Effects of Commonly Consumed Fruit Juices and Carbohydrates on Redox Status and Anticancer Biomarkers in Female Rats

Vibeke M. Breinholt, Salka E. Nielsen, Pia Knuthsen, Søren T. Lauridsen, Bahram Daneshvar, and Annemarie Sørensen

Abstract: Administration of apple juice, black currant juice, or a 1:1 combination of the two juices significantly decreased the level of the lipid peroxidation biomarker malondialdehyde in plasma of female rats, whereas the protein oxidation biomarker 2-amino-adipic semialdehyde, was significantly increased following administration of orange juice, black currant juice, or the 1:1 combination of apple and black currant juice. A significant increase in 2-amino-adipic semialdehyde was also observed in control rats given sucrose, fructose, and glucose in the drinking water at concentrations approximating the average carbohydrate levels in the employed fruit juices. None of the fruit juices were found to affect the activities of antioxidant enzymes in red blood cells or hepatic glutathione S-transferase. Hepatic quinone reductase activity, on the other hand, was significantly increased by grapefruit juice, apple juice, and black currant juice. The total daily intake of a selected subset of flavonoid aglycones ranged from 0.2 to 4.3 mg, and quercetin was found to be a minor constituent of all the juices investigated. In a parallel study, rats were fed quercetin at doses ranging from 0.001 to 10 g/kg of diet. However, no effects were observed on hepatic glutathione S-transferase or quinone reductase activities, plasma redox status, or the activity of red blood cell antioxidant enzymes. Overall, it thus seems more appropriate and less risky to obtain “an increased protected state” by increasing the consumption of fruits and vegetables rather than obtaining the potentially protective factors from enriched fractions of plant material or as synthetic or purified plant components.

Introduction

Many experimental animal studies, human intervention studies, and epidemiological studies suggest that inclusion of fruits and vegetables in a basal diet affords a significant protection against a wide range of common human cancer types (1–7). However, despite extensive research in the area of cancer chemoprevention for several decades, it is yet not possible to pinpoint specific food items or single dietary components that are particularly efficient in protecting against cancer. The observed protective effect of a high intake of fruits and vegetables is thus only weakly correlated to single dietary constituents or to distinct groups of phytochemicals. The findings that several cancer intervention studies with single or simple combinations of promising anticarcinogens have failed to show a cancer protective effect against several cancer types (8–12) indicate that the protective action of fruits and vegetables most likely arise from the combined exposure to several dietary components, rather than being the result of the action of single anticarcinogenic components. Overall, it thus seems more appropriate and less risky to obtain “an increased protected state” by increasing the consumption of fruits and vegetables rather than obtaining the potentially protective factors from enriched fractions of plant material or as synthetic or purified plant components.

Epidemiological investigations repeatedly show that regular consumption of dark green and cruciferous vegetables, tomatoes, and citrus fruits in particular is related to a reduced risk of several cancers (7,13–18). That fruit juices are readily accessible throughout the world, palatable to most people and a major source of flavonoids in several countries (19,20), warrants further studies on the health promoting effects of commercially available fruit juices.

The aim of the present study was to investigate the ability of four different fruit juices, containing a wide range of potentially cancer protective flavonoids, to affect selected biomarkers for redox status and phase 2 enzyme capacity in female rats, to gain information on the potentially health promoting effects of commercially available fruit juices.

The potential protective role of the most commonly occurring carboh-
Materials and Methods

The chemicals used in this study were obtained from the following: Sigma Chemical (St. Louis, MO): butylated hydroxytoluene, 2-thiobarbituric acid, NADPH, glutathione, FAD, purpureal, potassium periodate, β-naphthoflavone (BNF) (>97%), UDP-glucuronic acid, NADH, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, HEPES, Tween 20, glucose-6-phosphate, cytochrome c, menadione, glucose-6-phosphate dehydrogenase, and dicumarol; Aldrich Chemical (Steinheim, Germany): malondialdehyde and quercetin (>98%). Ethoxyresorufin, methoxyresorufin, pentoxyresorufin, benzoxylresorufin, and resorufin were obtained from Molecular Probes (Eugene, OR). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Riedel-de Häen (Seeze, Germany). The following commercial kits were used: superoxide dismutase (SOD) (Randox, cat. no. RS 505), glutathione peroxidase (GPx) (Randox, cat. no. RS 505), hemoglobin (Randox, cat. no. HG 980), fructose (BoehringerMannheim, kit. no. E0139106), and protein (Roche, cat. no. 0736783). All other chemicals were from Merck (Darmstadt, Germany).

Animals, Exposure Protocol, and Preparation of Subcellular Fractions

Forty female Wistar rats, aged 6–7 wk (106–138 g) from Møllegård Breeding Center Ltd. (Lille Skensved, Denmark), were acclimatized for 7 days while maintained on a powdered semisynthetic diet based on casein (18%) and carbohydrates (cornstarch, potato starch, dextrin, and sucrose) (68%) (21) and assigned to eight different treatment groups of 5 animals each. Animals were kept in a 12-h light and 12-h dark cycle at an average temperature and humidity of 22°C and 55%, respectively. The animals in Group 8 were administered BNF by gavage at a concentration of 0.1 g/kg of body weight (kg b.w.) in a total volume of 2 ml of dimethyl sulfoxide per kilogram of body weight during the last 4 days of the study. In Groups 1 (control rats) and 8, the animals were administered tap water, whereas the tap water in Groups 2–7 was substituted with fruit juices (Groups 2–6) or tap water containing different carbohydrates (Group 7) as shown in Table 1. During the study the animals had free access to the semisynthetic diet. After 7 days the animals were anesthetized and approximately 2–3 ml of blood was drawn from the heart. The animals were subsequently killed by decapitation. The liver was rinsed in iced cold phosphate-buffered saline, blotted dry on paper towel, weighed, and submerged in liquid nitrogen until storage at −80°C. The microsomal and cytosolic fractions from the liver were prepared as described by others (22). Aliquots of 0.5 ml were stored at −80°C until use. The protein concentration was determined by using the BCA method (23) adapted to a Cobas Mira (Roche Diagnostics System, Basel, Switzerland). The heparinized blood samples were separated into plasma and red blood cells (RBC) by centrifugation at 1,500 g for 10 min. The blood cells were washed three times with 1 volume of cold 0.9% NaCl followed by centrifugation at 1,500 g for 10 min. The washed cells were lysed with 1 volume of deionized water. Blood lysate and plasma were stored at −80°C until use.

In the quercetin dose-response study, 30 female Wistar rats (116–138 g) from Møllegård Breeding Center Ltd. (Lille Skensved, Denmark) were randomized according to weight into six groups of 5 animals and acclimatized for 7 days.

Table 1. Concentration of Total and Individual Flavonoids and Carbohydrates in Various Fruit Juicesa

<table>
<thead>
<tr>
<th>Flavonoid/Carbohydrate</th>
<th>Concentration of Flavonoids or Carbohydrates g/l Juice</th>
<th>Concentration of Flavonoids or Carbohydrates g/l Juice</th>
<th>Concentration of Flavonoids or Carbohydrates g/l Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Mean Flavonoid (mg) or Mean Carbohydrate (g) Intake/Animal/Day]</td>
<td>[Mean Flavonoid (mg) or Mean Carbohydrate (g) Intake/Animal/Day]</td>
<td>[Mean Flavonoid (mg) or Mean Carbohydrate (g) Intake/Animal/Day]</td>
</tr>
<tr>
<td></td>
<td>Group 2 (Grapefruit)</td>
<td>Group 3 (Apple)</td>
<td>Group 4 (Black Currant)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.003 (0.062)</td>
<td>0.002 (0.036)</td>
<td>0.004 (0.078)</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.196 (4.0)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>0.007 (0.14)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.003 (0.062)</td>
<td>n.d.</td>
<td>0.001 (0.019)</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>0.003 (0.062)</td>
<td>n.d.</td>
<td>Trace</td>
</tr>
<tr>
<td>Myricetin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.011 (0.21)</td>
</tr>
<tr>
<td>Phloretin</td>
<td>n.d.</td>
<td>0.011 (0.2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Glucose</td>
<td>22.8 (0.5)</td>
<td>23.2 (0.3)</td>
<td>51.4 (0.6)</td>
</tr>
<tr>
<td>Fructose</td>
<td>23.4 (0.5)</td>
<td>58.2 (0.5)</td>
<td>51.6 (1.1)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.0 (0.5)</td>
<td>15.9 (1.1)</td>
<td>27.6 (1.1)</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>0.212 (4.326)</td>
<td>0.013 (0.236)</td>
<td>0.016 (0.307)</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>66.2 (1.5)</td>
<td>97.3 (1.9)</td>
<td>130.4 (2.8)</td>
</tr>
</tbody>
</table>

a: The content of anthocyanins and catechins that may have been present and thus contributed to the overall flavonoid concentration in the juices was not determined in the present study. n.d., not detected.
while maintained on a powdered semisynthetic diet with free access to tap water. After acclimatization the animals received the semisynthetic diet containing 0, 0.001, 0.01, 0.1, 1, or 10 g of quercetin per kilogram of diet for 2 consecutive weeks. The animals were killed at Day 15 and the liver and blood processed as described above. Two samples from each animal were analyzed and each individual analysis was conducted in duplicate.

In both studies the intake of water or juice as well as food was recorded weekly. The body weights were likewise recorded once a week. Animal care and use complied with the recommendations of the National Institutes of Health (24).

**Determination of Drug-Metabolizing and Antioxidant Enzyme Activities**

The activities of cytosolic quinone reductase (QR) and glutathione S-transferase (GST) were determined on a Cobas Mira analyzer as described previously (25,26), according to the methods by Ernster (27) and Habig et al. (28). QR and GST activities were measured by using cytochrome c and CDNB as substrates, respectively. Automated assays for the activities of the antioxidant enzymes SOD, GPx, catalase (CAT), and glutathione reductase (GR) in blood hemolysate were performed on a Cobas Mira analyzer. SOD and GPx activities and hemoglobin were determined by using commercially available kits, whereas the activity of GR was determined by the method of Goldberg and Spooner (29). CAT activity was determined according to a method described by Wheeler et al. (30). The enzymatic activities in RBC were calculated relative to the amount of hemoglobin, measured by using Drabkins reagent (Randox Laboratories Ltd., Crumlin, UK).

**Assessment of Lipid and Protein Oxidation**

The biomarker of lipid peroxidation, malondialdehyde (MDA), and the protein oxidation biomarker, 2-amino adipic semialdehyde (AAS), were measured as described by Lauridsen and Mortensen (31) and Daneshvar and colleagues (32), respectively.

**Quantification of Flavonoids and Individual Carbohydrates in Fruit Juice**

All fruit juices were purchased from three different grocery stores in Denmark. A grapefruit juice from Cadiso (Frederikssund, Denmark) and a freshly squeezed orange juice from Irma (Rødovre, Denmark) containing high concentrations of the flavanones naringenin and hesperetin were selected for the study. Both juices contained additional flavonoids as shown in Table 1. The apple juice used was produced by Nutana (Bjæverskov, Denmark) and the black currant juice by Cadiso (Frederikssund, Denmark). The concentration of a selected set of flavonoids and carbohydrates present in the juices is given in Table 1.

Flavonoids were determined by applying fruit juice (5 ml) to a Bond Elute column (500 mg, Varian, Harbor City, CA), activated with 10 ml of methanol and 20 ml of water containing 1% formic acid. The column was rinsed with another 10 ml of water and finally the sample was eluted with 20 ml of methanol, evaporated in vacuo, and the reconstituted sample hydrolyzed in 1.2 M HCl. The hydrolysate was analyzed by liquid chromatography/mass spectrometry (LC/MS) as described previously (19). Fructose was determined enzymatically by the ultraviolet light method of Boehringer Mannheim (kit no. E0139106). Glucose and sucrose were determined by high-pH light method of Drabkins reagent with pulsed amperometric detection using a CarboPac MA1 column from Dionex (Sunnyvale, CA) as described by Andersen and Sørensen (33).

**LCMS Analysis of Plasma Flavonoids**

Aliquots of 300 µl of plasma were added, 250 ng of morin and daidzein as internal standards. The samples were enzymatically hydrolyzed essentially as described previously (34) by the addition of 50 µl of β-glucuronidase/arylsulfatase (0.55 U and 0.26 U, respectively) and incubation in a sealed vial for 1 h at 37°C under continuous shaking. After hydrolysis, the samples were added 2.0 ml of m-phosphoric acid and extracted with 2 ml of ethyl acetate. The samples were centrifuged for 10 min at 10,000 g at 4°C and the ethyl acetate phase was transferred to a new vial. This procedure was repeated twice. After pooling of the two organic phases the sample was evaporated to dryness under vacuum. The dry residue was stored under Argon (g) at −20°C until analysis. Prior to LC/MS the sample was reconstituted in 10% aqueous methanol containing 1% formic acid, giving a final volume of 250 µl, that was all injected onto the LC/MS. The methodology used for the LC/MS analysis of the samples with regard to quercetin and its metabolites isorhamnetin and tamarixetin has been described elsewhere (35).

**Statistical Analysis**

Statistical analyses were conducted by using SAS version 6.08 (SAS Institute Inc., Cary, NC). Analysis of variance with Dunnett’s test was employed for comparison between the means of the treated groups and the controls. A P value of less than 0.05 was used as the level of significance.

**Results**

The concentration of a selected subset of flavonoids and carbohydrates present in the investigated fruit juices is given in Table 1. The same brands of apple and black currant juice, as used in a previous human intervention study conducted in collaboration with our laboratory (36), were preselected for the study to be able to compare data between the two studies. The orange juice and the grapefruit used in the study were selected as they contained the highest concentration of total...
flavonoids in comparison with other juices analyzed. Depending on the juice the total daily intake of flavonoids per animal per day ranged between 0.2 and 4.3 mg.

In addition to the flavonoids, the concentrations of the three carbohydrates, sucrose, glucose, and fructose, were determined in the juices. The average daily intake of total and individual carbohydrates is presented in Table 1. From Table 1 it is evident that each fruit juice has a unique carbohydrate profile, and that the intake of both total and individual carbohydrates varied between the experimental groups. Despite the increased carbohydrate intake in groups 2–7 relative to the control Group 1 and the BNF Group 8, final body weights and the average weight gain did not differ among the various groups (data not shown). The relative and total liver weights in BNF-induced animals (Group 8), however, were significantly increased compared to Groups 2–7 (data not shown).

The antioxidant enzymes SOD, GR, GPx, and CAT measured in RBC were not found to be altered by any of the fruit juices or by quercetin administered in the diet at doses ranging from 0.001 to 10 g/kg of diet (Fig. 1A and B).

Hepatic QR was significantly elevated above the control level in rats administered grapefruit juice, apple juice, or black currant juice (Fig. 2). Hepatic QR in the apple plus black currant juice group also tended to be elevated compared to the control group; however, statistical significance was not reached (P > 0.05). Hepatic GST activity for these same juices was also above the control level but again statistical significance was not reached (P > 0.05). Across the employed dose range of quercetin, no effects were observed on GST or QR in the liver. BNF was found to significantly induce QR and GST (Fig. 2), whereas BNF had no effects on the remaining investigated biomarkers (data not shown).

Apple, black currant, and the 1:1 combination of these two juices were found to significantly decrease the lipid peroxidation biomarker MDA in plasma (Fig. 3A). Borderline significant (P = 0.07) reductions in plasma MDA in the orange and grapefruit juice groups were also observed. In contrast, increased levels of protein oxidation products, measured as AAS, were found in plasma of animals exposed to orange, black currant, and black currant plus apple juice. In the group of animals that received sucrose, glucose, and fructose in the drinking water (Group 7), the level of AAS was also significantly increased compared to the control. Neither the level of MDA, nor the level of AAS, was altered by quercetin at doses ranging from 1 mg to 10 g/kg of diet (Fig. 3B).

The bioavailability of quercetin and its major metabolites, isorhamnetin and tamarixetin, methoxylated at the 3 and 4 positions of quercetin, respectively, was analyzed for by LC/MS in the quercetin dose-response study as described in Materials and Methods. Quercetin and isorhamnetin could be detected at all doses investigated, whereas tamarixetin was only found at quercetin doses of 0.1 g/kg of diet or higher (Fig. 4). The concentrations of quercetin, isorhamnetin, and tamarixetin in plasma were found to increase in a dose-dependent fashion. The maximal plasma concentrations of the three flavonoids obtained at the 10 g/kg of diet dose were 2.3, 8.4, and 2.4 µM for quercetin, isorhamnetin, and tamarixetin, respectively. The average daily intake of quercetin (per kg b.w.) based on recorded food consumption data was estimated to be 0, 0.13, 1.3, 12.9, 128, and 1,397 mg, corresponding to total daily quercetin intakes of 0, 0.016, 0.161, 1.654, 16.6, and 177.4 mg per animal. The plasma flavonoid concentration in the juice study was not determined due to an insufficient amount of sample. However, neither quercetin, isorhamnetin, nor tamarixetin could be detected in urine from animals exposed to the various juices (data not shown), and it is thus questionable whether quercetin and its metabolites in plasma would have exceeded the detection limit of 2.5 ng/ml of plasma and thus allowed quantification of the three compounds.

Discussion

Several fruit juices were analyzed for their specific flavonoid content by LC/MS. From these analyses it was evident that the total flavonoid content varied markedly from brand to brand. Similar observations on other juices are re-
ported in the literature (37,38). The content of individual flavonoids was also found to vary within the same brand and type of juice. The concentration of quercetin (2 and 4 mg/l) in the currently employed batches of apple and black currant juice, respectively, was thus somewhat lower than the concentrations previously determined (7 and 6 mg/l) in juices of the same brand but from a different batch (36). Kaempferol was likewise found at a concentration of 0.2 mg/l in the apple juice used in the study by Young and colleagues (36), whereas this flavonoid could not be detected in the present study. The chalcone phloretin found in apple juice at a concentration of 11 mg/l in the present study, on the other hand, was not detected in the particular batch of apple juice used in the human intervention study. These results suggest that the flavonoid content of fruit juices vary considerably between different brands but also among different batches of juice. The observed differences presumably reflect variations in the quality of the fruits, the cultivar, the manufacturing practices used, storage conditions, and duration as well as the presence of other antioxidants in the juice that may function to stabilize the flavonoids. The flavonoid determinations in this and in the previously published juice intervention study (36) were conducted by the same laboratory and even by the same personnel, so only little variation from this source is expected. It should be noted that the employed fruit juices also contain anthocyanins, catechins, and other potentially active phytochemicals, and that the measured set of flavonoids only represents a fraction of the total flavonoid or phytochemical content.

It is noteworthy that the effects observed on the biomarkers of lipid and protein peroxidation, MDA and AAS, follow the same trend in the human study (36) and the present rat study, despite that both the total and the specific flavonoid content of the measured subset of flavonoids varied between the two studies. In both studies MDA was thus significantly decreased by the 1:1 combination of black currant and apple juice and plasma AAS was concordantly significantly increased. This observation strongly suggests that specific components in apple and/or black currant juice trigger identical mechanisms or pathways in humans and in the particular rat model used in the present study. The different qualities of the fruit juices in the two studies, judged from the content of specific flavonoids, furthermore suggest that other components of the fruit juice rather than the measured set of
flavonoids mediate the observed effects on redox status. The administration of either black currant or apple juice to rats suggested that both juices alone could reduce the level of lipid peroxidation, whereas only black currant juice administration increased the level of plasma AAS. Orange juice was also found to have this effect. That AAS was affected in the same direction in the human intervention study (36) and in the present rat study, despite that the juices used were qualitatively very different with respect to flavonoid content, might be a further indication that other juice components, such as carbohydrates, vitamin C, or other potentially prooxidative species, rather than the investigated flavonoids may be responsible for the observed effects on this particular protein oxidation marker. It has previously been speculated that vitamin C or other prooxidant compounds coexisting with vitamin C in fruits may, at least in part, be responsible for the prooxidant effect of fruit juices (36). The animals in Group 7, however, which received the pure carbohydrates sucrose, glucose, and fructose were also found to have an elevated level of plasma AAS compared to the control. This observation strongly suggests that carbohydrates present in the respective fruit juices may also be fully or in part responsible for the observed prooxidant effect on plasma proteins observed following fruit juice treatment. These results may be related to the well-established observations that high glucose levels result in nonenzymatic glycation of both extra- and intracellular proteins, accumulation of sorbitol via the aldose-reductase pathway, activation of protein kinase C isoforms, as well as reduced bioavailability of nitric oxide (39). The generation of reactive oxygen species may thus be a common downstream mechanism by means of which the multiple by-products of glucose exert their adverse effects on plasma proteins. More recent data furthermore suggest that sucrose enhances the number of preneoplastic lesions in azoxymethane-initiated rats (40) and act as a mutagen in rat colon (41). Further studies are thus strongly warranted to more firmly establish the role of carbohydrates and carbohydrate-containing products in mediating protein oxidation in humans, due to the central role refined and unrefined carbohydrates play in human nutrition today.

In several studies dealing with the health protective effects of flavonoids, a major focus has been on the flavonol quercetin, which is present at particularly high concentrations in onions, cruciferous vegetables, wine, and tea (42,43). In the present study, quercetin was found in all fruit juices investigated, but in relatively small amounts. The quercetin dose-response study conducted in the present investigation spanned a wide range of quercetin doses, including those obtained in rats dosed with the various fruit juices (0.04–0.08 mg per day). The results revealed that quercetin alone was not responsible for the observed alterations in redox status or phase 2 enzyme activity, as no effect on the respective biochemical markers was observed following daily quercetin intakes between 0.016 and 177.4 mg. However, it cannot be ruled out that quercetin together with other phytoprotectants present in the juices may play a role in mediating the observed effects.

Investigation of the activities of the two phase 2 enzymes QR and GST in liver and RBC antioxidative enzymes revealed that hepatic QR activity was significantly increased by grapefruit, apple, and black currant juice, whereas none of the remaining enzymes were affected by the juice administrations, by quercetin given in the diet as a pure compound, or by selected carbohydrates. It is noteworthy that administration of moderate doses of fruit juices for only 1 wk is sufficient to increase hepatic QR by 40–60% above the control. These data could be taken to suggest that fruit juices at reasonable dietary levels may favorably affect resistance to chemical carcinogens and other dietary or environmental toxicants detoxified by QR.

Overall, the present study provides evidence that commonly consumed fruit juices may alter important biochemical pathways involved in maintaining redox and phase 2 detoxification status in experimental animals. However, whether alterations of these redox and detoxification enzyme biomarkers do impact on the resistance of the animal toward chemical carcinogens, what particular fruit juice components are responsible for the observed effects, and whether these alterations impact on the cancer process in humans must still be clarified.

Acknowledgments and Notes

The authors would like to thank Anita Nielsen and Katrin Christiansen for excellent technical assistance. Address correspondence to V. M. Breinholt, Morkhøj Bygade 19, 2860 Soeborg, Denmark. Phone: +45 33 95 66 05; FAX: +45 33 95 66 99. E-mail: vib@fdir.dk.

Submitted 5 February 2002; accepted in final form 4 November 2002.
References
