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INFLUENCE OF CLOUDY APPLE JUICE ON N-NITROSODIETHYLAMINE-INDUCED LIVER INJURY AND PHASES I AND II BIOTRANSFORMATION ENZYMES IN RAT LIVER

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Abstract: Cloudy apple juice (CAJ) is a rich source of nutrients as well as non-nutrient components including high quantity of polyphenols, particularly oligomeric procyanidins, which are considered as potential chemo-preventive agents that protect against the action of chemical carcinogens. The aim of this study was to examine the effect of CAJ alone or in combination with hepatocarcinogenic N-nitrosodiethylamine (NDEA) on liver damage biomarkers, including DNA damage, and the phase I and II enzymes in rat. The forced feeding with CAJ alone for 28 days, has slightly reduced the activities of phase I enzymes, MROD (CYP1A2 biomarker) and PNPH (CYP2E1 biomarker), while phase II enzymes, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase-1 (NQO1), were elevated. Combined treatment of rats with CAJ and NDEA significantly reduced the levels of hepatic ALT and SDH (by ~100%) as compared to values from NDEA-treated animals. CAJ pretreatment further increased the PROD (CYP2B biomarker) and NQO1 activities increased by NDEA administration. Modulation of enzymes activities was accompanied by the changes in the proteins levels. These results indicate that CAJ may protect liver against damage induced by NDEA. Moreover, a significant decrease of SDH activity by CAJ may confirm its potential anti-diabetic activity.

Keywords: cloudy apple juice, cytochrome P450, GST, NDEA, NQO1, comet assay

Apples and apple juice are the most widely consumed fruit and fruit products in the Western diet. Early epidemiological studies have linked the consumption of apples with reduced risk of certain cancers, cardiovascular disease, asthma and diabetes (1). Experimental data indicated the antioxidant, anti-inflammatory and anti-mutagenic activities of apples or apple juice, which might be related to signal transduction pathways and carcinogen metabolism modulation (1, 2).

Apples are a rich source of phenolic constituents, which are distributed in the peel, core and pulp. The content and composition of phenolic compounds vary strongly depending on the apple variety, area of cultivation and time and year of harvest. The total polyphenol content of apples represents about 0.01 to 1% of the fresh weight. The main structural classes include hydroxycinnamic acids, dihydrochalcones, flavonols (quercetin glycosides), catechins and oligomeric procyanidins, as well as anthocyanins in red apples. Apples and apple juice are particularly good sources of oligomeric procyanidins (OPC) composed of (epi)catechin units which have recently gained interest because of potential health promoting effects. In apples OPC constitute 63-77% of all polyphenols (3). Several studies have shown higher cancer-preventive efficacy of cloudy apple juice in comparison to the clear product, which might be related to higher content of OPC (4, 5).

Apple OPC were found to inhibit colon cancer induced in rats by azoxymethane (6). This carcinogen similar to other N-nitroso compounds, including *N*-nitrosodiethylamine (NDEA), requires metabolic activation in order to exert tumorigenic activity. Metabolic activation is catalyzed by hepatic microsomal CYP450, mainly CYP2E1 and 2B (7).

In the case of N-nitrosoamines, including hepatocarcinogenic NDEA, cytochromes P450 mediated hydroxylation at position α to the nitroso group and

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formation of α-hydroxynitrosoamine have been considered a crucial step in the bioactivation to ultimate carcinogenic forms resulting in DNA alkylation, which might initiate the process of tumorigenesis. Moreover, it has been suggested that NDEA, besides being metabolized to reactive electrophiles, causes the generation of reactive oxygen species (ROS) leading to oxidative stress and cellular injury (8).

Phase II enzymes such as glutathione S-transferases (GSTs) conjugate activated phase I metabolites to endogenous ligands and enhance their detoxification and excretion. The reduction of elevated phase I enzymes activities to physiological levels and enhancing excretion of carcinogens *via* the upregulation of phase II enzymes are considered important chemoprevention strategies of cancers induced by exo- and endogenous carcinogens (3). This strategy refers also to apple juice.

Recently, Kujawska et al. (9) using the *in vivo* rat model with NDEA and carbon tetrachloride (CCl_4) challenge demonstrated a decrease in microsomal lipid peroxidation in the liver of rats treated with cloudy apple juice and NDEA in comparison to values obtained from animals given NDEA alone. The similar effect was observed in the case of CCl_4. However, DNA damage in the whole blood leukocytes was partially reduced only in animals treated with apple juice and NDEA but not in their counterparts exposed to juice and CCl_4.

Moreover, Soyalan et al. (10) described the elevation in the expression of the antioxidant response element-dependent genes in the distant colon of rats consuming cloudy apple juice, however, in the hepatic tissue, only NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione peroxidase were upregulated. The reported results showed that the effects of cloudy apple juice depend on metabolic pathway and tissue as well as on the kind of carcinogen tested.

The aim of our current study was to evaluate the effect of the long term treatment of rats with cloudy apple juice alone or in combination with NDEA on the phase I and II enzymes and hepatic DNA damage and the liver injury.

EXPERIMENTAL

Chemicals

NDEA, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, 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, ethidium bromide and Tris were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Normal melting point agarose was Prona Plus Agarose, Triton X-100 was purchased from Park Scientific, (Northampton, UK). Primary and secondary antibodies against CYP1A1/1A2, β-actin, GST α, GST μ, GST π, GST θ and NQO1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary and secondary antibodies against CYP2E1 were supplied by Oxford Biomedical Research (Oxford, MI, USA). Primary and secondary antibodies against CYP2B 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 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 γ glutamyl transferase (GGT) activities were provided by Pointe Scientific, Inc. (Canton, MI, USA).

All the other chemicals were commercial products of the highest purity available.

Animals and treatments

Male Wistar rats (6 weeks of age), provided by the University of Medical Sciences, Department of Toxicology Breeding Facility (Poznań, Poland), were housed in polycarbonate cages ($30 \times 20 \times 25$ cm; $4 \leq$ rats/cage), containing hardwood chip bedding. Commercial rat food (Labofeed H, ISO 9001 certified) and distilled water were available without restriction. The experimental animals were randomly divided into four experimental groups each of six rats.

The animals were treated by gavage with 10 mL of apple juice per kg body weight for 28 consecutive days. The chosen juice dose corresponds to approximately 500-600 mL of juice consumed daily by an average-weight adult individual. On day 27, NDEA was administered *i.p.* in a single tumor-initiating dose of 150 mg/kg body weight (11). Control groups of animals received distilled water without restriction. All the experiments were conducted according to the European guidelines for the care and use of laboratory animals and were approved by the Regional Ethics Committee (No. 33/2007).

Cloudy apple juice preparation

Apples Shampion before processing were stored for 3 months at temperature +1.5°C in normal atmosphere until complete starch degradation measured by iodine test. Only sound fruits after storage were used for juices production. Before processing, apples were ground using Fryma perforated disc mill with disc having circular openings of 6 mm (BASIS 91/55, Fryma-Maschinen AG, Rheinfelden, Switzerland).

Cloudy juices were pressed using a rack and frame press (Bucher, Niederweningen, Switzerland), ascorbic acid was added during apples grinding (200 mg/kg of apples). Raw cloudy juice was centrifuged using continuous flow disk stack centrifuge (LAB 102B-25, Alfa Laval, Brentford, Middlesex, UK) at 1500 rpm and hot filled at 96-98°C into 0.25 L bottles using plate heat exchanger (P20-VB, Alfa-Laval Food Engineering, Lund, Sweden). After 30 min, bottles were cooled in tap water and then stored in the dark at 4°C for 4 weeks prior to experiments.

Determination of phenolic compounds in CAJ (12)

Qualitative characterization of cloudy apple juice was performed by HPLC analyses using a Phenomenex Fusion RP column (250 × 4.6 mm; 4 µm) with a guard column . The mobile phase consisted of 10.2% acetic acid in 2 mmol/L sodium acetate (solvent A) and acetonitrile (solvent B). The flow rate was kept constant at 0.5 mL/min for a total run time of 73 min at 25°C. The system was run with a gradient program: 3% B (0-20 min); 3-17% B (20 min); 17-40% B (25 min); 40-90% B (3 min); 90-90% B (4 min); and 90-0% B (1 min).

Phenolic content in natural cloudy apple juices was 230.1 mg/L. Main group consisted of flavan-3-ols and oligomeric procyanidins (Fig. 1) followed by phenolic acids. Dihydrochalcones (phloridzin and phloretin xyloglucoside) are very specific compounds of apple juice (13, 14). These compounds are present mainly in apple seeds and peel (15, 16) and during fast processing of fruits in to cloudy juices remain in the apple pomace.

Preparation of liver homogenates and cytosolic and microsomal fractions

Twenty four hours after the last treatment, the rats were anesthetized by ketamine and 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, cholesterol, creatinine, BUN levels and ALT, AST, SDH, LDH, GGT activities. The livers were removed, 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 as described previously (17). Protein concentrations were determined by the method of Lowry et al. (18) using bovine serum albumin as the standard.

Phase I and II enzyme activity assays

The activities of ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and pentoxyresorufin-*O*-dependentylase (PROD) were measured as described previously (19, 20). The activity of *p*-nitrophenol hydroxylase (PNPH) was determined according to the Reinke and Moyer (21) protocol. Cytosolic NQO1 activity was assayed as described by Ernster (22) and modified by Benson et

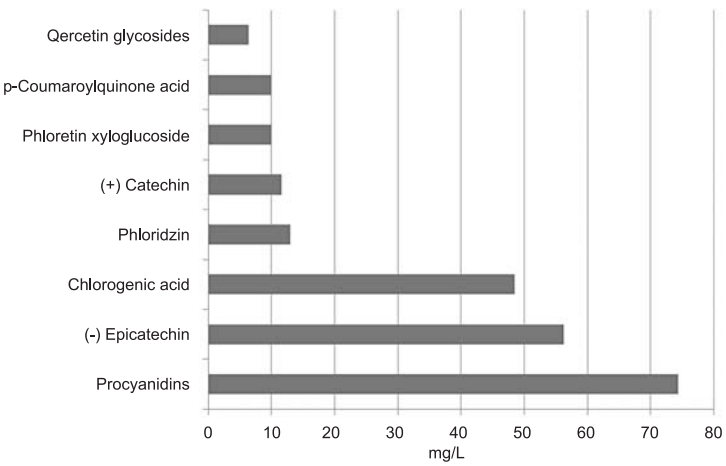

Figure 1. Composition of phenolic compounds in cloudy apple juice

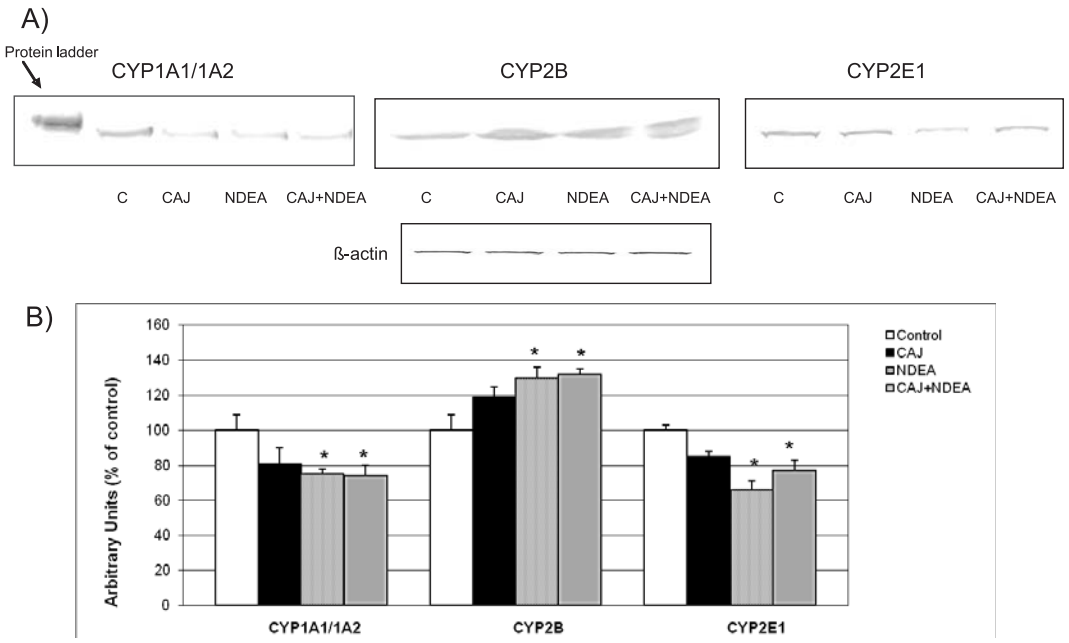

Figure 2. Effect of cloudy apple juice and NDEA on the expression of CYP1A1/1A2, CYP2B, CYP2E1 in microsomal fractions from rat liver. (**A**) Western blot analysis – representative blot is shown: C – control, CAJ – Cloudy apple juice, NDEA – N-nitrosodiethylamine, CAJ+NDEA – Cloudy apple juice + N-nitrosodiethylamine. The β-actin protein was used as an internal standard.(**B**) Data (mean ± SEM) present percentage of value obtained in control group, (expressed as arbitrary units), 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 * control group, $p < 0.05$

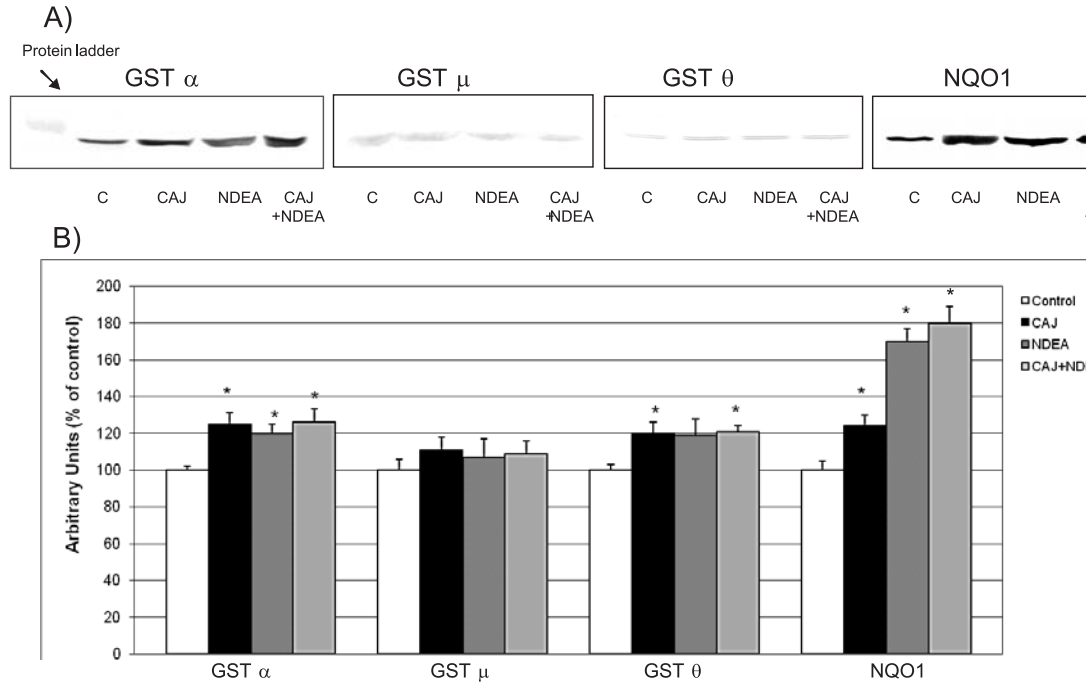

Figure 3. Effect of cloudy apple juice and NDEA on the expression of GST α , GST μ , GST π and NQO1 in cytosolic fractions from rat liver. (**A**) Western blot analysis – representative blot is shown: C – control, CAJ – Cloudy apple juice, NDEA – N-nitrosodiethylamine, CAJ+NDEA – Cloudy apple juice + N-nitrosodiethylamine.(**B**) Data (mean ± SEM) present percentage of value obtained in control group, (expressed as arbitrary units), 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 * control group, $p < 0.05$

al. (23) with NADPH as the electron donor and DPIP as the electron acceptor. The activity of GST was measured by the method of Habig et al. (24), using CDNB as a substrate.

Protein immunoblotting

Cytosolic and microsomal proteins (20-100 μg) were separated on 10 or 12% SDS-PAGE slab gels by the method of Laemmli (25). The proteins were transferred to nitrocellulose membranes using the method of Towbin et al. (26) and after blocking with 5% or 10% skimmed milk they were probed with mouse anti-rat CYP1A1/1A2, goat anti-rat CYP2B, goat anti-rabbit CYP2E1, rabbit anti-human GST α, goat anti-rat GST μ, rabbit anti-human GST π, mouse anti-human GST θ, goat anti-human NQO1 or rabbit anti-mouse β-actin antibodies. As the secondary antibodies in the staining reaction, the alkaline phosphatase-labeled anti-goat IgG, anti-mouse IgG or anti-rabbit IgG were used. The β-actin protein was used as an internal control. The amount of the 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 the liver homogenates according to the method described by Hartmann et al. (27). Samples embedded in the LMP agarose were submitted to the procedures of cell lysis, DNA unwinding, electrophoresis and neutralization and then they were dehydrated in the 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. For each sample 100 comets were scored. The comets were divided into 5 groups according to the degree of the DNA damage (28). 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 units were expressed as the percentage of the values received 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 con-

Table 1. Effect of cloudy apple juice and NDEA on the selected plasma enzymatic markers of rat liver function.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
ALT (IU/L)	35.12 ± 1.96	43.93 ± 1.81 (125)*	87.80 ± 2.80 (250)*	50.22 ± 2.09 (143)*,**
AST (IU/L)	63.24 ± 2. 11	59.44 ± 0.83 (94)	119.52 ± 2.20 (189)*	115.09 ± 2.56 (182)*
SDH (IU/L)	5.02 ± 0.41	4.03 ± 0.09 (80)*	30.67 ± 2.34 (611)*	25.04 ± 1.37 (499)*,**
LDH (IU/L)	189.80 ± 3.39	212.58 ± 4.83 (112)	265.72 ± 8.23 (140)*	216.37 ± 6.24 (114)
GGT (IU/L)	5.09 ± 1.01	6.27 ± 0.09 (123)*	9.11 ± 0.23 (179)*	7.53 ± 0.12 (148)*,**

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. Values in parentheses represent percent of control value. * Significantly different from control, $p < 0.05$. ** Significantly different from NDEA-treated rats, $p < 0.05$.

Table 2. Effect of cloudy apple juice and NDEA on the selected plasma biochemical markers of rat liver and kidney functions.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
Albumin (g/dL)	5.14 ± 0.09	5.12 ± 0.08 (100)	4.63 ± 0.11 (90)	5.10± 0.07 (99)
Bilirubin (mg/dL)	0.28 ± 0.04	0.29 ± 0.11 (104)	0.59 ± 0.06 (211)*	0.46±0.07 (164)*,**
Creatinine (mg/dL)	0.35 ± 0.04	0.38 ± 0.01 (109)	0.69 ± 0.05 (197)*	0.46±0.07 (131)*,**
BUN (mg/dL)	26.56 ± 0.21	26.83 ± 0.26 (101)	23.64 ± 0.21 (89)	24.87±0.31 (94)

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

trols was assessed by Tukey's *post hoc* test, with $p < 0.05$.

RESULTS

Selected biochemical markers of liver function in blood

The effects of CAJ and NDEA on selected liver function biochemical parameters (ALT, AST, ALP, SDH, LDH, GGT activities and albumin, bilirubin, creatinine, BUN) are presented in Tables 1 and 2. CAJ significantly increased ALT and GGT activities, but decreased SDH. Treatment of rats with a single dose of 150 mg/kg body weight of NDEA alone resulted in a statistically significant increase in the activity of all the tested enzymes in blood plasma in comparison to values from the control group (by 40-511%). Pretreatment with CAJ protected against the NDEA induced damage, reducing the activity of ALT, SDH, and GGT, and also bilirubin and creatinine levels.

Phase I enzymes in rat liver

The effects of CAJ and NDEA on cytochrome P450-dependent enzymes in rat liver are summarized in Table 3. Twenty eight days of forced feeding with cloudy apple juice alone significantly decreased the activities of MROD (the marker of CYP1A2) and PNPH (the marker of CYP2E1) by 19 and 20%, respectively, in comparison with the results from the control group of animals receiving water only. NDEA treatment reduced the activities of EROD (the marker of CYP1A1), MROD and PNPH by 40, 31 and 45%, respectively. The opposite effect was observed for PROD (the marker of CYP2B). As shown in Table 3, CAJ increased the activity of PROD by 25%, while NDEA enhanced it by 52%.

Pretreatment with CAJ further increased PROD activity elevated by NDEA treatment. Modulation of P450 enzymes activities was accompanied by changes in the relevant proteins levels. Western blot analysis with CYP2E1 and CYP1A1/1A2 specific antibodies (Fig. 2) revealed statistically significant decrease in the corresponding protein level in the NDEA-treated animals in comparison to the value achieved in the control rats. Densitometry of the bands presented in Figure 2 showed an enhanced level of CYP2B (by about 30%) in the liver of animals exposed to NDEA and the combined treatment with CAJ and NDEA.

Phase II enzymes in rats liver

The effects of CAJ administration alone or in combination with NDEA on phase II enzymes activities are presented in Table 4. Treatment with CAJ enhanced the activities of GST and NQO1. NDEA alone increased GST and NQO1 by 22 and 81%,

Table 3. Effect of cloudy apple juice and NDEA on the activity of cytochromes P450 in rat liver.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
EROD	35.14 ± 1.57	32.66 ± 1.48 (93)	21.08 ± 1.32 (60)*	23.54 ± 2.25 (67)*
MROD	31.76 ± 1.34	25.73 ± 0.86 (81)*	21.91 ± 0.55 (69)*	24.00 ± 0.9 (66)*
PROD	14.81 ± 0.88	18.05 ± 0.60 (125)*	22.51 ± 0.80 (152)*	25.77 ± 1.18 (174)*,**
PNPH	578.13 ± 54.83	462.50 ± 14.48 (80)*	319.08 ± 37.10 (55)*	384.88 ± 45.80 (60)*

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. EROD, MROD, PROD - pmol resorufin formed/min per mg of protein. PNPH - pmol p-nitrocatechol formed/min per mg of protein. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

Table 4. Effect of cloudy apple juice and NDEA on the activity of phase II enzymes in rat liver.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
GST	992.95 ± 41.44	1191.54 ± 35.03 (120)*	1211.39 ± 50.02 (122)*	1320.62 ± 43.84 (133)*
NQO1	96.08 ± 6.91	126.34 ± 3.90 (131)*	173.90 ± 17.79 (181)*	201.77 ± 9.17 (210)*,**

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. GST - nmol 1-chloro-2,4-dinitrobenzene conjugated formed/min per mg of protein. NQO1 - nmol 2,6-dichloroindophenol reduced/min per mg of protein. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

Table 5. Effect of cloudy apple juice and NDEA on the extent of DNA damage in homogenates of rat liver.

Treatment	DNA damage
Control	77.7 ± 3.19
CAJ	87.3 ± 2.36 (113)*
NDEA	123.7 ± 3.38 (159)*
CAJ + NDEA	120.0 ± 0.817 (154)*

Values are the means ± SEM from 6 animals. Each assay was run in duplicate. DNA damage in homogenates of rat liver in arbitrary units. Values in parentheses represent percent of control value.
* Significantly different from control, $p < 0.05$.

respectively. A similar effect was also observed in animals after the combined treatment with CAJ and NDEA with the exception of NQO1 which activity was further increased.

Figure 3 presents the immunoblots of GST isozymes and NQO1 and their quantitative analysis. GST π protein was not detected in the liver. CAJ increased the constitutive expression of GST μ, α and θ (by 11, 25 and 20%, respectively), but did not affect the NDEA induced GST proteins. The increased activity of NQO1 was accompanied by the elevated level of the enzyme protein as a result of cloudy apple juice or NDEA treatment as well. The pretreatment with CAJ did not significantly affect the level of NDEA-induced NQO1 protein.

Comet assay analysis of DNA damage in the liver homogenates

The effects of CAJ and NDEA treatments on DNA damage are presented in Table 5. CAJ administered to rats for 28 days moderately augmented the scale of DNA damage in the liver. NDEA alone increased the extent of DNA lesions by ~59%. Pretreatment of rats with CAJ did not significantly reduce DNA damage in NDEA-administered animals.

DISCUSSION

Several epidemiological studies suggest that fruits and vegetables in basal diet afford a significant protection against a wide range of common human cancers (29, 30). Some of the surveys showed that the protective effect of a high intake of fruits and vegetables was only weakly correlated with single dietary constituents or the distinct groups of phytochemicals. Moreover, cancer intervention studies with a single compound or simple combinations of promising anticarcinogens, often failed to show a cancer protective effect against several cancer types. These data indicate that the protective action most likely arise from the combined intake of several dietary components such as fruit or vegetables juices, rather than being a result of a single anticarcinogenic components (31, 32).

Apples and apple products, including juices and extracts, were included in health-related studies around the world due to their rich content of diverse phytochemicals, particularly specific classes of polyphenols. Although apple products were shown to exert beneficial effect in numerous pathologies, their preventive activity against chemically induced carcinogenesis was relatively less explored. Oszmiański et al. (33) found markedly higher content of procyanidins and pectins in cloudy apple juices, which was associated with higher radical-scavenging and antioxidant capacities. Additionally, Barth et al. (4, 5) demonstrated higher cancer-preventive efficacy of cloudy apple juice in comparison with clear juice. The authors suggested that these juices could modulate biochemical pathways by antagonistic, additive and/or synergic mechanisms.
In the present study, we focused on the evaluation of a possible interference of CAJ with NDEA-induced effects. This chemical is a very potent carcinogen that induces liver carcinomas and gastrointestinal tract neoplasms in rats (34). A prominent phenomenon during hepatocarcinogenesis is an alteration of the expression of drug-metabolizing enzymes. In the case of NDEA, cytochromes P450 CYP2E1- and 2B-mediated activation leads to the formation of electrophiles, which ethylate DNA and, if not repaired, to mutation. Moreover, the uncoupling of electron transfer and oxygen reduction from monooxygenation by CYP2B1 and CYP2E1 could result in the release of $O_2^{\cdot-}$ and H_2O_2 and cause liver injury (8, 35).

The results of our present study confirm our previous observations concerning NDEA effects in rat liver (36, 37).

The treatment with this compound resulted in an increase in hepatic DNA and liver tissues damage biomarkers, and the reduction in EROD, MROD and PNPH activities. At the same time NDEA induced CYP2B and phase II enzymes, GST and NQO1.

Twenty eight days administration of CAJ resulted in a decrease of MROD and PNPH activities. The expression of P450 isozymes, CYP1A1/A2, CYP2B and CYP2E1 was not changed in comparison with the values found in control group of animals. Since these P450 isoforms are involved in the activation of several classes of chemical carcinogens including polycyclic aromatic hydrocarbons and nitrosamines, their inhibition is

expected to block both the toxicity and carcinogenicity of these compounds. Such mechanisms of anticarcinogenic activity was proposed for several phytochemicals including organosulfur compounds and coumarins (38, 39). Decreased expression and activity of CYP1A as a result of apple juice extracts treatment was observed also by Pohl et al. (40) in Caco-2 colon cancer cells. Moreover, feeding with CAJ in our study increased the activity and protein level of phase II enzyme NQO1 and, to a lesser extent, of GST. Thus, the inhibition of CYP2E1 in concert with the induction of NQO1 might contribute to the potential anticarcinogenic activity of CAJ. We suggested a similar mechanism for chokeberry juice (36). Chokeberry juice like cloudy apples juice contains high amount of OPC. Since these compounds were shown to be responsible for the high antioxidant activity of CAJ, they might be responsible also for the effects observed in our current study.

On the other hand, CAJ slightly increased the activity and expression of CYP2B. This is a large P450 subfamily that encodes versatile catalysts of xenobiotics and steroid hydroxylation. Some of them induce phenobarbital-type response, epoxide hydrolase and GST (41). Thus, the induction of CYP2B may be related to tumorigenesis promotion, but also the enhancement of the detoxification of carcinogens.

Although CAJ feeding only slightly increased total GST activity, immunoblotting revealed an increase of GST α and θ. Increased level of GST α might be beneficial as this isoform contributes to the protection against ROS and the products of lipid peroxidation such as 4-hydroxynonenal (42), while GST θ class 1 and 2 (GSTT1 and GSTT2) react with a wide range of xenobiotics, including nitrosamines, what implicates their possible role in the prevention of carcinogenesis induced by NDEA. The possible involvement of GSTT2 in the anticarcinogenic effect of apples further supports the observation of Petermann et al. (43) that the induction of this GST isoform by apple polyphenols protects colon epithelial cells against genotoxic damage.

Consistently with our previous and the other authors observations (44, 45), the GST π protein was not detected in the liver, since this isoform is present in placental liver only, but not in adult rats (46). 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 (47).

Moreover, our recent study showed that phloretamide, one of apple components, activates the Nrf2/ARE pathway in human hepatocytes in culture (48). Thus, it is possible that this mechanism is also responsible for the induction of NQO1 and GST by apple juice in our current study.

Pretreatment with CAJ before the administration of NDEA reduced ALT, SDH and GGT activities and plasma bilirubin and creatinine levels. CAJ treatment alone caused minor, however significant rise in DNA damage, whereas NDEA induced larger DNA lesions. CAJ pretreatment before NDEA challenge did not protect hepatic DNA from decomposition. Among liver damage biomarkers, the reduction in SDH activity, which was also observed after feeding with CAJ only, seems to be the most interesting phenomenon.

Apples were identified as the unique flavonoid-rich food that might be protective against type 2 diabetes (49). SDH is a key enzyme in the polyol pathway converting sorbitol to fructose which level is significantly elevated in diabetes. The reduction in SDH activity may suggest that the antidiabetic effect of apples may be related to improvement of the polyol pathway as was shown for ursolic acid (50).

In our present experiment, CAJ pretreatment did not affect the expression and activity of CYPs increased by NDEA exposure, except for CYP2B which was elevated. Since, as it was mentioned above, the consequences of this CYP450 subfamily induction are complex, the explanation of this observation requires additional studies.

The most marked effect of NDEA on cytochrome P450 dependent enzymes (EROD, MROD, PNPH) was observed both on the levels of the enzymes activity and respective CYP proteins (CYP1A1/CYP1A2, CYP2E1), there was no significant correlation between the CYPs protein level and enzyme activities in the case of CAJ.

CAJ pretreatment further increased the expression and activity of NQO1 induced by NDEA. This enzyme is generally assumed to possess important protective properties, both by detoxifying carcinogenic compounds as well as by preventing the generation of oxygen radicals (51).

Collectively, the results of our present study indicate that metabolic alterations induced by cloudy apple juice may protect against liver damage and attenuate the effect of chemical carcinogens. Moreover, CAJ treatment decreased sorbitol dehydrogenase activity, a key enzyme of sorbitol pathway, particularly active in diabetic patients. Since cloudy apple juice is one of the most common diet components the results of our current study, although require further investigations, provide

rationale for its recommendation as chemopreventive food item.

Declaration of interest

The authors declare no conflicts of interest. All authors approved the final version submitted for publication.

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