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Effect of Chokeberry (*Aronia melanocarpa*) Juice on the Metabolic Activation and Detoxication of Carcinogenic *N*-Nitrosodiethylamine in Rat Liver

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Chokeberry is a rich source of polyphenols, which may counteract the action of chemical carcinogens. The aim of this study was to examine the effect of chokeberry juice alone or in combination with *N*-nitrosodiethylamine (NDEA) on phase I and phase II enzymes and DNA damage in rat liver. The forced feeding with chokeberry juice alone decreased the activities of enzymatic markers of cytochrome P450, CYP1A1 and 1A2. NDEA treatment also decreased the activity of CYP2E1 but enhanced the activity of CYP2B. Pretreatment with chokeberry juice further reduced the activity of these enzymes. Modulation of P450 enzyme activities was accompanied by the changes in the relevant proteins levels. Phase II enzymes were increased in all groups of animals tested. Chokeberry juice augmented DNA damage and aggravated the effect of NDEA. These results indicate that chokeberry may protect against liver damage; however, in combination with chemical carcinogens it might enhance their effect.

KEYWORDS: Chokeberry juice; cytochrome P450; GST; NDEA; NQO1

INTRODUCTION

The emphasis on dietary approaches for health promotion is backed by a large body of evidence accumulating over decades. Regular consumption of a variety of fruits and vegetables is, indisputably, a key factor that contributes to reduced risk of degenerative diseases and cancer. In the 1980s, it was assumed that 35% of various cancers were related to dietary factors, with a plausible contribution of diet to cancer ranging from 10 to 70%. More recent evaluation has supported the earlier assessment with a slightly narrower estimated range of 20−40% (*1*). Food contains both carcinogenic and anticarcinogenic substances. The latter are provided mostly by non-nutrient components of edible fruits and vegetables, while carcinogenic food contamination is principally a result of food processing and storage. *N*-Nitrosoamines are known hepatocarcinogens and have been implicated in the etiology of some other human cancers including cancer of the esophagus (*2*). They originate mainly from protein-containing foods and are also formed from nitrate precursors, which are abundant in leafy and root vegetables. Under the acidic conditions of the stomach, the amines present in food are activated by reactions with nitrates, leading to *N*-nitrosamine production (*3*). Cytochromes P450 (CYPs)-mediated hydroxylation at position α to the nitroso group and formation of α-hydroxylnitrosoamine has been considered a crucial step in the bioactivation of *N*-nitrosoamines to ultimate carcinogenic forms resulting in DNA alkylation, which might initiate the process of tumorigenesis.

N-Nitrosodiethylamine (NDEA), one of the most important environmental carcinogens of this class, has been suggested, besides being metabolized to reactive electrophiles, to cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury. In these reactions, several cytochromes P450 may be involved; thus, a prominent phenomenon during hepatocarcinogenesis is an alteration of the expression of drug metabolizing enzymes (*4*). These include phase I enzymes involved mainly in substrate hydroxylation catalyzed by cytochromes P450 and phase II enzymes converting the hydroxylated and other compounds to various polar metabolites.

Fruits and berries particularly are known as plant foods very rich in phenolic compounds. They contain a wide range of phenolic acids and flavonoids, which include anthocyanins, proanthocyanins, flavonols, and catechins. Chokeberry (*Aronia melanocarpa*, Elliot), native to eastern North America, now popular in Eastern and Northern Europe has been known as a very rich source of polyphenols, including anthocyanins, polymeric proanthocyanins, and phenolic acids, whose content is significantly higher than that of the blueberry, cranberry, and lingonberry crops (*5*).

Our earlier studies showed that treatment of rats or mice with structurally diverse phenolic acids, including chlorogenic and tannic acids, ingredients of chokeberry, significantly modulate the phase I and phase II enzymes involved also in NDEA activation and detoxication (*6*−*9*). Moreover, these compounds,

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which are considered strong antioxidants, also affected ROS formation and DNA damage in vitro and in vivo (*10*, *11*). Recently, it was also shown that anthocyanins, the abundant chokeberry components, exert their antioxidative effect through activation of phase II enzymes (*12*).

While dietary manipulation of metabolizing enzymes using isolated compounds seems a promising cancer chemoprevention approach, it may have serious consequences since these enzymes are normally involved in fundamental endogenous reactions and prominent cellular functions (*1*). Chokeberry fruits contain several classes of phenolic compounds, which in combination might differently affect the enzyme system involved in the metabolism of carcinogens, particularly in the presence of food-born carcinogens.

In order to elucidate if such interactions may occur, the aim of the study was to evaluate the effect of the long-term treatment of rats with chokeberry juice alone or in combination with NDEA on the phase I and phase II enzymes and DNA damage in liver.

MATERIALS AND METHODS

Chemicals. NDEA, ethoxyresorufin, methoxyresorufin, penthoxyresorufin, 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 agarose was Prona Plus Agarose, and Triton X-100 was purchased from Park Scientific (Northampton, UK). Primary and secondary antibodies against CYP1A1/1A2, β-Actin and NAD(P)H:quinone oxidoreductase-1 (NQO1) were 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, and purified standards of CYP2E1 and GST pi were supplied by Oxford Biomedical Research (Oxford, MI, USA). Primary and secondary antibodies against CYP2B1 were obtained from BD Biosciences (Woburn, MA, USA). All of 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 isozymes of the same family. Rainbow colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). Commercial reagent kits for the determination of albumin, bilirubin, creatinine, 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.

Animals and Treatments. Male Wistar rats (6 wks 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 rat food (Altromin GmbH, Germany) and distilled water were available without restriction. The animals were randomly divided into four experimental groups each of six rats.

The animals were treated by gavage with chokeberry juice (8 mL/kg b.w.) for 28 consecutive days. On day 27, NDEA was administered i.p. in a single dose of 150 mg/kg body wt. Control groups of animals received water. All of the experiments were conducted according to the Regional Ethics Committee Guidelines for Animal Experimentation.

Chokeberry Juice Preparation and Analysis. The 100% chokeberry juice was obtained by pressing from fruits cultivated in the ecological farm (ECOAR, Poland) and then pasteurized. Chokeberry juice was analyzed as described previously (*13*). Briefly, the separation of (−) epicatechin, chlorogenic, and neochlorogenic acids, flavonol glycosides, and anthocyanin glycosides was performed on a Synergi Fusion RP-80A 150 × 4.6 mm (4 μm) Phenomenex (Torrance, CA USA) column and detected with a Merck-Hitachi L-7455 diode array detector (DAD).

Table 1. Contents of Phenolic Compounds (mg/100 mL) in Chokeberry Juice

compounds	
neochlorogenic acid	49.21
chlorogenic acid	45.50
(−)epicatechin	1.48
p-coumaric acid derivatives	0.4
polymeric procyanidins	293.38
quercetin 3-*O*-rutinoside	1.68
quercetin 3-*O*-galactoside	2.83
quercetin 3-*O*-glucoside	2.25
quercetin 3-*O*-β-arabinosyl-β-glucoside	1.15
quercetin 3-*O*- α-rhamnosyl-β-galactoside	1.17
eriodictyol 3,7-*O*-diglucuronide	7.86
cyanidin 3-*O*-galactoside	12.49
cyanidin 3-*O*-arabinoside	0.71
cyanidin 3-*O*-glucoside	5.12
cyanidin 3-*O*-xyloside	0.59
cyanidin aglycon	0.22

The photo diode array spectra were measured over the wavelength range 240−600 nm in steps of 2 nm. Retention times and spectra were compared to those of pure standards within 200−600 nm. Anthocyanins and chlorogenic acids were quantified by including cyanidin 3-galactoside and chlorogenic acid (Polyphenols Laboratories, Norway), as external standards, while flavonols were assayed on the basis of standard rutin (Sigma Chemicals Co, St Louis MO, USA).

The analysis of proanthocyanidins was performed after thiolysis, and the resulting products were separated on Merck Purospher RP 18 endcapped column 250 × 4 mm, 5 μm (Merck, Darmstadt, Germany) assembled in a Waters (Milford, MA) system (DAD and scanning fluorescence detectors). The calibration curves (based on peak area at 280 nm) were established using flavan-3-ol and benzylthioether as the standards.

The established composition of chokeberry juice is presented in **Table 1**.

Preparation of Liver Homogenates and Cytosolic and Microsomal Fractions. Twenty-four hours after the last treatment, the rats were killed by decapitation, and the livers were removed. The tissue was rinsed in ice-cold buffered 0.2 M sucrose (pH 7.5) and homogenized in the same medium. Cytosolic and microsomal fractions were prepared by differential centrifugation, and protein concentrations were determined as described previously (*7*, *8*). Blood was collected by heart puncture into heparinized tubes and centrifuged (1000*g* for 10 min at 4 °C) to separate plasma for the determination of albumin, bilirubin, creatinine, and BUN levels, and ALT, AST, ALP, SDH, LDH, and GGT activities.

Phase I and Phase II Enzyme Activity Assays. The ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD), and penthoxyresorufin-*O*-depenthylase (PROD) activities were measured as described previously (*14*).The activities of *p*-nitrophenol hydroxylase (PNPH), cytosolic NQO1 (assayed with NADPH as the electron donor and DPIP as the electron acceptor), and GST using CDNB as a substrate were determined using protocols described in our earlier studies (*7*, *8*).

Protein Immunoblotting. Cytosolic and microsomal proteins (20−100 μg) were separated on 10% or 12% SDS−PAGE slab gels, and the proteins were transferred to nitrocellulose membranes (*8*, *15*). After blocking with 5% or 10% skimmed milk, the proteins were probed with mouse antirat CYP1A1/1A2, goat antirat CYP2B1, goat antirabbit CYP2E1, and rabbit antihuman GST alpha, goat antirat GST mu, rabbit antihuman GST pi, goat antihuman NQO1, and rabbit antimouse β-Actin antibodies. The β-Actin protein was used as an internal control. As the secondary antibodies in the staining reaction, the alkaline phosphatase-labeled antigoat IgG, antimouse IgG, or antirabbit IgG was used. The amount of immunoreactive product in each lane was determined by densitometric scanning using BioRad GS 710 Image Densitometer (BioRad Laboratories, Hercules, CA, USA). Values were calculated as relative absorbance units (RQ) per mg protein.

Comet Assay. Single cell gel electrophoresis in alkaline conditions (pH > 13) was performed in liver homogenates according to the method presented by Hartmann et al. (*16*). Samples embedded in the LMP agarose were submitted to the procedures of cell lysis, DNA unwinding, electrophoresis, and neutralization, and then were dehydrated in absolute

ethanol, dried, and stored at room temperature, protected from light. Just before microscopic evaluation, the slides were rehydrated and stained with ethidium bromide (0.05 mg/mL). Images of comets were captured with a digital camera. The comets were divided into 5 groups according to the degree of DNA damage (*17*). A total damage score for each sample on the slide was calculated by multiplying the number of cells classified to each grade of damage by the numeric value of the grade and summing over all grades. The results obtained in the arbitrary point units were expressed as the percentage of the values obtained in the control group.

Statistical Analysis. The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p < 0.05$.

RESULTS

Effect of Chokeberry Juice and NDEA on Selected Biochemical Parameters of Liver Function in Blood. The effects of chokeberry juice and NDEA on selected liver function biochemical parameters (ALT, AST, ALP, SDH, LDH, and GGT activities, and the concentration of albumin, bilirubin, creatinine, and BUN) are presented in **Tables 2** and **3**. Treatment of rats with a single dose of 150 mg/kg body wt of NDEA alone resulted in a statistically significant increase of all tested enzyme activities in blood plasma in comparison to that of the control group (by 37−538%). The concentration of bilirubin and creatinin were increased by 133% and 103%, respectively. Pretreatment with chokeberry juice significantly decreased SDH and GGT activities elevated by NDEA, although complete normalization to control group values was achieved only in the case of GGT. Moreover, treatment with chokeberry juice alone increased the SDH activity by 44%. Thus, pretreatment with chokeberry juice only partly protected against NDEA-induced damage.

Effect of Chokeberry Juice and NDEA on Phase I Enzymes in Rat Livers. The effects of chokeberry juice and NDEA on cytochrome P450-dependent enzymes in rat liver are summarized in **Table 4**. Twenty-eight days of forced feeding with chokeberry juice alone decreased the activities of EROD (the marker of CYP1A1) and MROD (the marker of CYP1A2) by 29 and 21%, respectively, in comparison with the control group of animals receiving water only. NDEA treatment reduced the activities of these enzymes by 51% and 67%, respectively. PNPH activity (marker of CYP 2E1) was reduced to a similar extent, while the activity of PROD (marker of CYP2B) was enhanced by 48%. Pretreatment with chokeberry juice further reduced the activity of PNPH in comparison with that of the control and NDEA treated group. A similar effect, but only in comparison with the NDEA treated group, was observed for PROD. Modulation of P450 enzyme activities was accompanied by changes in the levels of relevant proteins. Western blot analysis with CYP1A1/1A2- and CYP2E1-specific antibodies (**Figure 1**) revealed statistically significant decreases of the CYP1A1/1A2 and CYP2E1 protein levels in NDEA-treated animals in comparison with those in the control group. While densitometry of the bands presented in **Figure 1** showed a diminished level of these CYPs by about 40%, the expression of CYP2B was enhanced (by about 35%) in animals exposed to NDEA. Chokeberry juice did not change the effect of NDEA, but given alone decreased the CYP1A1/1A2 level by about 30%.

Effect of Chokeberry Juice and NDEA on Phase II Enzymes in Rat Livers. The effects of chokeberry juice administration alone or in combination with NDEA are presented in **Table 5**. Treatment with chokeberry juice only slightly increased the activity of GST, but NQO1 activity was increased by 219%. A similar effect was also observed in NDEA-treated animals. The combined treatment with chokeberry juice and NDEA resulted in a decrease

Table 2. Effect of Chokeberry Juice and NDEA on the Selected Plasma Enzymatic Markers of Rat Liver Function

treatment	ALT[a,b]	AST	SDH	LDH	GGT
control	32.8 ± 1.46	73.6 ± 1.85	4.5 ± 0.11	181.1 ± 2.72	4.4 ± 0.15
chokeberry juice	42.2 ± 1.52 (129)[c,d]	75.6 ± 0.53 (103)	6.5 ± 0.27 (144)[d]	183.6 ± 3.15 (101)	4.6 ± 0.22 (105)
NDEA	79.5 ± 1.37 (242)[d]	144.4 ± 2.97 (196)[d]	28.7 ± 0.97 (638)[d]	247.0 ± 8.23 (137)[d]	7.6 ± 0.23 (173) [d]
chokeberry juice + NDEA	69.4 ± 3.22 (212)[d]	136.6 ± 3.58 (186)[d]	24.5 ± 0.77 (544)[d,e]	205.9 ± 7.50 (114)	4.8 ± 0.13 (109)[d,e]

[a] Values are the means ± SEM from 6 animals. Each assay was run in triplicate. [b] ALT, AST, SDH, LDH, and GGT are expressed in IU/L. [c] Percent of control. [d] Significantly different from control, $p < 0.05$. [e] Significantly different from NDEA-treated rats, $p < 0.05$.

Table 3. Effect of Chokeberry Juice and NDEA on the Selected Plasma Biochemical Markers of Rat Liver Function

treatment	albumin[a,b]	bilirubin[c]	creatinin	BUN
control	4.54 ± 0.03	0.33 ± 0.01	0.32 ± 0.01	23.77 ± 0.25
chokeberry juice	4.60 ± 0.02 (101)[d]	0.33 ± 0.02 (100)	0.35 ± 0.01 (109)	24.32 ± 0.41 (102)
NDEA	3.97 ± 0.02 (87)	0.77 ± 0.04 (233) [e]	0.65 ± 0.04 (203)[e]	22.15 ± 0.18 (93)
chokeberry juice + NDEA	4.46 ± 0.01 (98)	0.62 ± 0.03 (188) [e,f]	0.37 ± 0.01 (116)[e,f]	22.44 ± 0.28 (94)

[a] Values are the means ± SEM from 6 animals. Each assay was run in triplicate. [b] Albumin, is expressed in g/dL. [c] Bilirubin, creatinin, and BUN are expressed in mg/dL. [d] Percent of control. [e] Significantly different from control, $p < 0.05$. [f] Significantly different from NDEA-treated rats, $p < 0.05$.

Table 4. Effect of Chokeberry Juice and NDEA on the Activity of Cytochromes P450 in Rat Liver

treatment	EROD[a,b]	MROD	PROD	PNPH[c]
control	20.13 ± 1.18	16.69 ± 0.72	9.55 ± 0.58	672.83 ± 66.84
chokeberry juice	14.30 ± 0.93 (71)[d,e]	13.14 ± 0.88 (79)[e]	8.09 ± 0.41 (85)	579.73 ± 43.73 (86)
NDEA	9.89 ± 1.27 (49)[e]	5.45 ± 0.76 (33)[e]	14.14 ± 1.67 (148)[e]	319.88 ± 13.81 (48) [e]
chokeberry juice + NDEA	8.53 ± 1.28 (42)[e]	6.52 ± 1.09 (39)[e]	10.78 ± 1.26 (113)[e,f]	210.83 ± 10.47 (31) [e,f]

[a] Values are the means ± SEM from 6 animals. Each assay was run in triplicate. [b] EROD, MROD, and PROD are expressed in pmol resorufin formed/min per mg. [c] PNPH is expressed in pmol *p*-nitrocatechol formed/min per mg. [d] Percent of control. [e] Significantly different from control, $p < 0.05$. [f] Significantly different from NDEA-treated rats, $p < 0.05$.

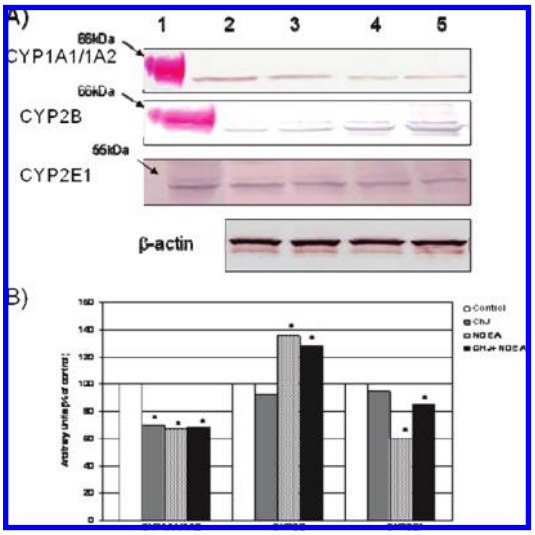

Figure 1. Effect of chokeberry juice and NDEA on the expression of CYP isozymes in rat liver. (**A**) A representative immunoblot from two independent experiments. (**B**) Data present the expression of CYP isozymes as percentage of control groups (mean ± SEM) from two separate experiments run in triplicate. Asterisks above the bars denote statistically significant differences from * control group, $p < 0.05$. CYP 1A1/1A2, CYP2B, CYP2E1 expression, (lane 1) protein molecular weight markers or purified standard CYP2E1; (lane 2) control; (lane 3) chokeberry juice (ChJ); (lane 4) NDEA; (lane 5) chokeberry juice + NDEA.

Table 5. Effect of Chokeberry Juice and NDEA on the Activity of Phase II Enzymes in Rat Liver

treatment	GST[a,b]	NQO1[c]
control	1101.51 ± 38.91	86.09 ± 5.59
chokeberry juice	1327.90 ± 34.00 (121)[d,e]	274.61 ± 17.81 (319)[e]
NDEA	1334.48 ± 59.71 (121)[e]	197.87 ± 9.93 (230)[e]
chokeberry juice + NDEA	1142.98 ± 54.60 (104)	177.08 ± 20.26 (206)[e,f]

[a] Values are the means ± SEM from 6 animals. Each assay was run in triplicate. [b] GST is expressed in nmol 1-chloro-2,4-dinitrobenzene conjugate formed/min per mg. [c] NQO1 is expressed in nmol 2,6-dichloroindophenol reduced/min per mg. [d] Percent of control. [e] Significantly different from control, $p < 0.05$. [f] Significantly different from NDEA-treated rats, $p < 0.05$.

of NQO1 activity in comparison with that of animals treated with either chokeberry juice or NDEA alone.

Figure 2 presents the immunoblots of GST isozymes and NQO1 and their quantitative analysis. Consistent with the observations of other authors (*18*) and our previous studies (*15*), the GST pi protein was not detected in the liver. Chokeberry juice increased the constitutive expression of GST mu and alpha (by 15% and 20%, respectively) but did not affect the NDEA-induced GST proteins. Increased activity of NQO1 was accompanied by an elevated level of the enzyme protein as a result of chokeberry juice or NDEA treatment. Pretreatment with chokeberry juice diminished the expression of NDEA-induced NQO1 protein to the level observed in the livers of the control group of rats.

Comet Assay Analysis of DNA Damage. The effects of chokeberry juice and NDEA treatments are presented in **Figure 3**. Chokeberry juice administered to rats for 28 days significantly augmented the scale of DNA damage in the liver and so did NDEA alone (by ~ 57%). In animals pretreated with chokeberry juice prior to NDEA administration, 59% aggravation of DNA damage was observed.

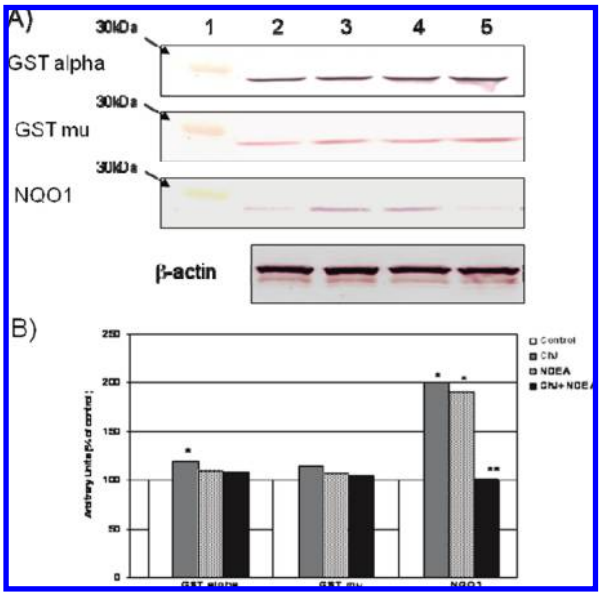

Figure 2. Effect of chokeberry juice and NDEA on the expression of GST isozymes and NQO1 in rat liver. (**A**) A representative immunoblot from two independent experiments. (**B**) Data present the expression of GST isozymes and of NQO1 as a percentage of control groups (mean ± SEM) from two separate experiments run in triplicate. Asterisks above the bars denote statistically significant differences from * control group and from ** NDEA-treated rats, $p < 0.05$. GST alpha, mu, and NQO1 expression, (lane 1) protein molecular weight markers; (lane 2) control; (lane 3) chokeberry juice (ChJ); (lane 4) NDEA; (lane 5) chokeberry juice + NDEA.

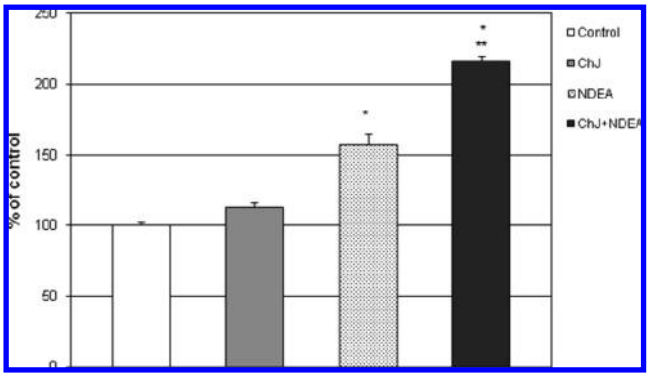

Figure 3. Effect of chokeberry juice (ChJ) and NDEA on the extent of DNA damage in rat liver. Data (mean ± SEM) are expressed as the percentage of value obtained in the control group (86 ± 2.2, arbitrary points). Asterisks above the bars denote statistically significant differences from * control group and from ** NDEA-treated rats, $p < 0.05$.

DISCUSSION

The focus of the present study was to evaluate the effect of chokeberry juice administration on hepatic phase I and II enzymes in rats and DNA damage, and its possible interference with NDEA action. It is well known that the fruits such as chokeberry are rich, besides other potential chemopreventive agents, in anthocyanins, plant pigments with anticarcinogenic, antioxidant, and anti-inflammatory activities (*19*). It has been shown that anthocyanins isolated from fruits of *Aronia melanocarpa* markedly inhibited the mutagenic activity of benzo(*a*)pyrene and 2-amino fluorene in the Ames test and the generation and release of superoxide radicals by human granulocytes. These data suggest that the antimutagenic effect of anthocyanins is

exerted not only through their free-radical scavenging action but also by inhibition of enzymes activating promutagens and converting mutagens to the DNA reacting derivatives (*20*). In the chokeberry juice used in this study, the concentration of anthocyanins (cyanidin glycosides and aglycon) ranged 4.5%.

NDEA is a very potent carcinogen that induces liver carcinomas and gastrointestinal tract neoplasms in rats (*21*). A prominent phenomenon during hepatocarcinogenesis is an alteration of the expression of drug-metabolizing enzymes. In the case of NDEA, cytochrome P450-mediated activation leads to the formation of electrophiles, which ethylate DNA and, if not repaired, to mutation. Some other probable mechanisms underlying the mutagenic potency of NDEA include the metabolism of exogenous chemicals involved in the generation of ROS and oxidative DNA damage. Microsomal generation of ROS is one of the most significant causes of liver injury. The existence of NADPH-dependent production of ROS by animal liver microsomes has been linked to CYPs. The uncoupling of electron transfer and oxygen reduction from monooxygenation by CYP2B1 and CYP2E1 could result in the release of $O_2^{\bullet -}$ and H_2O_2. A recent study has demonstrated that in this step CYP1A1, CYP3A, and CYP4A are also involved (*4*). Among these isozymes, CYP2E1 has been suggested to completely participate in NDEA induced hepatocarcinogenesis since *Cyp2e-1* null mice showed a lower incidence and multiplicity of tumors compared with that in wild-type mice in NDEA-induced hepatocarcinogenesis (*22*).

The results of our present study seem to confirm the uncoupling of electron transfer and oxygen reduction from microsomal monooxygenation. Treatment with a single dose of 150 mg/kg of NDEA (this dose was selected according to the protocol described in ref *23*) significantly reduced EROD, MROD, and PNPH activities. The latter is considered a marker of CYP2E1, and its activity assay is based on the hydroxylation of the substrate, *p*-nitrophenol. Moreover, the reduced activities of EROD, MROD, and PNPH were accompanied by decreased protein levels of CYP1A1/1A2 and CYP2E1, which might indicate the inhibitory effect of ROS on the transcription of genes encoding these enzymes. In contrast to the effect on these enzymes, NDEA induced CYP2B1, which plays a major role in the activation of longer chain nitrosodialkylamines such as *N*-nitrosodibutylamine (*24*). Diminished expression of CYP1A1/1A2 after treatment with a single dose of 200 mg/kg of NDEA was also observed by Liu et al. (*4*) in Sprague−Dawley rats. Moreover, it was demonstrated that the expression of CYP1A2 was reduced preferentially in rat liver bearing hyperplastic nodules.

In our study, treatment with NDEA significantly increased the activity and expression of phase II enzyme, NQO1. This enzyme is generally assumed to possess important protective properties, both by detoxifying some carcinogenic compounds as well as by preventing the generation of oxygen radicals. However, NQO1 not only may provide a cellular detoxifying system but also may accelerate the formation of ROS through the semiquinones pathway (*25*). Thus, both ROS production systems, those resulting from uncoupling of electron transfer and oxygen reduction from microsomal monooxygenation and the other involving the semiquinones pathway, might lead to increased oxidative DNA damage observed in comet assay in this study (**Figure 3**). Moreover, 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, while the increased level of bilirubin as a result of NDEA treatment might be related to heme oxygenase-1 induction as this enzyme is considered important to maintain liver homeostasis in stressful conditions due to the protective effects of the heme-derived metabolites including bilirubin (*26*).

Twenty eight day administration of chokeberry juice resulted in decreased activity and expression of P450 isozymes. Since these P450 are involved in the activation of several classes of chemical carcinogens including PAH and nitrosamines, their inhibition is expected to block both the toxicity and carcinogenicity of these compounds. Such a mechanism of anticarcinogenic activity is proposed for organosulfur compounds of garlic, which are competitive and mechanism-based inhibitors of CYP 2E1 (*27*). Similarly, naturally occurring coumarins selectively inhibiting CYP1A1 reduced PAH tumor initiating activity (*28*). Feeding with chokeberry juice substantially increased the activity and protein level of phase II enzyme NQO1 and, to a lesser extent, of GST. NQO1 catalyzes two-electron transfer from both reduced pyridine nucleotides to some redox azo dyes and from quinones. In this way, quinones are metabolized bypassing the formation of semiquinones and, subsequently, of superoxide anion radicals (*29*). Thus, the inhibition of CYP2E1 in concert with the induction of NQO1 might contribute to the potential anticarcinogenic activity of chokeberry juice.

Additionally, although feeding with chokeberry juice only slightly increased total GST activity, immunoblotting revealed an increase of GST alpha and mu, which might be beneficial as protection against ROS and the products of lipid peroxidation such as 4-hydroxynonenal (*30*).

Pretreatment with chokeberry juice before the administration of NDEA reduced the parameters of liver function damage elevated as result of NDEA treatment, although a significant decrease was observed for SDH, GGT, bilirubin, and creatinin, the marker of kidney damage.

A protective effect of chokeberry juice on liver cells exposed in vivo to carbon tetrachloride (CCl_4) or cadmium chloride ($CdCl_4$) has also been observed by Kowalczyk et al. (*31*) and Valcheva-Kuzmanowa et al. (*32*). These authors postulated that the protective effect of chokebery juice against chemically induced liver injury might be due to its ability to scavenge CCl_4- or $CdCl_4$-associated free radicals.

Chokeberry juice decreased the activity of PROD and CYP2B1 protein levels and further reduced the activity and expression of CYP2E1 already diminished by NDEA treatment. Considering the possibility of increased ROS production through microsomal pathways (as discussed earlier), the diminished activities of these P450 might explain the increased hepatic DNA damage in the animals treated with chokeberry juice and NDEA. This effect might be also a result of various polyphenolic antioxidants consumed with the juice that are capable of reducing of Fe(III) to Fe(II) according to the Fenton reaction in tissues challenged with NDEA (*33*).

Moreover, prolonged administration of chokeberry alone also caused greater hepatic DNA damage in comparison to that in controls. Such an effect could be ascribed to the potentially prooxidant activity in tissues exposed to plant polyphenols (*34*).

We suppose that the observed effects are related to polymeric procyanidins (protoanthocyanidines) as these active phytochemicals form the main polyphenol fraction (69%) of chokeberry fruit juice; however, some contribution from anthocyanins cannot be excluded. Polymeric procyanidins were shown to be responsible for the highest antioxidant activity of cloudy apple juice and its protection against colon cancer in rats (*35*). However, some studies demonstrated that phenolic acids such as chlorogenic acid can bind to cellular macromolecules, which may result in the DNA damage observed in our study.

Collectively, the results of our present study indicate that metabolic alterations induced by chokeberry feeding may protect

against liver damage but in combination with chemical carcinogens might aggravate their effect. This suggests that long-term uncontrolled administration of chokeberry juice could pose a potential health hazard, which has to be considered in dietary recommendation.

ABBREVIATIONS USED

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CDNB, 1-chloro-2,4-dinitrobenzene; CYPs, cytochromes P450; DAD, diode array detector; DPIP, 2,6-dichlorophenolindophenol; EROD, ethoxyresorufin-*O*-deethylase; GGT, gamma glutamyl transferase; GST, glutathione *S*-transferase; LDH, lactate dehydrogenase; LMP, low melting point; MROD, methoxyresorufin-*O*-demethylase; NDEA, *N*-nitrosodiethylamine; NQO1, NAD(P)H:quinone oxidoreductase-1; PNPH, *p*-nitrophenol hydroxylase; PROD, penthoxyresorufin-*O*-depenthylase; ROS, reactive oxygen species; SDH, sorbitol dehydrogenase.

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