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Fruit juice consumption modulates antioxidative status, immune status and DNA damage

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Abstract

Polyphenolic compounds exert a variety of physiological effects in vitro including antioxidative, immunomodulatory and antigenotoxic effects. In a randomized crossover study in healthy men on a low-polyphenol diet, we determined the effects of 2 polyphenol-rich juices (330 ml/d) supplemented for 2 weeks on bioavailability of polyphenols, markers of antioxidative and immune status, and reduction of DNA damage. Juices provided 236 mg (A) and 226 mg (B) polyphenols with cyanidin glycosides (A) and epigallocatechin gallate (B) as major polyphenolic ingredients. There was no accumulation of plasma polyphenols after two weeks of juice supplementation. In contrast, plasma malondialdehyde decreased with time during juice interventions. Moreover, juice consumption also increased lymphocyte proliferative responsiveness, with no difference between the two juices. Interleukin-2 secretion by activated lymphocytes and the lytic activity of natural killer cells were significantly increased by both juices. Juice intervention had no effect on single DNA strand breaks, but significantly reduced oxidative DNA damage in lymphocytes. A time-delay was observed between the intake of fruit juice and the reduction of oxidative DNA damage and the increase in interleukin-2 secretion. We conclude that consumption of either juice enhanced antioxidant status, reduced oxidative DNA damage and stimulated immune cell functions. However, fruit juice consumption for 2 weeks did not result in elevated plasma polyphenols in subjects after overnight fasting. Further studies should focus on the time-delay between juice intake and changes in measured physiological functions, as well as on active polyphenolic metabolites mediating the observed effects.

Keywords: Fruit; Antioxidant status; Immune system; DNA damage; Polyphenols

1. Introduction

Epidemiological data showed that a high consumption of vegetables and fruits is consistently associated with a low risk of cancer and cardiovascular disease [1,2]. Cell-culture systems and animal models have provided a wealth of information on the biological effects of phytochemicals from vegetables and fruits and on the mechanisms by which diets high in vegetables and fruits may reduce the risk of chronic diseases [3,4,5]. However, little is known from human experimental studies about the physiological effects of phytochemicals consumed as part of processed vegetables and fruits. Such studies serve as important links between nutritional epidemiological studies and the in-vitro and animal studies [6].

Polyphenols are the major phytochemicals in fruits and vegetables. High quantities of polyphenols are found in certain fruits and in tea. A variety of studies have shown that polyphenols such as flavonoids in vitro have antioxidative [7,8] and immunomodulatory activities [9]. Polyphenols further have a potential to prevent genotoxicity by reducing the exposure to oxidative and carcinogenic factors [10,11]. Few studies so far have looked at the physiological effects of dietary regimes providing reasonable amounts of polyphenols as part of regular foods [14,15]. No study measured antioxidant, immune, and DNA protecting effects during the same intervention period.

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Limited information is available on the absorption, distribution, metabolism, and excretion of polyphenols in man [16], especially about the bioavailability of polyphenols from fruits and vegetables in humans [17,18,19,13]. Therefore, the present study investigated the effect of daily consumption of fruit juices for two weeks on polyphenol appearence in plasma and excretion in urine. The major aim was to evaluate whether fruit juice consumption providing a variety of polyphenols at a physiological dose range affects a variety of biomarkers of antioxidative status, immune status, and DNA damage in healthy male subjects.

2. Methods and materials

2.1. Study design and diet

The randomized, crossover study was conducted during the months of January to April. The study was divided into 5 periods each lasting 2 weeks, resulting in a total study period of 10 weeks: week 1-2, run-in period, week 3-4, consumption of juices A or B (330 ml/day) , week 5-6 washout period, week 7-8 consumption of juices B or A (330 ml/day), week 9-10, washout period. The volunteers consumed the juices with their main dishes. Both juices are not commercially available so far. They contained a mixture of apple, mango and orange juice. In addition, juice A (76% w/w water) was rich in anthocyanin-providing aronia, blueberries, and boysenberries, while juice B (78% w/w water) contained flavanol-rich green tea, apricot, and lime. During the 10 week study period the subjects were instructed to exclude polyphenol-rich foods from their diet. A list of the food products which the subjects were not allowed to eat was provided. Food diaries were kept by the study subjects throughout the study period to calculate the fruit and vegetable intake and to check their compliance with these instructions.

2.2. Subjects

Twenty-seven non-smoking men (Table 1) with normal body weight were recruited for the study. All subjects were in good medical health as was determined by a screening history and medical examination. None were taking vitamin supplements or medications one month before or during the study. The study was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg and all participants gave their consent in writing. All participants were employees from the Research Center Karlsruhe.

2.3. Collection and preparation of blood samples

Fasting blood samples were taken at the beginning of the study and at the end of each experimental week in the morning between 7 and 9 h. Blood was drawn from an antecubital vein into prechilled tubes containing EDTA (1.6 g/L) or Li-heparin (Monovette-Sarstedt, Nümbrecht, Germany) and immediately placed on ice in the dark. Plasma was collected after centrifugation at 1500 x g for 10 min at 4°C. For the lipid peroxidation assay sucrose (15 g/L) was added to the plasma to prevent LDL aggregation and stored at −80°C until analysis. Tubes without anticoagulant (Serum-Monovette-Sarstedt) were used for serum collections. Blood was allowed to clot at RT for 30 min, was then centrifuged at 1500 × g for 10 min at RT and serum stored at −80°C until analysis.

Table 1
Physical characteristics of the subjects[1] (mean values and standard deviation)

Subjects	All		A/B		B/A	
	Mean	SD	Mean	SD	Mean	SD
n	27		14		13	
Age (years)	35	4	35	4	34	4
Height (cm)	183	5	183	6	183	5
Body mass (kg)	82	11	81	11	83	11
Body mass index (kg/m^2)	24	3	24	3	25	3
Body fat (%)	19	4	19	4	20	4

[1] A/B, denotes the subgroup of subjects after randomization who had juice A during the first and juice B during the second intervention period; B/A, juice B during the first and juice A during the second intervention period.

2.4. Collection and preparation of urine samples

Before and at the end of the second intervention period a subgroup of 17 volunteers collected the complete urine during 24 h in dark bottles. During the collection period urine was stored at 4°C and no preservatives were added. Exact sampling time and urine volume were determined.

2.5. Measurement of total phenolic compounds in urine

Total polyphenols in urine were measured using the Folin-Ciocalteau-method [20]. Diluted urine samples were directly assayed with gallic acid serving as standard. Results are expressed as total polyphenol (gallic acid equivalents) excretion per 24 h.

2.6. Sample preparation for analysis of polyphenols in beverages, plasma and urine

Twenty-five ml of fruit juices were diluted with water (1/1 v/v) and a 0.5 ml aliquot was extracted by ethylacetate or loaded onto a Sep-Pak C18 cartridge (500mg, Waters, Milford, MA). The cartridge was washed with 10 ml water. Analytes were eluted with 10 ml methanol. The eluted fraction was dried under nitrogen and redissolved in the HPLC mobile phase, and used for HPLC analysis. The total polyphenol content was determined after acid hydrolytic

cleavage or saponification of juice samples as described by McDonald et al. [21], and von Ropenack et al. [22]. Most polyphenols are excreted as conjugates of glycosides and sulfates, therefore we treated the plasma and urine samples with appropriate enzymes as described by Lee et al. [23]. Polyphenols were extracted by solid-phase extraction using a Sep-Pak C18 cartridge (500mg, Waters). The eluted fraction was dried under nitrogen and redissolved in the HPLC mobile phase, and used for HPLC analysis.

2.7. HPLC analysis of polyphenols

Individual polyphenols in the samples of beverages, plasma and urine were determined by reverse-phase HPLC using a Nova-Pak-C18, column (4μm, 4.6 $\times$ 250 mm) from Waters. Samples were analyzed using a Shimadzu photodiode detector at 280, 350, and 520 nm, a Shimadzu fluorescence detector (λex280/λem320nm) and a binary gradient. Solution A was 1% H_3PO_4 in H_2O, solution B was 100% methanol and the total flow rate was 0.6 ml/min. The linear gradient was changed progressively by increasing solution B from 10% to 50% at 40 min. This mixture was maintained from 40 to 70 min and finally reduced to 10% of solution B. Polyphenolic compounds were identified by comparing their retention time and UV-VIS spectra with those of standards. Catechin and epicatechin were also identified by fluorescence detection. The concentrations of polyphenols were calculated from the calibration curves made with standard solutions.

2.8. Antioxidant status

To measure "antioxidant power," the ferric reducing ability of plasma (FRAP)-assay has been used as described by Benzie & Strain [24] with minor modifications. Plasma malondialdehyde was determined as thiobarbituric acid reactive substances (TBARS) using a fluorometric method [25]. LDL preparation was performed by a short-run ultracentrifugation method based on non-equilibrium density-gradient ultracentrifugation [26] and LDL oxidation was performed by using a modification of the procedure described by Esterbauer et al. [27]. FRAP-assay, TBARS determination and LDL preparation and oxidation have been described previously [28]. The FOX-2 assay (ferrous oxidation of xylene orange) was used for determination of lipidhydroperoxides in serum as described byNouroozzadeh et al. [29]. Serum was incubated for 30 min at RT with the reaction mixture containing xylene orange and finally measured in a spectrophotometer (Lambda 15, Perkin Elmer, Überlingen, Germany) at 560 nm.

2.9. DNA damage

DNA damage (single strand breaks and oxidized DNA bases) was determined by the single cell microgel electrophoresis assay also known as the Comet assay [30]. For this, peripheral blood mononuclear cells (PBMC; 1×10^7 cells/L) were embedded into agarose on microscopical slides, lysed, subjected to alkaline unwinding and electrophoresis. After neutralization and staining with ethidium bromide, comet-like images resulting from the extension of DNA into the agarose are scored as a reflection of single strand breaks [31]. Levels of oxidized pyrimidine bases were detected with a lesion specific enzyme (endonuclease III) [32,33]. Using the imaging software of Perceptive Instruments (Halstead, UK) 50 images were evaluated per slide and the percentage of fluorescence in tail (tail intensity) was scored.

2.10. Immune status

2.10a. Isolation of PBMC and preparation of serum. Blood was drawn into K-EDTA tubes (proliferation and cytokine secretion) and lithium heparin tubes (natural killer cell lytic activity). PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) and resuspended in complete RPMI-1640 culture medium (Life Sciences, Eggenstein-Leopoldshafen, Germany), containing 5% (v/v) heat-inactivated FBS (Life Sciences), L-glutamine (2 mmol/L), penicillin (100.000 U/L) and streptomycin (100 mg/L). Serum from each subject was heat-inactivated for 30 min at 56°C.

2.10b. Lymphocyte proliferation. PBMC at 1×10^9 cells/L in medium containing 5% of either FBS or autologous serum were stimulated by the T cell mitogen concanavalin A (5 mg/L, ConA, Sigma) for 120 h at 37°C. Proliferation was measured using the thymidine analogue 5-bromo-deoxyuridine, which was quantified in PBMC by a cellular enzyme immunoassay as described earlier [34].

2.10c. Quantification of cytokine secretion. PBMC at 1×10^9 cells/L were cultured in medium containing 5% of either FBS or autologous serum and stimulated by 5 mg/L ConA for 48 h at 37°C (IL-2, IL-4). Cell-free supernatants were collected and stored at −80°C until analysis. IL-2 and IL-4 were measured by sandwich-ELISAs as described earlier [34].

2.10d. Lytic activity of NK cells. Lytic activity of NK cells was determined in cryopreserved PBMC, because measurement in freshly isolated PBMC was not possible for technical reasons. Although cryopreservation results in loss of total PBMC, NK cell activity and the percentage of CD16+ NK cells does not differ compared to fresh PBMC (35, personal observations). Lytic activity of NK cells against K562 target cells (effector:target ratios 50:1, 25:1, 12.5:1) was measured with a recently described flow cytometric method [36]. All the cryopreserved PBMC samples from one subject were measured on one day and cryopreserved controls from 2 additional subjects with known high and

low NK cell activity were included in the daily assays, which made it possible to control for interassay variability.

2.11. Statistical analyses

All statistical calculations were performed with the StatView program (SAS Institute 1998, Cary, NC) and GraphPad Prism 3.0 (GraphPad Prism Inc., San Diego, USA). Carry-over effects from intervention period 1 to intervention period 2 were determined by comparing the changes of each parameter during the intervention periods for each juice, respectively and by comparing the means of the end of the run in period and the end of the washout period. If no differences were present, the 2 intervention periods were seen as independent interventions and both groups were pooled to a sample size of 27. In this case, a two-factorial ANOVA was used to discriminate juice group and intervention time effects. If carry-over effects were found, differences in means were compared to the end of the run-in period. Normal distribution of the data was analyzed using the Kolmogorov-Smirnov normality test. Baseline data (end of depletion) versus post-treatment data within groups were analyzed using Student‘s paired t-test or Wilcoxon‘s rank test for data that were not normally distributed. Differences between treatment groups were analyzed using Student‘s t-test for independent samples (or the Mann-Whitney U test for data that were not normally distributed) on mean pre- to post-intervention differences. Statistical significance was accepted at the $P < 0.05$ level.

3. Results

3.1. Contents of polyphenols and carotenoids in the fruit juices

Table 2 shows the concentrations of individual polyphenols in the fruit juices as determined by gradient reverse-phase HPLC. The polyphenol profiles differed in the fruit juices tested. Juice A had high levels of cyanidin glycosides (210 mg/L) and chlorogenic acid (157 mg/L), whereas juice B had high concentrations of epigallocatechin gallate (155 mg/L) and gentisic acid (278 mg/L). Furthermore, juice B mainly contained flavanols and phenolic acids, whereas in juice A anthocyanins, flavonols and hydroxycynnamic acids were found. The sum of identified individual polyphenols in both juices was comparable, 714 mg/L (juice A) and 684 mg/L (juice B) - as investigated by HPLC. However, the total polyphenol content, measured using the Folin-Ciocalteau reagent, was much higher in juice A (6061 mg/L) than in juice B (1705 mg/L), indicating that in particular this juice contained a high percentage of polyphenols which were not detected by HPLC. Total carotenoid concentration in juice A was 4.2 mg/L and in juice B 2.9 mg/L with β-carotene being the major carotenoid in both juices.

Table 2
Polyphenol content of fruit juices (mg/L) (Mean values from ≤3 determinations)

	Juice A	Juice B
Flavanols		
(+)-Catechin	10	15
Epicatechin	22	33
Epicatechin glycosides	<LOD	19
Epigallocatechin (EGC)	<LOD	5
Epigallocatechin gallate (EGCG)	<LOD	155
Procyanidins	35	27
Anthocyanins		
Cyanidin glycosides	210[1]	<LOD
Flavonols		
Quercetin	5	1
Rutin	9	4
Isoquercitrin	37	<LOD
Quercitrin	15	3
Myricetrin	7	<LOD
Kaempferol	4	<LOD
Flavanones		
Hesperidin	13	47
Eriodictyol	28	40
Naringenin	26	16
Phenolic acids		
Gallic acid	14	10
Protocatechuic acid	44	<LOD
Gentisic acid	41	270[2]
Hydroxycinnamic acids		
Chlorogenic acid	157	30
Caffeic acid	23	6
Ferulic acid	10	1
Chalcones		
Phloridzin	4	2
Total (HPLC)	714	684
Total (Folin-Ciocalteau)	6061	1705

LOD, limit of detection
[1] estimated after acid hydrolysis
[2] estimated after saponification

3.2. Plasma and urinary polyphenols

Total polyphenol excretion in urine (mmol/24h) during the second juice consumption period as measured using the Folin-Ciocalteau method was 4.53 ± 1.09 (juice A) as compared with the end of the preceding washout period (3.48 ± 0.90, $P = 0.032$). Polyphenol excretion after consumption of juice B was 3.96 ± 0.81 as compared to 3.26 ± 0.84 at the end of the preceding washout period ($P = 0.11$). Twelve hours after the last ingestion of juices no polyphenols from either juices were detected in plasma or urine samples as investigated by HPLC. Also polyphenol metabolites such as glucuronides or sulfates were not detectable.

3.3. Biomarkers of antioxidant status

Fruit juice consumption modulated antioxidant status in healthy volunteers (Table 3), while no differences between

Table 3
Antioxidant status and lipid peroxidation of subjects consuming a low flavonoid diet supplemented with 2 fruit juices[1] (mean values and standard deviation for twenty-seven subjects)

	Juice A		Juice B	
	Mean	SD	Mean	SD
FRAP (μmol/L)				
Before	909	131	918	142
After	898	118	947	132
Lag time (min)				
Before	79	9	76	10
After	80	12	76	10
TBARS (μmol/L)				
Before	0.86	0.3	0.89	0.3
After	0.75	0.3[2]	0.77	0.2[3]
FOX2 (μmol/L)				
Before	1.5	0.3	1.4	0.3
After	1.4	0.2	1.5	0.2

[1] FRAP, ferric reducing ability of plasma; TBARS, thiobarbituric acid reactive substances; FOX2, ferrous oxidation of xylene orange assay 2.

Significantly different before intervention (two-tailed pairwise *t*-test): [2] $P < 0.05$, [3] $P < 0.01$.

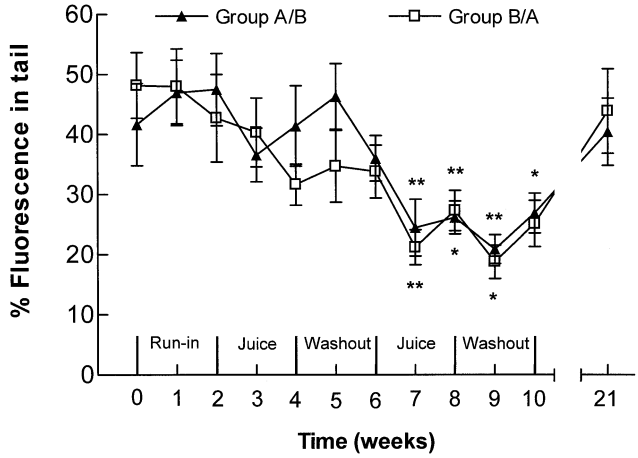

Fig. 1. Oxidized DNA bases in peripheral blood lymphocytes from subjects consuming a low-polyphenol diet supplemented with polyphenol-rich fruit juices (A/B n = 12, B/A n = 10, *p < 0.05, **p < 0.01 vs. week 2). Group A/B, denotes the subgroup of subjects after randomization who had juice A during the first and juice B during the second intervention period; Group B/A, juice B during the first and juice A during the second intervention period.

the tested juices were found. The ingestion of both juices reduced plasma TBARS significantly. Lipid peroxidation as determined by the "lag time" during LDL oxidation, lipid hydroperoxides as determined by the FOX2 assay, and antioxidant power measured as FRAP did not change, although there was a trend to increased antioxidant power (P = 0.07) after juice B.

3.4. Biomarkers of DNA damage

The supplementation of the low-polyphenol diet with the fruit juices had no effect on single strand breaks in PBMC (Comet assay data not shown). With a modification of the Comet assay, oxidative DNA damage was assessed in PBMC. While during the first juice supplementation period no significant effect on DNA bases was seen, the second supplementation period revealed significantly lower intensities of DNA base oxidation, which lasted throughout the final washout period (Fig. 1). Twenty-two weeks after the beginning of the study (11 weeks after the end of the last washout period), subjects were asked again to donate a blood sample, which was used to measure the level of oxidative DNA damage in their lymphocytes. At that point in time oxidative DNA damage in subjects from both groups was comparable with the baseline level (Fig. 1).

3.5. Biomarkers of immune status

The dietary intervention clearly modulated various immune functions in our subjects. T-helper (Th) lymphocyte cytokine secretion was specifically affected by the two fruit juices. During the first intervention period no significant effect on IL-2 secretion was observed, however, two weeks after the first juice supplementation period was stopped, IL-2 secretion increased significantly. During the second juice supplementation period and during the final washout period IL-2 secretion was significantly elevated and no differences between the juices were observed (Table 4). While IL-2 is secreted by the Th1 lymphocytes, IL-4 is primarily produced by Th2 lymphocytes. No significant effect of either of the fruit juices on IL-4 secretion was observed (data not shown).

Proliferative responsiveness of ConA-activated PBMC was also enhanced by fruit juice supplementation. During the run-in period lymphocyte proliferation increased significantly. At the end of the first juice supplementation period proliferative responsiveness showed a trend towards higher proliferation (A, p = 0.10; B = 0.06) which peaked during the first washout period (Table 4; B, p = 0.07). During the second juice supplementation period only subjects drinking juice B demonstrated increased lymphocyte responsiveness.

As in the case of lymphocyte proliferation, lytic activity of NK cells was affected by supplementing a low-polyphenol diet with polyphenol-rich fruit juices. During the run-in period a significantly increased lytic NK cell activity was seen which returned to baseline at the end of the run-in period. After one week of juice supplementation until the end of the whole study period subjects consuming first juice A followed by juice B showed significantly higher lytic NK cell activities compared with the end of the run-in period (week 2, Table 4). Subjects consuming first juice B followed by juice A showed significantly increased NK cell activity only during the first washout period.

Table 4
Proliferation and cytokine secretion capacity of peripheral blood mononuclear cells (activated with ConA 5 mg/L for 120 h or 48 h at 37°C) and lytic activity of NK cells of subjects consuming a low flavonoid diet supplemented with 2 fruit juices (A/B)[1] (mean values with their standard errors for fourteen (A/B) and thirteen (B/A) subjects, respectively)

Study period	Run-in						Juice				Washout				Juice				Washout			
Week	0		1		2		3		4		5		6		7		8		9		10	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Proliferation																						
Group A/B	1.1	0.1[5]	1.2	0.1[4]	1.5		0.1	0.1	1.9	0.2	2.0	0.2[3]	1.9	0.2	1.4	0.2	1.9	0.2[3]	1.5	0.1	1.6	0.1
Group B/A	1.0	0.1[3]	1.2	0.1[3]	1.4	0.1	1.4	0.1	1.8	0.2	1.9	0.2	1.5	0.3	1.4	0.2	1.6	0.3	1.4	0.2	1.7	0.1[3]
IL-2 (ng/L)																						
Group A/B	178	48	319	139	180	47	198	33	n.d.[2]		230	57	321	46[4]	222	44[3]	299	63[3]	445	88[5]	474	82[5]
Group B/A	146	42	320	100	183	31	217	48	n.d.		268	67	364	53[5]	230	36[3]	242	41[3]	426	75[4]	512	73[5]
NK-activity (%)																						
Group A/B	49	7	59	6[4]	45	5	58	6[3]	62	6[5]	66	6[5]	60	6[5]	61	6[5]	58	5[4]	56	6[4]	58	6[4]
Group B/A	45	4	62	4[4]	47	5	57	3	60	6	65	4[3]	59	4[3]	54	4	53	4	50	5	56	6

[1] A/B, denotes the subgroup of subjects after randomization who had juice A during the first and juice B during the second intervention period; B/A, juice B during the first and juice A during the second intervention period; Proliferation is presented as measured absorbance at 450 nm minus absorbance at 650 nm; IL-2, interleukin-2 secretion; NK-activity, lytic activity of natural killer cells with an effector:target ratio of 25:1.

[2] not detectable due to technical problems.

significantly different compared with end of run-in period (week 2); [3] $P < 0.05$, [4] $P < 0.01$, [5] $P < 0.001$ respectively.

4. Discussion

In the present study the consumption of both fruit juices providing 236 and 226 mg/day polyphenols resulted in improved antioxidant status, reduced level of oxidative DNA damage and enhanced immune functions. The observed physiological effects support the assumption that polyphenols in the two juices were bioavailable. In urine, after the consumption of the anthocyanin-rich juice A, a significant increase in total polyphenol excretion was observed. After consumption of flavanol-rich juice B, total polyphenol excretion had also increased but did not reach statistical significance when compared to the end of the washout period ($p = 0.11$). The difference in quantity and type of polyphenols in the two juices may be responsible for this finding. In fasted blood from our subjects we did not detect polyphenols by HPLC. Most polyphenols are known to peak in plasma 1-2 h after a single polyphenol intake [16]. We hypothesized that repeated ingestions of polyphenols would result in increased plasma polyphenol concentrations, as it has been shown for the ingestion of onions for one week, which results in elevated plasma quercetin concentrations [37]. However, in our study polyphenols were already eliminated from the circulation. Overall our data indicate that polyphenols and/or their metabolites from fruit juices were absorbed and excreted in part by the kidneys.

In vitro studies revealed, that polyphenols act as antioxidants as they inhibit LDL oxidation [38]. Polyphenol containing juices have not been well studied in humans with respect to LDL oxidation. In our human intervention study, LDL oxidation did not change after consumption of polyphenol-rich fruit juices. This is in agreement with findings from other comparable studies using polypheol-rich food products [39,40,41]. In contrast, other studies have reported reduced LDL oxidation in healthy volunteers after consumption of polyphenol-rich food or beverages [42,43,44,45,46]. The type of intervention and other different study conditions may be responsible for these conflicting results.

In our study, fruit juice consumption reduced plasma TBARS, which is also a measure of lipid peroxidation. A reduction of TBARS in humans has been reported after blackcurrant and apple juice consumption for seven days [13] and red wine consumption for two weeks [43], while others found no effect of polyphenol-rich food products on plasma TBARS [47,46,48,49]. Additionally, other measurements of oxidative stress, such as plasma F_2-isoprostane, protein carbonyls, and malondialdehyde-LDL autoantibodies did not change after high flavonoid diets [41,49]. The factors responsible for these inconsistent effects of polyphenol-rich diets on lipid peroxidation may be related to type and length of intervention and different study conditions, like being on a low-polyphenol diet or on a conventional diet and have to be elucidated in further studies.

Polyphenols may also improve antioxidant status [7]. In our study, juice supplementation was not effective, although juice B slightly increased “antioxidant power” measured by FRAP ($P = 0.07$). These results are in agreement with the study of Young et al. [13], where no changes in FRAP after blackcurrant and apple juice consumption were seen. During a short-time intervention with 2 fruit juices, only cranberry juice increased FRAP, indicating that specific polyphenols might be able to modulate antioxidant status [15]. In contrast to our results, Day et al. [42] found that the consumption of a red grape juice concentrate for one week increased total antioxidant capacity as measured by an enhanced chemiluminescence method. Additionally, the acute ingestion of alcohol-free red wine also increased plasma antioxidant capacity using the TRAP-assay (total radical-trapping antioxidant potential) [50]. However, Record et al.

[51] and van den Berg et al. [49] recently showed, that the consumption of an antioxidant-rich high-fruit and vegetable diet did not increase the antioxidant capacity of plasma. Besides the different study designs and interventions used in these trials, the assay used to measure antioxidant capacity may also be responsible for the observed differences [52].

The results of this study clearly show that juice consumption reduces the number of oxidized DNA bases in PBMC with no differences between the two juices. Post-intervention measurement of oxidized DNA bases revealed a similar level of oxidized DNA bases as compared to baseline, when subjects were neither receiving fruit juice supplementation nor consuming a low-polyphenol diet. This indicates that juice consumption was related to the reduction in DNA damage and the findings are in line with our previous studies on the protective activities of vegetable juices using a similar set of biomarkers [33]. In another human intervention study with diabetic subjects the intake of polyphenol-rich food (110 mg/day) for 14 days also significantly reduced DNA damage in PBMC [12]. Similarly, the antioxidant status measured as Trolox equivalent antioxidant capacity was not different between subjects with diets high or low in flavonoids [12]. These results are in contrast to the findings of a recent human intervention study with a high-quercetin diet, where no changes in oxidative DNA base damage were observed after the subjects had been eating this diet for two weeks [53]. Possibly, other polyphenols rather than the dominating quercetin may be important to reveal a protective effect against DNA base oxidation or the measurement of this parameter was performed too early during intervention. A 6-week supplementation with a single flavonol (500 mg rutin/day) in subjects consuming their regular diet did not affect significantly endogenous DNA strand breakage nor the resistance of PBMC to hydrogen-peroxide induced damage [47]. One may speculate that either the subjects‘ diet was already high enough in flavonoids or supplementing single types of flavonoids does not prevent DNA damage. The significant reduction in oxidative DNA damage in our study was seen after a period of 5 weeks after the beginning of the first juice supplementation period pointing to a time-delay between polyphenol intake and reduction in DNA damage. The reasons for this apparent latency period are not clear, but they could mean that reduced oxidative stress in the cells is not only the results of direct ROS scavenging by polyphenols, but rather the result of induced protective enzymes. Seasonal effects on DNA damage can not be excluded due to the experimental design using a cross over approach which lacks a placebo group. However, since the post-intervention measurement of oxidized DNA bases revealed a similar level of oxidized DNA bases as compared to baseline it is unlikely that seasonal effects are responsible for the time delayed changes in DNA damage. Finally our *in vitro* studies on the impacts of phytoprotectants in human lymphocytes and in colon cells do point out that these effects can be very specific for the type of compound investigated as well as for the target cells and the source of oxidative stress. For instance, some compounds, including anthocyanins and quercetin, are effecive in decreasing DNA damage and oxidized DNA bases by extra- [11,54] or intracellular mechanisms [55,56]. The effects of the polyphenols contained in the fruit juices used here is presently under investigation.

The immune system can provide markers for determining the biological health benefits of phytochemicals. Most studies so far have been done in in vitro systems demonstrating that pure flavonoids suppress a variety of immune mechanisms including lymphocyte proliferation, lytic activity of NK cells, and cytokine secretion [9]. The present study with healthy human subjects clearly showed that supplementing a low-polyphenol diet with polyphenol-rich fruit juices resulted in significantly increased lymphocyte responsiveness to mitogen-activation and in enhanced NK cell lytic activity. While quercetin in vitro ($\geq$1 mM) significantly decreased lytic activity of NK cells [57], in animal models quercetin (100 mg/kg) and catechin (125-500 mg/kg) also increased lytic activity of NK cells [57,58]. These results support the observations of the present study and suggest that immunosuppressive polyphenols used in the in vitro studies may be metabolized in animals and humans to polyphenols with different in vivo immunoactivities. In addition, the doses used in in vitro studies were much higher than the plasma polyphenol concentrations in the present study.

T-lymphocyte-specific cytokine secretion was also significantly affected by juice supplementation. As with the oxidative DNA damage, a time-delay was observed between intake of fruit juices and increase in IL-2 secretion. While IL-2 secretion by Th1-lymphocytes had nearly doubled by the end of the study period, IL-4 secretion by Th2-lymphocytes was not affected (data not shown). This clearly indicates that the fruit constituents do not generally interfere with immune cells, but specifically mediate their immunomodulatory effects.

An important observation of this study is the time delay between the intake of polyphenols and the measurable change in physiological functions. The mechanisms behind this time-dependency of the observed effects are currently not known. Our observation that some physiological parameters were significantly increased at the end of the first depletion period suggests that in future studies longer depletion periods should be used. Another consequence of our study design is that during the second intervention period subjects had already been on a low-polyphenol diet for 6 weeks, compared with the first intervention period with only 2 weeks of depletion. These differences between the 2 intervention periods may in part be responsible for the observed differences in juice effects in the intervention periods.

In addition, although no polyphenols were detectable in plasma from fasting subjects throughout the whole study period, physiologcal functions were significantly modified. This suggests that the different polyphenols were efficiently

metabolized and probably accumulated in body tissues. Since polyphenols were not detectable in fasting subjects we recently studied the bioavailability of anthocyanins, which predominate in Juice A, in humans [59]. We and others [60,61] showed that anthocyanin plasma concentrations are 10–100 times lower compared to other polyphenols [16] and are rapidly removed from circulation after a single anthocyanin administration. In order to induce physiological effects, despite low plasma concentrations, polyphenols may have to achieve specific tissue concentrations over a period of juice consumption. It may also be possible that polyphenolics even at very low plasma concentrations act synergistically and contribute to the observed physiological effects. Further studies should investigate in detail the kinetics of these polyphenols and the underlying mechanisms leading to the observed physiological effects.

In conclusion, the results from this human intervention study with polyphenol-rich fruit juices demonstrate significant physiological effects after supplementing a low-polyphenol diet with polyphenol-rich fruit juices. No clear differences were observed between the two types of juices suggesting that the observed effects of the fruit compounds are rather unspecific or are caused by compounds which were provided by both fruit juices (both juices contained extracts from apple, orange, and mango). Although it is difficult to deduce the biological significance of these physiological changes, one may speculate that these changes contribute over a long period of time to a reduction in the risk of developing common diseases such as cancer and cardiovascular disease [35].

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