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ORIGINAL ARTICLE

Hippophae rhamnoides attenuates nicotine-induced oxidative stress in rat liver

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Abstract

The effects of vitamin E and *Hippophae rhamnoides* L. (Elaeagnaceae) extract (HRe-1) on nicotine-induced oxidative stress in rat liver were investigated. Four groups, eight rats each, were used in this study, and the supplementation period was 3 weeks. The groups were: nicotine (0.5 mg/kg/day, intraperitoneal (i.p.)); nicotine plus vitamin E (75 mg/kg/day, intragastric (i.g.)); nicotine plus HRe-1 (250 mg/kg/day, i.g.); and the control group. The malondialdehyde and nitric oxide levels, glutathione peroxidase, glutathione *S*-transferase, glutathione reductase, superoxide dismutase, and total and non-enzymatic superoxide scavenger activities were measured spectrophotometrically in supernatants of the tissue homogenates. Nicotine increased the malondialdehyde level in liver tissue compared with control. This nicotine-induced increase in lipid peroxidation was prevented by both vitamin E and HRe-1. Superoxide dismutase activity was higher in the nicotine plus vitamin E-supplemented group compared with nicotine and control groups. Glutathione reductase activity was higher in the nicotine group compared with the control group. However, glutathione peroxidase activity in the control group was higher than the levels in the nicotine, and the nicotine plus HRe-1 supplemented groups. The nitric oxide level was higher in the nicotine group compared with all other groups. Total and non-enzymatic superoxide scavenger activities and glutathione *S*-transferase activity were not affected by any of the treatments. Our results suggest that *Hippophae rhamnoides* extract as well as vitamin E can protect the liver against nicotine-induced oxidative stress.

Keywords: Hippophae rhamnoides L.*; liver; nicotine; nitric oxide; oxidative stress; vitamin E*

Introduction

It is well known that nicotine, a major toxic component of cigarette smoke, induces oxidative stress *in vitro* (Yildiz et al., 1999) and *in vivo* (Helen et al., 2000; Kalra et al., 1991). Increased lipid peroxidation has been reported in Chinese hamster ovary cells (Yildiz et al., 1999), pancreatic tissue of rats (Wetscher et al., 1995) incubated with nicotine, and in liver, lung, and heart tissues (Helen et al., 2000), and also in brain tissue (Bhagwat et al., 1998) of intraperitoneal nicotine-administered rats. Increased lipid peroxidation is also reported in the blood of smokers (Altuntas et al., 2002; Kalra et al., 1991). In addition, transdermal nicotine patches used in the treatment of tobacco dependence in smokers can cause poisoning among children and adults (Ebbert et al., 2007; Woolf et al., 1996). It seems that people who smoke are exposed to nicotine-induced oxidative stress. In addition, smokers consume fewer green vegetables and fruits, which are rich in antioxidants, than non-smokers in both sexes (Beser et al., 1995). In a meta-analysis, it has also been found that smokers declare lower intakes of polyunsaturated fat, fiber, vitamin C, vitamin E, and β-carotene than

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non-smokers (Dallongeville et al., 1998). These differences in dietary habits may further exacerbate the nicotine-induced oxidative stress in smokers. Oxidative stress is considered to take part in the pathogenesis of various diseases, including alcoholic and non-alcoholic liver diseases, cancer, and diabetes (Gawrieh et al., 2004; Gul et al., 2000; Lieber, 2004; Messina, 1991; Vendemiale et al., 1999).

Vitamin E is an effective lipid-soluble, chain-breaking antioxidant, protecting cell membranes from peroxidative damage (Brigelius-Flohe & Traber, 1999). It is shown that vitamin E can also counteract against nicotine-induced lipid peroxidation in animals (Helen et al., 2000) and in humans (Brown et al., 1996). On the other hand, *Hippohae rhamnoides* L. (Elaeagnaceae) is a perennial plant native to Europe and Asia (Rousi, 1971) and also widely distributed in the fields of North and East Anatolia (Guliyev et al., 2004). Fruits of *Hippophae rhamnoides* have been used extensively in traditional medicine in Turkey as well as China and former Soviet republics (Guliyev et al., 2004). It contains tocopherols and tocotrienols (Andersson et al., 2008), and vitamin C (Gutzeit et al., 2008). It has antioxidant and various other pharmacological effects including antiulcerogenic, antimicrobial, radioprotective, antitoxic, and anticoagulant effects (Guliyev et al., 2004). For example, it has been reported that *Hippophae rhamnoides* extract (HRe-1) has antioxidant activity *in vitro* (Gao et al., 2000) and also *in vivo* (Cheng, 1992).

In the literature, there are studies where *Hippophae rhamnoides* extracts were used in the prevention or treatment of experimental and clinical liver injuries (Cheng, 1992; Gao et al., 2003; Liu et al., 2006). For example, the seed oil of *Hippophae rhamnoides* alleviated liver injury caused by CCl_4 in rats (Cheng, 1992; Liu et al., 2006). Also, sea buckthorn extract, which was taken for 6 months orally, shortened the duration for normalization of aminotransferases in cirrhotic patients (Gao et al., 2003). However, to the best of our knowledge, whether *Hippophae rhamnoides* extract prevents nicotine-induced lipid peroxidation in liver tissue of rats has not been studied before.

We have previously reported that HRe-1 prevents nicotine-induced lipid peroxidation in blood (Suleyman et al., 2002) but not in brain tissue (Gumustekin et al., 2003) of the rat. The aim of this study was to investigate the effects of HRe-1, and also vitamin E, on nicotine-induced oxidative stress in rat liver, specifically alterations in malondialdehyde (MDA) level as an oxidative stress parameter, and the activities of some antioxidant enzymes and also the nitric oxide (NO) level. The results of this study may have clinical implications, since HRe-1 is a non-toxic plant extract that can easily be used as a dietary supplement.

Materials and methods

Animals

Thirty-two rats (Sprague-Dawley strain with a body weight of 225 ± 28 g), fed with standard laboratory chow and water, were used in the study. They were randomly divided into four groups (eight rats per group) and placed in separate cages during the study. The groups were as follows:

- Group I: Nicotine [0.5 mg/kg/day, intraperitoneal (i.p.)];
- Group II: Nicotine [(0.5 mg/kg/day, i.p.) + vitamin E (75 mg/kg/day, intragastric (i.g.))];
- Group III: Nicotine (0.5 mg/kg/day, i.p.) + HRe-1 (250 mg/kg/day, i.g.);
- Group IV: Control group (received only the same amounts of vehicles, 0.9% NaCl solution, i.p., and corn oil, i.g.).

The supplementation period was 3 weeks. Animal experimentations were carried out in an ethically proper way by following the guidelines as set by the Ethical Committee of Atatürk University.

Preparation and administration of **Hippophae rhamnoides** *extract*

The ripe fresh fruits of *Hippophae rhamnoides* were collected from the Tortum area (altitude 1600 m), a town in Erzurum, Turkey. The fruits of *Hippophae rhamnoides* were removed from the branches, washed with tap water and dried, then crushed in a mortar and mixed. Fruit mash was placed in a glass jar and hexane was added in an equal volume. Forty-eight hours later, juice was obtained from the mixture by squeezing and centrifuging at 1000 × *g* for 15 min; clear supernatant was removed by a drip. Hexane was evaporated from the liquid by an evaporator (Rotavapor R110; Büchi, Switzerland). *Hippophae rhamnoides* extract (HRe-1, 500 mg/mL) was also mixed with corn oil (1/1, v/v), and administered orally by a stomach tube to group III for 3 weeks at 1 mL (250 mg)/kg/day.

Preparation and administration of nicotine

The hydrogen tartrate salt of nicotine (Sigma N-5260) was dissolved in 0.9% NaCl solution to obtain a 0.15 mg/mL concentration of nicotine. Then, the pH of the nicotine solution was adjusted to 7.4 by 0.1 N NaOH. Nicotine (0.5 mg/kg/day) was administered by intraperitoneal injection to groups I, II, and III for 3 weeks.

Preparation and administration of vitamin E

Vitamin E (Ephynal 300 capsule; Roche, France) was dissolved in corn oil (30 mg/mL) and administered

orally by a stomach tube (approximately 75 mg/kg/day) to group II for 3 weeks.

Sample collection

At the end of the experiment, the animals were anesthetized with ketamine-HCl (Ketalar, 20 mg/kg, i.p.). The animals were killed by exsanguination by cardiac puncture after thoracotomy. Then, liver tissue was carefully removed, rinsed in saline, and stored at –80°C until homogenization.

Preparation of supernatants for enzymes and MDA measurements

Homogenization of the liver tissue for enzyme activities and tissue MDA level

A piece of liver tissue (approximately 300 mg) was homogenized using an OMNI TH International, model TH 220 (Warrenton, VA, USA) homogenizer in 20 mM Tris-HCl, pH 7.4 (1/10 w/v) on ice for 10 s at the first speed level. Then, the homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C.

The supernatant was stored at –80°C in aliquots until biochemical measurements. Activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX), and NO and MDA levels were determined from these supernatants spectrophotometrically.

Assays of the enzyme activities

Glutathione peroxidase (GPX) activity was measured by coupled spectrophotometric assay at 340 nm from the oxidation of NADPH (reduced nicotinamide adenine dinucleotide phosphate) in the presence of H_2O_2 used as substrate (Paglia & Valentina, 1967). Glutathione *S*-transferase (GST) activity of the supernatant was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as described (Habig et al., 1974). Glutathione reductase (GR) activity was determined by coupled spectrophotometric registration at 340 nm, using glutathione disulfide (GSSG) as substrate and NADPH (Carlberg & Mannervik, 1985).

Determination of total superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), and superoxide dismutase (SOD) activity

TSSA and NSSA assays, as indicators of tissue antioxidant capacity, were performed in the samples before and after adding trichloroacetic acid (TCA, 20%), as described (Durak et al., 1998). First, TSSA is measured. In this method, the xanthine–xanthine oxidase complex produces superoxide radicals that react with nitroblue tetrazolium (NBT) to form a farmazone compound. TSSA activity is measured at 560 nm by detecting inhibition of this reaction. By using a blank reaction in which all reagents are present except the supernatant sample, and by determining the absorbance of the sample and blank, TSSA activity is calculated. Second, NSSA activity is measured in TCA-treated fractions prepared by treating part of the sample with a final concentration of 20% (w/v) TCA solution (to remove all enzymes and proteins), and centrifuging at 5000 × *g* for 30 min. After the elimination of proteins by this procedure, the NSSA activity assay is performed in the supernatant fraction. SOD activity is calculated as the difference between TSSA and NSSA (Durak et al., 1998).

Determination of the nitric oxide (NO) level

Liver tissue NO levels were measured using Griess reagent as previously described (Bories & Bories, 1995; Moshage et al., 1995). Griess reagent consists of sulfanilamide and *N*-(1-naphthyl) ethylenediamine. First, nitrate is converted to nitrite using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite to a deep purple azocompound; photometric measurement of the absorbance at 540 nm determines the nitrite concentration (sodium nitrate is used as a standard). NO levels are expressed as nmol/mg protein.

Measurement of MDA level

MDA, which is the final product of lipid peroxidation, was determined spectrophotometrically according to a similar method described (Ohkawa et al., 1979). Briefly, a mixture of 8.1% sodium dodecyl sulfate (SDS) (0.2 mL), 20% acetic acid (1.5 mL), and 0.9% thiobarbituric acid (1.5 mL) was added to the mixture to bring the total volume up to 4 mL. This mixture was incubated at 95°C for 1 h. After incubation, the tubes were left to cool under cold water, then 5 mL *n*-butanol/pyridine (15:1, v/v) was added, followed by mixing. The samples were centrifuged at 4000 rpm for 10 min. The organic phase, which accumulated at the top of the tube, was sampled, and sample absorbances were measured with respect to the blank at 532 nm. The concentration of MDA was calculated using 1.56×10^5 M^{-1} cm^{-1} as molar extinction coefficient.

Protein measurement

Protein concentration of the supernatant was measured by the Bradford method (Bradford, 1976). Biochemical measurements were carried out at room temperature using a spectrophotometer (Cecil CE 3041; Cambridge, UK).

Statistical analysis

The results given are the mean ± SD. One-way analysis of variance (ANOVA) with *post hoc* least significant difference (LSD) test was used to compare the group means, and $p < 0.05$ was considered statistically significant. SPSS for Windows (version 10.0.0) was used for statistical analysis.

Results

Nicotine increased the MDA level in liver tissue when compared with that of the normal control group. This nicotine-induced increase in lipid peroxidation was prevented by both vitamin E and HRe-1 (Figure 1).

The SOD activity was higher in the nicotine plus vitamin E supplemented group compared with the nicotine only and control groups. GR activity was higher in the nicotine group compared with the control group. However, GPX activity in the control group was higher than the levels in the nicotine, and the nicotine plus HRe-1 supplemented groups. The NO level was higher in the nicotine group compared with all other groups. Total and non-enzymatic superoxide scavenger activities and GST activity were not affected by any of the treatments (Table 1).

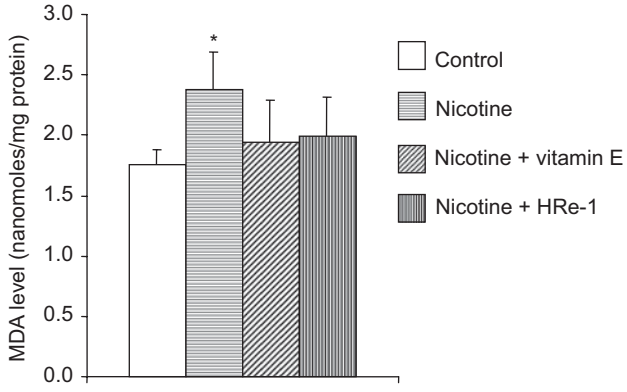

Figure 1. The effects of nicotine, and nicotine plus vitamin E or *Hippophae rhamnoides* L. extract (HRe-1) on malondialdehyde level in liver tissue of the rat. *Nicotine group vs. control ($p < 0.01$), nicotine + vitamin E ($p < 0.05$), and nicotine + HRe-1 ($p < 0.05$) groups. The results are mean ± SD. The group means were compared with one-way ANOVA with *post hoc* LSD test.

Discussion

Recent studies have reported decreased SOD, catalase, and GPX activities and also glutathione levels in liver tissue of nicotine-administered rats (s.c., 2.5 mg/kg, 5 days a week for 22 weeks) (Sudheer et al., 2008). On the other hand, the levels of inflammatory markers (nuclear factor κB (NF-κB) and cyclooxygenase-2 (COX-2) expression) were also found to be significantly increased in that study, which may be due to the excessive production of reactive oxygen species by nicotine (Sudheer et al., 2008). Reactive oxygen species are already known to upregulate the expression of NF-κB (Chen et al., 2001), which in turn is involved in regulation of the expression pattern of COX-2 mRNA. El-Sokkary et al. (2007) also reported decreased SOD activity and glutathione levels in liver of rats due to nicotine administration.

In this study, it is confirmed that nicotine induces oxidative stress in liver tissue, reflected as increased MDA level. Increased TBARS (thiobarbituric acid reactive substances), conjugated dienes, and hydroperoxide levels have been reported in heart, liver, and lung tissues of intraperitoneal nicotine-administered rats (Helen et al., 2000). Increased MDA (El-Sokkary et al., 2007), TBARS, and hydroperoxides (Sudheer et al., 2008) in liver tissue of nicotine-administered rats have also been reported. The possible mechanisms whereby nicotine increases oxidative stress include disruption of

Table 1. The effects of nicotine, and nicotine plus vitamin E or *Hippophae rhamnoides* L. extract (HRe-1) on total and non-enzymatic superoxide scavenger activities (TSSA and NSSA), antioxidant enzymes superoxide dismutase (SOD), glutathione reductase (GR), glutathione *S*-transferase (GST), and glutathione peroxidase (GPX) activities, and also nitric oxide (NO) level in liver tissue of the rat.

	Control	Nicotine	Nicotine + vitamin E	Nicotine + HRe-1
TSSA (U/mg protein)	39.93 ± 3.11	37.33 ± 5.54	43.00 ± 4.00	42.17 ± 6.27
NSSA (U/mg protein)	33.50 ± 5.26	31.17 ± 5.78	31.83 ± 2.64	32.50 ± 6.28
SOD (U/mg protein)	6.25 ± 4.79	6.25 ± 2.04	11.17 ± 3.66[a]	9.83 ± 3.60
GR (U/mg protein)	1.30 ± 0.29	1.93 ± 0.54[b]	1.74 ± 0.23	1.83 ± 0.58
GST (U/mg protein)	0.48 ± 0.15	0.70 ± 0.26	0.85 ± 0.30	0.72 ± 0.57
GPX (IU/mg protein)	0.41 ± 0.09[c]	0.32 ± 0.04	0.36 ± 0.08	0.31 ± 0.03
NO (nmol/mg protein)	5.97 ± 2.22	9.30 ± 1.35[d,e]	7.10 ± 1.42	6.07 ± 2.11

Note. Results are mean ± SD. Group means were compared with one-way ANOVA with *post hoc* LSD test.
[a]Nicotine + vitamin E vs. nicotine and control, $p < 0.05$.
[b]Nicotine vs. control, $p < 0.05$.
[c]Control vs. nicotine and nicotine + HRe-1, $p < 0.05$.
[d]Nicotine vs. nicotine + vitamin E, $p < 0.05$.
[e]Nicotine vs. nicotine + HRe-1 and control, $p < 0.01$.

the mitochondrial respiratory chain, leading to leakage from the electron transport chain in cardiomyocytes of rabbits (Gvozdjakova et al., 1992), depletion of cellular glutathione level in Chinese hamster ovary cells (Yildiz et al., 1999), and decreased activities of catalase and SOD in various tissues of the rat (Helen et al., 2000). It seems that superoxide anions and hydrogen peroxide are the main source of nicotine-induced free radical production, which depletes the cellular glutathione level, which has a central role in antioxidant defense in the cell (Gul et al., 2000).

Both vitamin E and HRe-1 protect the liver against nicotine-induced oxidative stress in our present study. Prevention of the increase in lipid peroxidation in liver tissue of intraperitoneal nicotine-injected rats by vitamin E supplementation has been reported (Helen et al., 2000). However, to the best of our knowledge, prevention of nicotine-induced lipid peroxidation in liver tissue by HRe-1 extract is reported for the first time in this study. It was also reported that seed oil of *Hippophae rhamnoides* markedly inhibited MDA formation in the liver induced by CCl_4, acetaminophen, and ethyl alcohol toxicity (Cheng, 1992). It also prevented the depletion of GSH in damaged liver induced by acetaminophen in the same study (Cheng, 1992). This glutathione-preserving effect of *Hippophae rhamnoides* could have taken part in the prevention of nicotine-induced oxidative stress in liver tissue of the rat in our present study. Flavonols such as quercetin and isorhamnetin, tocopherols such as α-tocopherol and β-tocopherol, carotenoids such as α-carotene and β-carotene, and vitamin C present in *Hippophae rhamnoides* could have taken part in the antioxidant effects of this plant extract (Andersson et al., 2008; Guliyev et al., 2004; Gutzeit et al., 2008).

Increased NO levels in the liver might have taken part in nicotine-induced oxidative stress in this study. Increased GR activity may be a compensatory increase against nicotine-induced oxidative stress. Increased SOD activity would have taken part in the prevention of nicotine-induced oxidative stress in the vitamin E supplemented group. Although not reaching statistical significance ($p = 0.094$), this increase might also have taken part in the prevention of nicotine-induced oxidative stress in the HRe-1 supplemented group.

Our results demonstrate that both vitamin E and HRe-1 can attenuate nicotine-induced oxidative stress in the liver. Ethanol and water extracts of sea buckthorn (*Hippophae rhamnoides*) were found to be non-toxic, and the LD_{50} value was reported to be more than 5 g/kg (Vijayaraghavan et al., 2006) and 10 g/kg (Saggu et al., 2007). Therefore, it is suggested that *Hippophae rhamnoides* extract as well as vitamin E can be used as a dietary supplement in order to prevent nicotine-induced oxidative stress in smokers.

Declaration of interest

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