Protective Effect of Red Beetroot against Carbon Tetrachloride- and *N*-Nitrosodiethylamine-Induced Oxidative Stress in Rats

MAŁGORZATA KUJAWSKA,[†] EWA IGNATOWICZ,[‡] MAREK MURIAS,[†]
MAŁGORZATA EWERTOWSKA,[†] KATARZYNA MIKOŁAJCZYK,[§] AND
JADWIGA JODYNIS-LIEBERT*,[†]

Department of Toxicology, Poznań University of Medical Sciences, 30 Dojazd Str.,
60-631 Poznań, Poland, Department of Pharmaceutical Biochemistry, Poznań University of Medical
Sciences, 4 Świecickiego Str., 60-761 Poznań, Poland, and Department of Fruit and Vegetables
Technology, Poznań University of Life Sciences, 32 Wojska Polskiego Str., 60-624 Poznań, Poland

The aim of the study was to investigate the potential protective effect of beetroot juice in a model of oxidative stress induced by *N*-nitrosodiethylamine (NDEA) and carbon tetrachloride (CCl_4). Male Wistar rats were treated with beetroot juice *per os*, 8 mL/kg/day for 28 days, and a single i.p. dose of the xenobiotics: 150 mg/kg NDEA or 2 mL/kg CCl_4. Simultaneously, two groups of rats not pretreated with juice were given only each of the xenobiotics. The level of microsomal lipid peroxidation in the liver, expressed as TBARS concentration, was increased several fold in rats administered only NDEA or CCl_4. TBARS were decreased by 38% only in rats pretreated with beetroot juice before the administration of CCl_4. In animals pretreated with juice and receiving NDEA, a further increase in TBARS occurred. All of the investigated antioxidant enzymes were inhibited by the administration of either toxicant alone by 26%–77% as compared to controls. Pretreatment with juice caused a partial recovery in the activity of glutathione peroxidase and glutathione reductase, by 35% and 66%, respectively. Superoxide dismutase activity was increased about 3-fold in animals pretreated with juice. Both xenobiotics caused a rise in plasma protein carbonyls, which were reduced by 30% in rats pretreated with juice and then injected with NDEA. Similarly, DNA damage in blood leukocytes caused by either toxicant was slightly diminished, by 20%, in the rats treated with juice before NDEA administration. It could be concluded that pretreatment with beetroot juice can counteract, to some extent, xenobiotic-induced oxidative stress in rats.

KEYWORDS: Rat; red beetroot; oxidative stress; *N*-nitrosodiethylamine; carbon tetrachloride; lipid peroxidation; protein carbonyls; antioxidant enzymes; comet assay

INTRODUCTION

Chemical-induced liver injury depends mostly on the oxidative stress in hepatic tissue and underlies the pathology of numerous diseases, including cancer. There is still a lack of effective therapeutics; hence, a treatment with antioxidants has been proposed for prevention and/or attenuation of injury. Such an approach has been defined as chemoprevention, and a large body of evidence from various experiments has supported its efficacy (*1*).

Human diet has been for long known as a rich source of structurally diversified active chemicals, mainly antioxidants,

e.g., vitamins and their precursors, polyphenols, and stilbenes that are bioavailable from edible fruits and vegetables. Red beetroot (*Beta vulgaris* L.) is a vegetable characteristic of the Eastern and Central European diet, and is also used as a popular folk remedy for liver and kidney diseases, for stimulation of the immune and hematopoietic systems, and as a special diet in the treatment of cancer (*2, 3*). Besides other active chemicals, beetroots contain a unique class of water-soluble, nonphenolic antioxidants, the betalains, including two classes of compounds, red betacyanins (principally betanin) and yellow betaxanthines (*2*). The antioxidant effects of betalains have been demonstrated mainly in various in vitro experiments (*2*). To date, very few investigations on beetroot activity have been performed in vivo. In an early study by Kapadia et al. (*4*), a commercial extract (betanin) was demonstrated to inhibit two-stage mouse lung and skin carcinogenesis. The same authors (*3*) investigated the cancer chemopreventive potential of betanin in three different experi-

* Corresponding author. Phone/Fax: 048 61 8470721. E-mail: liebert@ump.edu.pl.
† Department of Toxicology, Poznań University of Medical Sciences.
‡ Department of Pharmaceutical Biochemistry, Poznań University of Medical Sciences.
§ Poznań University of Life Sciences.

Protective Effect of Red Beetroot against Oxidative Stress

J. Agric. Food Chem., Vol. 57, No. 6, 2009 **2571**

mental skin and liver tumor models in mice. They showed that coadministration of betanin with model carcinogens exhibited a chemopreventive effect on experimental carcinogenesis. The effect was evident as a significant reduction in tumor incidence, multiplicity, and delay in tumor latency period. Agarwal et al. (5) reported on the hepatoprotective activity of beetroot ethanolic extract against CCl_4-induced liver injury in rats as assessed on the basis of routine serum markers of liver function: alanine aminotransferase and alkaline phosphatase activities, and the concentration of cholesterol and triglycerides.

The fraction of the ingested dose of betalains excreted unchanged into the human urine is extremely low. Kanner et al. (2) found that the amount of betanin excreted in human urine was 0.5%−0.9% of the dose ingested. The amount of betalains recovered in the urine of other volunteers receiving red beet juice was 0.28% of the dose. This suggests that either the betalains are only slightly bioavailable or that other pathways of betalain elimination may exist such as biliary excretion, enterohepatic circulation, and metabolism (6). However, the absorption of betalains seems to be affected by dietary source since the renal clearance of two betalains, namely, betanin and indicaxanthin, was much higher when ingested as cactus pear fruit: 3.7% and 76%, respectively (7).

Along with non-nutritive protective constituents, diet contains toxic chemicals resulting from food processing (e.g., acrylamide and nitrosamines) and/or pollutants (e.g., aflatoxins), and these diet-linked factors are considered, besides tobacco smoking, infection, and inflammation, the major factors of cancer occurrence (8).

Nitrosamines are a large group of food- and environment-born carcinogens, and *N*-nitrosodiethylamine (NDEA), a common representative of this class of chemicals, is present in salted and smoked fish and meat, alcoholic beverages, and cigarette smoke (9). Moreover, the acidic stomach environment, nitrite/nitrate from fertilizers, and traces of pesticides and pollutants promote endogenous synthesis of nitrosamines (10). The International Agency for Research on Cancer (IARC) classifies NDEA as a 2A chemical, a possible human carcinogen (9).

The metabolic activation of NDEA by hepatic microsomal cytochrome P450 is a critical step for the expression of its toxic and carcinogenic potential (10). The product of hydroxylation, α-hydroxynitrosamine, is converted into an intermediate, an ethyl diazonium ion, which is capable of DNA alkylation. In the NDEA activation reactions, there is also involvement of NADPH-reductase, which results in reactive oxygen species (ROS) generation and oxidative stress induction (10).

Liver injury induced by carbon tetrachloride is the best characterized system of xenobiotic-induced hepatotoxicity and is a commonly used model for screening the hepatoprotective activity of natural compounds. The hepatotoxic effect of CCl_4 is a result of its reductive dehalogenation by CYP2E1 into the highly reactive trichloromethyl and trichloromethyl peroxy free radicals. Both radicals form adducts with cellular macromolecules and abstract hydrogen from different molecules, thus initiating oxidation of lipids, proteins, and DNA. Although CCl_4 is not directly mutagenic, it can exert genotoxic effects under highly cytotoxic conditions through oxidative damage or endonuclease activation combined with sustained regeneration and increased cellular proliferation. CCl_4 has been shown to induce hepatocellular carcinomas in rodents by oral, inhalation, and parenteral exposure (11). Recently, the IARC has classified CCl_4 as a potential human carcinogen (12). As oxidative stress plays a central role in NDEA- and CCl_4-induced toxicity, the use of

phytochemicals with antioxidant activity can offer protection against this oxidative damage.

In view of the findings that NDEA and CCl_4 are capable of inducing oxidative damage and share common metabolic systems involved in their activation, our current study investigated the potentially protective effect of red beetroot juice in a model of oxidative stress induced by these two xenobiotics in rats.

MATERIALS AND METHODS

Chemicals and Red Beetroot Juice. Red beetroot var. Chrobry was a gift from Experimental Vegetable Plantation Nochowo (Poland). The juice was prepared in a household juice extractor. The content of betaxanthines was 79.3 mg/100 mL and of betacyanins 159.6 mg/100 mL as determined according to the method by Nilsson (13). Antioxidant activity of the juice measured in the ABTS radical cation decolorisation assay (14) was 23.5 μmol Trolox equivalents/mL.

The reagent kit for protein carbonyl determination was purchased from BioCell Corp. Ltd. (New Zealand). Agarose (normal melting point) was purchased from Prona, USA and all other chemicals were from Sigma-Aldrich (St Louis, USA) or from local chemical suppliers.

Experimental Design. A total of 48 male Wistar rats (240 ± 10 g) bred in the Department of Toxicology, Poznań University of Medical Sciences, were divided randomly into six groups, eight animals each. The rats were housed in an animal facility at 22 ± 1 °C with a 12 h light/dark cycle, controlled humidity, and circulation of air, and fed certified (ISO 9001) laboratory feed Labofeed H.

Groups II, V, and VI were given beetroot juice by gavage, 8 mL/kg b.w./day for 28 days. Control group I and groups III and IV were given the same volume of distilled water for 28 days. On day 27, a single dose of NDEA, 150 mg/kg b.w. (groups III and V), or CCl_4, 2 mL/kg b.w. (groups IV and VI), was administered intraperitoneally. After 24 h, the rats were anesthetized by ketamine, and blood was withdrawn from the heart. A portion of whole heparinized blood was separated for the comet assay. The remaining blood was centrifuged (3000 rpm, 15 min, +4 °C), and the resulting plasma was stored at −80 °C. The livers were removed, rinsed with ice-cold 1.15% KCl, and homogenized in buffered sucrose solution (Tris, pH 7.55). Microsomal and cytosol fractions were prepared by differential centrifugation according to the standard procedure. Protein concentration in the fractions was determined using the Folin−Ciocalteu reagent. Liver homogenate for glutathione determination was prepared in phosphate buffer, pH 7.4.

The experiment was performed according to the Local Animal Ethics Committee guidelines for animal experimentation.

Biochemical Assays. Lipid peroxidation in the liver was assayed both in native microsomes and after stimulation with Fe^{2+}/ascorbate. The level of microsomal lipid peroxidation (LPO) was evaluated by measuring thiobarbituric acid reactive substances (TBARS). The results were expressed in nmol malondialdehyde per mg protein (15). Reduced glutathione was assayed in the liver homogenate with Ellman's reagent (16). Antioxidant enzymes were assayed in the liver cytosol. The superoxide dismutase (SOD) assay was based on the inhibition of spontaneous epinephrine oxidation, while catalase (CAT) activity was determined by following the rate of H_2O_2 reduction (17). Glutathione peroxidase (GPx) activity was determined according to Mohandas et al. (18), with hydrogen peroxide as a substrate. The disappearance of NADPH at 340 nm was a measure of the enzyme activity. Glutathione reductase (GR) activity was assayed by measuring NADPH oxidation at 340 nm using oxidized glutathione as a substrate (18). Protein carbonyl concentration in plasma was determined by an ELISA procedure according to the test kit manufacturer's instructions.

Alkaline comet assay in whole blood leukocytes was performed according to the protocol of Olive and Banath (19). After cell lysis, DNA unwinding, electrophoresis, and neutralization, the slides were dehydrated in absolute ethanol, dried, stored at room temperature, and protected from light. Before evaluation, the slides were rehydrated and stained with ethidium bromide. Images of comets from a Zeiss fluorescence microscope (magnification 400×) were captured with a digital camera. The comets were divided into 5 groups according to

Table 1. Effect of Red Beetroot Juice Pretreatment on Microsomal Lipid Peroxidation and Reduced Glutathione in the Liver of Rats Receiving NDEA or CCl_4

treatment	microsomal lipid peroxidation		GSH [μmol/g tissue]
	uninduced [nmol TBARS/min/mg protein]	Fe^{2+}/ascorbate -induced [nmol TBARS/min/mg protein]	
controls	0.37 ± 0.07^a	2.87 ± 0.63^a	1.45 ± 0.19^a
beetroot juice	0.43 ± 0.07	2.34 ± 0.32	1.47 ± 0.14
NDEA	$1.07 \pm 0.21^{a,b}$ [↑186]	$14.87 \pm 3.54^{a,b}$ [↑418]	$2.46 \pm 0.29^{a,b}$ [↑69]
CCl_4	$3.33 \pm 0.51^{a,c}$ [↑787]	20.93 ± 2.54^a [↑629]	1.99 ± 0.27^a [↑37]
beetroot juice + NDEA	1.39 ± 0.31^b [↑30]	21.88 ± 7.06^b [↑47]	2.03 ± 0.27^b [↓18]
beetroot juice + CCl_4	2.05 ± 0.37^c [↓138]	18.22 ± 4.40	2.01 ± 0.22

Results are the mean $\pm$ SD, $n = 8$. Control rats were given water. [a] Controls are compared with toxicant-treated groups. [b] The NDEA-treated group is compared with the juice + NDEA-treated group. [c] The CCl_4-treated group is compared with the juice + CCl_4-treated group. Values with the same superscripts are significantly different, $p < 0.05$. The values in parentheses express % of change.

Table 2. Effect of Red Beetroot Juice Pretreatment on Hepatic Antioxidant Enzymes in Rats Receiving NDEA or CCl_4

treatment	SOD [U/mg]	CAT [U/mg]	GPx [nmol NADPH/min/mg protein]	GR [nmol NADPH/min/mg protein]
controls	11.2 ± 2.7^a	11.6 ± 2.0^a	1277 ± 120^a	124.1 ± 9.5^a
beetroot juice	10.5 ± 2.0	10.8 ± 2.6	1243 ± 177	123.9 ± 12.0
NDEA	$4.5 \pm 1.0^{a,b}$ [↓60]	6.2 ± 1.0^a [↓46]	$519 \pm 101^{a,b}$ [↓59]	$28.9 \pm 6.1^{a,b}$ [↓77]
CCL_4	14.4 ± 1.6^c	$4.5 \pm 0.3^{a,c}$ [↓61]	903 ± 60^a [↓29]	$91.3 \pm 8.7^{a,c}$ [↓26]
beetroot juice + NDEA	17.6 ± 2.1^b [↑295]	6.1 ± 0.6	862 ± 108^b [↑66]	43.3 ± 9.0^b [↑48]
beetroot juice + CCl_4	19.1 ± 2.7^c [↑32]	5.4 ± 0.3^c [↑12]	952 ± 116	123.1 ± 13.8^c [↑35]

Results are mean $\pm$ SD; $n = 8$. Control rats were given water. [a] Controls are compared with toxicant-treated groups. [b] The NDEA-treated group is compared with the juice + NDEA-treated group. [c] The CCl_4-treated group is compared with the juice + CCl_4-treated group. Values with the same superscripts are significantly different, $p < 0.05$. Values in parentheses express % of change.

the degree of DNA damage (*20*). A total damage score for the slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades.
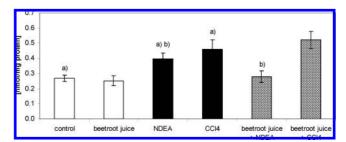
Statistical Analysis. The data were expressed as mean $\pm$ SD. One way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons were used. $P \leq 0.05$ was considered to be the limit of significance.

RESULTS

Treatment of rats with either carcinogen used in the experiment increased hepatic microsomal lipid peroxidation 3–9-fold in the uninduced assay and 5–7-fold in the Fe^{2+}/ascorbate stimulated assay, as compared to the control group (**Table 1**). In rats treated with beetroot juice before the administration of a single dose of NDEA, the level of TBARS was higher by 30% (uninduced LPO) and by 47% (Fe^{2+}/ascorbate induced LPO), as compared to the values obtained in rats given NDEA alone. Pretreatment with beetroot juice appeared to be more efficient in attenuating oxidative stress in rats receiving CCl_4. In this group, the TBARS level in the uninduced LPO assay was 38% lower than that of animals given the carcinogen alone. Fe^{2+}/ascorbate-induced lipid peroxidation was not changed by the beetroot juice pretreatment (**Table 1**).

The concentration of hepatic GSH was increased in the rats dosed with NDEA or CCl_4 by 69% and 37%, respectively, as compared to the control. Pretreatment with beetroot juice caused a slight decrease (by 18%) in GSH concentration only in rats receiving NDEA as compared to the values obtained from animals given the carcinogen alone (**Table 1**).

All investigated antioxidant enzymes were inhibited in animals treated with the carcinogens alone (except SOD in CCl_4-treated rats) by 26%–77% as compared to controls. Pretreatment with beetroot juice caused a partial recovery in GR activity by 48% and by 35% in rats given NDEA or CCl_4, respectively, and a marked recovery in GPx activity, by 66%, in rats given NDEA. SOD activity in animals pretreated with the juice and

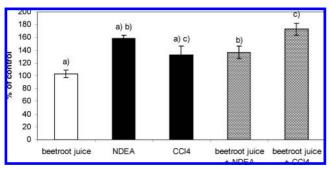
Figure 1. Effect of red beetroot juice pretreatment on protein carbonyl content in the plasma of rats receiving NDEA or CCl_4. Results are mean $\pm$ SD, $n = 8$. Control rats were given water. a) Controls are compared with toxicant-treated groups. b) NDEA-treated group is compared with juice + NDEA-treated groups. Values with the same superscripts are significantly different, $p < 0.05$.

receiving NDEA or CCl_4 was significantly increased, by 295% and 32%, respectively, and in the beetroot/NDEA group, the activity exceeded levels observed in control rats. Catalase was the only enzyme that had no activity reduction by pretreatment of rats with beetroot juice (**Table 2**).

In rats treated with NDEA or CCl_4 alone, the plasma concentration of protein carbonyls (POCs) was higher by 47% and 71%, respectively, as compared to the control group (**Figure 1**). Pretreatment with beetroot juice resulted in a 30% decrease in the POCs content in animals receiving NDEA. Pretreatment with the juice did not affect the POCs value in rats administered CCl_4.

Beetroot juice administration to rats did not cause the DNA damage in the whole blood leukocytes (**Figure 2**). In rats receiving only NDEA or CCl_4, marked DNA damage was measured in the blood leukocytes, by 58% and 33%, respectively, compared to the control group. In animals receiving beetroot juice prior to the NDEA treatment, a significant reduction of DNA damage, by 21%, was found in comparison to animals receiving NDEA alone. However, in rats treated with

Protective Effect of Red Beetroot against Oxidative Stress

J. Agric. Food Chem., Vol. 57, No. 6, 2009   **2573**

Figure 2. Effect of red beetroot juice pretreatment on DNA damage in whole blood leukocytes in rats receiving NDEA or CCl_4. Results are expressed in % of control value (sum of arbitrary points: 120). Results are mean $\pm$ SD, $n = 6$. Control rats were given water. a) Controls are compared with toxicant-treated groups, b) NDEA-treated group is compared with juice + NDEA-treated group, c) CCl_4-treated group is compared with juice + CCl_4-treated group. Values with the same superscripts are significantly different, $p < 0.05$.

beetroot juice prior to the CCl_4 exposure a 40% increase in DNA damage was observed.

DISCUSSION

In all of the above cited animal experiments, the extracts of beetroot, isolated fractions, or single betalains were used (*2−5*). In our experiment, animals were treated with the crude natural beetroot juice to mimic one of the forms in which red beet is mainly consumed, i.e., as juice, pickled, or as a cooked vegetable. Beside betalains, red beet contains small amounts of other compounds displaying remarkable antioxidant properties such as *cyclo*-dopa-glucoside, hydroxycinnamic acids, and their derivatives (*6*). Hence, simultaneous ingestion of multiple components can provide an additive or synergistic effect.

The majority of oxidative stress parameters tested in our experiment were affected by beetroot juice. Our investigation revealed that pretreatment with beetroot juice attenuated the microsomal lipid peroxidation evoked by CCl_4 in the liver of rats. The reason for the enhancement of hepatic TBARS levels induced by NDEA administration after pretreatment with the juice is not clear. It is known that redox active compounds can act as pro-oxidants under some conditions. This is particularly the case in metal ion-catalyzed system. In the healthy body, metal ions appear to be largely sequestered in forms unable to catalyze free radical reactions, whereas injury to tissues may release iron or copper (*21*). Therefore, it could be hypothesized that betalains administered for 28 days in a relatively high dose might exert a pro-oxidant effect in rats challenged with NDEA. However, such an effect was not observed in rats that received CCl_4. The likely explanation might be different mechanism of liver damage caused by each toxicant.

In our experiment, both toxicants caused an increase in hepatic GSH concentration. In the majority of reports, the depletion of liver GSH after administration of CCl_4 to rodents has been observed (*11*). However, Sheweita et al. (*22*) found about 50% increase in the GSH hepatic content in rats given CCl_4. Similarly, in the previous study we observed slight but statistically significant increase in the hepatic GSH content in rats treated with CCl_4 (unpublished data). In another report, we found no effect of CCl_4 on GSH hepatic level (*23*). These discrepancies could be due to the different routes of administration and different experimental protocols.

There are also discrepancies in reports concerning the level of hepatic GSH in NDEA-treated rats. Some authors found the

depletion of GSH (*24*), others reported a marked increase in the GSH level (*25*). Classic hepatotoxins such as CCl_4 or bromobenzene cause very significant GSH depletion soon after administration. After a phase of GSH deficiency, the level of GSH increases leading to hypercompensation (*26*). This is likely the case in the rats treated with CCl_4 and NDEA in our experiment. Pretreatment with the juice counteracted the NDEA-stimulated increase in hepatic GSH content, which may be interpreted as an effect of attenuation of oxidative stress.

Consistent with other authors' findings (*27*), the activity of all assayed antioxidant enzymes was decreased in rats given either toxicant alone, except SOD in the CCl_4-treated group. Pretreatment with the juice attenuated the decrease in SOD and GR activity, while CAT remained unaffected. The loss of GPx activity was counteracted only in rats treated with the juice and NDEA. It should be emphasized that the activity of SOD in animals pretreated with the juice and dosed with NDEA exceeded the activity observed in control animals. Treatment with the juice alone did not affect antioxidant enzymes; its action was demonstrated only in prooxidant-challenged rats.

Apart from the lipid peroxidation assay in liver tissue, we assessed oxidative damage to other macromolecules, namely, DNA damage in leukocytes and protein carbonyl groups in plasma. We intended to test whether these markers might be useful in investigations regarding the protective effect of beetroot juice on xenobiotic-induced oxidative stress. As both markers are measured relatively noninvasively, they might be applied in human nutrition intervention studies.

Protein carbonyls have a major advantage over lipid peroxidation products as oxidized proteins are generally more stable. POCs form early and circulate in the blood for longer periods (*28*). Both toxicants used in the experiment generate free radicals in the process of their biotransformation; hence, their effect on plasma proteins was similar, i.e., an increase in protein carbonyl groups. However, it is not clear why the beetroot juice was effective only against the oxidative damage of proteins evoked by NDEA and did not affect this marker in animals treated with CCl_4. It could be suggested that despite the common primary step of activation, these two xenobiotics exert effects of diverse magnitude and quality on plasma proteins.

The comet assay is a simple and sensitive method of demonstrating DNA damage. In the alkaline version (pH > 13), the assay is considered to detect strand breaks, which may result from the appearance of apurinic/apyrimidinic sites that are labile under the alkaline treatment. The breaks may also transiently occur during the cellular repair process (*19, 20*). DNA damage appearing upon the NDEA or CCl_4 treatment may have a diversified character. Besides the direct oxidation of bases, a set of other impairments may occur: ethylation (bioactivated NDEA is an alkylating agent), formation of adducts with products of lipid peroxidation, cross-linking (*29*), and endonuclease activation in the case of CCl_4 (*11*).

Thus, the extent of DNA damage observed in whole blood leukocytes of rats treated with NDEA or CCl_4 is a result of a balance between the action of xenobiotic metabolites and the activity of numerous possible counteracting systems, such as enzymatic repair of the lesions and endogenous antioxidant defense.

In hepatic DNA of animals exposed to NDEA, various forms of alkylated bases, mainly ethylguanines and ethylthymidines, were found. These adducts revealed diverse mispairing properties and susceptibility to enzymatic repair (*30*). The repair mechanism depends on the transfer of the alkyl group on the protein acceptor, the O^6-alkylguanine-DNA alkyltransferase.

2574 *J. Agric. Food Chem.,* Vol. 57, No. 6, 2009

Kujawska et al.

Alkylated forms of this enzyme protein are not stable and undergo rapid degradation; hence, the direct action of alkylating agents brings about a depletion of this repair mechanism (*31*). In our experiment, the applied dose of NDEA (150 mg/kg b.w.) was probably high enough to attenuate the cellular repair system.

Ueno et al. (*32*) suggested that ROS generated by the NDEA treatment activate the NFκB-dependent pathways of neutrophil stimulation and enhance pro-inflammatory cytokine release and NO production. As a result, these cells produce and release additional amounts of ROS, which strengthen the rate of oxidative damage. Thus, any action capable of limiting neutrophil stimulation could reduce the oxidizing effect of NDEA. Winkler et al. (*33*) found that beetroot juice was capable of reducing ROS generation in stimulated blood mononuclear cells in vitro.

Our experiment demonstrated that the intraperitoneal administration of NDEA to rats caused a significant rise in the DNA damage rate observed in the whole blood leukocytes. It might be suggested that this effect could be ascribed to the action of reactive metabolites passing from the liver to the circulatory system or to the appearance of stimulated nuclear cells in circulation. The reduction of DNA damage observed in rats receiving beetroot juice may result from the antioxidant action of beetroot.

The in vivo investigation of CCl$_4$ activity confirms hepatic DNA damage primarily due to the reactions of trichloromethyl and/or trichloromethyl peroxy radicals directly or via peroxidation products; however, the results of in vitro testing are inconsistent (*11*). Although Kadiiska et al. (*34*) did not find DNA strand breaks in rat blood leukocytes after CCl$_4$ treatment (1200 mg/kg b.w.), in our current experiment a marked rise in comet length was found in rats injected with CCl$_4$. Moreover, 28 day pretreatment with beetroot juice enhanced CCl$_4$-induced DNA damage.

As in the rats treated with beetroot juice prior to NDEA administration, a significant reduction of DNA damage was found, and the adverse direction of change in the beetroot/CCl$_4$ group may suggest different mechanisms of inducing lesions to nucleic acids, even though the two carcinogens share the initial step of metabolic activation. Hence, it cannot be excluded that antioxidants of beetroot juice are ineffective against CCl$_4$-induced damage to DNA.

The protective effect of beetroot juice was more pronounced in rats treated with NDEA than in those given CCl$_4$ as assessed by comet assay in blood leukocytes and plasma carbonyl group content, and the restored activity of some hepatic antioxidant enzymes.

It could be suggested that the damage evoked by CCl$_4$ was so severe that it could not be reversed even when the rats were pretreated with beetroot juice. However, such an interpretation is contradicted by the reduction in microsomal lipid peroxidation in the liver by pretreatment with the juice in rats challenged with CCl$_4$ but not with NDEA.

The different effects of beetroot juice on various markers of oxidative insult assayed in our experiment are not an unusual finding. According to Halliwell (*35*), biomarkers of DNA, lipid, and protein damage should be used in parallel because it is perfectly feasible for an antioxidant to alter one but not the others. There are compounds that increase oxidative DNA damage in vivo even though they are powerful inhibitors of lipid peroxidation; the carcinogen diethylstilbestrol is one of such compounds.

The protective effect of beetroot juice on macromolecules assayed in blood, namely, protein and DNA, was much weaker in comparison with alterations in parameters analyzed in the liver. Greater damage was caused by toxicants used in the target tissue, that is, liver, hence the response of surrogate markers in the blood, both to oxidative insult, and the protective action of beetroot was less pronounced.

In summary, pretreatment with red beetroot juice can counteract xenobiotic-induced oxidative stress in rats as assessed by restoration of the activity of the majority of antioxidant enzymes in the liver and, to some extent, diminishing oxidative injury of plasma protein as well as a decrease in DNA damage in leukocytes.

ABBREVIATIONS USED

b.w., body weight; CAT, catalase; ELISA, enzyme-linked immunosorbent assay; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, reduced glutathione; IARC, International Agency for Research on Cancer; ICAM-1, Inter-Cellular Adhesion Molecule-1; i.p., intraperitoneal; LDL, low density lipoprotein; LPO, lipid peroxidation; NADPH, nicotinamide adenine dinucleotide phosphate-reduced form; NDEA, *N*-nitrosodiethylamine; NFκB, nuclear factor kappa B; POCs, protein carbonyls; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Gonzales, C. A. Nutrition and cancer: the current epidemiological evidence. *Br. J. Nutr.* **2006**, *96*, S42–S45.

(2) Kanner, J.; Harel, S.; Granit, R. Betalains: a new class of dietary cationized antioxidants. *J. Agric. Food Chem.* **2001**, *49*, 5178–5185.

(3) Kapadia, G. J.; Azuine, M. A.; Sridhar, R.; Okuda, Y.; Tsuruta, A.; Ichiishi, E.; Mukainake, T.; Takasaki, M.; Konoshima, T.; Nishino, H.; Tokuda, H. Chemoprevention of DMBA-induced UV-B promoted, NOR-1-induced TPA promoted skin carcinogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot. *Pharmacol. Res.* **2003**, *47*, 141–148.

(4) Kapadia, G. J.; Tokuda, H.; Konoshima, T.; Nishino, H. Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract. *Cancer Lett.* **1996**, *100*, 211–214.

(5) Agarwal, M.; Srivastava, V. K.; Saxena, K. K.; Kumar, A. Hepatoprotective acitivity of *Beta vulgaris* against CCl$_4$-induced hepatic injury in rats. *Fitoterapia* **2006**, *77*, 91–93.

(6) Frank, T.; Stintzing, F. K.; Carle, R.; Bitsch, I.; Quaas, G.; Bitsch, R.; Netzel, M. Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans. *Pharmacol. Res.* **2005**, *52*, 290–297.

(7) Tesoriere, L.; Allegra, M.; Butera, D.; Livrea, M. A. Absorption, excretion, and distribution of dietary antioxidant betalains in LDLs: potential health effects of betalains in humans. *Am. J. Clin. Nutr.* **2004**, *80*, 941–945.

(8) Sugimura, T. Nutrition and dietary carcinogens. *Carcinogenesis* **2000**, *21*, 387–395.

(9) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; IARC: Lyon, France, 1987; Vol. 17, Supp. 7.

(10) Verna, L.; Whysner, J.; Williams, G. M. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity and tumor initiation. *Pharmacol. Ther.* **1996**, *71*, 57–81.

(11) Manibusan, M. K.; Odin, M.; Eastmond, D. A. Postulated carbon tetrachloride mode of action: a review. *J. Environ. Sci. Health* **2007**, *25*, 185–209.

Protective Effect of Red Beetroot against Oxidative Stress

J. Agric. Food Chem., Vol. 57, No. 6, 2009 **2575**

(12) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; IARC: Lyon, France, 1999; Vol. 71.

(13) Nilson, T. Studies into the pigments in beet root (*Beta vulgaris var rubra* L.). *Lantbrukshogsk. Ann* **1970**, *36*, 179–219.

(14) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.

(15) Sanz, M. J.; Ferrandiz, M. L.; Cejudo, M.; Terencio, M. C.; Gil, B.; Bustos, G.; Ubeda, A.; Gunasegaran, R.; Alcaraz, M. J. Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. *Xenobiotica* **1994**, *24*, 689–699.

(16) Sedlak, J.; Lindsay, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **1968**, *25*, 192–205.

(17) Jodynis-Liebert, J.; Murias, M.; Błoszyk, E. Effect of sesquiterpene lactones on antioxidant enzymes and some drug-metabolizing enzymes in rat liver and kidney. *Planta Med.* **2000**, *66*, 199–205.

(18) Mohandas, J.; Marshall, J. J.; Duggin, G. G.; Horvath, J. S.; Tiller, D. J. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. *Cancer Res.* **1984**, *44*, 5086–5091.

(19) Olive, P. L.; Banath, J. P. The comet assay: a method to measure DNA damage in individual cells. *Nat. Protoc.* **2006**, *1*, 23–29.

(20) Collins, A. R. The comet assay for DNA damage and repair. *Mol. Biotechnol.* **2004**, *26*, 249–261.

(21) Morton, L. W.; Abu-Amsha Caccetta, R.; Puddey, I. B.; Croft, K. D. Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. *Clin. Exp. Pharmacol. Physiol.* **2000**, *27*, 152–159.

(22) Sheweita, S. A.; El-Gabar, A.; Bastawy, M. Carbon tetrachloride-induced changes in the activity of phase II drug-metabolizing enzyme in the liver of male rats: role of antioxidants. *Toxicology* **2001**, *165*, 217–224.

(23) Jodynis-Liebert, J.; Matławska, I.; Bylka, W.; Murias, M. Antioxidant Activity of *Aquilegia vulgaris* in CCl4-Induced Liver Injury in Rats. In *Recent Progress in Medicinal Plants*; Govil, J. N., Singh, V. K., Ahmad, K., Eds.; Studium Press, LLC: Houston, TX, 2006; Vol. 14 Biopharmaceuticals, pp 59−70.

(24) Bansal, A. K.; Bansal, M.; Soni, G.; Bhatnagar, D. Protective role of vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem.-Biol. Interact.* **2005**, *156*, 101–111.

(25) Jeena, K. J.; Joy, K. L.; Kuttan, R. Effect of *Emblica officinalis, Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer Lett.* **1999**, *136*, 11–16.

(26) Summer, K. H.; Klein, D.; Lichtmannegger, J.; Wolff, T. 2-Chloroacetophenone is an effective glutathione depletor in isolated rat hepatocytes. *Arch. Toxicol.* **1996**, *71*, 127–129.

(27) Rodriguez, C.; Mayo, J. C.; Sainz, R. M.; Antolin, I.; Herrera, F.; Martin, V.; Reiter, R. J. Regulation of antioxidant enzymes: a significant role of melatonin. *J. Pineal. Res.* **2004**, *36*, 1–9.

(28) Dalle-Donne, I.; Giustarini, D.; Colombo, R.; Rossi, R.; Milzani, A. Protein carbonylation in human diseases. *Trends Mol. Med.* **2003**, *4*, 169–176.

(29) Bartsch, H.; Nair, H. Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch. Surg.* **2006**, *391*, 499–510.

(30) Schut, H. A.; Castonguay, A. Metabolism of carcinogenic amino derivatives in various species and DNA alkylation by their metabolites. *Drug Metab. Rev.* **1984**, *15*, 753–839.

(31) Pegg, A. E. Repair of O(6)-alkylguanine by alkyltransferases. *Mutat. Res.* **2000**, *462*, 83–100.

(32) Ueno, S.; Aoki, D.; Kubo, F.; Hiwatashi, K.; Matsushita, K.; Oyama, T.; Maruyama, I.; Aikou, T. Roxithromycin inhibits constitutive activation of nuclear factor κB by diminishing oxidative stress in a rat model of hepatocellular carcinoma. *Clin. Cancer. Res.* **2005**, *11*, 5645–5650.

(33) Winkler, C.; Wirleitner, B.; Schroecksnadel, K.; Schennach, H.; Fuchs, D. *In vitro* effects of beet root juice on stimulated and unstimulated peripheral blood mononuclear cells. *Am. J. Biochem. Biotechnol.* **2005**, *1*, 180–185.

(34) Kadiiska, M. B.; Gladen, B. C.; Baird, D. D.; Germolec, D.; Graham, L. B.; Parker, C. E.; Nyska, A.; Wachsman, J. T.; Ames, B. N.; Basu, S.; Brot, N.; FitzGerald, G. A.; Floyd, R. A.; George, M.; Heinecke, J. W.; Hatch, G. E.; Hensley, K.; Lawson, J. A.; Marnett, L. J.; Morrow, J. D.; Murray, D. M.; Plastaras, J.; Roberts, L. J., II; Rokach, J.; Shigenaga, M. K.; Sohal, R. S.; Sun, J.; Tice, R. R.; Van Thiel, D. H.; Wellner, D.; Walter, P. B.; Tomer, K. B.; Mason, R. P.; Barrett, J. C. Biomarkers of oxidative stress study II. Are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? *Free Radical Biol. Med.* **2005**, *38*, 698–710.

(35) Halliwell, B. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutr. Rev.* **1999**, *57*, 104–113.

JF803315D