b) groups. The histopathological changes such as necrosis, degeneration, desquamation, disorganisation and reduction in germinal cells, atrophy in tubules, thickening in basal membrane, interstitial oedema and congestion, multinuclear syncytial cell formation and spermatogenic arrest were observed only in CCl_4 (Fig. 4c) and CCl_4 + CBO (Fig. 4d) groups. Almost all ST in testes of CCl_4 group contained a great number of spermatogonia, but with a very few number of spermatocytes and spermatids when compared to control group. However, an increase in the numbers of spermatocytes and spermatids in addition to spermatogonia was observed in ST of CCl_4 + CBO group in comparison with the CCl_4 group. In addition, the degree of lesions was significantly ($P < 0.001$) worse only in CCl_4 group than in CCl_4 + CBO group (Table 3). Significant ($P < 0.001$) decreases in diameters of ST, GCLT and Johnsen's testicular score were determined only in CCl_4 group as compared to the control group. However, CBO administration to CCl_4-treated animals significantly ($P < 0.001$) improved the CCl_4-induced damages in these parameters (Table 4).

Figure 5 illustrates apoptosis, demonstrated by TUNEL staining, in the testis of control and treated groups. The apoptotic cell index of CCl_4 group was significantly ($P < 0.001$) higher than that of control group. However, a significant ($P < 0.001$) decrease was observed in apoptotic cell index of CCl_4 + CBO group as compared to the CCl_4 group only (Table 4).

Discussion

In this study, we demonstrated for the first time that long-term CBO consumption provided a marked

Table 1 Mean ± SEM values of malondialdehyde (MDA), reduced glutathione (rGSH) levels and glutathione-peroxidase (GSH-Px) and catalase (CAT) activities

Oxidative stress markers				
Groups	MDA (nmol g protein^{-1})	rGSH (nmol g protein^{-1})	GSH-Px (IU g protein^{-1})	CAT (kg protein^{-1})
Control	7.32 ± 0.47^{a}	6.12 ± 0.67^{A}	1.75 ± 0.52	46.37 ± 14.13^{B}
CBO	5.45 ± 0.23^{a}	8.33 ± 0.19^{B}	2.05 ± 0.50	96.59 ± 23.78^{B}
CCl_4	15.94 ± 1.25^{b}	7.22 ± 0.24^{AB}	0.85 ± 0.25	17.06 ± 1.36^{A}
CCl_4 + CBO	6.49 ± 1.09^{a}	6.06 ± 0.27^{A}	1.23 ± 0.57	19.78 ± 8.93^{A}

CBO, cinnamon bark oil; CCl_4, carbon tetrachloride.
The mean values having different superscripts (a and b: $P < 0.001$; A and B: $P < 0.01$) within the same column significantly differ from each other.

Table 2 Mean ± SEM values of sperm parameters

	Sperm parameters				
			Abnormal sperm rate (%)		
Groups	Sperm motility (%)	Epididymal sperm concentration (million/right cauda epididymis)	Head	Tail	Total
Control	77.20 ± 3.03[c]	87.66 ± 3.68[c]	3.66 ± 0.76[a]	6.50 ± 1.11[a]	10.16 ± 1.74[a]
CBO	89.70 ± 0.55[c]	118.00 ± 6.90[d]	3.71 ± 0.80[a]	4.71 ± 0.74[a]	8.42 ± 1.21[a]
CCl_4	26.65 ± 6.23[a]	23.25 ± 11.30[a]	18.00 ± 1.08[c]	20.25 ± 3.44[b]	38.25 ± 9.03[c]
CCl_4 + CBO	58.00 ± 8.00[b]	53.80 ± 13.43[b]	9.40 ± 0.81[b]	9.20 ± 1.11[a]	18.60 ± 1.24[b]

CBO, cinnamon bark oil; CCl_4, carbon tetrachloride.
The mean values having different superscripts within the same column significantly differ from each other (a–d: $P < 0.001$).

Table 3 The degree of some pathological lesions in testicular tissues of different treatment groups

Groups				
Lesions	Control	CBO	CCl_4	CCl_4 + CBO
Necrosis in germinal cells	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.86 ± 0.14[c]	2.29 ± 0.18[b]
Atrophy in seminiferous tubules	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.57 ± 0.20[c]	2.00 ± 0.00[b]
Thickening in tubule basal membrane	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.71 ± 0.18[c]	1.86 ± 0.26[b]
Degeneration in germinal cells	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.57 ± 0.20[c]	1.43 ± 0.20[b]
Desquamation in germinal cells	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.86 ± 0.14[c]	2.14 ± 0.26[b]
Reduction in germinal cell counts	0.00 ± 0.00[a]	0.00 ± 0.00[a]	3.00 ± 0.00[c]	2.43 ± 0.20[b]
Disorganisation in germinal cells	0.00 ± 0.00[a]	0.00 ± 0.00[a]	3.00 ± 0.00[c]	2.57 ± 0.20[b]
Vacuolisation in germinal cells	0.00 ± 0.00[a]	0.00 ± 0.00[a]	1.29 ± 0.29[b]	0.00 ± 0.00[a]
Interstitial oedema and congestion	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.43 ± 0.20[c]	1.43 ± 0.20[b]
Multinucleated syncytial cell formation	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.86 ± 0.14[c]	1.86 ± 0.14[b]
Spermatogenic arrest	0.00 ± 0.00[a]	0.00 ± 0.00[a]	2.71 ± 0.18[c]	1.71 ± 0.42[b]

CBO, cinnamon bark oil; CCl_4, carbon tetrachloride.
The mean values having different superscripts within the same row significantly differ from each other (a–c: $P < 0.001$).

protection on CCl_4-induced reproductive dysfunction in male rats.

It is known that monitoring body weight provides information on the general health level, which can be important for interpretation of reproductive effects. Androgens stimulate the growth by inducing the protein synthesis (Fernandes *et al.*, 2007). In addition, it is well known that testis, epididymis and accessory sex organs need a permanent androgenic stimulation for their normal growth and functions (Klinefelter & Hess, 1998). Reduced body and testis weights (Castilla-Cortazar *et al.*, 2004; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009) and decreased testosterone level (Khan & Ahmed, 2009; Khan, 2012) have been reported by some studies. In the present study, CCl_4 caused significant decreases in body weight and also weights of testes, epididymides and accessory sex glands. These decreases in the weights of body and reproductive organs observed herein may possibly be explained by CCl_4-induced decreased testosterone concentration (Khan & Ahmed, 2009; Khan, 2012).

Many compounds, metabolised by cells, cause an increase in the levels of electrophilic radicals that can react with oxygen giving rise to reactive oxygen species (ROS), one of the main sources of free radicals like H_2O_2, singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$) or peroxynitrite. When ROS begin to accumulate, cells exhibit a defensive mechanism using various antioxidant enzymes. The main detoxifying systems for peroxides are CAT and GSH. Of them, CAT is an antioxidant enzyme, which destroys H_2O_2 that can form a highly reactive $^{\bullet}OH$ in presence of iron as a catalyst. By participating in the glutathione redox cycle, GSH together with GSH-Px convert H_2O_2 and lipid peroxides to nontoxic products (Turner & Lysiak, 2008). CCl_4 administration significantly increased testicular MDA level and significantly decreased testicular CAT activity in this study. It has been reported that CCl_4 causes an increase in testicular tissue LPO level (Abraham *et al.*, 1999; Fadhel & Amran, 2002; Castilla-Cortazar *et al.*, 2004; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012) and a decrease in testicular antioxidant enzyme activities (Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012). The CCl_4-induced oxidative damage in testes may be depend on the increased free radicals mediated by

Table 4 Mean ± SEM values of diameters of seminiferous tubules (ST), germinal cell layer thickness (GCLT), Johnsen testicular score and TUNEL-positive apoptotic cell index

Variables				
Groups	Diameter of ST (μm)	GCLT (μm)	Johnsen testicular score (1–10)	TUNEL-positive apoptotic cell index (%)
Control	255.85 ± 1.84^c	105.23 ± 1.05^c	10.00 ± 0.00^c	0.73 ± 0.28^a
CBO	264.55 ± 2.01^d	102.37 ± 0.94^c	10.00 ± 0.00^c	0.75 ± 0.29^a
CCl_4	191.51 ± 2.73^a	48.55 ± 1.14^a	4.00 ± 0.37^a	4.14 ± 0.19^c
CCl_4 + CBO	225.58 ± 1.79^b	61.83 ± 0.93^b	7.50 ± 3.42^b	2.35 ± 0.13^b

CBO, cinnamon bark oil; CCl_4, carbon tetrachloride.
The mean values having different superscripts within the same column significantly differ from each other (a–d: $P < 0.001$).

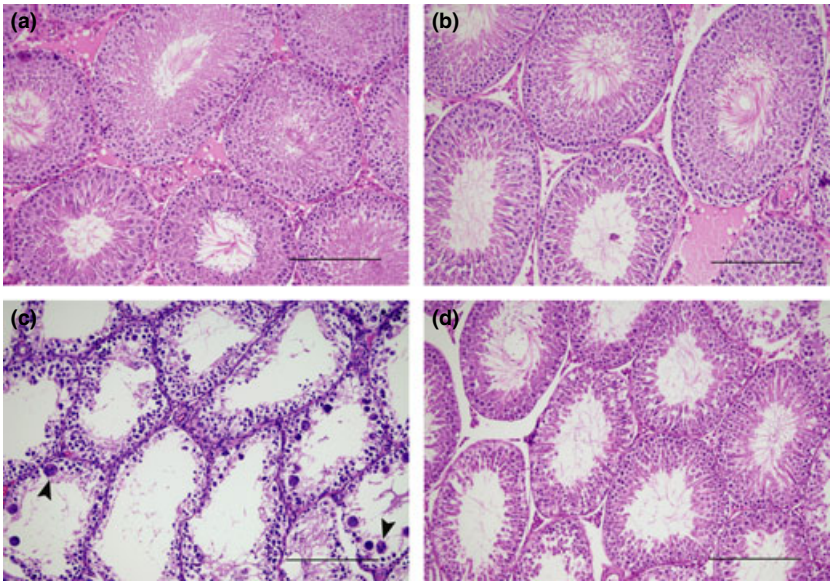

Fig. 4 Representative photomicrographs of histopathological structure of testis in different treatment groups (CBO, cinnamon bark oil; CCl_4, carbon tetrachloride; calibration bar = 200 μm). (a) Haematoxylin and eosin staining in control group. (b) Haematoxylin and eosin staining in CBO-treated group. (c) Haematoxylin and eosin staining in CCl_4-treated group (arrows show multinuclear syncytial cells). (d) Haematoxylin and eosin staining in CCl_4 + CBO-treated group.

CYP activity (Abraham *et al.*, 1999; Sheweita *et al.*, 2001), which was also identified in testes (Jiang *et al.*, 1998) and responsible for metabolic bioactivation of CCl_4, in the present study.

Reactive oxygen species (ROS) can attack the unsaturated bonds of the membrane lipids in an autocatalytic process, with the genesis of peroxides, alcohol and lipidic aldehydes as by-product of the reaction. Thus, the increase in free radicals in cells can induce the LPO by oxidative breakdown of PUFAs in the membranes of cells (Henkel, 2005; Turner & Lysiak, 2008). Spermatozoa are especially susceptible to peroxidative damage because of high concentration of PUFAs and low antioxidant capacity. Obviously, peroxidation of sperm lipids destroys the structure of lipid matrix in the membranes of spermatozoa; it is associated with rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases (Sikka, 1996). In the present study, significant decreases in sperm motility and concentration and significant increases in head, tail and total abnormal sperm rates were observed in CCl_4 group when compared to control group. These findings are in agreement with the earlier reports that reduced sperm count, motility (Khan, 2012) and also increased sperm shape abnormalities (Abdou *et al.*, 2012; Khan, 2012) have been reported in CCl_4-treated rats. Increased lipid peroxidation and decreased antioxidant enzyme activity may be responsible for impaired sperm quality observed in this study.

It has been reported that long-term CCl_4 administration (from 20 days to 16 weeks) leads to severe damage

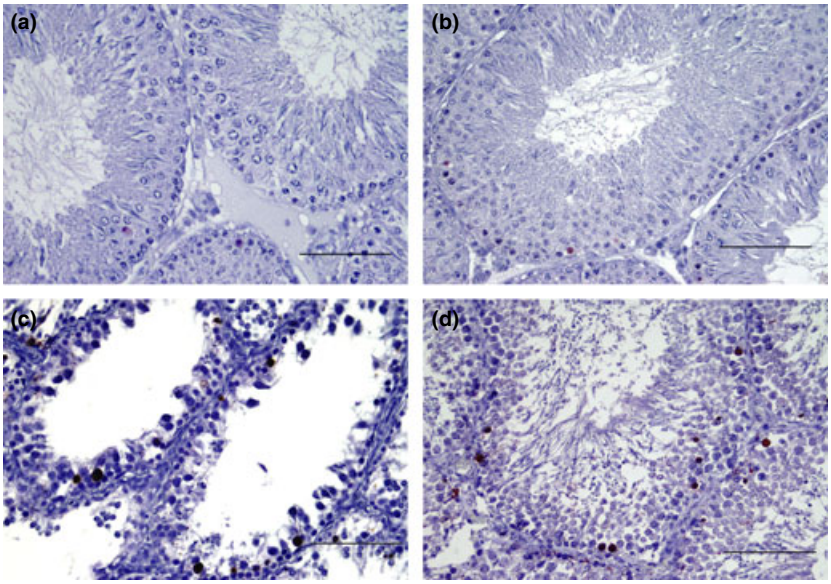

Fig. 5 Representative photomicrographs of apoptotic cells by TUNEL method in the testis of different treatment groups (CBO, cinnamon bark oil; CCl_4, carbon tetrachloride, calibration bar = 500 μm). (a) TUNEL staining in control group. (b) TUNEL staining in CBO-treated group. (c) TUNEL staining in CCl_4-treated group (marked reduction in germinal cells and brown-red-stained cells is the apoptotic ones. Marked increase is seen in the apoptotic index that calculated by dividing total apoptotic cell number into total germinal cell number in 25 seminiferous tubules). (d) TUNEL staining in CCl_4 + CBO-treated group (marked increase in germinal cells and brown-red-stained cells is the apoptotic ones. Marked decrease is seen in the apoptotic index that calculated by dividing total apoptotic cell number into total germinal cell number in 25 seminiferous tubules).

to the spermatogenic cycle such as exfoliation of the germinal epithelium, depletion and degeneration of germ cells, shrinkage of the tubules, vacuolisation of germinal epithelium and meiotic arrest (Kalla & Bansal, 1975; Horn *et al.*, 2006; Khan & Ahmed, 2009; Khan, 2012). However, short-term administration (10–15 days) of CCl_4 has no marked adverse effect on testicular structure (Kalla & Bansal, 1975; Castilla-Cortazar *et al.*, 2004), but it alters hematotesticular barrier (Castilla-Cortazar *et al.*, 2004). Similarly, prominent histopathological damages such as necrosis, degeneration, desquamation, disorganisation, reduction in germinal cells, spermatogenic arrest and marked decreases in diameters of ST, GCLT and Johnsen's testicular score were determined in CCl_4 group only as compared to the control group herein. Besides, the apoptotic cell index of CCl_4 group was found to be markedly higher than that of control group. Apoptosis is an indicator of DNA damage in the cells including testicular germ cells, and an increase in free radicals results in increased testicular apoptotic germ cell (Maheshwari *et al.*, 2009). It has been reported that CCl_4 administration induces testicular DNA damage testicular apoptosis (Abdou *et al.*, 2012; Khan, 2012), which is an agreement with our findings. Increased lipid peroxidation induced by CCl_4 administration may possibly cause testicular histopathological damages and increase in testicular apoptotic index.

Cinnamon has been used as a spice and has several biological activities including radical scavenging activity. The most important volatile oils derived from cinnamon are *C. zeylanicum* bark and leaf oils, *C. cassia* (cassia oil) and *C. camphora* (Jayaprakasha & Rao, 2011). The antioxidant and free radical scavenging activity of bark oil extracted from *C. zeylanicum* have been reported in different experimental studies (Ciftci *et al.*, 2010; El-Baroty *et al.*, 2010; Yüce *et al.*, 2013). In addition, *C. zeylanicum* consumption has been reported to improve significantly the sperm quality, reproductive organ weights (Yüce *et al.*, 2013), LH, FSH and testosterone concentrations (Modaresi *et al.*, 2009; Hemayatkhah Jahromi *et al.*, 2011) in healthy animals, and also improve sperm and reproductive organ damages (Hafez, 2010; Shalaby & Mouneir, 2010) in diabetic rats. Besides, some herbal antioxidants were used to prevent the CCl_4-induced testicular oxidative stress (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012). However, there is no evidence about the protective effect of CBO on testicular oxidative stress, histopathological lesions and testicular apoptosis induced by CCl_4. Therefore, this is the first report regarding the protectiveness of CBO on CCl_4-induced reproductive dysfunction in males. In this study, long-term CBO administration to CCl_4-treated rats significantly decreased the increments in testicular LPO, abnormal sperm rates,

testicular histopathological lesions and testicular apoptotic cell index, and significantly increased the reductions in body, testis and epididymis weights, sperm concentration and motility when compared to the CCl_4 group. The increase in CAT and GSH-Px activities following CBO administration to CCl_4-treated rats was statistically insignificant. This status may be explained by excessive utilisation of these enzymes to reduce the LPO level. In addition, antioxidants have been reported to reduce the toxic effects exerted by CCl_4 through inhibition of CYP system that activates CCl_4 into its active metabolite, trichloromethyl radical (Sheweita *et al.*, 2001). The improvements observed in these parameters may possibly be related to the potent antioxidant and radical scavenging activity of CBO, and also inhibition of CYP activity.

In conclusion, the findings of the present study clearly suggest that CBO has protective effect on CCl_4-induced damages in male reproductive system. This protective effect of CBO seems to be closely involved with the scavenging free radicals and suppressing LPO.

Acknowledgements

The authors acknowledge the financial support from Firat University, Scientific Research Projects Unit (FUBAP); Project number: 2070.

References

Abdou HS, Salah SH, Hoda FB, Abdel Rahim EA (2012) Effect of pomegranate pretreatment on genotoxicity and hepatotoxicity induced by carbon tetrachloride (CCl_4) in male rats. *J Med Plants Res* 6:3370–3380.

Abraham P, Wilfred G, Cathrine SP (1999) Oxidative damage to the lipids and proteins of the lungs, testis and kidney of rats during carbon tetrachloride intoxication. *Clin Chim Acta* 289:177–179.

Aebi H (1983) Catalase. In: Methods in Enzymatic Analysis. Bergmeyer HU (ed.). Academic Press, New York, USA, pp 276–286.

Azab KS, Mostafa AHA, Ali EMM, Abdel-Aziz MAS (2011) Cinnamon extract ameliorates ionizing radiation-induced cellular injury in rats. *Ecotoxicol Environ Saf* 74: 2324–2329.

Bennett JP, Vickery BH (1970) Rats and mice. In: Reproduction and Breeding Techniques for Laboratory Animals. Hafez ESE (ed). Lea and Febiger, Philadelphia, USA, pp 299–315.

Castilla-Cortazar I, Diez N, Garcia-Fernandez M, Puche JE, Diez-Caballero F, Quiroga J, Diaz-Sanchez M, Castilla A, Casares AD, Varela-Nieto I, Prieto J, Gonzalez-Baron S (2004) Hematotesticular barrier is altered from early stages of liver cirrhosis: effect of insulin-like growth factor 1. *World J Gastroenterol* 10:2529–2534.

Ciftci M, Simsek UG, Yuce A, Yilmaz O, Dalkilic B (2010) Effects of dietary antibiotic and cinnamon oil supplementation on antioxidant enzyme activities, cholesterol levels and fatty acid compositions of serum and meat in broiler chickens. *Acta Vet Brno* 79:33–40.

El Denshary ES, Al-Gahazali MA, Mannaa FA, Salem HA, Hassan NS, Abdel-Wahhab MA (2012) Dietary honey and ginseng protect against carbon tetrachloride-induced hepatonephrotoxicity in rats. *Exp Toxicol Pathol* 64:753–756.

El-Baroty GS, Abd El-Baky HH, Farag RS, Saleh MA (2010) Characterization of antioxidant and antimicrobial compounds of cinnamon and ginger essential oils. *Afr J Biochem Res* 4:167–174.

Fadhel ZA, Amran S (2002) Effects of black tea extract on carbon tetrachloride-induced lipid peroxidation in liver, kidneys, and testes of rats. *Phytother Res* 16:S28–S32.

Fernandes GSA, Arena AC, Fernadez CDB, Mercadante A, Barbisan LF, Kempinas WG (2007) Reproductive effects in male rats exposed to diuron. *Reprod Toxicol* 23:106–112.

Hafez DA (2010) Effect of extracts of ginger goots and cinnamon bark on fertility of male diabetic rats. *J Am Sci* 6:940–947.

Hemayatkhah Jahromi V, Parivar K, Forozanfar M (2011) The effect of cinnamon extract on spermatogenesis hormonal axis of pituitary gonad in mice. *Iranian J Appl Anim Sci* 1:99–103.

Henkel R (2005) The impact of oxidants on sperm functions. *Andrologia* 37:205–206.

Horn MM, Ramos AR, Winkelmann L, Matte US, Goldani HA, Silveira TR (2006) Seminiferous epithelium of rats with food restriction and carbon tetrachloride-induced cirrhosis. *Int Brazilian J Urol* 32:94–99.

Hudson CE, Schulte BA, Sutter TR, Norris JS (2001) Steroid hormones modulate expression of cytochrome P450 enzymes in male hamster reproductive tract and leiomyosarcomas. *Carcinogenesis* 22:763–770.

Jayaprakasha GK, Rao LJM (2011) Chemistry, biogenesis, and biological activities of Cinnamomum zeylanicum. *Crit Rev Food Sci Nutr* 51:547–562.

Jayaprakasha GK, Ohnishi-Kameyama M, Ono H, Yoshida M, Rao LJ (2006) Phenolic constituents in the fruits of Cinnamomum zeylanicum and their antioxidant activity. *J Agric Food Chem* 54:1672–1679.

Jiang Y, Kuo C-L, Pernecky SJ, Piper WN (1998) The detection of cytochrome P450 2E1 and its catalytic activity in rat testis. *Biochem Biophys Res Commun* 246:578–583.

Johnsen SG (1970) Testicular biopsy score count a method for registration of spermatogenesis in human normal values and results in 335 hypogonadal males. *Hormones* 1:2–25.

Kalla NR, Bansal MP (1975) Effect of carbon tetrachloride on gonadal physiology in male rats. *Acta Anat (Basel)* 91: 380–385.

Karakus E, Karadeniz A, Simsek N, Can I, Kara A, Yildirim S, Kalkan Y, Kisa F (2011) Protective effect of *Panax ginseng* against serum biochemical changes and apoptosis in liver of

rats treated with carbon tetrachloride (CCl4). *J Hazard Mater* 195:208–213.

Kempinas WDG, Suarez JD, Roberts NL, Strader L, Ferrell J, Goldman JM, Klinefelter GR (1998) Rat epididymal sperm quantity, quality, and transit time after guanethidine-induced sympathectomy. *Biol Reprod* 59:890–896.

Khan RA (2012) Protective effects of Launaea procumbens on rat testis damage by CCl4. *Lipids Health Dis* 11:103.

Khan MR, Ahmed D (2009) Protective effects of *Digera muricata* (L.) Mart. on testis against oxidative stress of carbon tetrachloride in rat. *Food Chem Toxicol* 47:1393–1399.

Klinefelter GR, Hess RA (1998) Toxicity of the excurrent ducts and accessory organs in the male. In: Reproductive and Developmental Toxicology. Korach KS (ed). Marcel Dekker, New York, USA, pp 553–591.

Lawrence RA, Burk RF (1976) Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 71:952–958.

Liu Y, Dettin LE, Folmer J, Zirkin BR, Papadopoulos V (2007) Abnormal morphology of spermatozoa in cytochrome P450 17 alpha-hydroxylase/17, 20-lyase (CYP17) deficient mice. *J Androl* 28:453–460.

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

Maheshwari A, Misro MM, Aggarwal A, Sharma RK, Nandan D (2009) Pathways involved in testicular germ cell apoptosis induced by H_2O_2 *in vitro*. *FEBS J* 276:870–881.

Manjrekar AP, Jisha V, Bag PP, Adhikary B, Pai MM, Hegde A, Nandini M (2008) Effect of *Phyllanthus niruri* Linn. treatment on liver, kidney and testes CCL_4 induced hepatotoxic rats. *Indian J Exp Biol* 46:514–520.

Modaresi M, Messripour M, Rajaei R (2009) The effect of cinnamon (bark) extract on male reproductive physiology in mice. *Armaghan Danesh* 14:67–77.

Nebert DW, Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* 360:1155–1162.

Placer ZA, Cushman LL, Johnson BC (1966) Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem* 16:359–364.

Prasad KN, Yang B, Dong X, Jiang G, Zhang H, Xie H, Jiang Y (2009) Flavonoid contents and antioxidant activities from Cinnamomum species. *Inn Food Sci Emerg Tech* 10:627–632.

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.

Shah AH, Al-Shareef AH, Ageel AM, Qureshi S (1998) Toxicity studies in mice of common spices, Cinnamomum zeylanicum bark and Piper longum fruits. *Plant Foods Hum Nutr* 52:231–239.

Shalaby MA, Mouneir SM (2010) Effect of zingiber officinale roots and cinnamon zeylanicum bark on fertility of male diabetic rats. *Global Veterinaria* 5: 341–347.

Sheweita SA, El-Gabar MA, Bastawy M (2001) Carbon tetrachloride changes the activity of cytochrome P450 system in the liver of male rats: role of antioxidants. *Toxicology* 169:83–92.

Sikka SC (1996) Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front Biosci* 1: 78–86.

Soliman AM, Fahmy SR (2011) Protective and curative effects of the 15 KD isolated protein from the *Peganum harmala* L. seeds against carbon tetrachloride induced oxidative stress in brain, testes and erythrocytes of rats. *Eur Rev Med Pharmacol Sci* 15:888–899.

Türk G, Sönmez M, Aydın M, Yüce A, Gür S, Yüksel M, Aksu EH, Aksoy H (2008) Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. *Clin Nutr* 27:289–296.

Türk G, Çeribaşı AO, Şahna E, Ateşşahin A (2011) Lycopene and ellagic acid prevent testicular apoptosis induced by cisplatin. *Phytomedicine* 18:356–361.

Turner TT, Lysiak J (2008) Oxidative stress: a common factor in testicular dysfunction. *J Androl* 29:488–498.

U.S. EPA. IRIS. (2010) Toxicological review of carbon tetrachloride (External Review Draft). U.S. Environmental Protection Agency, Washington, DC, EPA/635/R-08/005A. Available online at: www.epa.gov/iris/toxreviews/0020tr.pdf (accessed September 2012).

Vernet P, Aitken RJ, Drevet JR (2004) Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* 216:31–39.

Xu J-Y, Su Y-Y, Cheng J-S, Li S-X, Liu R, Li W-X, Xu G-T, Li Q-N (2010) Protective effects of fullerenol on carbon tetrachloride-induced acute hepatotoxicity and nephrotoxicity in rats. *Carbon* 48:1388–1396.

Yüce A, Türk G, Çeribaşı S, Sönmez M, Çiftçi M, Güvenç M (2013) Effects of cinnamon (*Cinnamomum zeylanicum*) bark oil on testicular antioxidant values, apoptotic germ cell and sperm quality. *Andrologia* 45:248–255.