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Effects of quercetin and fish n-3 fatty acids on testicular injury induced by ethanol in rats

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Summary

The aim of this study was to investigate the effects of quercetin and fish n-3 fatty acids on the changes in testis induced by ethanol. Forty-five rats divided into five groups, control, ethanol, ethanol+quercetin, ethanol+fish n-3 fatty acids and ethanol+quercetin+fish n-3 fatty acids. At the end of 8 weeks, all the rats were sacrificed. Degenerative changes in histopathological analyses, the decreased body weight gain and seminiferous tubule diameters in ethanol group have been observed. TUNEL assay also showed an increase in apoptotic cell number. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), xanthine oxidase (XO) and testosterone levels were decreased as well as the levels of malondialdehyde (MDA) and nitric oxide (NO) were increased in ethanol group. Histopathological changes caused by ethanol have been improved by quercetin and fish n-3 fatty acids. It was also found that protection was provided by increasing SOD, CAT and GSH-Px activities in groups administered quercetin, fish n-3 fatty acids and quercetin+fish n-3 fatty acids, and by decreasing the levels of MDA and NO in groups administered both quercetin and fish n-3 fatty acids together. These results suggest that quercetin and fish n-3 fatty acids are beneficial agents to reduce testicular injury induced by ethanol except for testosterone levels.

Introduction

Ethanol is among the most widely abused drugs (Sivasankaran *et al.*, 2008). Ethanol diffuses into all tissues due to its solubility in both water and lipids and affects most vital functions of the body (Lieber, 2005). Numerous studies have shown the hampering effects of ethanol on the hypothalamohypophysial–testicular axis (Srikanth *et al.*, 1999). Ethanol shows toxic effect to testicular tissue, and its chronic use leads to both endocrine and reproductive failure (Rosenblum *et al.*, 1989; El-Sokkary *et al.*, 1999).

Both clinical and experimental studies have shown that chronic ethanol abuse leads to testicular atrophy, feminisation, impaired steroidogenesis, infertility, low sperm count, impaired sperm motility, impotence, loss of libido, premature or delayed ejaculation, decreased testosterone production, increased serum estradiol levels and gynecomastia (Cicero *et al.*, 1980; Van Thiel *et al.*, 1980; Esquifino *et al.*, 1989; Villalta *et al.*, 1997; Srikanth *et al.*, 1999; Zhu *et al.*, 2000; Quintans *et al.*, 2005; Maneesh *et al.*, 2006).

Histological examinations of the testes of chronic alcoholics have revealed decreased diameter of the seminiferous tubules, resulting primarily from the loss of germ cells (Van Thiel *et al.*, 1975). In addition, ethanol abuse leads to histopathological changes such as vacuolisation and lipid droplets in seminiferous tubules and germ cells, tubular degeneration and atrophy, irregular diameter of the seminiferous tubules, interstitial dilated blood vessels, diminished Sertoli, Leydig and germ cell counts (Kurus *et al.*, 2009; Martinez *et al.*, 2009).

Chronic high doses of alcohol consumption result in many pathophysiological changes in cellular function caused by the alcohol itself and by the effects of its metabolism like acetaldehyde, nicotinamide adenine dinucleotide, free radicals and oxidative stress (Amanvermez *et al.*, 2005). Free radical formation and lipid peroxidation are

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potentially important mediators in testicular physiology and toxicology (Peltola *et al.*, 1996). Testicular mitochondrial membranes enriched with polyunsaturated lipids may be the principally affected sites for putative peroxidation injury and contribute to the testicular membrane injury, resulting in gonadal dysfunction, common in alcohol abuse and chronic use (Villalta *et al.*, 1997). Previous studies have shown that ethanol exposure may result in increased oxidative stress with formation of lipid peroxides and free radicals (Grattagliano *et al.*, 1997; El-Sokkary *et al.*, 1999; Oh *et al.*, 2002; Maneesh *et al.*, 2006). Ethanol-induced oxidative stress is linked to the metabolism of ethanol (Zima *et al.*, 2001). Deleterious action of ethanol involves the *in situ* biotransformation to acetaldehyde (Quintans *et al.*, 2005). Ethanol metabolism produces an oxidative stress within the testes, which is documented by an increase in testicular malondialdehyde (MDA) and a reduction in testicular glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) levels (Grattagliano *et al.*, 1997; Maneesh *et al.*, 2006; Kurus *et al.*, 2009).

Oxidative stress is suggested to be the main factor that leads to apoptosis in male germ cells and thus causes male infertility (Aitken & Baker, 2006). Apoptosis of testicular germ cells occurs under physiological conditions and serves to prevent overproduction of normal germ cells and maturation of abnormal germ cells (Kerr, 1992; Bartke, 1995). In rats, there is evidence that chronic and acute ethanol exposure of adults can increase testicular germ cell apoptosis (Zhu *et al.*, 2000; Koh & Kim, 2006).

Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid (ALA) are known as omega-3 polyunsaturated fatty acids (n-3 PUFA). Omega-3 (n-3) is an essential fatty acid (EFA, C20:5n-3) found in large amounts in fish oil. It contains EPA (C20:5n-3), and DHA (C22:6n-3). ALA is found in vegetable sources such as soybean and linseed oil (Sarsilmaz *et al.*, 2003). After certain enzyme-catalysed chain reactions, EPA can be produced from ALA and then DHA can be produced from EPA, but only 5% of ALA can enter this metabolic pathway. On the other hand, the tissue EPA level increases when ALA is taken in the diet, while the DHA level is not changed (Mantzioris *et al.*, 2000). Previous studies have shown that fish n-3 fatty acids have antioxidants, anti-inflammatory and anti-apoptotic properties (Zararsiz *et al.*, 2006a,b; Calder, 2010).

Flavonoids are polyphenolic compounds that occur in a variety of foods and beverages from vegetable origin. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a prime example of such a flavonoid found in broccoli, apple and, especially, in onions, as the glycosidic form (Hertog *et al.*, 1992). Quercetin shows antioxidant, anticarcinogenic, anti-inflammatory and antiviral properties. Quercetin diminishes reactive oxygen species (ROS) and oxidative stress, inhibits xanthine oxidase (XO) and lipid peroxidation, displays free radical scavenging activity and inhibits the DNA damage caused by H_2O_2 (Da Silva *et al.*, 1998; Johnson & Loo, 2000). Quercetin might indirectly affect sperm quality through the stimulation of the sex organs, both at the cellular and organ levels (Taepongsorat *et al.*, 2008).

To date, no such studies have been performed on the antioxidant and anti-apoptotic potential of quercetin and fish n-3 fatty acids on ethanol-induced testicular toxicity. In this study, we intended to determine the possible beneficial effects of quercetin and fish n-3 fatty acids on histopathological changes, apoptosis and oxidative stress caused by ethanol administration in rat testis.

Materials and methods

Animals and treatments

The experiment was performed in accordance with the guidelines for Animal Research from National Institutes of Health, was approved by Afyon Kocatepe University Animal Experiments Local Ethics Committee (numbered as 2009/154) and was in accordance with the 'Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals prepared by the Afyon Kocatepe University Animal Experiments Local Ethics Committee'. Forty-five adult male Wistar albino rats aged 3 months and weighing 275–325 g obtained from the Laboratory of Animal Production Unit of Suleyman Demirel University were used in the experiment. The rats were kept in special cages in ventilated rooms with 21 ± 2 °C temperature, 60 ± 10% humidity, 12-h light/12-h dark cycle and fed as described. A standard diet and tap water were provided *ad libitum*.

Experimental design

Following an adaptation period of 5 days, the rats were divided into five groups of nine rats each. These groups were as follows: (i) Group A, control group (saline, 3 g kg^{-1}); (ii) Group B, ethanol (40% 3 g kg^{-1}) group (Sigma-Aldrich, absolute ethyl alcohol 99% solution, Steinheim, Germany); (iii) Group C, ethanol (40% 3 g kg^{-1}) + quercetin (270 mg kg^{-1}) group (quercetin hydrate, Sigma-Aldrich, quercetin hydrate ≥95%); (iv) Group D, ethanol (40% 3 g kg^{-1}) + fish n-3 fatty acids (400 mg kg^{-1}) group (New Life EFA S-1200; Eurocaps Limited, England, UK). The fatty acid composition of the EFA S-1200 capsule (1200 mg) includes EPA (396 mg), DHA (264 mg) and other fatty acids (60 mg); (v) Group E, ethanol (40% 3 g kg^{-1}) + quercetin (270 mg kg^{-1}) + fish

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n-3 fatty acids (400 mg kg^{-1}) group. All effective substances were administered by intragastric gavage for 8 weeks. Two rats in Group E died during the study period.

Rats were weighed at the beginning and at the end of the study. At the end of the procedure, the rats of all groups were anaesthetised with an intraperitoneal injection of ketamine–xylazine (90–10 mg kg^{-1}). All rats were sacrificed under anaesthesia. Blood sample was obtained from left ventricle of rats at the time of death. The testes of rats were removed immediately, dissected from the surroundings tissues and weighed. For biochemical analyses, the left testes were harvested and stored at −25 °C. Right testes were fixed using 4% paraformaldehyde solution with phosphate-buffered saline (PBS) for histopathological examination.

Body weight gain and relative testis weight

Body weight gain was calculated as the difference between rats' body weight measured at the beginning and at the end of the experimental protocol. The relative testis weight was calculated by dividing testis weight (g) by body weight (kg) and expressed as g kg^{-1}.

Histological examination

The testes of all rats were removed immediately after collection of the blood. The testes were dissected from the surrounding tissue and weighed. Then testicular tissue specimens were fixed in 4% paraformaldehyde solution with PBS, embedded in paraffin, sectioned at 5-μm thickness and then stained with haematoxylin and eosin (H–E). Histological specimens were examined in light microscopy (Olympus CX31; Olympus Co., Tokyo, Japan). To assess seminiferous tubules, diameters of 100 tubules from each group were randomly chosen and measured with an ocular micrometre adjusted to the microscopy. For each tubule, the diameter was measured four times and then a mean value was calculated.

Evaluation of germ cell apoptosis

TUNEL staining: The right testis was fixed in 4% paraformaldehyde solution with PBS. After fixation step, the testes were dehydrated through ethanol and xylene and embedded and blocked in paraffin. Five-micrometre-thick sagittal sections of testes were cut by a microtome and mounted on poly-L-lysine-coated glass slides. The FragEL™ DNA Fragmentation Detection Kit, Calorimetric–Terminal deoxynucleotidyl transferase (TdT) Enzyme (Cat. No. QIA33; Calbiochem, Darmstadt, Germany) was used for detection for apoptosis according to the manufacturer's instructions. Briefly, residues of digoxigenin nucleotide were catalytically added by proteinase K to the 3X–OH ends of double- or single-stranded DNA. The labelling product was visualised using diaminobenzidine (DAB), which yielded brown granules mainly localised to apoptotic cells. Following this, the sections were counterstained with 1% methyl green. Omission of TdT or digoxigenin nucleotide gave completely negative results, which were not shown.

TUNEL staining was scored in a semiquantitative manner to determine the differences between the control group and the experimental groups. The numbers of the positive staining were also recorded as absent (−), a few (±), few (+), medium (++), high (+++) and very high (++++).

Biochemical analysis

After weighing the left testis, tissues were homogenised in five volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 0.50 ml l^{-1} Triton X-100 with a homogenizator (Ultra-Turrax T 25 Basic; IKA, Staufen, Germany) for 3 min at 13,500 rpm. Nitric oxide (NO) and MDA measurements were taken at that stage of homogenisation. The homogenate was then centrifuged at 3200 × ***g*** for 30 min to remove debris. Supernatant was taken and used in the determination XO, GSH-Px and CAT activities. Then, the supernatant was extracted using ethanol/chloroform mixture (5 : 3, v/v). After second centrifugation at 3200 × ***g*** for 40 min, the upper ethanol phase was taken and used in SOD activity determination. All procedures were performed at +4 °C.

Protein determination

Tissue protein was measured spectrophotometrically according to the method of Lowry *et al.* (1951). All samples were assayed in duplicate.

SOD determination

Superoxide dismutase activity was determined according to the method of Sun *et al.* (1988), with a slight modification by Durak *et al.* (1993). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 ml of ethanol/chloroform mixture (5 : 3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as units per milligram of tissue protein (U mg^{-1} protein).

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GSH-Px determination

Glutathione peroxidase activity was measured by the method of Paglia & Valentine (1967). The enzymatic reaction in the tube, which contains the following items, NADPH, reduced GSH, sodium azide and glutathione reductase, was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Results were expressed as unit per gram of tissue protein (U g^{-1} protein).

CAT determination

Catalase activity was measured according to Aebi's method (1974). The principle of the assay is based on the determination of the rate constant k (dimension: s^{-1}, k) of the H_2O_2 decomposition at 240 nm. By measuring the absorbance changes per minute, the rate constant of the enzyme was determined. Results were expressed as k (rate constant) per gram of tissue protein (k g^{-1}protein).

MDA determination

Malondialdehyde levels, an indicator of free radical generation, which increase at the end of the lipid peroxidation, were estimated by the double-heating method of Draper & Hadley (1990). The principle of the method is the spectrophotometric measurement of the colour generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g l^{-1} trichloroacetic acid solution was added to 0.5 ml of supernatant in each centrifuge tube, and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 × ***g*** for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g l^{-1} TBA solution in a test tube, and the tube was placed in a boiling water bath for 15 min. The solution was then cooled in tap water, and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient $\varepsilon = 1.56 \times 105$ $cm^{-1}M^{-1}$) and expressed as nanomoles per gram protein (nmol g^{-1} protein).

NO determination

As the biological half-life of NO is very short and the measurement is very difficult in biological specimens (Sahin *et al.*, 2002), tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. The method for determination of testis nitrite levels was based on the Griess reaction (Cortas & Wakid, 1990). Samples were initially deproteinised with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured by spectrophotometry (UV-Pharmaspec 1700; Shimadzu) at 545 nm after the conversion of NO_2^- to NO_3^- by copperised cadmium (Cd) granules. A standard curve was established by a set of serial dilutions (10^{-8}–10^{-3} M) of sodium nitrite. Linear regression was calculated by using the peak area from nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as micromole per gram of tissue protein (μmol g^{-1} protein).

XO determination

Xanthine oxidase activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbency at 293 nm, according to Prajda & Weber's (1975) method. Tissue homogenate was incubated (50 μl) for 30 min at 37 °C in 2.85 ml of medium containing phosphate buffer (pH 7.5, 50 mM) and xanthine (0.067 mmol final concentration in each tube). The reaction was stopped by the addition of 0.1 ml 100% (w/v) of TCA, and the mixture was centrifuged at 5000 × ***g*** for 15 min. With resultant clear supernatant, absorption at 293 nm was measured against blank. A calibration curve was constructed by using 10–50 milliunits ml^{-1} concentration of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 μmol of uric acid formed per minute at 37 °C, pH 7.5, and expressed in units per gram of protein (U g^{-1} protein).

Serum testosterone assay

Blood was collected and serum was separated and stored at −20 °C for analysis. Total serum testosterone levels were measured by chemiluminescent enzyme immunoassay (Testosterone EIA Kit, Catalog No. 582701; Cayman Chemical Company, Ann Arbor, MI, USA). Results were expressed as pg ml^{-1}.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (SPSS 16.0; SPSS Inc., Chicago, IL, USA). All biochemical and histopathological data were presented as means ± SEM. Distribution of the groups was analysed with Shapiro–Wilk test. Because of the lesser number of rats in each group, the results showed abnormal distribution, so the data were considered to be nonparametric. Therefore, they were analysed using a Kruskal–Wallis *H*-test. Differences between two groups were determined with Mann–Whitney *U*-test. As diameters of seminiferous tubules were found to be normally distributed,

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within-group comparisons were made using Student's *t*-test. *P* value of <0.05 was considered statistically significant.

Results

Body weight gain, testis weight and relative testis weight

The values of the body weight gain, testis weight and relative testis weight in five groups are summarised in Table 1. In spite of the similar food intake provided by pair feeding, the mean body weight gain was significantly lower in the ethanol group in relation to the control group ($P = 0.005$). Group B animals showed the same mean body weight gain compared with Groups C, D and E.

Contrasting to this, testis weight showed a trend in opposite direction: heavier testis in ethanol group compared with control group. However, the difference in means did not reach statistical significance. Mean testis weight was significantly higher in Group B than in Groups C, D and E (*P* values 0.012, 0.019 and 0.039 for right testis, 0.031, 0.034 and 0.023 for left testis respectively) (Table 1).

Relative testis weight was significantly increased in ethanol group compared with control group ($P = 0.015$).

Histological findings

The values of the diameters of the seminiferous tubules in five groups are summarised in Table 2. Diameters of the seminiferous tubules in ethanol group were decreased compared with those of the control group ($P = 0.001$). In addition, diameters in Groups C, D and E were higher compared with Group B, but the difference was not statistically significant.

Table 2 Mean ± SEM diameters of seminiferous tubules in control (A), ethanol (B), ethanol + quercetin (C), ethanol + fish n-3 fatty acids (D), and ethanol + quercetin + fish n-3 fatty acids (E) groups

Groups	*n*	Diameters of seminiferous tubules (μm)
A	9	333.800 ± 26.875
B	9	309.750 ± 20.589[a]
C	9	311.950 ± 24.130
D	9	312.450 ± 31.571
E	7	315.100 ± 26.092

[a]Significant difference compared to control group ($P < 0.001$).

The control group showed normal histological architecture of the testis and regular morphology of the seminiferous tubules and interstitial area. Ethanol exposure caused severe histopathological changes such as degeneration, disorganisation, atrophy and vacuolisation in seminiferous tubules, intense intercellular spaces in germinative epithelium and corrugation in basal plate. The seminiferous tubules showed irregular arrangement of spermatogenic cell line and germ cell degeneration. Atrophic Leydig cells were observed. All of these changes were ameliorated by administration of quercetin and fish n-3 fatty acids in Groups C, D and E (Fig. 1).

Evaluation of germ cell apoptosis

We observed apoptotic germ cells by the TUNEL method. Within the testis of control group rats, only a few TUNEL-positive cells were observed. These results showed that the number of apoptotic cells were greater in ethanol-treated rats than in control group rats. On the other hand, the numbers of apoptotic cells were reduced in Groups C, D and E rats than in ethanol-treated rats' testis (Fig. 2). Semiquantitative comparison of the numbers of TUNEL-positive cells in testicular tissues for each group is shown in Table 3.

Table 1 Body weight gain, right and left testis weight, and relative testis weight in control (A), ethanol (B), ethanol + quercetin (C), ethanol + fish n-3 fatty acids (D), and ethanol + quercetin + fish n-3 fatty acids (E) groups

Groups	*n*	Body weight gain (g)	Right testis weight (g)	Left testis weight (g)	Relative testis weight (g kg^{-1})
A	9	23.222 ± 10.485	1.557 ± 0.132	1.576 ± 0.129	9.64 ± 1.30
B	9	10.667 ± 4.301	1.647 ± 0.084	1.666 ± 0.097	10.91 ± 0.85
C	9	10.333 ± 6.595	1.528 ± 0.102	1.544 ± 0.104	10.09 ± 0.99
D	9	10.444 ± 2.963	1.526 ± 0.113	1.545 ± 0.123	10.14 ± 1.34
E	7	13.0 ± 4.761	1.499 ± 0.132	1.512 ± 0.126	9.83 ± 1.15
P values					
A–B		0.005	NS	NS	0.015
B–C		NS	0.012	0.031	NS
B–D		NS	0.019	0.034	NS
B–E		NS	0.039	0.023	NS

The data represents mean ± SEM. NS, not significant ($P > 0.05$).

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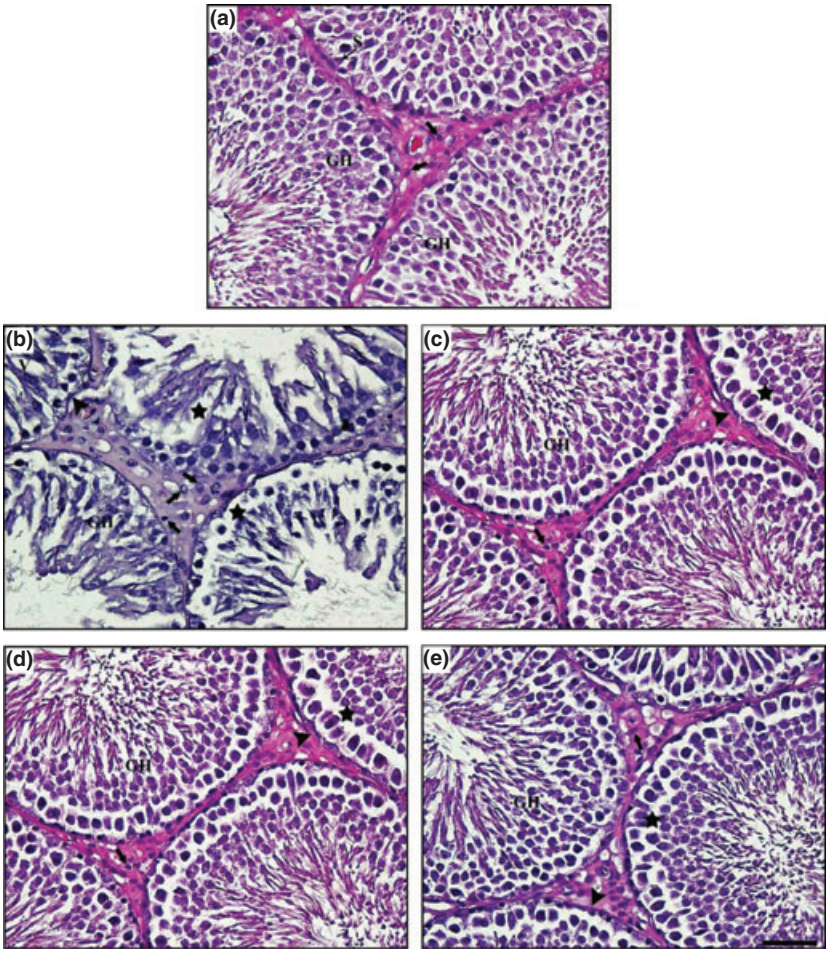


Fig. 1 Light microscopy of testicular tissue in different groups. (A) In controls, normal testicular architecture was seen; (B) after ethanol, severe testicular damage was noted; (C) ethanol + quercetin, (D) ethanol + fish n-3 fatty acids and (E) ethanol + quercetin+ fish n-3 fatty acid treatment prevented testicular damage. (GH: germinal cells, S: Sertoli cells, V: vacuoles, asterisk: decomposition, arrow: Leydig cells, arrow head: corrugation in basal plate), (H&E, magnification = X40, scale bar = 50 μm).

Biochemical analysis

Tissue levels of testicular SOD, GSH-Px, CAT, XO, MDA and NO for each group and statistical comparisons are given in Tables 4 and 5. According to these tables, SOD, GSH-Px, CAT and XO activities were significantly decreased and MDA and NO levels increased in ethanol group compared with control group (*P* values 0.047, 0.047, 0.001 and 0.001, and 0.004 and 0.024 respectively). SOD, GSH-Px and CAT activities and MDA levels were significantly increased and NO levels were decreased in Group C compared with Group B (*P* values 0.004, 0.003, 0.002 and 0.004, and 0.031 respectively). SOD, GSH-Px, CAT and XO activities and MDA levels were significantly increased in Group D compared with Group B (*P* values 0.005, 0.009, 0.005, 0.038 and 0.004 respectively). GSH-Px, CAT and XO activities were significantly increased and MDA and NO levels were decreased in Group E compared with Group B (*P* values 0.007, 0.001 and 0.001, and 0.004 and 0.004 respectively).

On the other hand, XO activities and NO levels were significantly increased in Group D compared with Group C (*P* values 0.002 and 0.005 respectively). CAT and XO activities were significantly increased and MDA levels were decreased in Group E compared with Group C (*P* values 0.003, 0.001 and 0.001 respectively) and Group D (*P* values 0.010, 0.002 and 0.001 respectively). NO levels were significantly decreased in Group E compared with Group D ($P = 0.002$).

Serum testosterone levels

A significant decrease was observed in the serum testosterone levels of ethanol group in comparison with the control group ($P < 0.05$) (Table 6). On the other hand, it was very striking that the difference between the serum testosterone values of Groups C, D and E in comparison with the Group B was significant ($P < 0.05$) (Table 6).

Discussion

Ethanol exposure is known to suppress male reproductive activity in laboratory animals and humans (Martinez

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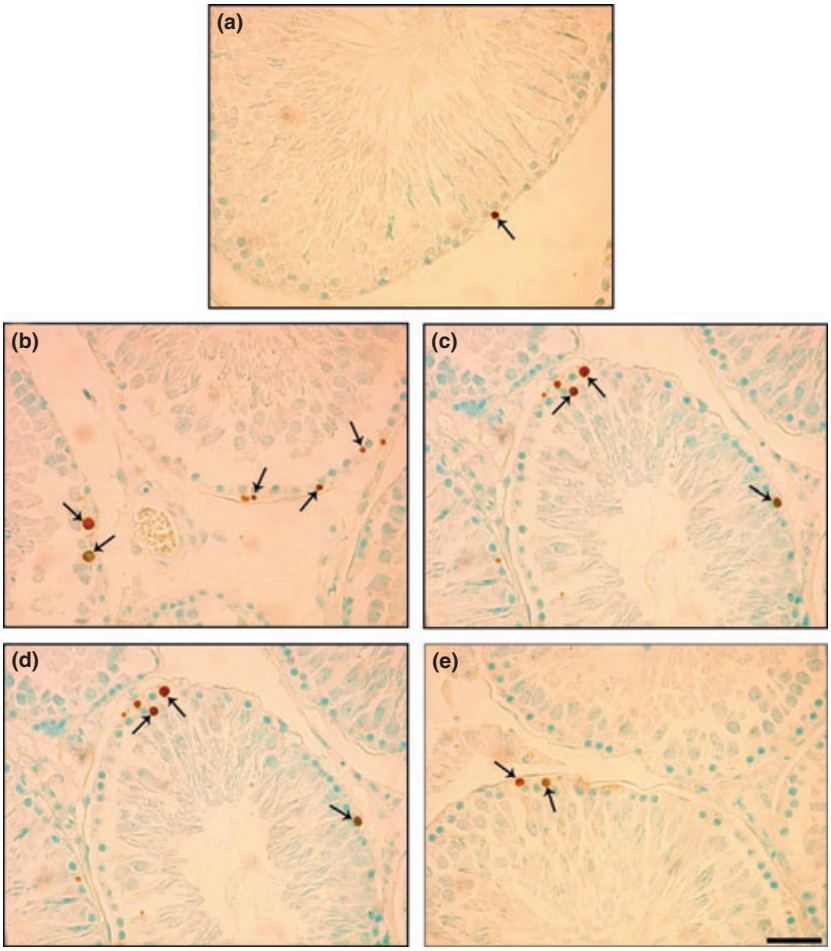


Fig. 2 TUNEL staining of testis in control (Group A), ethanol (Group B), ethanol + quercetin (Group C), ethanol + fish n-3 fatty acids (Group D) and ethanol + quercetin + fish n-3 fatty acids (Group E). A, In a control group, a few TUNEL-positive germ cells (arrow) are observed in the seminiferous epithelium. B, After ethanol administration, positivity is mainly observed in germ cells of testis. TUNEL-positive germ cells, labelled as brown nuclei, had markedly decreased in Groups C and D compared with Group B. E, Quercetin + fish n-3 fatty acid-labelled germ cells are rarely observed in the seminiferous epithelium. Scale bar = 50 μm.

et al., 2009). It has been reported that acute ethanol administration results in testicular injury (Dahlgren *et al.*, 1989), while chronic ethanol consumption results in testicular atrophy, imbalance of testosterone production, impaired reproductive performance, enhanced testicular germ cell apoptosis and increased oxidative stress and lipid peroxidation in both humans and experimental animals (Rosenblum *et al.*, 1989; Grattagliano *et al.*, 1997; Zhu *et al.*, 2000; El-Sokkary, 2001; Kurus *et al.*, 2009). In our study, we examined the effects of quercetin and fish n-3 fatty acids on ethanol-induced testicular injury.

In this study, ethanol-applied rats also had lower body weight gain when compared with control rats. Klassen & Persaud (1978) and Weinberg & Vogl (1988) presented a significantly small body weight gain in alcoholic animals. Oh *et al.* (2002) reported that isocaloric substitution of carbohydrates by ethanol for 6 weeks resulted in a decrease in body weight gain, despite similar food intake between ethanol and control groups. However, the administration of quercetin, fish n-3 fatty acids and quercetin + fish n-3 fatty acids did not restore the difference in body weight gain due to ethanol.

The literature shows controversy regarding the body weight gain and testis weight. Agreeing with our results, Salonen & Huhtaniemi (1990), Grattagliano *et al.* (1997), Martinez *et al.* (2000) and Oh *et al.* (2002) did not observe difference in testis weight between control group and ethanol group. On the other hand, Weinberg & Vogl (1988) and Maneesh *et al.* (2005) presented a significantly reduced testis weight in the alcoholic animals. Previous studies showed that rats exposed for 2 months to high doses of ethanol (5%), accounting for 36% of the total caloric intake, did not show changes in testicular weight (Salonen & Huhtaniemi, 1990). However, testicular atrophy was observed only when ethanol was administered for longer periods and when it accounted for 46% of the total caloric intake (Shirai & Ikemoto, 1992). On the other hand, testis weight was lower in quercetin, fish n-3 fatty acid and quercetin + fish n-3 fatty acids groups compared with ethanol group.

The finding that relative testis weight was higher in ethanol-consuming rats than in controls is contrary to previous data indicating that reduced relative testis weight is typically associated with ethanol consumption

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Table 3 Semiquantitative comparison of the positive staining of TUNEL cell numbers in testicular tissues for each group. Control (A), ethanol (B) and ethanol treated with quercetin (C), fish n-3 fatty acids (D), and quercetin + fish n-3 fatty acids (E)

Groups	A	B	C	D	E
TUNEL-positive germ cells	+	++++	+++	+++	++

The numbers of the positive staining were recorded as absent (−), a few (±), few (+), medium (++), high (+++), and very high (++++) (n = 9 for each group, but n = 7 for Group E).

Table 4 The results of activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in control (A), ethanol (B), ethanol + quercetin (C), ethanol + fish n-3 fatty acids (D), and ethanol + quercetin + fish n-3 fatty acids (E) groups in rats' testis

Groups	SOD (U mg^{-1} protein)	GSH-Px (U g^{-1} protein)	CAT (k g^{-1} protein)
A	1.053 ± 0.167	1.241 ± 0.237	0.229 ± 0.014
B	0.879 ± 0.095	1.021 ± 0.149	0.163 ± 0.010
C	1.155 ± 0.167	1.357 ± 0.214	0.180 ± 0.008
D	1.090 ± 0.170	1.234 ± 0.130	0.180 ± 0.014
E	0.991 ± 0.152	1.235 ± 0.077	0.198 ± 0.011
P values			
A–B	0.047	0.047	0.001
B–C	0004	0.003	0.002
B–D	0.005	0.009	0.005
B–E	NS	0.007	0.001
C–D	NS	NS	NS
C–E	NS	NS	0.003
D–E	NS	NS	0.010

The data represents mean ± SEM. NS, not significant ($P > 0.05$).

(Weinberg & Vogl, 1988). The reason for this condition is that ethanol group has less body weight gain in spite of more testis weight compared with control group. On the other hand, Oh *et al.* (2002) and Maneesh *et al.* (2005) reported no significant differences between the ethanol and the control groups in relative testis weight.

The present study shows that ethanol consumption induced histopathological and morphometric changes in rat testis. In this study, ethanol-induced testis revealed presence of marked disorganisation of germ cells, degeneration, atrophy and vacuolisation in seminiferous tubules, intense intercellular spaces in germinative epithelium, corrugation in basal plate, irregular arrangement of spermatogenic cell line and germ cell degeneration in the seminiferous tubules and atrophic Leydig cells in the interstitial region. Our findings were in agreement with previous studies on ethanol-induced testicular injury in rats (El-Sokkary, 2001; Kurus *et al.*, 2009; Martinez *et al.*, 2009). All these changes were ameliorated by the administration of quercetin, fish n-3 fatty acids and quercetin + fish n-3 fatty acids.

Table 5 The results of malondialdehyde (MDA) and nitric oxide (NO) levels and activity of xanthine oxidase (XO) in control (A), ethanol (B), ethanol + quercetin (C), ethanol + fish n-3 fatty acids (D), and ethanol + quercetin + fish n-3 fatty acids (E) groups in rats' testis

Groups	MDA (nmol g^{-1} protein)	NO (μmol g^{-1} protein)	XO (U g^{-1} protein)
A	6.159 ± 0.663	0.374 ± 0.095	0.914 ± 0.080
B	7.144 ± 0.421	0.491 ± 0.094	0.559 ± 0.097
C	8.349 ± 0.876	0.370 ± 0.099	0.515 ± 0.052
D	8.455 ± 0.837	0.547 ± 0.101	0.646 ± 0.070
E	5.597 ± 0.812	0.347 ± 0.040	0.802 ± 0.050
P values			
A–B	0.004	0.024	0.001
B–C	0.004	0.031	NS
B–D	0.004	NS	0.038
B–E	0.004	0.004	0.001
C–D	NS	0.005	0.002
C–E	0.001	NS	0.001
D–E	0.001	0.002	0.002

The data represents mean ± SEM. NS, not significant ($P > 0.05$).

Table 6 Mean ± SEM total serum testosterone levels (pg ml^{-1}) in control (A), ethanol (B), ethanol + quercetin (C), ethanol + fish n-3 fatty acids (D), and ethanol + quercetin + fish n-3 fatty acids (E) groups in rats' serum

Groups	*n*	Testosterone (pg ml^{-1})
A	9	735.667 ± 52.870
B	9	676.333 ± 46.933[a]
C	9	602.889 ± 38.985[b]
D	9	579.556 ± 55.815[b]
E	7	598.000 ± 49.227[b]

[a]Significant difference compared to control group ($P < 0.05$) using Mann–Whitney *U*-test.
[b]Significant difference compared to ethanol group ($P < 0.05$) using Mann–Whitney *U*-test.

In our study, ethanol group had decreased diameters of the seminiferous tubules compared with control group, all in conjunction with the literature (Weinberg & Vogl, 1988; Grattagliano *et al.*, 1997; El-Sokkary, 2001; Maneesh *et al.*, 2005; Kurus *et al.*, 2009). In the testicular tissue, diameters of the seminiferous tubules are used to assess histopathological damage. Seminiferous tubule atrophy and the decrease in spermatogenic cells were morphological indicators of spermatogenesis failure (Cameron *et al.*, 1985). Van Thiel *et al.* (1980) reported decreased diameter of the seminiferous tubules, resulting primarily from the loss of germ cells. The loss in germ cell number is most obvious for the more mature germ cells.

Apoptosis described as programmed cell death is a physiological phenomenon characterised by cellular morphological and biochemical alterations that cause a cell to commit suicide (Vaux & Flavell, 2000). Animal studies

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have suggested that apoptosis is a key regulator of spermatogenesis in normal and pathological states (Kerr, 1992). An altered apoptotic process has been observed to be closely associated with male infertility (Lin *et al.*, 1997). The testis is sensitive to a variety of stressors and exposure to agents that induce germ cell apoptosis (Maneesh *et al.*, 2005). In a study, ethanol was shown to produce oxidative stress within the testis and enhance cell death (Rosenblum *et al.*, 1989). Results of Koh & Kim (2006) showed that ethanol administration markedly increases apoptotic cell death in testicular germ cells. Maneesh *et al.* (2005) observed that ROS induced apoptosis in the testis primarily with spermatocytes and spermatogonia. The testis has been shown to be highly susceptible to ethanol as it crosses blood–testis barrier and depresses spermatogenesis. In the present study, apoptosis occurred predominantly in spermatogonia, the most ethanol-sensitive cell type as reported by other previous studies (Zhu *et al.*, 2000; Maneesh *et al.*, 2005). The results of our study showed that the number of apoptotic cells was greater in ethanol group rats than control group rats. The findings of Zhu *et al.* (2000) suggested that ethanol exposure enhances apoptosis of germ cells within rat testes. Ethanol increased the number of apoptotic spermatogonia as well as spermatocytes. In study of Koh & Kim (2006), the number of TUNEL-positive cells was significantly increased in the testicular germ cells of ethanol-treated rats. Ethanol-induced elevation of germ cell apoptosis may contribute to male infertility associated with ethanol abuse (Zhu *et al.*, 2000). Our findings suggested that ethanol may contribute to the suppression of male reproductive activity through an enhancement of cell death in rat testes.

The antioxidants protect germ cells against oxidative DNA damage. The number of apoptotic cells was reduced by the administration of quercetin, fish n-3 fatty acids and quercetin + fish n-3 fatty acids. Several reports have demonstrated that quercetin (Ishikawa & Kitamura, 2000; Aktoz *et al.*, 2010; Liu *et al.*, 2010) and fish n-3 fatty acids (Zararsiz *et al.*, 2006b; Ozen *et al.*, 2008) possess anti-apoptotic properties.

Reactive free radicals are known to induce apoptosis as a consequence of oxidative damage of DNA (Lotem *et al.*, 1996). ROS are regularly formed during the process of normal respiration. However, the production is kept at physiologically low levels by intracellular free radical scavengers (Sikka, 2001). Under an elevated oxidative stress status, ROS cause cellular injury via several mechanisms including lipid peroxidation and oxidative damage of proteins and DNA (Buttke & Sandstrom, 1994). Oxidative stress is recognised as a strong mediator of apoptosis. Mitochondria play an important role in the apoptotic process. The mitochondrial dysfunction induced by oxidative stress can lead to the release of cytochrome c and then caspase activation, which results in apoptotic cell death (Leon *et al.*, 2005). Oxidative stress in the testis is one of the major factors that induce germ cell apoptosis (Kasahara *et al.*, 2002).

Testicular membranes are rich in polyenoic fatty acids that are prone to undergo peroxidative decomposition, and it is likely that the resulting lipid peroxidation contributes to the membrane injury and gonadal dysfunction that occur as a result of alcohol abuse or chronic use (El-Sokkary *et al.*, 1999). The interaction of ethanol with testicular subcellular membranes contributes to ethanol-induced gonadal dysfunction in rats (El-Sokkary, 2001). Acute and chronic ethanol administration leads to increased free radical and lipid peroxidation (Bekpinar & Tugrul, 1995; Oner-Iyidogan *et al.*, 2001; Lieber, 2005; Kurus *et al.*, 2009). Quercetin can act as a ROS scavenger. It scavenges free radicals and stabilises free electrons because of its particular chemical structures including a dihydroxylated B-ring capable of readily donating electron to stabilise radical species, the C2–C3 unsaturation bond in conjugation with a 4-oxo function in the C-ring and some functional catechol groups capable of chelating transition metal ions (Rice-Evans *et al.*, 1996). It is possible that n-3 EFA was having its effect through some mechanisms besides its direct incorporation into the cell membrane. One possibility is that EPA, a member of n-3 EFA, is capable of inhibiting phospholipase A_2 enzyme. This may lead to stabilisation of membrane structure and diminishing of ROS production and lipid peroxidation (Sarsilmaz *et al.*, 2003).

Malondialdehyde is one of the products formed after lipid peroxidation and is a commonly used parameter for the determination of lipid peroxidation and oxidative damage (Songur *et al.*, 2003). In our study, MDA levels were significantly higher in ethanol group rats as compared to control group rats. This showed that ethanol consumption had caused lipid peroxidation in the testis, thus leading to oxidative damage. Previous studies showed that the testicular levels of lipid peroxide were increased by ethanol (Bekpinar & Tugrul, 1995; El-Sokkary *et al.*, 1999). El-Sokkary (2001) suggested that lipid peroxidation is a consequence of ethanol metabolism, which may be involved, at least in part, in testicular dysfunction in ethanol-treated rats. Ethanol generates oxygen radicals, increases MDA levels and generally impairs the antioxidative defence system (El-Sokkary *et al.*, 1999). The administration of fish n-3 fatty acids caused an increase in MDA levels as compared to ethanol group. It is a known fact that testicular membranes are rich in fatty acids. Therefore, it may be surmised that an extra PUFA load by fish n-3 fatty acid supplementation may increase lipid metabolism species, especially thiobarbituric

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acid-reactive substances (TBARS). Our finding agrees with other previous study. Previous studies suggested that intake of a diet high in fish oil results in high n-3 PUFA content in membrane lipids and enhances lipid peroxidation in organs and blood of experimental animals and humans (Garrido *et al.*, 1989; Van Den Berg *et al.*, 1991; Kaasgaard *et al.*, 1992; Allard *et al.*, 1997). In addition, MDA levels were significantly increased quercetin group compared with ethanol group. The findings of Liu *et al.* (2010) showed that quercetin prevented ethanol-induced oxidative damage in rat primary hepatocytes by inhibiting lipid peroxidation reaction. In our study, quercetin did not inhibit ethanol-induced lipid peroxidation in testis. This situation may be related to the dose and the duration of quercetin treatment. The administration of fish n-3 fatty acids + quercetin caused a decrease in MDA levels as compared to ethanol group. We think that fish n-3 fatty acids + quercetin have a positive effect on the structure of membrane phospholipids after ethanol administration, so the levels of MDA decreased in Group E. The reduced level of lipid peroxides by quercetin plus fish n-3 fatty acids in the ethanol-fed rat testes suggested a biological effectiveness of quercetin plus fish n-3 fatty acids against ethanol-driven disorders. Differences in the susceptibility of cells to ethanol toxicity may be explained by the difference in their activities of antioxidants and related enzymes.

To protect tissues against the deleterious effects of ROS, cells possess numerous enzymatic and nonenzymatic antioxidants. The activity of SOD with the protein environment prevents the metal-catalysed reaction between O_2 and H_2O_2 (Ozen *et al.*, 2008). In the current study, a decrease in testicular tissue SOD levels occurred after ethanol consumption in ethanol group compared with control group. The oxidative stress developed after ethanol consumption may cause this induction for SOD activities. The SOD levels were increased in Groups C and D compared with ethanol group. Additionally, the reason for equivalence in SOD levels seen in Groups A, C and D could be the maintenance of the levels of SOD by fish n-3 fatty acids and quercetin via scavenging ROS produced during oxidative stress.

Peroxides, especially H_2O_2, are metabolised by CAT. Additionally, cellular membranes, membrane and cellular proteins and DNA are protected from the lipid peroxidation (Choi-Kwon *et al.*, 2004). In other words, CAT works as an antioxidant in the cell as well as during oxidative stress. The CAT activities increase during oxidative stress. In our study, CAT activities were decreased in ethanol group compared with control group. The administration of quercetin and fish n-3 fatty acids caused a significant increase in CAT activities in Groups C, D and E as compared to ethanol group rats. The reason may be the prevention of ROS in Groups C, D and E via quercetin and fish n-3 fatty acids.

Nitric oxide is involved in the cascade of metabolic events. NO appears to be intimately involved in superoxide-mediated tissue injury. The presence of free radicals potentiates systems in the cell that may lead to increased production and release of NO. The concurrent production of mitochondrial superoxide and cytoplasmic NO leads to the rapid formation of peroxynitrite. While decreasing the steady-state concentration of superoxide, peroxynitrite itself is the major toxic product leading to extensive damage (Irmak *et al.*, 2003). The results of the present study indicate that ethanol consumption induces significant increase in peroxynitrite levels in the testis. In contrast, quercetin (Group C) and quercetin plus fish n-3 fatty acids (Group E) might reduce NO levels in ethanol consumption.

Glutathione peroxidase plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of the cell membranes, spermatogenesis and sperm morphology and motility (Maneesh *et al.*, 2005). We also analysed the capacity of testicular antioxidant defence system using glutathione after ethanol consumption. The activities of GSH-Px in the testis were decreased in ethanol group compared with control group. The administration of quercetin and fish n-3 fatty acids caused a significant increase in GSH-Px activities in Groups C, D and E as compared to ethanol group rats.

In our study, XO activities were decreased in ethanol group compared with control group. This finding is contrary to the findings of previous study. Grattagliano *et al.* (1997) showed that the testicular activities of XO were increased by ethanol. The administration of quercetin and quercetin plus fish n-3 fatty acids caused a significant increase in XO activities in Groups D and E as compared with ethanol group rats.

Husain *et al.* (2001) reported that decreased GSH-Px and SOD activities indicate the ethanol-induced oxidative stress. Several studies reported that decreased tissue SOD, CAT and GSH-Px activities and elevated MDA levels obviously reveal the damage caused by free radicals in alcoholic group (Maneesh *et al.*, 2005; Kurus *et al.*, 2009). Biochemical evaluations in our study support the literature. Our findings showed that ethanol caused a decrease in SOD, CAT and GSH-Px activities and impairs antioxidant system. On the other hand, ethanol causes oxidative damage by increasing NO level and eventually leads to lipid peroxidation, which increases MDA level. When our biochemical results are evaluated collectively, it is noted that, while quercetin or fish n-3 fatty acid treatments alone strengthen the antioxidant system, they are not successful in the prevention of oxidative damage and lipid peroxidation by ethanol. But, quercetin decreases

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NO level and protects from ethanol-induced oxidative damage. When quercetin plus fish n-3 fatty acids were administered, a considerable reduction was observed in the oxidative damage and lipid peroxidation together with decreasing NO and MDA levels. Histopathological data support this synergistic effect.

Evidence for an inhibitory role of ethanol on the hypothalamic–pituitary–gonadal axis in male rats was described in numerous studies (Cicero *et al.*, 1980; Esquifino *et al.*, 1989). Murono & Fisher-Simpson (1986) suggested that ethanol metabolism to acetaldehyde by alcohol dehydrogenase activity reduced androgen secretion by rat Leydig cells. Ethanol has direct adverse effects on Leydig cell's morphology and function (Weinberg & Vogl, 1988). *In vitro* studies using isolated Leydig cells indicated that ethanol inhibited testosterone synthesis in rats (Orpana *et al.*, 1990). In our study, testosterone was decreased in ethanol group compared with control group. Previous studies showed that ethanol treatment decreases serum testosterone in rats (Weinberg & Vogl, 1988; Shirai & Ikemoto, 1992; Emanuele *et al.*, 1999). Giltay *et al.* (2012) reported that ALA (2 g day^{-1}), EPA–DHA (400 mg day^{-1}, ratio 3 : 2) and EPA–DHA (400 mg day^{-1}) plus ALA (2 g day^{-1}) supplementation for 41 months did not affect serum total testosterone compared with placebo in men who had had a myocardial infarction. In a study, groups fed tuna oil (60 g day^{-1} for 7 months, containing 7% of EPA and 33% of DHA) had significantly lower concentrations of testosterone in testis (Castellano *et al.*, 2011). In our study, fish n-3 fatty acids did not improve ethanol-induced lower testosterone levels. Sebokova *et al.* (1990) suggested that modification of the phospholipid profile of the testicular membrane by dietary fat could affect the number of gonadotrophin receptors implicated in the regulation of steroidogenesis. Quercetin inhibited the adverse effects of several toxic agents on the testosterone levels in rats (Ciftci *et al.*, 2012; Farombi *et al.*, 2012). Ciftci *et al.*, 2012 reported that quercetin [20 mg $(kg\ day)^{-1}$ for 60 days] prevented the negative effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the testosterone levels in rats. Likewise, Farombi *et al.* (2012) showed that quercetin [10 mg $(kg\ day)^{-1}$ for 5 days] inhibited the adverse effects of cadmium on the testosterone levels in rats. However, in our study, quercetin did not improve ethanol-induced lower testosterone levels. This situation may be related to the dose and the duration of quercetin treatment.

In this study, we found that histopathological alterations in ethanol-consuming rats due to the oxidative stress and lipid peroxidation were prominent in testicular tissue. Quercetin and fish n-3 fatty acids addition to diet ameliorate these alterations mainly by radical scavenging activity and antioxidant system strengthening effect. In particular, treatment with quercetin plus fish n-3 fatty acids prior to ethanol exposure was mostly effective in face of testicular morphology except for testosterone levels. Resultantly, antioxidants in quercetin and fish n-3 fatty acids may prevent ethanol-induced testicular damage.

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