Beetroot juice protects against N-nitrosodiethylamine-induced liver injury in rats

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Abstract

Red beetroot, a common ingredient of diet, is a rich source of a specific class of antioxidants, betalains. Our previous studies have shown the protective role of beetroot juice against carcinogen induced oxidative stress in rats. The aim of this study was to examine the effect of long term feeding (28 days) with beetroot juice on phase I and phase II enzymes, DNA damage and liver injury induced by hepatocarcinogenic N-nitrosodiethylamine (NDEA). Long term feeding with beetroot juice decreased the activities of enzymatic markers of cytochrome P450, P4501A1/1A2 and P4502E1, NDEA treatment also reduced the activities of these enzymes, but increased the activity of CYP2B. Moreover, combined treatment with beetroot juice and NDEA enhanced significantly CYP2B only. Modulation of P450 enzyme activities was accompanied by changes in the relevant proteins levels. Increased level and activity of NQO1 was the most significant change among phase II enzymes. Beetroot juice reduced the DNA damage increased as the result of NDEA treatment, as well as the biomarkers of liver injury. Collectively, these results confirm the protective effect of beetroot juice against oxidative damage shown in our previous studies and indicate that metabolic alterations induced by beetroot feeding may protect against liver damage.

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1. Introduction

Chronic liver damage is a worldwide common pathology characterized by inflammation and fibrosis that can lead to chronic hepatitis, cirrhosis and cancer (Köhle et al., 2008). N-nitroso compounds are well known hepatic carcinogens and causes of liver necrosis (Tricker et al., 1991). Human exposure occurs mainly through the smoked or salted meat and fish and alcoholic beverages consumption, tobacco smoking and through the use of certain cosmetics and pharmaceutical products. Additionally, a fraction of N-nitrosodiethylamine (NDEA) can be produced endogenously in the acidic stomach environment upon ingestion of nitrite/nitrate and pesticide-polluted food. NDEA is metabolized mainly by hepatic microsomal CYP450 isozymes (phase I enzymes), especially CYP2E1, which results in the formation of products capable of DNA alkylation. Moreover, NDEA is oxidized with NADPH-reductase to yield oxygen reactive species (ROS) and oxidative stress induction. Detoxification of NDEA proceeds through reactions catalyzed by phase II enzymes in which various polar metabolites appear (Verna et al., 1996). The role of phase I and II enzymes in carcinogen metabolism was corroborated in numerous experiments and reviewed convincingly (Guengerich, 2003a; Tan and Spivack, 2009), hence the idea of impact on carcinogen bioactivity through suppression of phase I and induction of phase II reactions gained interest as a possible cancer chemoprevention strategy. Diet rich in fruit and vegetable provides numerous phytochemicals active in these metabolic pathways. In Central and Eastern Europe red beetroot (Beta vulgaris var. rubra) is a popular vegetable, known for a long time for its beneficial health effects: stimulation of hematopoietic and immune systems, kidney and liver protection and as a special diet during cancer treatment, due to antioxidant, anti-inflammatory, hepatoprotective and antitumor properties (Escribano et al., 1998; Kapadia et al., 2003; Winkler et al., 2005; Georgiev et al., 2010). Besides diverse polyphenols red beetroot contain betalains, a family of non-phenolic and water-soluble anti-oxidants which comprise red betacyanins and yellow betaxanthins (Kanner et al., 2001). The few reports published on red beetroot chemopreventive action described an important potential of this vegetable and its products: inhibition of 7-methoxyresorufin demethylase, marker of CYP1A2 (Platt et al., 2010) and increase in phase II detoxification mechanisms in various experimental
settings. In this regard Lee et al. (2005) found induction of quinone reductase in the murine hepatoma cells upon exposure to beetroot extract in vitro and in vivo. In the studies on chemical carcinogenesis in animal models, beetroot showed protective effect in mouse skin and lungs (Kapadia et al., 1996) and in mouse skin and liver (Kapadia et al., 2003). In our previous studies we have shown a protective role of beetroot juice against NDEA-induced oxidative stress in rats (Kujawska et al., 2009) and in isolated neutrophils (Zielinska-Przyjemska et al., 2009). The aim of this study was the evaluation of the effect of long term (28 days) treatment of rats with beetroot juice alone or in combination with NDEA on phase I and phase II enzymes, DNA damage and liver injury.

2. Materials and methods

2.1. Chemicals

NDEA, ethoxyresorufin, methoxyresorufin, pentoxysorufin, resorufin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, p-nitrophenol, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenolindophenol (DPIP), dicoumarol, NADP, NADPH, dithiothreitol, sucrose, low melting point (LMP) agarose, bovine serum albumin and Tris were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), normal melting point (NMP) agarose was Prana Plus Agarose, Triton X-100 was purchased from Park Scientific, (Northampton, UK). Primary and secondary antibodies against CYP1A1/1A2, α-actin and NAD(P)H:quinone oxidoreductase-1 (NQO1) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), Primary and secondary antibodies against CYP2E1, glutathione S-transferase (GST) alpha, GST pi and GST mu were supplied by Oxford Biomedical Research (Oxford, MI, USA). Primary and secondary antibodies against CYP2B1 were obtained from BD Biosciences (Woburn, MA, USA). All the antibodies used in these experiments were specific for their respective proteins, and according to the information provided by suppliers there was no cross-reactivity within the isoforms of the same family. Rainbow colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). Commercial reagent kits for determination of albumin, bilirubin, creatinin, blood urea nitrogen (BUN) and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT) activities were provided by Pointe Scientific, Inc. (Canton, MI, USA). All other chemicals were commercial products of the highest purity available.

2.2. Animals and treatments

Male Wistar rats (6 weeks of age), provided by University of Medical Sciences, Department of Toxicology Breeding Facility (Poznań, Poland), were housed in polycarbonate cages (30 × 20 × 25 cm; 4 rats/cage), containing hardwood chip bedding. Commercial, ISO 9001 certified rat food (Labofeed H) and distilled water were available without restriction. The experimental animals were randomly divided into groups with 6 rats/cage. Seven groups were available without restriction. The experimental animals were randomly divided into four experimental groups each of six rats. For 28 consecutive days groups II and IV were treated with gavage with 8 mL/kg body weight of crude natural beet-root juice per day, groups I (controls) and III received the same volume of water. The effects of beetroot juice and NDEA on cytochrome P450-dependent enzymes in rat liver were summarized in Table 2. Twenty-eight days of forced feeding with beetroot juice alone...
decreased activities of EROD (marker of CYP1A1), MROD (marker of CYP1A2) and PNPH (marker of CYP2E1) by 17%, 20% and 29%, respectively, in comparison to the control group of animals (group I). NDEA treatment (group III) reduced the activities of these enzymes by 34%, 47% and 33% respectively. The opposite effect was observed for PROD (marker of CYP2B). NDEA increased the PROD activity by 65% in comparison to controls (group I). Pretreatment with beetroot juice further enhanced the activity of PROD in comparison with the NDEA treated group (group III). Modulation of P450 enzymes activities was accompanied by the parallel changes in relevant proteins levels. Western blot analysis with CYP1A1/1A2, CYP2E1 and CYP2B specific antibodies (Fig. 1) revealed statistically significant decrease in the CYP1A1/1A2 and CYP2E1, but an increase in CYP2B protein level in NDEA treated animals in comparison with the corresponding values in the control group of animals (group I). While densitometry of the bands presented in Fig. 1 showed diminishment by about 20–30% level of these CYPs, the expression of CYP2B was enhanced (by about 35%) in animals exposed to NDEA (group III).

3.3. Phase II enzymes in the liver

The effects of beetroot juice administration alone or in combination with NDEA on GST and NQO1 activities are presented in Table 2. Treatment with beetroot juice increased the activities of GST and NQO1 by 30% and 67%, respectively. A similar effect for NQO1 was observed in NDEA treated animals (group III). A combined treatment with beetroot juice and NDEA resulted in an increase in the NQO1 activity in comparison with that in the control group (group I).

Fig. 2 presents the immunoblots of GST isozymes and NQO1 and their quantitative analysis. Consistently with our previous and the other authors observations (Krajka-Kuźniak et al., 2008; Nijhoff et al., 1993) the GST pi protein was not detected in the liver. Beetroot juice increased the constitutive expression of GST μ (by 20%), however, the treatment with beetroot and NDEA or with NDEA only did not induced GST protein. Increased activity of NQO1 was accompanied by an elevated level of the enzyme protein as a result of beetroot juice treatment (group II).

3.4. Comet assay analysis of DNA damage in the liver

The effects of beetroot juice and NDEA treatments are presented in Fig. 3. Beetroot juice administered to rats for 28 days did not cause a DNA damage in the liver. In the rats receiving NDEA, only the elevated (by 57%) DNA damage was found in the liver. Pretreatment with beetroot juice prior to the NDEA administration resulted in a significant reduction in DNA damage (by 20%) in the rat liver.

4. Discussion

Red beetroot contains a specific class of antioxidants collectively named betalains, which have been shown to have anticarcinogenic and anti-inflammatory potential (Lee et al., 2005). Beside betalains, red beetroot also contains small amounts of other antioxidants, derivatives of ferrulic acid, hydroxycinnamic acid and cyclo-dopa-glucoside, so consumption of a natural product can provide an additive or synergistic effect (Frank et al., 2005). Most of the studies performed so far were concentrated on betalains or beetroot extracts (Wettasinghe et al., 2002; Nilsson, 1970). In our study, we applied a crude natural beetroot juice which not only mimics a natural edible beetroot product, but is also consumed in this form. The antioxidant activity of red beetroot juice in rats was confirmed in our previous study (Kujawska
et al., 2009). In this regard it was showed that pretreatment with beetroot resulted in partial recovery of glutathione peroxidase and glutathione reductase, diminished by treatment with NDEA. Moreover, beetroot juice increased about 3-fold the activity of superoxide dismutase and reduced the level of plasma protein carbonyls increased by NDEA treatment. The aim of this study was to evaluate the effect of beetroot juice administration to rats on hepatic phase I and II enzymes and DNA damage and its possible interference with NDEA action. NDEA induces carcinoma and gastrointestinal tract neoplasms in rats (Bartsch and Montesano, 1984). Different cytochrome P450-dependent monooxygenases are considered to be key enzymes involved in the activation of NDEA. Metabolic activation can lead to formation of DNA adducts, impairment of bases and DNA breaks (Aiub et al., 2011). The latter may result from the action of ROS. Treatment of rats with the initiating dose of NDEA in this study significantly reduced the activities of EROD, MROD and PNPH, markers of CYP1A1, CYP1A2 and 2E1, respectively. This observation may indicate the possibility of uncoupling of electron transfer and oxygen reduction from monoxygenation by these P450s, resulting in ROS generation (Nesnow et al., 2011). The reduced activities of these enzymes were accompanied by decreased levels of CYP1A1/1A2 and CYP2E1 proteins suggesting the inhibitory effect of ROS on the transcription of genes encoding these enzymes. This suggestion is supported by the fact that CYP1A1 expression was greatly decreased by oxidative stress or glutathione depletion (Morel and Barouki, 1999). The negative regulation of transcription by ROS was also shown for CYP2E1 gene (Morel et al., 2000). Moreover, treatment with NDEA in our current study increased the activity and expression of phase II enzyme, NQO1 which may further accelerate the formation of ROS (Ross, 2004). Thus, as a result of possible ROS generation, increased oxidative DNA damage was observed in the liver accompanied by enhanced levels of liver damage biomarkers in blood plasma, particularly AST and ALT activities, a sensitive marker of hepatocyte injury. Twenty-eight days of administration of beetroot juice alone or in combination with NDEA resulted in decreased activity and expression of CYP1A1/1A2 and 2E1. These P450 isoforms beside nitrosamines are involved in the activation of several classes of chemical carcinogens, including PAH (Guengerich, 2003b). Beetroot juice feeding also increased the activity and pro-

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EROD $^{ab}$</th>
<th>MROD</th>
<th>PROD</th>
<th>PNPH $^{c}$</th>
<th>GST $^{d}$</th>
<th>NQO1 $^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.53 ± 1.57</td>
<td>27.07 ± 1.44</td>
<td>9.71 ± 0.54</td>
<td>635.17 ± 30.15</td>
<td>1000.11 ± 73.40</td>
<td>102.41 ± 8.84</td>
</tr>
<tr>
<td>Beetroot juice</td>
<td>32.14 ± 1.11 (83)$^{f,g}$</td>
<td>21.60 ± 0.35 (80)$^{g}$</td>
<td>9.57 ± 0.25 (99)$^{g}$</td>
<td>449.14 ± 29.77 (71)$^{g}$</td>
<td>1304.83 ± 71.80 (130)$^{g}$</td>
<td>171.30 ± 15.09 (167)$^{g}$</td>
</tr>
<tr>
<td>NDEA</td>
<td>25.32 ± 0.44 (65)$^{g}$</td>
<td>14.23 ± 0.78 (53)$^{g}$</td>
<td>16.05 ± 0.79 (165)$^{g}$</td>
<td>428.13 ± 29.12 (67)$^{g}$</td>
<td>1122.34 ± 47.88 (112)$^{g}$</td>
<td>169.46 ± 9.65 (165)$^{g}$</td>
</tr>
<tr>
<td>Beetroot juice + NDEA</td>
<td>25.28 ± 0.88 (65)$^{g}$</td>
<td>16.59 ± 1.01 (61)$^{g}$</td>
<td>19.23 ± 1.34 (198)$^{g,h}$</td>
<td>481.86 ± 41.74 (76)$^{g}$</td>
<td>987.83 ± 66.18 (99)$^{g}$</td>
<td>160.85 ± 14.39 (157)$^{g}$</td>
</tr>
</tbody>
</table>

$^{a}$ Values are means ± SEM from 6 animals. Each assay was run in triplicate.
$^{b}$ EROD, MROD, PROD, are expressed in pmol resorufin formed/min per mg of protein.
$^{c}$ PNPH, is expressed in pmol p-nitrocatechol formed/min per mg of protein.
$^{d}$ GST, is expressed in nmol 1-chloro-2,4-dinitrobenzene conjugated formed/min per mg of protein.
$^{e}$ NQO1, is expressed in nmol 2,6-dichloroindophenol reduced/min per mg of protein.
$^{f}$ Values in parentheses represent percent of control.
$^{g}$ Significantly different from control, $p < 0.05$.
$^{h}$ Significantly different from NDEA treated group, $p < 0.05$.

Fig. 1. Effect of beetroot juice and NDEA on the expression of CYP1A1/1A2, CYP2B, CYP2E1 in microsomal fractions from rat liver. (A) Western blot analysis – representative blot is shown: C – control, BJ – Beetroot juice, NDEA – N-nitrosodiethylamine, BJ + NDEA – Beetroot juice + N-nitrosodiethylamine. The beta-actin protein was used as an internal standard. (B) Data (mean ± SEM) present percentage of value obtained in control group, from 6 different animals per each experimental group ($n = 6$). Measurements were performed at least three times. Protein expression was quantified by densitometry analysis. Asterisk above bar denote statistically significant differences from $\sim$ control group, $p < 0.05$. 

...
tein level of phase II enzymes NQO1 and GST. Thus, it can be assumed that the inhibition of CYPs, particularly the above mentioned 1A1, 1A2 and 2E1 and the induction of NQO1, might contribute to the potential antiproliferative activity of beetroot juice. Such an assumption, although contradictory to explanation of the NDEA effects above, is based on the fact that NQO1 catalyzes the two-electron transfer from both reduced pyridine nucleotides to some redox azo dyes and quinones. Two-electron reduction of quinones to hydroquinones by NQO1 usually leads to their excretion from a biological system and bypasses one-electron reduction that can generate ROS (Winski et al., 1998). Hydroquinones are also potentially reactive metabolites, and if not conjugated and/or excreted, may undergo bioactivation via auto-oxidation or by the rearrangement reactions. Thus, the overall contribution of NQO1 to cellular toxicity/detoxification systems will depend on the reactivity and properties of the hydroquinone that is generated (Ross et al., 2000). It is possible that the induction of NQO1 by NDEA alone may increase its carcinogenicity while beetroot juice treatment will favor the detoxification. Additionally, beetroot juice feeding increased the GST mu, which often is linked with reduced mutagenicity of chemical carcinogens (Guyonnet et al., 2001). Moreover, in contrast to other isoforms, GST mu expression was confirmed in human cells (Konsue and Ioannides, 2008), which may indicate the relevance of our observations to humans.

GST pi was not detected in the liver, since this isoform is present in placental liver, but not in adult rats (Aliya et al., 2003). Its induction requires the application of two-stage carcinogenesis protocol in which initiation achieved with NDEA application is followed by treatment with promoter or partial hepatectomy (Shimamoto et al., 2011).

Beetroot juice pretreatment also increased the activity of PROD and CYP2B1 protein levels induced by NDEA. Although it is believed that induction of all CYP isoforms could contribute to increased amounts of ROS (Paolini et al., 2001), it is also possible that selective increase in certain CYPs may overcome uncoupling of catalytic cycle of the others, namely CYP2E1 in case of NDEA.
Such an explanation is supported by a significant reduction in DNA damage observed in this study in the animals treated with beetroot juice and NDEA, and may lead to the conclusion that beetroot juice components may act as anti-initiating agents in rat liver hepatocarcinogenesis models.

Microsomal generation of ROS is one of the most significant causes of liver injury. Thus, the most significant and somehow in concert with reduced DNA damage, pretreatment with beetroot juice before the administration of NDEA reduced the parameters of liver function damage elevated as a result of NDEA treatment. NDEA treatment increased the plasma levels of most liver damage biomarkers, including SDH, released into circulation after cellular damage. The enhanced level of SDH in blood plasma indicates the destruction of mitochondrial membranes, which might be an additional source of reactive species from the mitochondrial electron transport chain. Pretreatment with beetroot juice before the administration of NDEA reduced the level of all parameters of liver function damage, elevated as a result of NDEA treatment, but the most significant decrease was observed for ALT, SDH, GGT and bilirubin. The latter may be related to reduction in heme-oxygenase-1 activity as this enzyme is considered important to maintain the liver homeostasis in stressful conditions due to the protective effects of the heme-derived metabolites such as bilirubin (Cantoni et al., 2003). Moreover, beetroot juice also reduced the level of creatinin, the marker of kidney damage. Thus, the protective activity of beetroot juice against liver damage may be comparable to that of garlic extracts or silymarin, which by maintaining the integrity of the plasma membrane suppresses the leakage of enzymes and proteins (Pradep et al., 2007; Gedik et al., 2005).

In summary, the results of our present study confirm the protective activity of beetroot juice against oxidative damage shown in previous studies (Kujawski et al., 2009; Zielińska-Przyjemskas et al., 2009) and indicate that metabolic alterations induced by beetroot feeding may protect against liver damage. In combination with chemical carcinogens, beetroot juice may reduce their effects. A detailed mechanism of this protective activity requires further studies. Since beetroot juice is one of the human diet components, the results of our current study provide arguments for its recommendation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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Aliya, S., Reddamma, P., Thyagaraju, K., 2003. Does glutathione S-transferase Pi (GST-Pi) a marker of kidney damage. Thus, the protective activity of beetroot juice against oxidative damage shown in previous studies (Kujawski et al., 2009; Zielińska-Przyjemskas et al., 2009) and indicate that metabolic alterations induced by beetroot feeding may protect against liver damage. In combination with chemical carcinogens, beetroot juice may reduce their effects. A detailed mechanism of this protective activity requires further studies. Since beetroot juice is one of the human diet components, the results of our current study provide arguments for its recommendation.