Anthocyanin/Polyphenolic–Rich Fruit Juice Reduces Oxidative Cell Damage in an Intervention Study with Patients on Hemodialysis

Thomas M. Spormann,[1] Franz W. Albert,[3] Thomas Rath,[3] Helmut Dietrich,[4] Frank Will,[4] Jean-Pierre Stockis,[2] Gerhard Eisenbrand,[1] and Christine Janzowski[1]

[1]Division of Food Chemistry and Toxicology, Department of Chemistry, and [2]Department of Mathematics, University of Kaiserslautern; [3]Westpfalz-Klinikum, Medizinische Klinik III, Kaiserslautern, Germany; and [4]Section of Wine Analysis and Beverage Technology, Geisenheim Research Center, Geisenheim, Germany

Abstract

Hemodialysis patients face an elevated risk of cancer, arteriosclerosis, and other diseases, ascribed in part to increased oxidative stress. Red fruit juice with high anthocyanin/polyphenol content had been shown to reduce oxidative damage in healthy probands. To test its preventive potential in hemodialysis patients, 21 subjects in a pilot intervention study consumed 200 mL/day of red fruit juice (3-week run-in; 4-week juice uptake; 3-week wash-out). Weekly blood sampling was done to monitor DNA damage (comet assay ± formamidopyrimidine-DNA glycosylase enzyme), glutathione, malondialdehyde, protein carbonyls, trolox equivalent antioxidant capacity, triglycerides, and DNA binding capacity of the transcription factor nuclear factor-κB. Results show a significant decrease of DNA oxidation damage ($P < 0.0001$), protein and lipid peroxidation ($P < 0.0001$ and $P < 0.001$, respectively), and nuclear factor-κB binding activity ($P < 0.01$), and an increase of glutathione level and status (both $P < 0.0001$) during juice uptake. We attribute this reduction in oxidative (cell) damage in hemodialysis patients to the especially high anthocyanin/polyphenol content of the juice. This provides promising perspectives into the prevention of chronic diseases such as cancer and cardiovascular disease in population subgroups exposed to enhanced oxidative stress like hemodialysis patients. (Cancer Epidemiol Biomarkers Prev 2008; 17(12):3372–80)

Introduction

Epidemiologic studies provide convincing evidence that consumption of diets rich in fruits and vegetables is associated with the prevention or delay of chronic degenerative diseases such as cancer, cardiovascular disease, atherosclerosis, type 2 diabetes, and end-stage renal disease (1, 2). In the pathogenesis of these diseases, chronically increased formation of reactive oxygen species and the resulting imbalance between prooxidative and antioxidative processes are considered to be major causative factors (3). Patients on maintenance hemodialysis, known to face an increased risk for cardiovascular diseases, accelerated atherosclerosis, and cancer, suffer from increased oxidative stress due to the enhanced elimination of antioxidants during hemodialysis, malnutrition (dietary restrictions), reduced antioxidant enzyme activities, and treatment of anemia with iron and erythropoietin (4-8). Also contributing to oxidative stress is the dialyzer membrane inducing release of reactive oxygen species and proinflammatory cytokines (4, 8). Standard supplementation with antioxidant vitamins and acetylcysteine, commonly used in hemodialysis therapy for improvement of oxidative status, often has failed to protect efficiently against oxidative cell damage (9-11).

Increased uptake of food-based antioxidants is a promising alternative measure to reduce oxidative cell damage and stress response. Many fruits and vegetables, in addition to antioxidant vitamins, are rich in flavonoids/polyphenols, acting as powerful antioxidants *in vitro* and *in vivo*, which scavenge diverse reactive oxygen species or inhibit their formation (e.g., by chelating prooxidative metal ions; refs. 12, 13). In addition, other biological activities such as modulation of cell growth, cellular defense, and signal transduction mechanisms might contribute to the anti-inflammatory and anticarcinogenic properties of these compounds (14-17). Although adverse health effects cannot be excluded after consumption of unphysiologically high dosage of individual compounds taken as dietary supplements (18), they are unlikely to arise from the consumption of flavonoid-rich food. Red and dark-colored fruits/berries and their respective juices contain high amounts of anthocyanins in complex mixtures (19) whose antioxidant activities have been shown in cell culture studies and in animal trials (20, 21). Epidemiologic studies indicate a lowered incidence of inflammatory diseases in populations consuming polyphenol-rich foods (22). With healthy subjects, the antioxidant effects of the juices of red grapes/berries and of other polyphenol-rich food have previously been

Received 4/21/08; revised 9/5/08; accepted 9/22/08.

Grant support: Karin Nolte Foundation.

Requests for reprints: Christine Janzowski, Department of Chemistry, Division of Food Chemistry and Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Strasse 52, 67663 Kaiserslautern, Germany. Phone: 49-631-205-2532; Fax: 49-631-205-3085. E-mail: janzo@rhrk.uni-kl.de

doi:10.1158/1055-9965.EPI-08-0364

reported in intervention studies (23-25). For hemodialysis patients, however, information on protective effects due to the antioxidant efficacy of polyphenol-rich food is barely available.

The present study aimed to characterize the antioxidative potential of a mixed red fruit juice in hemodialysis patients. A 10-week pilot intervention study was done in a 3-phase design (run-in, juice intervention, and wash-out) with 10 blood samplings, thus allowing for minimization of intraindividual variations, because each patient serves as his own control. It also allowed for continuous monitoring of biomarkers throughout the study, including the phasing out of effects during the wash-out phase. Biomarkers of oxidative cell damage (DNA damage, malondialdehyde, protein carbonyls), selected redox-sensitive cell response [glutathione level/status, nuclear factor-κB (NF-κB)-DNA binding activity], and the trolox equivalent antioxidant capacity (TEAC) were determined in blood, plasma, or peripheral blood mononuclear cells (PBMC). Additionally, triglycerides were quantified in plasma to compare with malondialdehyde levels.

Materials and Methods

Subjects and Study Design. This study was approved by the local ethic committee [Rheinland-Pfalz no. 83736102 (3579)]. All investigated subjects gave their informed written consent.

Clinically stable hemodialysis patients (n = 21; ages 21-79 y; non-smokers; body mass index 26.04 ± 3.83 kg/m^2; 14 males) recruited from Westpfalz-Klinikum GmbH, Kaiserslautern and Kuratorium für Dialyse und Nierentransplantation e. V., Kaiserslautern, were dialyzed with cellulose acetate, polysulfone, triacetate, polyamide, or polyethylene glycol membranes three times a week for four to five hours. Fifteen patients were treated with iron medication (71%) and four were diabetics (19%).

Exclusion criteria were: hematocrit <30%; hemoglobin <10 g/dL, >13 g/dl; C-reactive protein >30 mg/L; serum ferritin <100 μg/dL, > 800 μg/dL; change of erythropoietin dose >30% in the preceding 8 wk; potassium >6 mg/dL; and ineffective dialysis [Kt/V (clearance × duration of dialysis/content of body water) <1,3]. The types of kidney disease were shrunken kidneys (n = 7), diabetic nephropathy (n = 3), analgetic nephropathy (n = 2), chronic glomerulonephritis (n = 2), binephrectomy (n = 2), Wegener's granulomatosis (n = 1), hypoplasia of the kidney (n = 1), polycystic kidney disease (n = 1), hypertensive nephropathy (n = 1), and unknown genesis (n = 1). Patients were treated with established therapeutic regimens, including antihypertensive, anticoagulative, antiosteopathic, and cholesterol-lowering drugs. Furthermore, patients received antianemia treatment encompassing erythropoietin and iron preparations. In addition, some individuals also received vitamin D (n =13) and calcium preparations (n = 12). The total study period was 10 wk, subdivided into a 3-wk run-in phase (R, adaptation to restricted diet, see below), a 4-wk juice uptake phase (J), and a 3-wk wash-out phase (W). The patients, restricted in uptake of water and components (e.g., potassium and phosphate), consumed 200 mL of fruit juice daily in two 100-mL portions (1st in the morning, 1 to 2 h before hemodialysis; 2nd in the evening) in the juice uptake phase. Patients were instructed to keep their usual dietary habits for the duration of the study, except for avoiding intake of vitamin supplements and foods rich in anthocyanins (e.g., red berries/juices, red wine, red onions, red beans, red cabbage). Food records were completed by the participants every week (day before blood sampling) to document individual dietary habits and to monitor their compliance with the instructions.

Fruit Juice. Mixed fruit juice was produced from red grape juice (40%), blackberry juice (20%), sour cherry juice (15%), black currant juice (15%), and elderberry juice (10%) at the Geisenheim Research Center. The juice was analyzed for standard parameters, including TEAC, ascorbic acid (reductometrically), anthocyanins [high-performance liquid chromatography (HPLC)/diode array detection], and total phenolics [folin reaction, using (+)-catechin as a standard] as described (24).

Blood Sampling, Processing, and Storage. Blood sampling was done after each experimental week (1-10). Samples were drawn immediately before the beginning of the dialysis session, 1 to 2 h after juice ingestion.

Venous blood samples were collected in 6.5-mL EDTA tubes and stored at room temperature until the individual sampling period was completed. Thereafter, 50-μL samples were immediately submitted for determination of DNA oxidation damage. Glutathione was measured in whole blood (0.1 mL): immediately after collection, protein was precipitated by adding 10% (v/v) 5-sulfosalicylic acid (5-SSA; 0.4 mL), followed by shock-freezing and storage in liquid nitrogen until analysis. Blood was centrifuged (2.2 mL, 300 × g, 5 min, room temperature) to determine malondialdehyde, TEAC, protein carbonyls, and triglycerides. For malondialdehyde analysis, butylated hydroxytoluene (0.05% v/v) was added to plasma (200 μL) and the mixture was stored at −80°C. For determination of TEAC, plasma (100 μL) was submitted for protein precipitation with 100 μL 5-SSA, centrifuged (10,000 × g, 10 min, 4°C), and stored at −80°C. To measure protein carbonyls and triglycerides, plasma (900 μL) was frozen and stored at −80°C until analysis. For determination of NF-κB, aliquots of blood (4 mL) were centrifuged (400 × g, 30 min, room temperature) on Histopaque 1077 (Sigma-Aldrich) and used to collect PBMCs and prepare nuclear extracts as described (26). In 3 μL nuclear extract, proteins were determined using the Bradford reagent, and the residual nuclear extract was stored at −80°C until analysis. All determinations of biomarkers were done blinded.

Determination of DNA Damage. Alkaline single-cell gel electrophoresis was done according to Collins et al. (27), with slight modifications (24). Briefly, aliquots of whole blood (6 μL) were mixed with low-melting agarose (65 μL), and distributed onto an agarose-coated microscope slide (two gels per slide). After removing the cover glass, slides were immersed in a lysis solution for 1 h at 4°C, washed, drained, and covered with 50 μL of either enzyme buffer or formamidopyrimidine-DNA glycosylase enzyme (FPG; obtained from Dr. A.R. Collins, Institute for Nutrition Research, University of Oslo, Norway) to differentiate between strand breaks, including

some alkali-labile sites (one slide), and total DNA damage (strand breaks plus FPG-sensitive sites, two slides). The gels were incubated for 30 min at 37°C and subsequently for 20 min at 4°C, pH 13.5 (DNA unwinding), followed by horizontal gel electrophoresis at 4°C for 20 min. Then the slides were washed, stained with ethidium bromide (40 μL, 10 μg/mL), and viewed microscopically with a Zeiss Axioskop 10. Slides were analyzed by computerized image analysis (Perceptive Instruments), scoring 2 × 50 images per slide (two gels per slide). DNA migration was directly expressed as mean tail intensity (TI%) from one or two slides. In each experiment, analysis of isolated lymphocytes (obtained from one healthy subject and stored at −80°C) was included for assay control. Data are presented as DNA strand breaks (without FPG treatment) and total DNA damage (with FPG treatment). The coefficient of variation (CV; intra-assay) was 7.3% when processing with FPG.

Determination of Glutathione. Total glutathione (tGSH), the sum of reduced glutathione (GSH) and oxidized glutathione (GSSG), and GSSG were measured in whole blood by photometric determination of 5-thio-2-nitrobenzoate, in a kinetic assay, according to Müller et al. (8). Samples were thawed and centrifuged (12,000 × *g*, 10 min). Using a 96-well plate for the tGSH assay, supernatants (final dilution 1:50 in 5% SSA, 10 μL) were added to 190 μL of a freshly prepared reaction mixture [4 μL 20 mmol/L NADPH, 20 μL 6 mmol/L 5,5′-dithiobis-(2-nitrobenzoic acid), 2 μL 50 units/mL glutathione reductase and 164 μL phosphate buffer, containing 106 mmol/L K_2HPO_4, 18.3 mmol/L KH_2PO_4 and 6.29 mmol/L Na_2EDTA]. The 5-thio-2-nitrobenzoate formation rate of samples and GSH standards (0-160 μmol/L) were monitored in a microplate reader (MWGt, Sirius HT Injector, 412 nm; MWG Biotech) after 5 min. To measure GSSG, supernatants (final dilution 1:25, 500 μL) and GSSG standards (0-40 μmol/L) were reacted with 100 μL 50% triethanolamine and 20 μL 2-vinylpyridine for 1 h in a thermo mixer at 26°C. Aliquots of 20 μL were assayed as described for tGSH determination, using a 10-min reaction time period. Results were obtained from three independent determinations, each done in duplicate; tGSH and GSSG levels were expressed as μmol/L GSH and GSSG, respectively, and were used to calculate GSH and glutathione status (GSH status = GSH in % of tGSH). The CV was 2.5% (intra-assay).

Determination of Malondialdehyde. Malondialdehyde was determined in plasma by high-performance liquid chromatography (HPLC)/fluorescence after derivatization with thiobarbituric acid as described (24), with slight modifications. Using a malondialdehyde detection kit (Sobioda, Montbonnot-Saint-Martin), 20 μL sample or standard (1,1,3,3-tetraethoxypropane, 0-5 μmol/L) were mixed with thiobarbituric acid/perchloric acid (160 μL, 2:1) and heated for 1 h at 95°C. After cooling and centrifugation, *n*-butanol (400 μL) was added and mixed for 1 min. After further centrifugation (5 min, 2,600 × *g*), the butanol phase (2 × 150 μL) was used to measure the thiobarbituric acid derivate of malondialdehyde by HPLC/fluorescence [Jasco; HPLC system: PU 1580, DG 1580-53, LG 1580-02, FP 1520, AS 1550; excitation at 532 nm, emission at 553 nm, injection volume 50 μL; HPLC column: Macherey-Nagel: Nucleosil 100, 5 μm, RP-18ec, 250 × 4 mm; isocratic flow of 12.5 mol/L Na_2HPO_4 (pH 7.4) and methanol (53:47), 0.9 mL/min, retention time of malondialdehyde 3.0 min]. Each HPLC analysis was done in duplicate. Results are expressed as μmol/L malondialdehyde. The recovery of this method was >95%, the CV was 3.4% (intra-assay).

Determination of Triglycerides. Triglycerides in plasma were quantified photometrically (28) using a commercial enzymatic colorimetric test (Fluitest TG; triglyceride ''GPO-PAP''-kit; Biocon Diagnostik), according to the instructions of the manufacturer. Sample, triglyceride standard, or blank (10 μL each) was mixed with 1 mL of working reagent and incubated for 10 min at room temperature. For determination of the resulting chinonimine dye, 200 μL of each solution were pipetted into a 96-well plate and measured in quadruplicate at 546 nm in a multiplate reader (MWGt; Sirius HT injector). Triglyceride concentrations are expressed as mmol/L plasma. The CV of the method was 1.3% (intra-assay).

Determination of Protein Carbonyls. Protein carbonyls were determined as hydrazones (29) with a protein carbonyl detection kit (Cayman Chemical), according to the instructions of the manufacturer, with slight modifications. Aliquots of plasma (100 μL; ''sample'') were reacted for 1 h in the dark at room temperature with 400 μL of 2,4-dinitrophenylhydrazine solution (in 2.5 mol/L HCl) under vortexing every 15 min. A control (100 μL plasma) was treated similarly, except for replacing the 2,4-dinitrophenylhydrazine solution with 400 μL 2.5 mol/L HCl. All subsequent steps were carried out at 4°C. Proteins were precipitated with 1 mL 20% trichloroacetic acid and centrifuged at 10,000 × *g* for 10 min; the pellet was resuspended in 10% trichloroacetic acid and washed (twice in ethanol/ethylacetate 1:1 and once in ethanol) with intermittent ultrasonic treatment and centrifugation. The pellet was redissolved in 0.5 mL 6 mol/L guanidine hydrochloride and centrifuged (13,000 × *g*,10 min); then 220 μL of the solution were analyzed in a 96-well plate at 370 nm in a microplate reader (MWGt, Sirius HT injector), and 20 μL of the solution were used for protein determination with the Bradford reagent. Carbonyl content (corrected absorbance/0.011 $\mu mol^{-1}/L^{-1}$) is expressed as nanomoles of carbonyl per milligram of protein. Results were obtained from three independent determinations, each done in duplicate. The CV was 4.7% (intra-assay).

Determination of NF-κB. The NF-κB DNA-binding activity was analyzed in nuclear extracts of PBMCs with a commercial ELISA kit (TransAM NF-κB p65), according to the instructions of the manufacturer (Active Motif). Briefly, nuclear extract (26) containing 5 μg protein was diluted to 20 μL with lysis buffer and mixed with 30 μL binding buffer per well in an oligonucleotide-coated 96-well plate. After 1 h incubation at room temperature with mild agitation and subsequent washing (three times), the primary antibody (NF-κB p65 antibody) was added and the mixture was incubated for 1 h. After rewashing (three times), the secondary antibody (anti-rabbit horseradish peroxidase–conjugated IgG; Active Motif) was added, followed by 1 h incubation, washing

(four times), and addition of developing and stop solution. NF-κB binding activity was quantified photometrically at 450 nm (MWGt, Sirius HT injector) and expressed as relative optical density, using HeLa nuclear extract (Active Motif) as reference (optical density = 1). The CV was 5.0% (intra-assay).

Determination of Antioxidant Capacity. TEAC was determined with the ABTS [2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] radical cation decolorization assay, according to Weisel et al. (24). Briefly, protein-free plasma (diluted 1:50 with PBS, 2 μL) was added to ABTS solution, preactivated for 12 to 16 h in the dark by 2.45 mmol/L potassium persulfate, and diluted with PBS to reach an absorbance of the radical cation of 0.70 ± 0.02 at 734 nm. Two microliters of each solution were mixed with 98 μL PBS and 200 μL of the final ABTS solution (at 30°C) in a 96-well plate. Absorption was measured after 6 min incubation at 30°C in a multiplate reader (MWGt, Sirius HT injector, 734 nm). Trolox standards (0-2 mmol/L in DMSO) and a PBS blank were run in each assay. TEAC values are expressed as mmol/L Trolox, equivalent to the antioxidant capacity of the plasma. Results were obtained from one to two independent determinations, each done six times. The CV of the method was 5.7% (intra-assay).

Statistics. Results from 21 hemodialysis patients are reported as means and SD. Anderson-Darling test was used for analysis of normal distribution. Differences of markers between run-in/juice uptake (R/J), juice uptake/wash-out (J/W), and run-in/wash-out (R/W), including all blood sampling time points, were analyzed with one-sided paired *t*-test (differences normally distributed) or one-sided Wilcoxon-test (differences without normal distribution). For analysis of independent predictors (normal distribution), ANOVA was used (two-way ANOVA for patients; three-way ANOVA for gender, diabetes, iron infusion).

Results and Discussion

Antioxidant Capacity and Constituents of the Juice. The anthocyanin-rich mixed fruit juice exhibited an especially high antioxidant activity, corresponding to a TEAC value of 31.3 mmol/L (equivalent to 5.5 g/L of vitamin C) and a high concentration of total phenols (3,478 mg/L catechin equivalents) and anthocyanins (301 mg/L cyanidin-3-glucoside equivalents). Cyanidin-3-sambubioside at 67.1 mg/L and malvidin-3-glucoside at 48.4 mg/L were identified as major constituents, mainly originating from elderberries and red grapes (19), followed by peonidin-3-glucoside at 29.5 mg/L (mainly from red grapes; ref. 30), cyanidin-3-glucoside at 27.8 mg/L (mainly from blackberries; ref. 19), delphinidin-3-rutinoside at 27.7 mg/L (mainly from black currants; ref. 19), cyanidin-3-rutinoside at 24.9 mg/L (mainly from black currants; ref. 19), cyanidin-3-(2^G-glucoside-rutinoside) at 21.4 mg/L, and delphinidin-3-glucoside at 15.5 mg/L. The juice processing technology applied minimizes anthocyanin losses during juice production, thus safeguarding the high antioxidant capacity achieved by the berry species selected (31). By comparison, other fruit juices such as apple juice or orange juice usually display much lower antioxidant capacities (<5 mmol/L Trolox; refs. 32, 33). Because ascorbic acid was present only in moderate amounts (268 mg/L), corresponding to a TEAC value of 1.5 mmol/L Trolox, it does not seem to make a major contribution to the antioxidant effectiveness of the study juice.

DNA Damage. The time course of DNA strand breaks (including some alkali-labile sites) and total DNA damage (strand breaks plus FPG sensitive sites) in WBC during the intervention study is shown in Fig. 1. Mean DNA strand breaks (0.55 ± 0.22 TI%) during run-in were slightly but not significantly decreased during juice uptake and wash-out. In FPG-treated samples, TI% values were 6- to 9-fold higher, compared with those representing only DNA strand breaks. Thus, oxidation damage largely accounts for the total DNA damage measured. The 4-week intervention with the polyphenolic-rich fruit juice resulted in a highly significant decrease of total DNA damage (mean TI% down from 5.22 to 2.85), which was maximally expressed in the second week of juice consumption. In the 3-week wash-out phase, the amount of total DNA damage steadily reincreased, but without reaching the initial level of the run-in phase.

The extent of DNA strand breaks and total DNA damage during the run-in phase agreed well with results reported in earlier studies with hemodialysis patients using different dialyzer membranes (8). DNA strand breaks in patients were rather similar compared with healthy probands (run-in mean, 0.39 TI%; ref. 24). In FPG-treated samples, however, elevated DNA damage was observed (run-in phase mean TI%, 5% in the present study versus 3% for healthy probands; ref. 24), reflecting the known increased oxidative stress of patients undergoing hemodialysis. In healthy subjects, intervention with red mixed fruit juice and other polyphenol-rich juices also had resulted in a significant reduction of DNA oxidation damage, which, in addition to radical scavenging, was ascribed to increased synthesis of cellular antioxidants (e.g. glutathione) and/or enhanced DNA

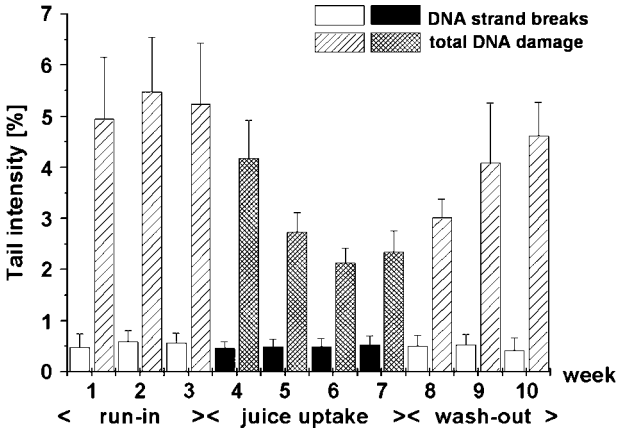

Figure 1. DNA strand breaks (including some alkali-labile sites) and total DNA damage (strand breaks plus FPG-sensitive sites) in PBMCs during run-in (*R*), juice uptake (*J*), and wash-out (*W*) phases in mean ± SD. No significant changes for DNA strand breaks ($P > 0.05$); significance for total DNA damage: R/J, $P < 0.0001$ (one-sided Wilcoxon test); J/W, $P < 0.0001$ (one-sided, paired *t* test).

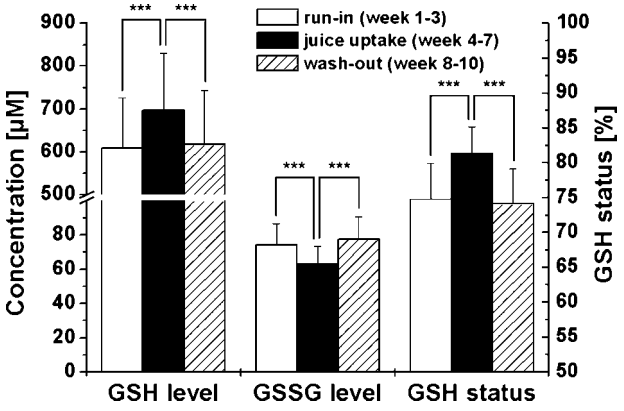

Figure 2. GSH level, GSSG level, and GSH status in whole blood samples during run-in (*R*), juice uptake (*J*), and wash-out (*W*) phases in mean ± SD. Significance for GSH level (one-sided, paired *t* test): R/J, $P < 0.0001$; J/W, $P < 0.0001$; GSSG level: R/J, $P < 0.0001$ (one-sided, paired *t* test); J/W, $P = 0.0001$ (one-sided Wilcoxon test); GSH status: R/J, $P < 0.0001$ (one-sided, paired *t* test); J/W, $P = 0.0001$ (one-sided Wilcoxon test).

repair (24, 34). In agreement with the present study, Bub et al. (23) observed a delay in decrease of total DNA damage during the juice uptake phase, which was interpreted as a result of antioxidant enzyme induction. Other interventions, however, did not show such preventive effects on DNA damage by ingestion of fruits and fruit juices (35, 36). Thus, observations in healthy probands are not consistent yet and further studies are required for a conclusive evaluation. In hemodialysis patients some reduction of DNA oxidation damage was previously achieved by vitamin E supplementation (37). To the best of our knowledge, interventions in hemodialysis patients by polyphenol-rich diets such as the present one, assessing protection against DNA damage, have not been done so far.

Glutathione. tGSH and GSSG level and GSH status in whole blood are shown in Fig. 2. Blood samples drawn in the run-in phase showed large interindividual variations for tGSH and GSSG, ranging from 352 to 858 μmol/L and from 50 to 105 μmol/L, respectively. During juice uptake, the tGSH level significantly increased, as compared with the run-in phase, and the GSSG level was significantly lowered, with maximal effects expressed already in the first week of juice intervention. These changes resulted in a significant increase of the GSH status during the intervention (means up from 74.8% to 81.4%). In the first week of the subsequent wash-out phase, all values reached the initial level of the run-in phase. These short-term alterations suggest a rapid reaction of the glutathione homeostasis system on the polyphenol/anthocyanin intervention.

The mean tGSH and GSSG levels in the run-in phase (608 and 74 μmol/L, respectively) are in line with the known decreased tGSH and the elevated GSSG levels in the blood of hemodialysis patients, as compared with the respective levels in the blood of healthy subjects reported by us from a similarly designed intervention (run-in means, 644 μmol/L tGSH and 43 μmol/L GSSG; ref. 24) and by others (7, 38). The observed 7% increase of glutathione status during juice uptake in the present study is comparable with the results from healthy subjects (6% increase in juice intervention; ref. 24). In the patients, this increase was due to both elevation of tGSH level and decrease of GSSG level, whereas in healthy subjects no modulation of GSSG was observed. The hypothesis of impaired glutathione synthesis as a causative factor for tGSH deficiency in hemodialysis patients is supported by accumulation of cysteine (7, 39). Additionally, formation of adducts with aldehydes originating from lipid peroxidation might also contribute to the decline of GSH levels (40). The known polyphenol-mediated increase of tGSH *in vitro* (15) suggests that the tGSH elevation in the present study is due to activation of γ-GCL, the rate-limiting enzyme of glutathione synthesis. A similar induction of GSH-related enzymes and elevation of tGSH level have been reported in hemodialysis patients receiving supplementation with antioxidant vitamins A, C, and E (41) and with the flavonoid silybinin (38).

Malondialdehyde and Triglycerides. The concentrations of the lipid peroxidation marker malondialdehyde, analyzed in butylated hydroxytoluene–supplemented plasma to avoid artifactual formation during clean-up, were significantly lowered during juice uptake, compared with the run-in phase (Fig. 3) and remained decreased in the wash-out phase.

The plasma malondialdehyde levels in the run-in phase were comparable with values described previously for hemodialysis patients (mean 0.83 nmol/mL; ref. 7), which had been shown to be significantly higher as compared with healthy subjects (mean 0.72 nmol/mL; ref. 7); this reflects the increased oxidative burden of the patients. Vitamin E supplementation has been found to lower the malondialdehyde levels in hemodialysis patients as well (42), suggesting that at least in populations with increased oxidative stress malondialdehyde seems to be a reliable biomarker. In intervention

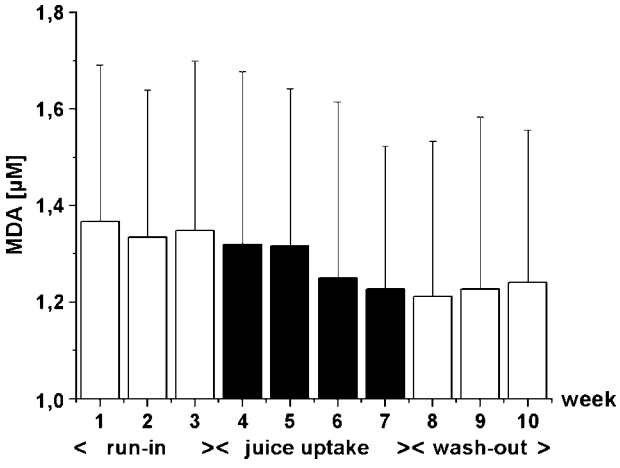

Figure 3. Malondialdehyde concentrations in plasma during run-in (*R*), juice uptake (*J*), and wash-out (*W*) phases in mean ± SD. Significance (one-sided, paired *t* test): R/J, $P < 0.001$; J/W, $P < 0.001$.

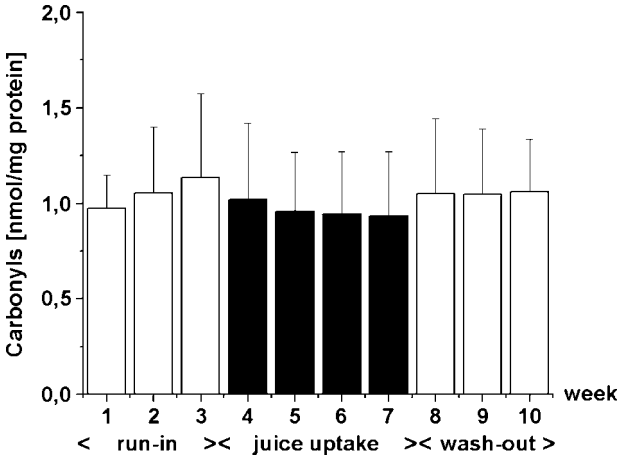

Figure 4. Protein carbonyls in plasma during run-in (*R*), juice uptake (*J*), and wash-out (*W*) phases in mean ± SD. Significance (one-sided, paired *t* test): R/J, $P < 0.0001$; J/W, $P < 0.0001$.

studies with healthy subjects, the value of malondialdehyde as a biomarker for lipid peroxidation is subject to controversial discussion, especially with respect to lack of specificity (43, 44). For example, malondialdehyde concentration in the plasma of nonsmoking men (23) was reduced by polyphenol-rich juice but was not decreased in another juice intervention study with healthy subjects (24). In a study with composite berry juice, ingestion of a single portion (400 mL) resulted in decreased plasma malondialdehyde content (45). The antioxidant effectiveness of red grape juices and grape seed extracts on plasma low density lipoprotein oxidation, another frequently used lipid peroxidation marker, was also shown in intervention studies with both healthy subjects and hemodialysis patients (25).

Plasma concentrations of triglycerides showed large interindividual variations, ranging from 0.42 to 3.31 mmol/L; no significant differences ($P > 0.05$) between the study phases were observed. Between malondialdehyde and triglyceride concentration, no correlation was found, as already described previously in a study with cancer patients, suggesting that the extent of malondialdehyde formation is not dependent on the concentration of plasma triglycerides (46).

Protein Carbonyls. Protein carbonyls, a common marker of protein oxidation, were found to be significantly diminished in plasma during the 4-week consumption of fruit juice (mean concentrations down from 1.09 to 0.96 nmol/mg protein); in the following wash-out, the concentrations increased but did not reach the initial level of the run-in phase (Fig. 4).

The observed elevated carbonyl concentrations agree well with the known increased oxidation of plasma proteins in patients with enhanced oxidative stress (diabetes or neurodegenerative diseases), as compared with healthy subjects (47). In hemodialysis patients, such elevated protein oxidation is also associated with i.v. iron treatment (48). In contrast to our results with juice, antioxidant supplements such as the ω-3 fatty acid docosahexaenoic acid or vitamins C and E were not found to decrease the protein carbonyl content in hemodialysis patients (10, 11). Further juice intervention studies in hemodialysis patients utilizing the protein carbonyl content as biomarker have not been described yet.

NF-κB. NF-κB, an oxidative stress-sensitive transcription factor, regulates the expression of many genes involved in the control of immune and inflammatory response (49). In mammalian cell lines, flavonoid-mediated down-regulation of NF-κB DNA binding activity has been reported (50, 51). In the present intervention study, the DNA binding activity of NF-κB was significantly reduced in nuclear extracts of PBMCs during juice uptake and wash-out, as compared with the run-in phase (reduction by 19 and 25%, respectively; Fig. 5).

Enhanced activation of NF-κB, reported for patients with type I and II diabetes, diabetic nephropathy, and on maintenance hemodialysis was at least in part attributed to oxidative stress and inflammation activated by the receptor for advanced glycation end products, followed by induction of intracellular mitogen-activated protein kinase activity (52, 53). A down-regulation, but not complete inhibition, of NF-κB DNA binding activity was obtained by the antioxidant α-lipoic acid in patients with diabetic nephropathy (52). This is in line with the partial inhibition observed in the present study that supports the protective antioxidant/anti-inflammatory efficacy of polyphenolic fruit juice constituents. Because no decrease of NF-κB DNA binding activity was detected in interventions with healthy subjects consuming polyphenol-rich vegetable and fruit products (24, 54), the modulation of NF-κB activation might be utilized as a biomarker to monitor the expression of NF-κB–dependent genes, known to be related to inflammation, particularly in populations with enhanced oxidative stress.

Antioxidant Capacity. The TEAC of protein-free plasma varied between 0.82 and 2.25 mmol/L Trolox

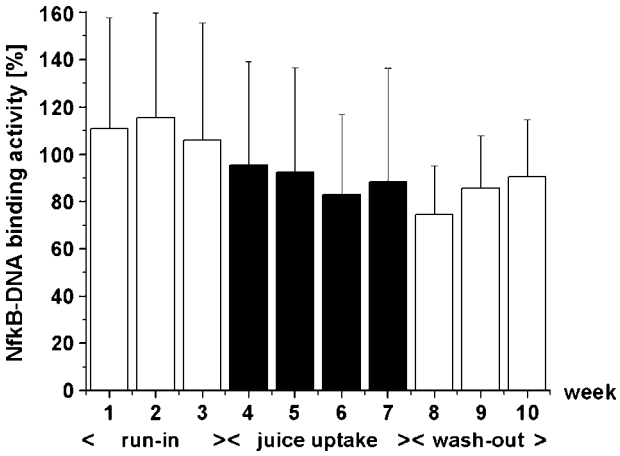

Figure 5. DNA binding activity of NF-κB, measured in nuclear extract of isolated PBMCs during run-in (*R*), juice uptake (*J*), and wash-out (*W*) phases in mean ± SD. Significance (one-sided, paired *t* test): R/J, $P < 0.01$; J/W, $P < 0.001$. HeLa-Control = 100%.

Table 1. Influence of independent predictors on glutathione, malondialdehyde, protein carbonyls, and NF-κB

Variable	*P*
Glutathione	
GSH level	
Phases*	<0.0001
Patients*	<0.0001
Gender†	0.06
Iron infusion†	<0.0001
Diabetes†	<0.05
GSSG level	
Phases	<0.0001
Patients	<0.0001
Gender	0.052
Iron infusion	0.179
Diabetes	<0.0001
GSH status	
Phases	<0.0001
Patients	<0.0001
Gender	0.121
Iron infusion	0.058
Diabetes	<0.0001
MDA	
Phases	<0.01
Patients	<0.0001
Gender	0.737
Iron infusion	<0.0001
Diabetes	<0.0001
Carbonyls	
Phases	<0.0001
Patients	<0.0001
Gender	<0.0001
Iron infusion	<0.01
Diabetes	<0.0001
NF-κB	
Phases	<0.01
Patients	<0.001
Gender	0.084
Iron infusion	<0.001
Diabetes	<0.01

Abbreviation: MDA, malondialdehyde.
*Two-way ANOVA.
†Three-way ANOVA.

and was not significantly modulated in the course of the study (data not shown). Similarly, no effect of a diet rich in fruit and vegetables on the TEAC of human plasma was found in other trials (24, 55), except for a study with healthy volunteers which consumed a single portion (400 mL) of composite berry juice (45) resulting in elevated TEAC levels. It should be kept in mind that for assessment of antioxidant efficacy in biological systems such as plasma, TEAC appears not the best choice, because it is limited to only indicate direct antioxidant capacity and not the activation of cellular antioxidant defense systems, which here seems to have greater relevance (43).

Influence of Independent Predictors. The influence of study phases and patient characteristics on biomarker results were investigated by ANOVA (Table 1). Study phases, individual patients, and diabetes significantly affected glutathione, malondialdehyde, protein carbonyls, and NF-κB. Additionally, malondialdehyde and protein carbonyls were significantly altered by gender, and an influence of iron infusion was found for tGSH, NF-κB, malondialdehyde, and protein carbonyls. Total DNA damage was not investigated, because values were not normally distributed.

Taken together, the results of the pilot study show markedly reduced oxidative stress–related biomarkers in blood, plasma, and PBMCs of hemodialysis patients during the 4-week intervention with the flavonoid/anthocyanin–rich fruit juice. Most distinct effects were observed for DNA oxidation damage, glutathione, and content of protein carbonyls. Malondialdehyde and NF-κB DNA binding activity were also found to be significantly decreased. Concomitant with this antioxidant/anti-inflammatory efficacy, glutathione level and status were highly significantly elevated by juice uptake, probably due to the known enhanced expression of γ-GCL by flavonoids/polyphenols. It is to be taken in consideration that these beneficial antioxidant effects were evoked by a rather low juice uptake (200 mL/day) as compared with similar intervention studies with healthy probands. Complete renal failure of the hemodialysis patients does not allow urinary excretion of polyphenols and their metabolites, but they can be excreted during hemodialysis into the dialysate (56). Because hemodialysis is done only three times a week, interim accumulation of polyphenols may result in sustained biological effectiveness of the juice. Yet, an exhalative elimination through metabolic breakdown to CO_2, as shown in probands for a high oral dose of quercetin (100 mg), cannot be excluded (57). Because the food records of the patients showed no alteration in their food intake habits over the whole course of the study, except for the juice consumption, and the body mass index of the patients remained unchanged as well, no indications for unknown ''seasonal'' effects were obtained. This supports the conclusion that the observed biomarker alterations during the intervention are caused by the juice.

The allocation of the predominant antioxidant effectiveness to the polyphenol fraction of the juice is further supported by our previous findings in an intervention with healthy subjects (24). In this study, substantial antioxidative efficacy in plasma/blood was seen for original red berry juice (verum), whereas its counterpart, the same juice depleted from the polyphenol fraction (control), had no antioxidative effects on the respective markers of oxidative damage and antioxidant protection. This suggests that polyphenols are responsible for the observed antioxidant effects of such juice in healthy volunteers and are likely to exhibit an antioxidant potential also in hemodialysis patients, even though bioavailability in healthy volunteers (24, 25) cannot definitely be transferred to the situation in hemodialysis patients.

In conclusion, consumption of antioxidant berry juices appears to be a promising preventive/therapeutic measure to reduce oxidative stress in patients suffering from end-stage renal disease and other reactive oxygen species–related diseases. Overall, our results favor the use of such berry juices for effective prevention of long-term sequelae of chronically enhanced oxidative cell damage such as cancer or cardiovascular disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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We thank Tamara Bakuradze, Natalie Ruppel, Veronica Blatz, Christoph Seubert, and Eva Hanke for excellent assistance, and the nursing staff of Westpfalzklinikum and Kuratorium für Dialyse und Nierentransplantation (KfH), Kaiserslautern, for help with the blood sampling. We gratefully acknowledge the cooperation and support of Dres G. Scherer, W. Kress, and A. Rieder-Haaff (KfH), and Dres H. Borchardt and D. Budiman (Westpfalzklinikum) for the recruitment of patients.

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