The effects of *Ziziphus jujuba* fruit extract on catalase activity and lipid peroxidation in the heart and erythrocytes of rats following chronic ethanol consumption

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Abstract

Chronic ethanol consumption leads to oxidative stress in the heart and erythrocytes of rats. As *Ziziphus jujuba* fruit has been shown to have potent antioxidants, such as flavonoids, we conducted this study to evaluate the effects of aqueous fruit extracts from *Z. jujuba* on rat hearts and erythrocytes following chronic ethanol consumption. Twenty-eight male Wistar rats were randomly divided into the following groups: control, *Z. jujuba* fruit extract treated (200 mg/kg, p.o.), ethanol (4 g/kg, p.o.) and *Z. jujuba* plus ethanol. The animals were treated orally for consecutive 8 weeks. At the end of experiment, catalase (CAT) activity and thiobarbituric acid reactive substances (TBARS) concentration (an indicator of lipid peroxidation) were measured in the heart and erythrocytes of rats. The results showed that the concentration of TBARS was significantly lower, and CAT activity higher in erythrocyte homogenates of ethanol-treated rats that were pretreated with fruit extract, compared with rats treated with ethanol alone. However, the fruit extract had no effect on TBARS concentration or CAT activity in rat heart tissue. This finding indicates that the antioxidant properties of *Z. jujuba* fruit extract protect erythrocytes against ethanol-induced oxidative stress, but are not sufficient to protect the heart.

Introduction

Chronic ethanol consumption may lead to several metabolic disorders, including hepatic and extrahepatic diseases (Lieber, 2000; Farren and Tipton, 1999). These disorders are initiated by oxidative stress, as has been found in the heart (Kasdallah-Grissa *et al*., 2006; Balasubramaniyan and Nalini, 2007) and blood erythrocytes (Wasu and Muley, 2009; Zapora *et al*., 2009), and lead to cellular damage (Nordmann *et al*., 1992). Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and their removal by available antioxidant systems (Floyd and Hensley, 2002). Ethanol-induced oxidative stress is linked directly to its metabolic pathways. Each metabolic pathway of ethanol (e.g., alcohol dehydrogenase, microsomal ethanol oxidizing system, and catalase) is able to produce ROS (Rao *et al*., 1996), and to diminish enzymatic/nonenzymatic antioxidant defence systems (Zima *et al*., 2001).

It has been shown that antioxidants can prevent ethanol-induced disturbances in the heart (Mansouri *et al*., 2001; Balasubramaniyan and Nalini, 2007, Kasdallah-Grissa *et al*., 2006) and erythrocytes (Zapora *et al*., 2009). The major antioxidant enzymes in mammals are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The heart tissue may be more susceptible to oxidative injury, as antioxidant enzyme activity in the heart is lower than in other tissues (Chen *et al*., 1994). Erythrocytes are also particularly susceptible to oxidative damage because of the high polyunsaturated fatty acid content in their membranes, and the high concentration of oxygen and haemoglobin, which is a potentially powerful promoter of oxidative processes (Cimen, 2008).

Zizyphus jujuba (*Rhamnaceae*) is widely distributed in Iran and the fruit of this plant has gained wide attention in native herbal medicine for the treatment of a broad range of disorders. Chemical analysis of the fruit has shown the presence of antioxidant chemicals such as flavonoids (quercetin and kaempferol) and phloretin derivatives (Pawlowska *et al*., 2009). The aim of the present study, therefore, was to evaluate the antioxidant effects of aqueous fruit extracts from *Z. jujuba* on CAT activity and thiobarbituric acid reactive substances (TBARS)

concentration, an indicator of lipid peroxidation, in the heart and erythrocytes of rats subjected to chronic ethanol exposure.

Materials and Methods

Preparation of extract

Freshly ripened fruits of *Z. jujuba* were purchased from local herbal shops of Khoramabad, 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 in total) by grinding with a mechanical set. The extract was centrifuged at 4°C for 20 min at 4000*g*, and the supernatant was collected, lyophilized and stored at –20°C until required. A solution was prepared with distilled water at a concentration of 100 mg/ml on the day of experiment.

Experimental design

We used 28 male Wistar rats weighing 220-250 g. These animals were kept under standard laboratory conditions with a 12-h light/dark cycle and *ad libitum* food and water throughout the experiments, which were approved by a local ethics committee. All animals were treated humanely and in compliance with the recommendations of the Animal Care Committee for the Lorestan University of Medical Sciences (Khoramabad, Iran). All experimental procedures were carried out between 0400–2000 h. The animals were randomly divided into four groups. One group received ethanol (the ethanol group), the second group was treated with the extract of *Z. jujuba* fruit (the *ziziphus* extract group), the third group received extract 30 min before the ethanol administration (the *ziziphus* extract plus ethanol group), and a control group received equivalent volumes of saline at the respective time points (the control group). Ethanol was diluted to 40% in saline and administered at a dose of 4 g/kg. Fruit extract of *Z. jujuba* was administered at a dose of 200 mg/kg. The animals were treated daily by oral gavage for eight consecutive weeks. The doses of ethanol and aqueous extract were chosen on the basis of our previous research on liver and kidney (unpublished data). In that study, after the administration of *Z. jujuba* fruit extract to different groups at the increasing doses of 50, 100 and 200 mg/kg, rats were allowed food and water *ad libitum* and all animals were observed for 72 h, for possible mortality and behavioral changes. The optimum dose with respect to antioxidant enzymes in the liver and kidney was 200 mg/kg. We did not observe any mortality up to the dose of 200 mg/kg of *Z. jujuba* fruit extract. Thus, we selected 200 mg/kg for this study.

Tissue preparation for protein measurement and enzyme assay

After 8 weeks, the animals were killed by cervical decapitation under ether anesthesia. Blood was collected from the jugular vein, and the hearts were rapidly excised, rinsed in saline to remove blood, frozen immediately in liquid nitrogen, and stored at -80°C for biochemical assays. The blood samples were centrifuged at 1000*g* for 15 min. The cells were washed three times with ice cold saline. Samples of heart and erythrocytes were stored at -70°C until required for analysis.

Measurement of lipid peroxidation

The concentration of TBARS in the heart and erythrocytes indicated the level of lipid peroxidation. Tissue TBARS was determined by following the procedure described by Subbarao *et al.* (1990). Briefly, 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 37ºC for 20 min and stopped by the addition of 600 µl cold hydrochloric acid (0.8 M) containing 12.5% trichloroacetic acid. Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4ºC for 1 h. To measure the amount of TBARS produced by the homogenate, the cooled reaction was centrifuged 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. The blanks for all TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate. TBARS results were expressed as nmol per milligram of tissue protein (nmol/mg protein).

Determination of CAT activity

Tissue CAT activity was measured according to the method of Aebi (1984). In brief, tissue sections were homogenized in triton X-100 1% (Merck, Darmstadt, Germany) and the homogenates were diluted with phosphate buffer (pH 7.0). The reaction was initiated by the addition of hydrogen peroxide (H_2O_2) to the reaction mixture and the level of enzyme activity was quantified according to the ability of tissue CAT to break down H_2O_2 by monitoring the decrease in absorbance at 240 nm against a blank contains phosphate buffer instead of substrate. The value of log A1/A2 for a measured interval was used for unit definition owing to the first-order reaction of enzyme. One unit of CAT is the amount of enzyme that decomposes 1.0 mmol of H_2O_2 per minute at pH 7.0 and 25ºC.

Protein measurement

Protein content of the supernatants for the enzyme assay, and homogenates for TBARS concentration was determined using the Lowry colorimetric method, with bovine serum albumin as the standard (Lowry *et al*., 1951).

Statistical analysis

Values are given as mean ± SEM. The differences among the four groups for each parameter were

analysed using a one-way ANOVA, followed by *Tukey*'s post-hoc test. The level of statistical significance was set at $P<0.05$.

Results

The effects of *Z. jujuba* fruit extract and ethanol on TBARS concentration in the heart and erythrocytes are shown in Table 1. The TBARS concentration was significantly higher in erythrocyte homogenates of ethanol-treated rats than in the control group, whereas, no difference was seen in the heart tissue of ethanol-treated and control rats. When rats were pretreated with the fruit extract, the effect of ethanol on the TBARS concentration in the erythrocyte was significantly attenuated ($P<0.05$).

Table 2 shows CAT activity in rat erythrocytes and heart after administration of ethanol and *ziziphus* extract. A statistically significant ($P<0.05$) decrease in CAT activity in the erythrocytes and heart was observed after ethanol administration, whereas CAT activity was increased in the control group. Administration of *ziziphus* fruit extract before ethanol prevented the decrease in CAT activity in erythrocytes of *ziziphus* and ethanol-treated rats, whereas, no change was observed in the heart tissue.

Table 1: TBARS concentration (mean ± SEM) in erythrocytes and heart from control, ethanol and *Z. jujuba* fruit extract-treated rats

	Heart nmol/mg protein	Erythrocytes nmol/mg protein
Control	1.89±0.15[a]	7.67±0.62[a]
Ethanol	2.05±0.26[a]	10.54±0.46[b]
Z. jujuba fruit extract	1.24±0.09[b]	3.32±0.35[c]
Z. jujuba fruit extract plus ethanol	1.64±0.08[a,b]	4.27±0.39[c]

Means with different superscripts (a, b, c) within each column are significantly different ($P<0.05$).

Table 2: CAT (mean ± SEM) activity in erythrocytes and heart from control, ethanol and Z. jujuba fruit extract-treated rats.

	Heart U/mg protein	Erythrocytes U/mg protein
Control	23.82±0.47[a]	16.35±0.90[a]
Ethanol	34.36±0.81[b]	9.90±0.94[b]
Z. jujuba fruit extract	24.01±0.83[a]	19.88±1.02[c]
Z. jujuba fruit extract plus ethanol	33.06±1.01[b]	17.39±0.55[c]

Means with different superscripts (a, b, c) within each column are significantly different ($P<0.05$).

Discussion

Our data support the hypothesis that fruit extract of *Z. jujuba* can prevent oxidative effects of ethanol in rat erythrocytes, and also decrease lipid peroxidation by scavenging ROS. The observed increase in TBARS concentration of erythrocytes, as a marker of oxidative stress and lipid peroxidation (McCall and Frei, 1999) following chronic administration of ethanol is consistent with earlier reports in rats (Wasu and Muley, 2009; Zapora *et al*., 2009). The present study shows a significant increase in CAT activity in hearts from rats receiving ethanol, and indicates that ethanol consumption induces oxidative stress, as monitored by the elevation in CAT activity. In this sense, oxidative stress in ethanol-treated rats is indicated by an increase in the antioxidant enzyme CAT in our study. Therefore, it seems the increase in CAT activity of ethanol-treated rats is a compensatory mechanism that upregulates the enzyme and its activity against destructive effects of ethanol. This finding is consistent with several studies that revealed an increase in myocardial CAT activity after chronic administration of ethanol in rats (Zhang *et al*., 2003; Antonenkov and Panchenko, 1988). Recent evidence has shown that transgenic mice with cardiac overexpression of antioxidant catalase displayed preserved cardiac mechanical function against ethanol-induced damage (Zhang *et al*., 2003). The probable role of catalase in the pathogenesis of alcoholic cardiomyopathy is questionable, as catalase seems to have a minor role, compared with GPx, in the detoxification of H_2O_2 in the rat heart (Simmons and Jamall, 1989). Administration of *Z. jujuba* extract did not prevent the increase in cardiac CAT activity in the *Z. jujuba* plus ethanol group. By contrast, erythrocyte CAT activity in the *Z. jujuba* group was significantly higher, compared with the other groups, because mammalian erythrocytes have large amounts of CAT activity (Carone *et al*., 1993). In this study we found a mild, but not statistically significant increase in heart TBARS concentration in ethanol-fed rats, compared with the control group. This finding is in contrast with other studies showing that chronic intraperitoneal or intragastric administration of ethanol at doses of 3-6.3 g/kg daily leads to a significant increase in TBARS levels in murine heart (Kasdallah-Grissa *et al*., 2006; Balasubramaniyan and Nalini, 2007). As lipids or lipid metabolism seems to have a primary role in the changes in membraneous fatty acid composition (Ribiere *et al*., 1992), it may be suggested that the discrepancies between the data previously reported and our data concerning ethanol-induced changes in heart lipid peroxidation are linked to the much higher dietary lipid level in the previous reports as compared with the present finding. The finding that changes in heart TBARS concentration observed in these rats was not significant could also result, at least partly, from an ethanol-induced increase in myocardial aldehyde dehydrogenase activity (Remla *et al*., 1991), an enzyme which uses malonaldehyde as substrate although, we did not measure its activity in this study. It has been revealed that ethanol metabolism is accompanied by formation of free radicals and by changes in antioxidant status, which lead to induction of oxidative stress that is dangerous to erythrocytes (Zima *et al*., 2001). ROS, such as superoxide, H_2O_2 and hydroxyl radicals, along with a compromised antioxidant capacity contributes to the extensive damage to cellular carbohydrates, proteins, lipids and nucleic acids (Paradis *et al*., 1997).

Erythrocytes are particularly susceptible to oxidative damage because of the high polyunsaturated fatty acid content in their membranes and the high concentration of oxygen and haemoglobin, the latter being a potentially powerful promoter of oxidative processes (Cimen, 2008). The reactions of ROS, such as superoxide radicals and H_2O_2 with haemoglobin, destabilize the haem and globin structure and release free iron ions that have a significant role in the further generation of ROS. Both haem compounds and free iron ions can be active catalysts of lipid peroxidation processes. Moreover, a direct increase in membrane permeability induced by ethanol may cause an increased susceptibility to lipid peroxidation (Bogdanska *et al*., 2005). Our data indicate that chronic alcohol ingestion decreased CAT activity in erythrocytes. A decrease in CAT activity in erythrocyte was also reported in rats chronically intoxicated with ethanol (Wasu and Muley, 2009; Zapora *et al*., 2009). Mammalian erythrocytes have large amounts of CAT, which is a haem-containing enzyme that catalyses the conversion of H_2O_2 to water and oxygen (Carone *et al*., 1993). CAT has a dual function: it has a catalytic role in the decomposition of H_2O_2 (involvement in natural enzymatic antioxidant defence system), and a peroxidic role in which the peroxide is used to oxidize a range of hydrogen donors (Temel *et al*., 2002). Erythrocytes seem to have high CAT activity, compared with other cells, because of the high exposure of erythrocytes to molecular oxygen. The diminution in erythrocyte CAT activity during chronic ethanol intoxication may be caused by many factors. It may be caused by inhibition of protein molecule biosynthesis, which was earlier observed in ethanol intoxication (Bengtsson *et al*., 1984). Both ROS and acetaldehyde, which are produced through ethanol metabolism, can readily react with amino and sulfhydryl groups of protein molecules (Dean, 2008) and thus disturb their structure. All the above reactions lead to changes in the structure and function of protein molecules, including enzymes. If the decrease in the erythrocyte antioxidant abilities and accumulation of TBARS in ethanol intoxication acetaldehyde and ROS (Zapora *et al*, 2009), antioxidant preparations can be used to prevent these deleterious effects. In this study, it was further observed that administration of aqueous fruit extract of *Z. jujuba*, significantly prevented the ethanol-induced changes in the CAT activity ($P<0.05$, 43%) and TBARS concentration ($P<0.05$, 59.4%) in erythrocytes. These effects may be attributable to the antioxidant activity. *Z. jujuba* fruit contains a large amount of polyphenols, mainly quercetin, kaempferol and phloretin, which possess strong antioxidant properties (Pawlowska *et al*., 2009). This is why the *Z. jujuba* given with ethanol may directly and indirectly prevent changes in erythrocytes caused by alcohol metabolism. Zapora *et al*., (2009) suggested that phenolic antioxidants attenuate the effects of ethanol on lipid peroxidation and CAT activity in rat erythrocytes. Quercetin and its derivatives are known scavengers of superoxide anion free radicals. The mechanism of their scavenging free radicals and inhibiting lipid peroxidation is by chelating transition metal ions to inactive ion compounds, inhibiting biochemical reactions of ions in the formation of many free radicals, in which the iron ion compounds still maintain free radical scavenger activity (Kuo *et al*., 1998). It has been clearly indicated that lipid peroxidation significantly increases by accumulation of H_2O_2 (a ROS) in a concentration-dependent manner (Garcia *et al*., 2005). CAT can decompose H_2O_2 to water. Thus, it seems that the increase of CAT activity by *Z. jujuba* extract in this study causes more rapid conversion of H_2O_2 to H_2O and preventing H_2O_2 accumulation and availability to shift for lipid peroxide production. This is further supported by the fact that administration of *Z. jujuba* fruit extract lowered the concentration of TBARS in the erythrocytes of rats that received ethanol. On the other hand, flavonoids preferentially enter the hydrophobic core of the membrane where they exert a membrane stabilizing effect by modifying the lipid packing order (Arora *et al*., 2000). Consequently, the slower pace of free radical reactions leads to inhibition of lipid peroxidation (Tsuchiya, 1999).

In conclusion, our findings demonstrate that *Z. jujuba* fruit extract increases CAT activity and protects erythrocytes against lipid peroxidation during chronic ethanol intoxication. These effects may be attributable to the antioxidant properties of flavonoids that are present in the *Z. jujuba* fruit extract.

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