First International Journal of Andrology
ANDROLOGIA

ORIGINAL ARTICLE

Quercetin attenuates carbon tetrachloride-induced testicular damage in rats

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

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

Keywords
Apoptosis—carbon tetrachloride—lipid peroxidation—quercetin—spermatozoa-testis

Correspondence
Assoc. Prof. Dr Gaffari Türk, PhD, Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, 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: July 19, 2013

doi: 10.1111/and.12159

Summary

This study was conducted to investigate the effect of quercetin on carbon tetrachloride (CCl_4)-induced sperm damages, testicular apoptosis and oxidative stress in male rats. Group 1 served as control, group 2 was treated with only quercetin, group 3 was treated with only CCl_4 and group 4 received CCl_4 + quercetin. All administrations were performed by gavage and maintained for 10 weeks. CCl_4 administration caused significant decreases in absolute and relative reproductive organ weights, sperm motility, concentration and testicular glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and significant increases in lipid peroxidation (LPO) level, abnormal sperm rate and testicular apoptotic cell index, along with some histopathological damages when compared to the control group. However, administration of CCl_4 together with quercetin provided statistically significant improvements in LPO level, abnormal sperm rate, the degree of histopathological lesions and testicular apoptotic cell index when compared to only CCl_4 group. In addition, improvements observed in absolute and relative weights of reproductive organs, sperm motility and concentration, and testicular GSH-Px and CAT activities in group 4 were statistically insignificant when compared to only CCl_4 group. In conclusion, quercetin has antiperoxidative effect, and its oral administration attenuates the CCl_4-induced some damages in male reproductive organs and cells by decreasing the LPO.

Introduction

Carbon tetrachloride (CCl_4) has been used as a model toxicant and has been the focus of many *in vitro* and *in vivo* toxicological studies. The primary site of CCl_4 toxicity and carcinogenesis is the liver, and it consistently causes liver toxicity, resulting in fatty degeneration, cellular necrosis, fibrosis and cirrhosis in multiple species and through multiple routes of exposure (Manibusan *et al.*, 2007). CCl_4 also causes structural and functional damages in other organs of body such as kidney (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008), lung (Abraham *et al.*, 1999; Ögetürk *et al.*, 2009) and brain (Soliman & Fahmy, 2011) apart from liver toxicity. It has been reported that metabolism of CCl_4 via cytochrome P450 (CYP) to highly reactive free radical metabolites plays a critical role in the postulated mode of action. The free radicals initiate lipid peroxidation (LPO) by attacking polyunsaturated fatty acids (PUFAs) in membranes, setting off a free radical chain reaction sequence. LPO is known to cause membrane disruption, resulting in the loss of membrane integrity and the leakage of microsomal enzymes. By-products of LPO can form protein and DNA adducts and may contribute to hepatotoxicity and carcinogenicity respectively (Manibusan *et al.*, 2007).

Spermatozoa require a high PUFA content to provide the plasma membrane with the fluidity essential at fertilisation. However, this makes spermatozoa particularly vulnerable to attack by reactive oxygen species (ROS) that have clear associations with reduced fertility (Wathes *et al.*, 2007). In addition, as the testis, prostate (Jiang

et al., 1998), epididymis (Hudson *et al.*, 2001) and germ cells (Liu *et al.*, 2007) contain CYP isozymes, it is possible that CCl_4 causes oxidative damage in lipids of these tissues and cells (Abraham *et al.*, 1999). Acute or chronic CCl_4 administration has been reported to cause morphological, structural and functional damages in reproductive system through oxidative toxicity in male laboratory animals (Kalla & Bansal, 1975; Horn *et al.*, 2006; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Abdou *et al.*, 2012; Khan, 2012; Yüce *et al.*, 2013).

Endogenous antioxidants are capable of quenching the LPO reaction. When antioxidants are depleted, however, opportunities for LPO are enhanced (Manibusan *et al.*, 2007). Therefore, natural antioxidant administrations are likely to improve the LPO-induced damages in the structures and functions of the testis and spermatozoa (Vernet *et al.*, 2004). CCl_4-induced some damages in reproductive system have been reported to be prevented by some herbal antioxidants through their scavenging properties on LPO in male rats (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012; Yüce *et al.*, 2013). Quercetin, is one of the dietary bioflavonoids, has been reported to have beneficial health effects. Till date, most of the research has been focused on the antioxidant properties of quercetin, as it is believed to prevent LPO. However, there are conflicting reports about whether quercetin has pro-oxidant or antioxidant effect on male reproduction and fertility (Ranawat *et al.*, 2013b). While some authors reported that quercetin has deleterious effect on male reproductive function through its pro-oxidant effect (Farombi *et al.*, 2013; Ranawat *et al.*, 2013a) and the other mechanism of action (Khanduja *et al.*, 2001); whereas many researchers reported that quercetin has improvement effect on male reproductive dysfunction by means of its antioxidant activity (Zhang, 2005; Khaki *et al.*, 2010; Ben Abdallah *et al.*, 2011, 2012; Ciftci *et al.*, 2012; Farombi *et al.*, 2012a,b; Kanter *et al.*, 2012; Moretti *et al.*, 2012; Zribi *et al.*, 2012) and its other effect (Izawa *et al.*, 2008; Taepongsorat *et al.*, 2008; Abarikwu *et al.*, 2012). In addition, quercetin has been reported to possess beneficial effect on CCl_4-induced liver fibrosis by enhancing the antioxidant enzyme activity (Pavanato *et al.*, 2003, 2007) and decreasing the LPO (Pavanato *et al.*, 2007). However, there is no evidence about the deleterious or beneficial effect of quercetin on male reproductive dysfunction induced by CCl_4 according to our knowledge. Therefore, this study was conducted to investigate whether quercetin has improvement/deleterious effect on CCl_4-induced negative changes in sperm quality, testicular apoptosis, oxidative stress and histopathological lesions.

Materials and methods

Animals and experimental design

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 Experimental Research Centre of Firat University (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 drinking water was given *ad libitum*.

Olive oil was used as vehicle because CCl_4 (99.9%, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) is an oil-dissolved chemical. Quercetin (100 g, Molekula Ltd., Wimborne Dorset, UK) was also dissolved in alkaline solution (0.01 N NaOH, pH ≈ 12) because it is hardly dissolved in natural conditions. The final pH of this solution after the supplementation of quercetin was ≈8. The rats were randomly divided into four groups; each containing 7 rats. The volume of the drugs received by rats in each group was 1 ml (0.5 ml olive oil + 0.5 ml slightly alkaline solution). Pure olive oil + slightly alkaline solution (pH ≈8) was daily administered by gavage to rats in the first group, and they served as *control*. 150 mg kg^{-1} quercetin + pure olive oil was daily given by gavage to rats in the second group and named as *quercetin*. The rats in third group were treated with 0.25 ml kg^{-1} $week^{-1}$ CCl_4 + daily slightly alkaline solution and named as *CCl_4*. The rats in fourth group received 0.25 ml kg^{-1} $week^{-1}$ CCl_4 and 150 mg kg^{-1} day^{-1} quercetin and named as *CCl_4+quercetin*. All administrations maintained for 10 weeks. The doses of CCl_4 (Horn *et al.*, 2006; Yüce *et al.*, 2013) and quercetin (Taepongsorat *et al.*, 2008) given to rats in this study 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 in rats is approximately 1 week (Kempinas *et al.*, 1998), 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 in oil suspension and quercetin in slightly alkaline solution 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 9-fold 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).

Testicular tissue LPO level and antioxidant enzyme activities

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. The 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 kg protein^{-1}, where k is the first-order rate constant (Aebi, 1983). Protein concentration was determined using the method of Lowry *et al.* (1951).

Epididymal sperm analyses

All sperm analyses were made 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 & eosin. Twenty-five seminiferous tubules (ST) were randomly examined per section, their diameters and germinal cell layer thicknesses (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 (+++).

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) according to the manufacturer's instructions. The fixed testicular tissues in Bouin's solution were embedded in paraffin and sectioned at 4-μm thicknesses. 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 re-washing 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 the TdT enzyme. Finally, sections were counterstained with Mayer's haematoxylin, rinsed in tap water and mounted with glycerol. TUNEL-positive apoptotic cell index was calculated as follow:

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

Data analysis

Data are presented as the 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 analysis of variance (ANOVA) 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, Chicago, IL, USA).

Results

Reproductive organ weights

The mean values of absolute and relative reproductive organ weights are given in Table 1. Only quercetin administration did not have any significant harmful or useful effect on reproductive organ weights in comparison with the control group. CCl_4 administration caused significant reductions in absolute and relative weights of testis ($P < 0.001$, $P < 0.01$), epididymis ($P < 0.001$), right cauda epididymis ($P < 0.01$), seminal vesicles ($P < 0.05$) and prostate ($P < 0.05$) as compared to the control group. With the exception of epididymal weight, the decreased values in the absolute and relative reproductive organ weights of group given CCl_4 only were brought to the near values to control group by quercetin administration to CCl_4-treated rats. However, the improvements in these organ weights of rats in CCl_4+quercetin group were not statistically different from CCl_4 group only.

Testicular tissue LPO level and antioxidant enzyme activities

Testicular tissue LPO, demonstrated as MDA, and rGSH level, GSH-Px and CAT activities of all the groups are given in Table 2. Only quercetin administration had no significant effect on LPO and antioxidants when compared to the control group. Only CCl_4 administration caused significant ($P < 0.001$) increase in MDA level and significant ($P < 0.01$) decreases in GSH-Px and CAT activities when compared to the control group. In addition, CCl_4 tended to decrease rGSH level, but this reduction did not reach the statistical significance when compared to the control group. However, quercetin administration to CCl_4-treated rats significantly ($P < 0.001$) decreased the CCl_4-induced increment in MDA level. Although the increases observed in GSH-Px and CAT activities following quercetin administration to CCl_4-treated rats were not statistically significant in comparison with the alone CCl_4 group, these activities in CCl_4+quercetin group were found as a near value to control group.

Epididymal sperm parameters

Epididymal sperm concentration, sperm motility and abnormal sperm rate in all groups are presented in Table 3. Only quercetin administration had no significant effect on sperm parameters in comparison with the control group. Significant decreases ($P < 0.001$) in sperm motility and concentration, and 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, quercetin consumption by rats treated with CCl_4 provided significant ($P < 0.001$) improvements in all abnormal sperm rates as compared to the CCl_4 group only. Although the increase observed in sperm motility following quercetin administration to CCl_4-treated rats were not statistically significant in comparison with the alone CCl_4 group, this parameter in CCl_4+quercetin group was found as a near value to control group. Administration of quercetin together with CCl_4 tended to increase the reductions in sperm concentration, but this increase did not reach the statistical significance when compared to the CCl_4 group only.

Testicular histopathological lesions and apoptotic cell index

No histopathological lesions (Table 4) were observed in testicular tissues in control (Fig. 1a) and quercetin (Fig. 1b) 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. 1c) and CCl_4+quercetin (Fig. 1d) groups. Almost all ST in testes of CCl_4 group were 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+quercetin group in comparison with the CCl_4 group. In addition, the degree of lesions was significantly ($P < 0.001$) worse in only CCl_4 group than in CCl_4+quercetin group (Table 4). 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, quercetin administration to CCl_4-treated animals significantly ($P < 0.001$) improved the CCl_4-induced damages in these parameters (Table 5).

Table 1 Mean ± SEM values of absolute and relative reproductive organ weights (CCl_4 = carbon tetrachloride)

Groups	Parameters									
	Absolute weight (g)					Relative weight (g/body weight × 100)				
	Testis	Epididymis	Right cauda epididymis	Seminal vesicles	Ventral prostate	Testis	Epididymis	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	1.443 ± 0.032^{AC}	0.503 ± 0.011^{A}	0.169 ± 0.006^{a}	0.805 ± 0.045^{x}	0.419 ± 0.017^{x}	0.442 ± 0.008^{a}	0.154 ± 0.004^{A}	0.050 ± 0.002^{a}	0.251 ± 0.025^{x}	0.133 ± 0.006^{x}
Quercetin	1.507 ± 0.057^{A}	0.507 ± 0.028^{A}	0.173 ± 0.011^{a}	0.787 ± 0.114^{x}	0.405 ± 0.062^{x}	0.467 ± 0.010^{a}	0.157 ± 0.004^{A}	0.053 ± 0.002^{a}	0.220 ± 0.034^{x}	0.148 ± 0.029^{x}
CCl_4	0.968 ± 0.125^{B}	0.283 ± 0.014^{B}	0.108 ± 0.016^{b}	0.296 ± 0.009^{y}	0.090 ± 0.014^{y}	0.335 ± 0.043^{b}	0.098 ± 0.005^{B}	0.035 ± 0.005^{b}	0.084 ± 0.002^{y}	0.036 ± 0.005^{y}
CCl_4 + Quercetin	1.190 ± 0.099^{BC}	0.365 ± 0.061^{B}	0.138 ± 0.023^{ab}	0.460 ± 0.237^{xy}	0.288 ± 0.144^{xy}	0.410 ± 0.026^{ab}	0.124 ± 0.014^{B}	0.047 ± 0.006^{ab}	0.147 ± 0.065^{xy}	0.092 ± 0.040^{xy}

The mean differences between the values bearing different superscript letters within the same column are statistically significant (A, B and C; $P < 0.001$, a and b; $P < 0.01$, x and y; $P < 0.05$).

Fig. 2 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 control group. However, a significant ($P < 0.001$) decrease was observed in apoptotic cell index in CCl_4+quercetin group as compared to the CCl_4 group only (Table 5).

Discussion

Numerous studies have shown that oral (Abdou *et al.*, 2012; Yüce *et al.*, 2013) and parenteral (Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Khan, 2012) CCl_4 exposure causes morphological and functional reproductive disorders through oxidative toxicity in male rodents. Many antioxidants have been used to overcome these reproductive disorders (Fadhel & Amran, 2002; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012; Yüce *et al.*, 2013). Quercetin has been reported to possess both antioxidant and pro-oxidant properties on the male reproductive system of rodents and humans (Ranawat *et al.*, 2013b). In this study, we examined the changes in reproductive organ weights, sperm parameters, testicular tissue oxidative stress markers, testicular histological structure and apoptotic germ cells in order to observe whether has quercetin pro-oxidant otherwise antioxidant effect on CCl_4-induced reproductive disorders in adult male rats.

CCl_4 toxicity

In the present study, CCl_4 administration caused significant decreases in absolute and relative weights of all reproductive organs studied. It has been reported that CCl_4 administration results in reduced weights of testis (Castilla-Cortazar *et al.*, 2004; Manjrekar *et al.*, 2008; Khan & Ahmed, 2009; Yüce *et al.*, 2013), epididymis and accessory sex organs (Yüce *et al.*, 2013) as well as decreased testosterone level (Khan & Ahmed, 2009; Khan, 2012). Disturbances in the synthesis of androgens have been reported to be able to cause negative changes in reproductive organ weights, because permanent androgenic stimulation is necessary for normal growth and functions of testes, epididymides and accessory sex organs (Klinefelter & Hess, 1998). In addition, it is well known that testicular LPO is strongly associated with the testicular dysfunction including steroidogenesis (Turner & Lysiak, 2008). These decreases in reproductive organ weights observed in the present study may possibly be explained by CCl_4-induced decreased testosterone concentration in conjunction with the increased LPO, as evidenced by increased MDA herein.

Table 2 Mean ± SEM values of malondialdehyde (MDA), reduced glutathione (rGSH) levels and glutathione peroxidase (GSH-Px) and catalase (CAT) activities (CCl_4 = carbon tetrachloride)

	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	8.72 ± 0.34^{A}	7.02 ± 0.54	2.35 ± 0.54^{a}	42.16 ± 4.91^{a}
Quercetin	7.54 ± 0.87^{A}	6.95 ± 0.79	3.25 ± 0.39^{a}	45.67 ± 6.01^{a}
CCl_4	18.91 ± 0.96^{B}	5.85 ± 0.52	0.93 ± 0.18^{b}	15.31 ± 1.77^{b}
CCl_4 + quercetin	5.97 ± 0.63^{A}	7.09 ± 0.62	2.10 ± 0.30^{ab}	29.36 ± 1.53^{ab}

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a and b: $P < 0.001$; A and B: $P < 0.01$).

Table 3 Mean ± SEM values of sperm parameters (CCl_4 = carbon tetrachloride)

	Parameters				
			Abnormal spermrate (%)		
Groups	Sperm motility (%)	Epididymal sperm concentration (million/right cauda epididymis)	Head	Tail	Total
Control	73.33 ± 4.22^{ac}	90.50 ± 5.59^{a}	3.77 ± 0.56^{a}	6.00 ± 1.02^{a}	9.77 ± 1.87^{a}
Quercetin	81.17 ± 3.89^{a}	111.71 ± 5.28^{a}	4.00 ± 0.62^{a}	5.29 ± 1.25^{a}	9.29 ± 1.61^{a}
CCl_4	37.50 ± 4.79^{b}	20.25 ± 8.99^{b}	17.00 ± 2.16^{b}	18.00 ± 2.24^{b}	35.00 ± 4.14^{b}
CCl_4 + quercetin	52.50 ± 10.31^{bc}	47.00 ± 9.74^{b}	7.25 ± 2.10^{a}	8.75 ± 2.36^{a}	16.00 ± 4.38^{a}

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a, b, c and d: $P < 0.001$).

Oxidative stress results from the free oxygen radicals, which include superoxide anion (O_2^-), H_2O_2 and the hydroxyl ion ($^{\bullet}OH$), in excess of the enzymatic and nonenzymatic antioxidants of the stressed tissue. Free radicals have high affinity to cell membrane lipids, especially PUFAs, leading to tissue damage due to the LPO (Aitken & Roman, 2008; Turner & Lysiak, 2008). CCl_4 has been reported to cause an increase in by-products of LPO (Abraham *et al.*, 1999; Fadhel & Amran, 2002; Castilla-Cortazar *et al.*, 2004; Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012; Yüce *et al.*, 2013) and a decrease in enzymatic and nonenzymatic antioxidants in testicular tissue (Khan & Ahmed, 2009; Soliman & Fahmy, 2011; Khan, 2012). In this study, CCl_4 administration caused significant increase in testicular MDA level and significant decrease in testicular GSH-Px and CAT activities when compared to the control group. The CCl_4-induced oxidative stress in testes may be depend on the increased free radicals mediated by CYP activity, which was also identified in testes (Jiang *et al.*, 1998), in the present study.

Free radicals can be produced in large amounts by spermatozoa, mitochondria and a variety of enzymes including the xanthine- and NADPH oxidases, and the CYP isozymes in the testis under pathologic conditions. Spermatozoa and other cells within the testis remain vulnerable to oxidative stress due to the abundance of highly PUFAs and the presence of potential free radical-generating systems (Aitken & Roman, 2008). In addition, they are also vulnerable to oxidative damage during the epididymal transit due to the maturational changes in sperm plasma membrane (Vernet *et al.*, 2004). Thus, excessive generation of free radicals in pathologic conditions can induce the LPO by oxidative breakdown of PUFAs in the membranes of cells. Obviously, peroxidation of sperm lipids destroys the structure of lipid matrix in the membranes of spermatozoa, and 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 (Aitken & Roman, 2008; Turner & Lysiak, 2008). In the present study, significant decreases in epididymal sperm concentration and motility, 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 and sperm motility as well as increased sperm shape abnormalities has been reported in CCl_4-treated rats (Khan, 2012; Yüce *et al.*, 2013). Increased LPO and decreased antioxidant enzyme activity, as evidenced by increased MDA level and decreased GSH-Px and CAT activities in this study, may be responsible for impaired sperm quality observed in CCl_4-treated rats.

Table 4 The degree of some pathological lesions in testicular tissues of different treatment groups (CCl_4 = carbon tetrachloride)

Lesions	Control	Quercetin	CCl_4	CCl_4+quercetin
Necrosis in germinal cells	ND	ND	2.92 ± 0.12[a]	1.29 ± 0.36[b]
Atrophy in seminiferous tubules	ND	ND	2.40 ± 0.18[a]	1.14 ± 0.40[b]
Thickening in tubule basal membrane	ND	ND	2.84 ± 0.28[a]	1.71 ± 0.18[b]
Degeneration in germinal cells	ND	ND	2.65 ± 0.06[a]	1.71 ± 0.18[b]
Desquamation in germinal cells	ND	ND	2.07 ± 0.20[a]	1.00 ± 0.00[b]
Reduction in germinal cell counts	ND	ND	2.90 ± 0.10[a]	1.14 ± 0.40[b]
Disorganisation in germinal cells	ND	ND	2.70 ± 0.14[a]	1.71 ± 0.29[b]
Vacuolisation in germinal cells	ND	ND	1.59 ± 0.24[a]	0.57 ± 0.20[b]
Interstitial oedema and congestion	ND	ND	2.58 ± 0.15[a]	1.86 ± 0.14[b]
Multinucleated syncytial cell formation	ND	ND	3.00 ± 0.00[a]	0.43 ± 0.20[b]
Spermatogenic arrest	ND	ND	2.82 ± 0.20[a]	1.43 ± 0.37[b]

ND, Not detected.
[a]Different from both control and quercetin groups ($P < 0.001$).
[b]Different from CCl_4 group ($P < 0.001$).

Marked histopathological damages such as necrosis, degeneration, desquamation, disorganisation, reduction in germinal cells, spermatogenic arrest, multinuclear syncytial cell formation and significant decreases in diameters of ST, GCLT and Johnsen's testicular score were determined in CCl_4 group as compared to the control group in this study. Similar findings including exfoliation of the germinal epithelium, depletion and degeneration of germ cells, shrinkage of the tubules, vacuolisation of germinal epithelium and meiotic arrest have been reported following long-term (from 20 days to 16 weeks) CCl_4 administration in rats (Kalla & Bansal, 1975; Horn *et al.*, 2006; Khan & Ahmed, 2009; Khan, 2012; Yüce *et al.*, 2013). Apoptosis is known to be a programmed cell death for controlling the spermatogonial population within the testis. However, increased rate of apoptotic germ cells in pathologic conditions disrupts this programme leading to excessive cell death (Blanco-Rodriguez, 1998). Excessive generation of free radicals-induced DNA damage results in increased testicular apoptotic germ cell (Maheshwari *et al.*, 2009). It has been reported that long-term CCl_4 administration causes testicular DNA damage (Khan, 2012) and increase in testicular apoptotic cell number (Yüce *et al.*, 2013). The apoptotic cell index in CCl_4 group was found to be significantly higher than control group in the present study. Increased LPO level and decreased antioxidant activity following CCl_4 administration may possibly cause the testicular histopathological

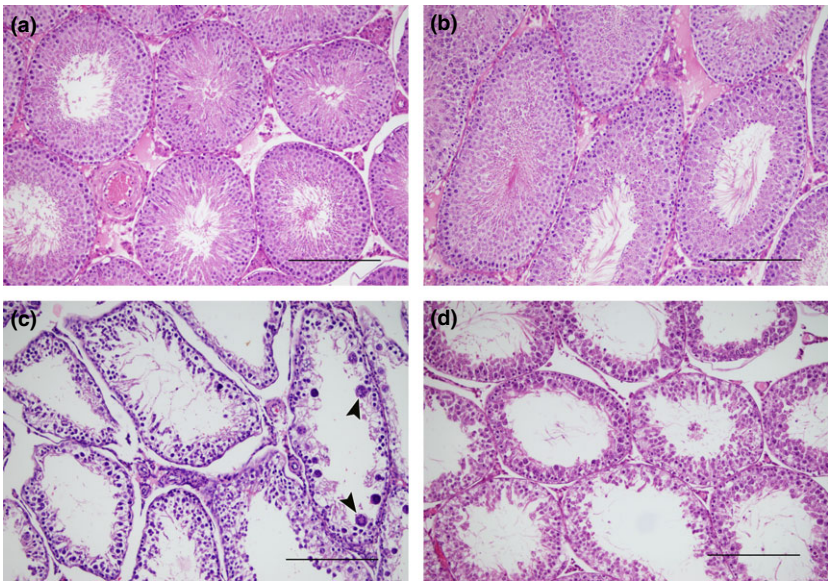

Fig. 1 Representative photomicrographs of histopathological structure of testis in different treatment groups (CCl_4 = carbon tetrachloride, calibration bar = 200 μm). (a) Haematoxylin and eosin staining in control group. (b) Haematoxylin and eosin staining in quercetin-treated group. (c) Haematoxylin and eosin staining in CCl_4-treated group (arrows show multinuclear syncytial cells). (d) Haematoxylin and eosin staining in CCl_4 + quercetin-treated group.

Table 5 Mean ± SEM values of diameters of seminiferous tubules (ST), germinal cell layer thickness (GCLT), Johnsen testicular score and TUNEL-positive apoptotic cell index (CCl_4 = carbon tetrachloride)

	Variables			
Groups	Diameter of ST (μm)	GCLT (μm)	Johnsen testicular score (1–10)	TUNEL-positive apoptotic cell index (%)
Control	260.53 ± 1.82[a]	100.94 ± 0.93[a]	9.90 ± 0.07[a]	1.73 ± 0.28[a]
Quercetin	263.78 ± 1.31[a]	101.20 ± 0.83[a]	9.70 ± 0.08[a]	2.81 ± 0.34[a]
CCl_4	189.16 ± 2.08[b]	46.85 ± 1.44[b]	4.26 ± 0.25[b]	14.28 ± 1.73[b]
CCl_4+quercetin	223.91 ± 2.10[c]	71.31 ± 1.51[c]	6.83 ± 0.65[c]	9.89 ± 0.97[c]

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a, b, c and d: $P < 0.001$).

damages and the increase in testicular apoptotic cell index.

Beneficial effects of quercetin

Quercetin has the ability to prevent the oxidation of low-density lipoproteins by scavenging free radicals and chelating transition metal ions, thereby aiding in the prevention of various diseases, such as cancer, atherosclerosis and chronic inflammation. However, there are controversial reports in the literature highlighting the antioxidant as well as a pro-oxidant effect of quercetin when male reproduction and fertility are considered (Ranawat *et al.*, 2013b). Many studies (Zhang, 2005; Izawa *et al.*, 2008; Taepongsorat *et al.*, 2008; Khaki *et al.*, 2010; Ben Abdallah *et al.*, 2011, 2012; Abarikwu *et al.*, 2012; Ciftci *et al.*, 2012; Farombi *et al.*, 2012a,b; Kanter *et al.*, 2012; Moretti *et al.*, 2012; Zribi *et al.*, 2012) have reported that quercetin has stimulating or protective effect; whereas few studies (Khanduja *et al.*, 2001; Farombi *et al.*, 2013; Ranawat *et al.*, 2013a) have mentioned from its deleterious effect on male reproduction. Currently, it has been reported in an article reviewed by Ranawat *et al.* (2013b) that the reason for the conflicting biological effects of quercetin might be related to its administration dose and the redox state of the cell. Although antioxidant property of quercetin has been reported to provide significant improvements in increased LPO level and decreased enzymatic and nonenzymatic

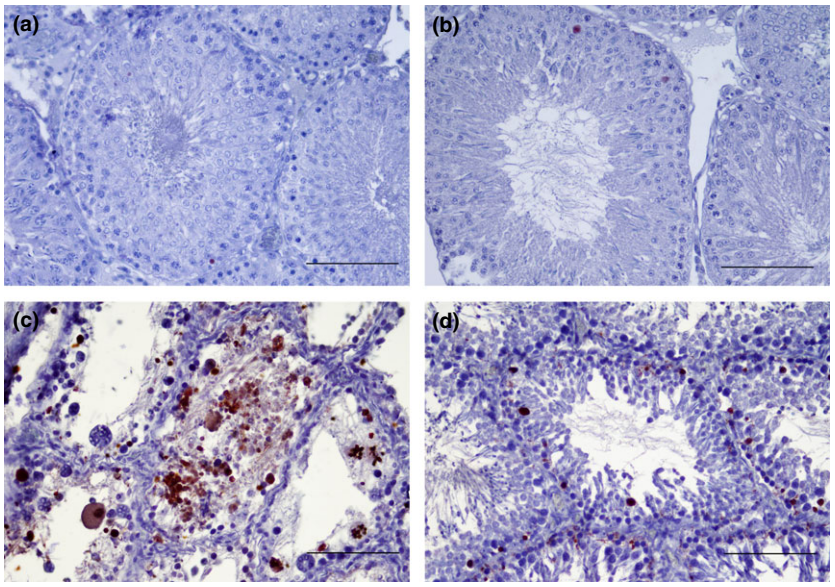

Fig. 2 Representative photomicrographs of apoptotic cells by TUNEL method in the testis of different treatment groups (CCl_4 = carbon tetrachloride, calibration bar = 100 μm). (a) TUNEL staining in control group. (b) TUNEL staining in quercetin-treated group. (c) TUNEL staining in CCl_4-treated group (marked reduction in germinal cells and brown-red-stained cells are the apoptotic ones. Marked increase is seen in the apoptotic cell index that calculated by dividing total apoptotic cell number to total germinal cell number in 25 seminiferous tubules). (d) TUNEL staining in CCl_4 + quercetin-treated group (marked increment in germinal cells and brown-red-stained cells are the apoptotic ones. Marked decrease is seen in the apoptotic cell index that calculated by dividing total apoptotic cell number to total germinal cell number in 25 seminiferous tubules).

antioxidants in testicular tissue as well as deteriorated sperm parameters, testicular histopathological lesions, DNA damages and decreased testosterone level induced by various chemicals (Khaki *et al.*, 2010; Ben Abdallah *et al.*, 2011, 2012; Ciftci *et al.*, 2012; Farombi *et al.*, 2012a; Kanter *et al.*, 2012; Moretti *et al.*, 2012); whereas some authors (Farombi *et al.*, 2013; Ranawat *et al.*, 2013a) have reported that pro-oxidant property of quercetin causes an increase in LPO level and a decrease in antioxidant enzymes as well as damages in spermatozoa and other reproductive parameters. On the other hand, although it has been reported that repeated doses of antioxidants could reduce the toxic effects exerted by CCl_4 upon the liver, and probably other organs, through inhibition of CYP system that activates CCl_4 into its active metabolite, trichloromethyl radical (Sheweita *et al.*, 2001). No comprehensive scientific study has been performed about the improvement or deleterious effect of quercetin on CCl_4-induced testicular oxidative stress, sperm damages, histopathological lesions and testicular apoptosis so far. In this regard, this is the first comprehensive report on the effectiveness of quercetin on CCl_4-induced reproductive dysfunction in males. In this study, while long-term quercetin administration to CCl_4-treated rats significantly decreased the increments in testicular LPO, abnormal sperm rates, the degree of testicular histopathological lesions and testicular apoptotic cell index, it provided numerical increase, though not significant, in GSH-Px and CAT activities, reproductive organ weights, sperm concentration and motility when compared to the CCl_4 group. These numerical increases observed in some parameters of CCl_4+quercetin group were found as close values to the control group. The improvements observed in these parameters may possibly be related to the antiperoxidative effect of quercetin rather than its pro-oxidant effect and also its inhibitory effect on CYP activity/expression.

Conclusion

In conclusion, the findings of this study clearly suggest that quercetin has attenuating effect on CCl_4-induced damages in sperm shape abnormalities, testicular histopathological lesions and apoptosis. This effect of quercetin seems to be closely involved with the scavenging of free radicals and suppressing LPO as well as its inhibitory effect on CYP activity. In addition, the findings of this study support the results of previous reports suggesting that quercetin has antioxidant effect.

Acknowledgement

The authors acknowledge for financial support from Firat University, Scientific Research Projects Unit (FUBAP; project no. 2070).

References

Abarikwu SO, Pant AB, Farombi EO (2012) The protective effects of quercetin on the cytotoxicity of atrazine on rat Sertoli-germ cell co-culture. *Int J Androl* 35:590–600.

Abdou HS, Salah SH, Hoda FB, Abdel Rahim EA (2012) Effect of pomegranate pretreatment on genotoxicity and hepatotoxicity induced by carbon tetrachloride (CCl4) 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, NY, USA, pp. 276–286.

Aitken RJ, Roman SD (2008) Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* 1:15–24.

Ben Abdallah F, Zribi N, Ammar-Keskes L (2011) Antioxidative potential of quercetin against hydrogen peroxide induced oxidative stress in spermatozoa *in vitro*. *Andrologia* 43:261–265.

Ben Abdallah F, Fetoui H, Zribi N, Fakhfakh F, Keskes L (2012) Quercetin attenuates lambda cyhalothrin-induced reproductive toxicity in male rats. *Environ Toxicol* doi: 10.1002/tox.20762 [Epub ahead of print].

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

Blanco-Rodriguez J (1998) A matter of death and life: the significance of germ cell death during spermatogenesis. *Int J Androl* 21:236–248.

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

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.

Farombi EO, Abarikwu SO, Adesiyan AC, Oyejola TO (2013) Quercetin exacerbates the effects of subacute treatment of atrazine on reproductive tissue antioxidant defence system, lipid peroxidation and sperm quality in rats. *Andrologia* 45:256–265.

Farombi EO, Adedara IA, Akinrinde SA, Ojo OO, Eboh AS (2012a) Protective effects of kolaviron and quercetin on cadmium-induced testicular damage and endocrine pathology in rats. *Andrologia* 44:273–284.

Farombi EO, Ekor M, Adedara IA, Tonwe KE, Ojujoh TO, Oyeyemi MO (2012b) Quercetin protects against testicular toxicity induced by chronic administration of therapeutic dose of quinine sulphate in rats. *J Basic Clin Physiol Pharmacol* 23:39–44.

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.

Izawa H, Kohara M, Aizawa K, Suganuma H, Inakuma T, Watanabe G, Taya K, Sagai M (2008) Alleviative effects of quercetin and onion on male reproductive toxicity induced by diesel exhaust particles. *Biosci Biotechnol Biochem* 72:1235–1241.

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.

Kanter M, Aktas C, Erboga M (2012) Protective effects of quercetin against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testis. *Food Chem Toxicol* 50:719–725.

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.

Khaki A, Fathiazad F, Nouri M, Khaki AA, Maleki NA, Khamnei HJ, Ahmadi P (2010) Beneficial effects of quercetin on sperm parameters in streptozotocin-induced diabetic male rats. *Phytother Res* 24:1285–1291.

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.

Khanduja KL, Verma A, Bhardwaj A (2001) Impairment of human sperm motility and viability by quercetin is independent of lipid peroxidation. *Andrologia* 33:277–281.

Klinefelter GR, Hess RA (1998) Toxicity of the excurrent ducts and accessory organs in the male. In: Reproductive and Developmental Toxicology. Korach KS (ed). New York, NY, USA, Marcel Dekker Inc, 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 17alpha-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 H2O2 *in vitro*. *FEBS J* 276:870–881.

Manibusan MK, Odin M, Eastmond DA (2007) Postulated carbon tetrachloride mode of action: a review. *J Environ Sci Health Part C* 25:185–209.

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 CCL4 induced hepatotoxic rats. *Indian J Exp Biol* 46:514–520.

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.

Ögetürk M, Çolakoğlu N, Kuş MA, Kuş İ, Sarsılmaz M (2009) Protective efficiency of caffeic acid phenethyl ester in carbon tetracloride-induced experimental lung injury (Article in Turkish). *Fırat Unv J Health Sci* 23:57–61.

Pavanato A, Tunon MJ, Sanchez-Campos S, Marroni CA, Llesuy S, Gonzalez-Gallego J, Marroni N (2003) Effects of quercetin on liver damage in rats with carbon tetrachloride-induced cirrhosis. *Dig Dis Sci* 48:824–829.

Pavanato MA, Marroni NP, Marroni CA, Llesuy SF (2007) Quercetin prevents oxidative stress in cirrhotic rats. *Dig Dis Sci* 52:2616–2621.

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

Ranawat P, Kaushik G, Saikia UN, Pathak CM, Khanduja KL (2013a) Quercetin impairs the reproductive potential of male mice. *Andrologia* 45:56–65.

Ranawat P, Pathak CM, Khanduja KL (2013b) A new perspective on the quercetin paradox in male reproductive dysfunction. *Phytother Res* 27:802–810.

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.

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.

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.

Taepongsorat L, Tangpraprutgul P, Kitana N, Malaivijitnond S (2008) Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats. *Asian J Androl* 10:249–258.

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, Çeribası AO, Sahna E, Atessahin 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.

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

Wathes DC, Abayasekara DRE, Aitken RJ (2007) Polyunsaturated fatty acids in male and female reproduction. *Biol Reprod* 77:190–201.

Yüce A, Türk G, Çeribaşı S, Güvenç M, Çiftçi M, Sönmez M, Özer Kaya Ş, Çay M, Aksakal M (2013) Effectiveness of cinnamon (*Cinnamomum zeylanicum*) bark oil in the prevention of carbon tetrachloride-induced damages on male reproductive system. *Andrologia* doi: 10.1111/and.12072 [Epub ahead of print].

Zhang YMC (2005) Protective effect of quercetin on aroclor 1254-induced oxidative damage in cultured chicken spermatogonial cells. *Toxicol Sci* 88:545–550.

Zribi N, Chakroun NF, Ben Abdallah F, Elleuch H, Sellami A, Gargouri J, Rebai T, Fakhfakh F, Keskes LA (2012) Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. *Cryobiology* 65:326–331.