Antioxidant effects of aqueous fruit extract of *Ziziphus jujuba* on ethanol-induced oxidative stress in the rat testes

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(Received 6 Jul 2010; revised version 30 Oct 2010; accepted 23 Nov 2010)

Summary

The aim of the present study was to evaluate the antioxidant effects of aqueous fruit extract of *Ziziphus jujuba* on ethanol-induced oxidative stress and to examine its protective effects on sperm motility and plasma membrane integrity of male Wistar rats. Adult male rats (n = 28) were divided into four equal groups: the first group served as untreated controls, the second group of rats was given ethanol (4 g/kg), and group 3 received aqueous fruit extract of *Z. jujuba* (200 mg/kg). The final group of rats were supplemented by oral ingestion of ethanol (4 g/kg), which was preceded 60 min earlier by an oral administration of *Z. jujuba* extract (200 mg/kg). Fruit extract of *Z. jujuba* could prevent the reduction of concentration, motility and plasma membrane integrity of the sperm in treated rats with *Z. jujuba* extract plus ethanol ($P<0.05$). Glutathione peroxidase and superoxide dismutase activities significantly increased in the animals ingested with *Z. jujuba* extract prior to ethanol compared to the ethanol group. Decrease of thiobarbituric acid reactive substances (TBARS) level was statistically significant in the animals that ingested the fruit extract of *Z. jujuba* prior to ethanol compared to the ethanol group ($P<0.05$). Our findings suggest that aqueous fruit extract of *Z. jujuba* possesses beneficial effects on ethanol-induced sperm toxicity, subsequently enhancing sperm motility and plasma membrane integrity.

Key words: Antioxidant, *Ziziphus jujuba*, Ethanol, Rat, Testes

Introduction

It is generally accepted that excessive ethanol consumption can produce oxidative stress and induces testicular damage (Maneesh *et al*., 2005; Kasdallah-Grissa *et al*., 2006; Turner and Lysiak, 2008). With regards to this, the fatty acids are an essential requirement for the male germ cell to maintain sperm functions (Henkel, 2005). Therefore, oxidative stress in a tissue like testis, with its high rates of metabolism and cell replication, can be especially damaging. In addition, testicular cell membranes are rich with polyunsaturated fatty acids and thus susceptible to oxidative injury, which leads the spermatozoa to infertility due to defective sperm function (Kim *et al*., 2003; Turner and Lysiak, 2008).

In recent years, it has been suggested that plant origin antioxidants could be of value in the prevention of fundamental cellular disturbances resulting from alcohol-induced oxidative stress in testis and other organs (Hussein *et al*., 2006; Kasdallah-Grissa *et al*., 2006; Dahiru and Obidoa, 2007). Al-Reza *et al*. (2009) showed antioxidant effects of seed essential oil and

organic extracts from *Ziziphus jujuba* for the food industry. Also, flavonoids of *Z. jujuba* L. and *Z. spina-christi* (L.) fruits were considered for possible protective effects on human health (Pawlowska *et al*., 2009).

Fruits of *Z. jujuba* are edible and different parts of this plant possess multiple medicinal properties such as antifertility, analgesic, and antidiabetes (Al-Reza *et al*., 2010). Thus, we decided to investigate the potential antioxidant effects of aqueous fruit extract of *Z. jujuba* on the parameters of sperm motility and plasma membrane integrity by evaluation of antioxidant enzymes activity and lipid peroxidation levels following ethanol-induced oxidative stress in the rat testes.

Materials and Methods

Preparation of extract

Fresh ripened fruits of *Z. jujuba* were purchased from local herbal shops of Khorramabad, Iran during the months of October to November 2008. Fruits were authenticated at the botany department of Lorestan University. Seeds were separated from fruits and about 700 g of pulp material was extracted three times with distilled water (1500 ml totally) by grinding with a mechanical set. This was centrifuged at 4°C for 20 min at 4000 g and the supernatant was collected, lyophilized and stored at -20°C until use (Al-Reza *et al*., 2009). A solution was prepared with distilled water at a concentration of 100 mg/ml on the day of experiment.

Animals

All investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. All animals were treated humanely and in compliance with the recommendations of the Animal Care Committee for the Lorestan University of Medical Sciences (Khorramabad, Iran). The experiment was carried out on 220-250 g adult male Wistar rats (n = 28) bred in the vivarium of the Pasteur Institute in Iran. All animals were allowed free access to standard chow diet and tap water *ad libitum*. All of the rats were housed under standard conditions in a room in groups of seven rats per cage at a temperature of 21-24°C and constant 12 h light/dark cycle. All of the experimental procedures were carried out between 08.00-11.00 am.

Experimental design

The animals were divided into four groups (n = 7 per group), weight gain and food consumption were determined at weekly intervals and treated daily for eight weeks as in the order listed below:

Group I-received 2 ml normal saline (control)
Group II-received ethanol (4 g/kg)
Group III-received aqueous fruit extract of *Z. jujuba* (200 mg/kg)
Group IV-received aqueous fruit extract of *Z. jujuba* (200 mg/kg) and after 60 min, feeding with ethanol solution (4 g/kg)

Doses of ethanol and aqueous extract of *Z. jujuba* were determined by review of previous study about *Z. mauritiana* and ethanol (Dahiru and Obidoa, 2007), all of the treatments were applied orally by gavage. One day after the last gavage, the rats were killed upon diethyl ether anesthesia (May and Baker Ltd, Dagenham, UK) by decapitation. Immediately after rat killing both testes were removed and carefully cleaned of fat and adhering, then the right epididymis in all groups was separated for sperm evaluation and simultaneously all testes were stored in liquid nitrogen prior to analysis for testicular antioxidant enzymes activities and thiobarbituric acid reactive substances (TBARS) content.

Sperm evaluation

Rat spermatozoa were obtained by the method of Cancel *et al*. (2000). In brief, 5 mm of right cauda epididymis was minced in 2 ml of physiological saline and incubated at 37°C for 45 min to allow dispersion of spermatozoa. The obtained spermatozoa from the four groups were assessed for concentration, total sperm motility (TSM), forward progressive movement (FPM) and plasma membrane integrity (PMI). The concentration of spermatozoa was determined after adding of 50 µl of sperm into the 1 ml of formalin-saline to achieve a dilution rate of 1:20. Approximately 10 µl of the diluted sperm suspension was transferred

to each counting chamber of the haemocytometer and the total number of spermatozoa per ml was counted with the help of light microscope (Kheradmand *et al*., 2009a). The TSM (cells showing any kind of movement) and FPM percentage (of the motile spermatozoa showing progressive movement) were assessed according to the method as previously described by Sonmez *et al*. (2005). The fluid obtained from cauda epididymis was diluted to 2 ml with PBS and an aliquot of this suspension was placed on the microscope slide covered with a cover slip and examined visually under a phase contrast microscope (Leica, USA) at a magnification of 400. Motility estimations were performed from four different fields in each sample and the mean of the four estimations was used as the final motility score. Samples for motility evaluation were kept at 37°C. To evaluate the PMI, hypoosmotic swelling (HOS) test was applied. Assessment of functional integrity of the sperm membrane was determined by HOS-water test according to the method as described previously by Sliwa and Macura (2005). In short, 10 µl of sperm was added into 0.4 ml of distilled water and incubated for 5 min at 37°C. The swelling reaction was measured by counting of sperm with curled tail using a phase-contrast microscope at magnification of 400. All of the above examinations were performed by the same person with counting of at least 200 spermatozoa.

Analysis and measurements

Tissue preparation for enzyme assay

Rat testes were rapidly thawed and manually homogenized in cold phosphate buffer (pH = 7.4) and debris removed by centrifugation at 3500 g for 10 min (Centrifuge 5415 R; Rotofix 32A, Hettich, Tuttlingen, Germany). Supernatants were recovered and used for antioxidant enzymes activities, lipid peroxidation value and protein measurement.

Measurement of GPx activity

The activity of GPx was evaluated with Randox GPx detection kit according to the manufacturer's instructions. GPx catalyze the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) against blank at 340 nm. One unit (U) of GPx was defined as 1 µmol of oxidized NADPH per min per milligram of tissue protein. The GPx activity was expressed as milliunit per milligram of tissue protein (mU/mg protein).

Measurement of SOD activity

The activity of SOD was evaluated with Randox SOD detection kit according to the manufacturer's instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve and expressed as unit per milligram of tissue protein (U/mg protein).

Measurement of lipid peroxidation

The level of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the testis. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described by Subbarao *et al*. (1990). In short, 40 µl of homogenate was added to 40 µl of 0.9% NaCl and 40 µl of deionized H_2O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µl of cold 0.8 mol/l hydrochloride acid (HCL), containing 12.5% trichloroacetic acid (TCA). Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 h. In order to measure the amount of TBARS produced by

the homogenate, the cooled reaction was spun at 1500 g in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56×10^5/mol.cm. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Protein measurement

Protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as standard (Lowry *et al*., 1951).

Statistical analysis

The results were analyzed by SPSS 12.0 software. The homogenicity of variances were assessed using Levene static test when the variance was homogenous, the data were compared using One-Way ANOVA. To determine the differences among groups, Tukey's test was applied as post hoc (Petrie and Watson, 1999). The significant difference was set at $P<0.05$.

Results

The mean values (±SEM) of the total sperm motility, forward progressive movement, plasma membrane integrity (HOS test) percentage and sperm count of the four groups of rats are presented in Table 1. These values are significantly different between the control and ethanol-ingested groups ($P<0.05$). Although all of the measured parameters in *Ziziphus*-treated rats are generally higher than those of the control animals, these enhancements are not statistically significant ($P<0.05$). In contrast, when *Ziziphus* extract was administered prior to ethanol, it could increase the level of sperm parameters to the control levels.

Changes in antioxidant enzymes activities including GPx, SOD, as well as the TBARS level in the rat testicular tissue are shown in Table 2. Chronic administration of ethanol increased the TBARS level, while decreasing the GPx and SOD activities in the testes of rats. The mean activity of testicular GPx was significantly ($P<0.05$) lower in the ethanol-treated group compared to the control animals. Although administration of *Ziziphus* extract induced a potent increase in GPx activity, no statistically significant changes were observed in the mean level of TBARS and SOD activity in the testes of this group compared to the control group ($P<0.05$).

Pretreatment of rats with *Ziziphus* extract prior to ethanol decreased the lipid peroxidation (as indicated by the TBARS level). However, administration of *Ziziphus* extract to the ethanol-treated rats prevented the reduction of GPx activity and enhanced it to a normal level, so that there was a statistically significant difference between the ethanol-treated group and the *Ziziphus* extract plus ethanol group. Also, the activity of SOD did not remarkably alter following the pretreatment of rats with the *Ziziphus*

Table 1: Mean ± SEM percentages of sperm motility and HOS test as well as sperm concentration in the animal groups

Groups	Total motility %	Progressive movement %	HOS test %	Concentration × 10^6
Control	$61.6 \pm 3.4^{*}$	$55.4 \pm 3.65^{*}$	$65.4 \pm 2.71^{*}$	$40.8 \pm 2.22^{*}$
Ethanol	$41 \pm 2.52^{**}$	$32 \pm 2.42^{**}$	$42 \pm 2.21^{**}$	$22.8 \pm 2.67^{**}$
Ziziphus extract	$64 \pm 3.96^{*}$	$58.2 \pm 3.58^{*}$	$67.4 \pm 2.44^{*}$	$44.2 \pm 2.63^{*}$
Ziziphus extract and ethanol	$60 \pm 2.00^{*}$	$55.8 \pm 2.20^{*}$	$61 \pm 1.81^{*}$	$48.8 \pm 2.63^{*}$

*, **: Means in the same columns with different superscripts differ statistically ($P<0.05$)

Table 2: Mean ± SEM of testicular antioxidant enzymes activity (GPx and SOD) as well as TBARS level in the animal groups

Groups	GPx (mU/mg protein)	SOD (U/mg protein)	TBARS (nmol/mg protein)
Control	$144.96 \pm 9.97^{*}$	$12.97 \pm 1.12^{*}$	$28.09 \pm 3.64^{*}$
Ethanol	$90.84 \pm 7.93^{**}$	$7.65 \pm 1.30^{**}$	$48.47 \pm 4.65^{**}$
Ziziphus extract	$260.97 \pm 10^{***}$	$13.72 \pm 2.36^{*}$	$17.19 \pm 4.10^{*}$
Ziziphus extract and ethanol	$138.96 \pm 9.89^{*}$	$9.14 \pm 1.03^{**}$	$30.49 \pm 4.51^{*}$

*, **, ***: Means in the same columns with different superscripts differ statistically ($P<0.05$)

extract prior to ethanol compared to the ethanol-ingested animals.

Discussion

Our data support the hypothesis that the fruit extract of *Z. jujuba* reduces ethanol-induced oxidative stress in the rat testes. In the present study, it was found that activities of GPx and SOD were significantly decreased in the testis of ethanol-ingested rats compared to the control group and chronic ethanol consumption resulted in a significant increase in the TBARS concentration of rat testis. Indeed, pre treatment with *Z. jujuba* could promote the sperm parameters to the normal range as it was administered orally before ethanol ingestion.

The present data indicate that chronic ethanol consumption induces oxidative stress as monitored by the reduction of important antioxidant enzymes GPx and SOD activities and an increase of the lipid peroxidation level in the rat testis (Mirault *et al*., 1991; Turner and Lysiak, 2008). It also supports and extends previous reports suggesting that ethanol intoxication generally impairs the testicular antioxidant defense system and induces lipid peroxidation in experimental animals and humans (Maneesh *et al*., 2005; Kasdallah-Grissa *et al*., 2006). In this content, our finding further indicates that pre treatment of rats with fruit extract of *Ziziphus* significantly prevents the ethanol suppressive effects on the testicular antioxidant enzyme (GPx) activity and prevents an increase in TBARS level, moreover, reversing them to the normal value.

It has been suggested that the possible antioxidant activities of extracts were due to the presence of tannins (Adzu *et al*., 2001), carotenes (Guil-Guerrero *et al*., 2004) and flavonoids (Pawlowska *et al*., 2009) in some *Ziziphus* species. Carotenoids were found to inhibit free radicals-induced lipid peroxidation and β-carotene is one of the most efficient quenchers of singlet oxygen. It can also prevent lipid peroxidation by inhibiting the activity of lipoxygenase towards linoleate (Lomnitski *et al*., 1993; Hekimoglu *et al*., 2009). Quite recently, Hekimoglu *et al*. (2009) showed that lycopene (a carotene found in tomato) has a potent protective effect against ischemia-reperfusion that is induced by oxidative stress in rat testis, attributing to its ability to react with the oxygen metabolites. Therefore, it is possible that the fruit extract of *Ziziphus*, which possesses a remarkable carotenoid, attenuates ethanol-induced oxidative stress and lipid peroxidation in this study by two pathways; First, by increasing the activity of GPx and therefore rapid conversion of H_2O_2 to H_2O and preventing H_2O_2 accumulation and second, by quenching the hydroxyl radicals that trap HO leading to oxidative breakdown of the carotenoids molecule (Al-Reza *et al*., 2009). Thus, it can be concluded that the fruit extract of *Ziziphus* may protect the membrane of testicular cells against ethanol-induced oxidative damage and appears to be a good candidate in the prevention of ethanol-induced injuries in testis.

Herein, we observed a non-significant stimulation of SOD activity and a mild reduction in TBARS level in the testes of *Ziziphus* extract-treated rats compared to the control animals. Simultaneously, ingestion of *Ziziphus* extract to animals induced a significant increase in the GPx level. Our data are in agreement with previous studies that have shown the administration of antioxidants such as resveratrol, ascorbate or cocoa rich in flavanols improve testicular total antioxidant capacity and its function (Orozco *et al*., 2003; Juan *et al*., 2005). These findings confirm the fact that oxidative stress is a consistent feature of testicular physiology (Turner and Lysiak, 2008).

The present study also shows that chronic administration of ethanol resulted in sperm count decrease, impaired sperm motility and plasma membrane integrity. Our data are in accordance with previously described detrimental effects of ethanol on sperm characteristics in the treated rats (Emanuele and Emanuele, 1998; Srikanth *et al*., 1999; El-Sokkary, 2001). Fruit extract of *Ziziphus* significantly prevented the sperm quality impairment in the rats given ethanol plus extract. Recently, it has been reported that carotenoids have a protective effect against ROS mediated perturbation in sperm

quality in rats (Hekimoglu *et al*., 2009) and the relation between the oxidant/antioxidant status of testicular tissue and sperm quality has been well documented (Aurich *et al*., 1997; Ball *et al*., 2001).

In our recent studies, we proved that chronic injection of ghrelin (as a new antioxidant agent) significantly increases functional plasma membrane integrity (PMI) in rat spermatozoa and enhances antioxidant enzymes activity in the rat testes (Kheradmand *et al*., 2009a; Kheradmand *et al*., 2009b). In addition, a high positive correlation has been shown between the sperm PMI and total sperm motility (Kheradmand *et al*., 2009a). Therefore, restoration of sperm quality following pretreatment of rats with *Ziziphus* extract prior to ethanol may be attributable to antioxidant properties of *Ziziphus* extract.

In conclusion, this work provides the novel evidence that aqueous fruit extract of *Z. jujuba* has a protective effect in ethanol-induced oxidative stress in testes of Wistar rats by increasing antioxidant enzymes activity and decreasing TBARS levels. Therefore, these alterations enable enhanced sperm motility and plasma membrane integrity.

Acknowledgements

This research was financially supported by grant No.: 08760629 from the Lorestan University Research Council and Lorestan University of Medical Sciences. We are most grateful to Dr. N. Tanideh (The member of Stem Cell and Transgenic Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran) for his kind cooperation.

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