

Addition of strawberries to the usual diet increases postprandial but not fasting non-urate plasma antioxidant activity in healthy subjects

Anna Prymont-Przyminska,¹ Piotr Bialasiewicz,² Anna Zwolinska,³ Agata Sarniak,¹ Anna Wlodarczyk,² Jaroslaw Markowski,⁴ Krzysztof P. Rutkowski⁴ and Dariusz Nowak^{5,*}

¹Department of General Physiology, ²Department of Sleep Medicine and Metabolic Disorders, ³Cell-to-Cell Communication Department and

⁵Department of Clinical Physiology, Medical University of Lodz, Lodz, Poland

⁴Fruit Storage and Processing Department, Division of Pomology, Research Institute of Horticulture, Skierniewice, Poland

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Strawberries can augment plasma antioxidant activity, but this may be confounded by selection of methods, time of blood sampling and concomitant dietary restrictions. We examined the effect of strawberry consumption on ferric reducing ability (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (DPPH-test) of native and non-urate plasma in healthy subjects on their usual diet. Eleven subjects consumed strawberries (500 g daily) for 9 days. Fasting and 3-h postprandial plasma and 24-h urine collection were obtained before, during and after strawberry course for FRAP, DPPH-test and polyphenols determination. Fifteen subjects served as a control in respect to plasma antioxidant activity changes and effect of 300 mg of oral ascorbate. First, 5th and 9th strawberry dose increased 3-h postprandial DPPH-test by 17.4, 17.6 and 12.6%, and FRAP by 15.5, 25.6 and 21.4% in comparison to fasting values in non-urate plasma ($p < 0.05$). In native plasma only a trend was observed to higher postprandial values for both tests. Strawberries increased urinary urolithin A and 4-hydroxyhippuric acid whereas plasma polyphenols were stable. No changes of FRAP and DPPH-test were noted in controls and after ascorbate intake. Strawberries transiently increased non-urate plasma antioxidant activity but this cannot be attributed to direct antioxidant effect of polyphenols and ascorbate.

Key Words: strawberry, plasma antioxidant activity, phenolics, dietary antioxidants, bioactive compounds, supplements and functional foods

Strawberry (*Fragaria × ananassa*) fruits (culinary term) are highly appreciated by consumers due to sweetness, flavor, fragrance, bright red color and juicy texture. They are frequently consumed either fresh, frozen or as an additive to dairy products, ice creams, chocolates and pies. Therefore, strawberry world production revealed increasing trend during the last decade reaching about 4.6×10^6 tons in 2011 year. There is a substantial body of evidence that regular strawberry consumption can have pro-healthy effect.^(1–3) This may include cardiovascular protection, e.g., reduction of atherosclerotic markers in subjects with metabolic syndrome or decrease in low-density lipoprotein peroxidative damage;^(4–7) anti-inflammatory activity (attenuation of the postprandial inflammatory response);^(6,8,9) attenuation of the risk of oxidative stress by augmentation of plasma antioxidant defense^(7,10–13) and suppression of baseline formation of reactive oxygen species by blood phagocytes.⁽¹¹⁾ These plausibly pro-healthy effects of strawberries can be attributed to the presence of numerous polyphenols such as anthocyanins (e.g., pelargonidin, cyanidin), ellagic acid and ellagitannins, phenolic acids (chlorogenic acid, hydroxycinnamic acid), catechin, quercetin and vitamins

(ascorbic acid, folates).^(1–3) Apart from the direct antioxidant and anti-inflammatory properties polyphenols can modulate the expression of genes involved in cell metabolism and survival, enzymatic antioxidant defense and systems of DNA protection and repair.^(1,2) Although, the enhancing effect of strawberry consumption on plasma antioxidant activity in humans seems extensively investigated^(7,10–14) there are some controversies and unresolved issues regarding this effect: (A) – Consumption of 500 g strawberries daily for 16 days increased fasting plasma ferric reducing ability (FRAP) while had no effect on plasma ability to decolorize 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation (TEAC assay) in healthy subjects on low-polyphenols diet.⁽¹³⁾ Conversely, in another study the same dose of strawberries consumed for 9 days did not alter fasting plasma FRAP or its ability to decompose 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (DPPH-test) in healthy subjects also on low-polyphenols diet.⁽¹⁰⁾ On the other hand, under the same conditions a rise in DPPH-test was observed in fasting plasma deprived of uric acid (non-urate DPPH-test);⁽¹⁰⁾ (B) – Withdrawal of strawberries from the diet resulted in the decrease of non-urate DPPH-test to baseline values within 6 days,⁽¹⁰⁾ but in the other study increased fasting FRAP values persisted for 30 days after cessation of strawberry consumption;⁽¹³⁾ (C) – 30 days consumption of 500 g strawberries daily did not alter fasting plasma FRAP and DPPH-test (but increased non-urate DPPH-test) in healthy subjects on their usual diet,⁽¹²⁾ while plasma FRAP (but not TEAC assay) measured 3 and 4 h after single consumption of 1,000 g and 240 g of strawberries, respectively, increased substantially in healthy subjects.^(12,14) These inconsistencies suggest that discovery of a positive effect of strawberries consumption on plasma antioxidant activity may depend on various factors including selection of the method used for plasma antioxidant activity measurement, time of blood sampling after strawberry intake and concomitant dietary restrictions. Therefore, to resolve these issues we decided to analyze the effect of daily consumption of 500 g strawberries for 9 days on antioxidant activity of fasting plasma samples and those collected 3 h after fruits intake in healthy subjects on their usual diet. FRAP and DPPH-test were used for the assessment of total antioxidant activity of native plasma samples and those deprived of uric acid (non-urate) along with determination of selected phenolic acids and total phenolic concentrations in plasma. Furthermore the effect of a single oral dose of 300 mg of ascorbic acid, comparable to that ingested with 500 g of strawberries on plasma antioxidant activity was evaluated.

*To whom correspondence should be addressed.
E-mail: dariusz.nowak@umed.lodz.pl

Materials and Methods

This study was an extension of our previously published results of interventional trials in which consumption of strawberries (500 g daily) increased fasting non-urate plasma DPPH radical scavenging activity^(10,11) while had no effect on FRAP.⁽¹¹⁾ It was executed just after interim analysis of these variables, therefore, the same strawberry pulp was used for dietary intervention.

Strawberries. The strawberry 'Honeoye' fruits were harvested at commercial maturity at plantation managed according to standards of integrated food production. They were washed, destemmed, mixed in order to obtain a uniform experimental sample, frozen, disintegrated at -25°C , then packed in polyethylene bags in quantities of 500 ± 1 g and stored at the same temperature (-25°C) until consumption but no longer than 4 months. The detailed characteristics of fruits; content of anthocyanins (e.g., cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, pelargonidin 3-*O*-rutinoside), ellagic acid and other phenolics (e.g., catechin, chlorogenic acid, quercetin glycosides) and phytochemicals stability during storage were described elsewhere.⁽¹¹⁾

Study Population. The study group (strawberry consumers) involved 11 volunteers [F/M – 1/10, mean age 41 ± 11 years, mean body weight 80 ± 7 kg, mean body mass index (BMI) = 25.1 ± 1.7 kg/m²]. The inclusion criteria were age between 20 and 60 years and a written informed consent before initiating the study procedures. The control group consisted of 7 subjects (F/M – 2/5, mean age 47 ± 8 years, mean body weight 80 ± 10 kg, BMI 26.1 ± 1.5 kg/m²). Additional group for evaluation of single 300 mg oral dose of vitamin C on plasma antioxidant activity involved 8 subjects (F/M – 3/5, mean age 41 ± 10 years, mean body weight 74 ± 10 kg, BMI 24.1 ± 2.3 kg/m²). All volunteers were the members of our university scientific staff and were apparently healthy nonsmokers. They were employed at departments located in the close neighborhood of Department of Clinical Physiology where the clinical part of study was carried out. All subjects agreed to keep their dietary habits constant during the study period and to comply with instructions related to participation in the study. The exclusion criteria included pregnancy, alcohol and illicit drug abuse, fructose intolerance, strawberry hypersensitivity; moreover, any history of infectious or inflammatory diseases, use of any vitamins or food supplements, or any systemic treatment with antibiotics within 3 months prior to the study.

Study protocol. The study had an open-label design with run-in and washout periods and consisted of 5 visits at the 0th, 3rd, 7th, 11th and 14th day of observation. During the whole study period (14 days) volunteers ($n = 11$) were on their usual diet. After a 3-day run-in period (a visit on the 3rd day), volunteers started to eat a daily portion of 500 g of strawberries (strawberry pulp thawed to room temperature, consumed between 11:00 and 11:30 AM) for 9 days until a visit on 11th day. The washout period following this visit lasted 3 days (until a visit on 14th day). Fasting venous blood (10 ml) was collected between 8:30 and 9:30 AM into vacutainer tubes with lithium heparin (Becton Dickinson, Franklin Lakes, NJ) for plasma antioxidant activity measurement (FRAP and DPPH-test) and into tubes containing EDTA for other assays. The same blood collection procedure was repeated 3 h after strawberry pulp consumption at 2:30 PM. Blood was centrifuged (10 min, $1,500 \times g$, 4°C) and the plasma was stored at -80°C until assayed but not longer than 3 months. All volunteers had a light snack (roll with butter and cheese and a cup of tea) within one hour after fasting blood collection. The controls ($n = 7$) underwent all the aforementioned procedures, with the exception of the strawberry pulp intake. At the first visit volunteers received a diary card to record daily consumption of selected food products and beverages rich in polyphenols as previously described.⁽¹¹⁾ Strawberry consumers were additionally asked to perform 24-h urine collection at each visit. It started from 11:30 AM, right after

fruits intake, till 11:30 AM the next day. One gram of ascorbic acid and 130 mg of NaN_3 per urinary container were used as a preservative. Evaluation of a single 300 mg oral dose of vitamin C on plasma antioxidant activity was executed in the same manner as in the case of strawberries, however, it was performed only during the one visit. The Medical University of Lodz Ethics Committee approved the study protocol (RNN/62/09/KE).

Measurement of plasma DPPH radical scavenging activity. Plasma DPPH-test was measured according to our recently described procedure.⁽¹⁵⁾ Briefly, 5 μl of 10 mmol/L of a DPPH radical solution in methanol was added to a cuvette containing 970 μl of methanol and continuously stirred and, after a 3-min incubation period at 20°C , the baseline absorbance at wavelength 517 nm (A_{517}) was measured with an Ultrospec III, using Spectro-Kinetics software (LKB Biochrom Pharmacia, Cambridge, England). Then, 25 μl of deproteinized plasma was added and mixed; the A_{517} decrease related to the DPPH radical decomposition over 30 min of incubation period was then recorded. As a negative control 25 μl samples of 9.50 mol/L acetonitrile solution was used. All individual results were obtained from triplicate measurements and plasma scavenging effect (PSE%) was calculated according to the formula $\text{PSE}\% = [1 - A_{517} \text{ plasma sample} / A_{517} \text{ negative control}] \times 100\%$.^(15,16)

Measurement of FRAP. Measurement was conducted following the procedure originally described by Benzie and Strain,⁽¹⁷⁾ with some modifications,^(16,18) in which Fe^{3+} to Fe^{2+} ion reduction at low pH caused the formation of a colored ferrous-2,4,6-tripirydyls-triazine (TPTZ) complex, resulting in an increase in absorbance at 593 nm (A_{593}). Briefly, 30 μl of plasma was mixed with 90 μl of deionized water and then added to 900 μl of a FRAP reagent (prewarmed to 37°C) while sample absorbance at 593 nm was continuously measured over 10 min (end point assay) at 37°C (Ultrospec III, with Spectro-Kinetics software, LKB Biochrom Pharmacia, Cambridge, England). As a control served 120 μl samples of water. FRAP reagent was prepared just before the assay by adding 10 ml of 300 mmol/L acetate buffer (pH 3.6), 1 ml of 10 mmol/L TPTZ in 40 mmol/L HCl, and 1 ml of 20 mmol/L aqueous FeCl_3 solution in the given order. Calibration was performed with a FRAP reagent containing FeCl_2 (total sample volume 1.02 ml, final concentrations from 5 to 1,000 $\mu\text{mol/L}$, 25 concentration points). Absorbance was linear ($r = 0.98$, $p < 0.01$). Individual results were obtained from triplicate measurements and were expressed as a concentration of Fe^{3+} ions reduced into Fe^{2+} after 10 min of incubation of plasma samples with FRAP reagent. Calculations were done according to the formula: $Y [\mu\text{mol/L}] = 1687.9X - 3.3$, Y being the concentration of Fe^{3+} reduced ions while X the difference between A_{593} of assayed sample and A_{593} of a blank.⁽¹⁶⁾

Preparation of non-urate plasma. Samples of plasma (200 μl) were incubated with 1.2 U/ml uricase and 24 U/ml catalase for 10 min at 37°C in order to decompose uric acid.⁽¹⁹⁾ Preliminary experiments with 6 plasma specimens with uric acid concentration ranging from 5.2 to 6.8 mg/dl revealed complete decomposition of uric acid already after 5 min from uricase addition. Catalase was added for rapid decomposition of H_2O_2 (generated during uricase catalyzed oxidation of uric acid by oxygen) to avoid the risk of suppression of plasma antioxidant activity. Catalase alone had no effect on the results of FRAP and DPPH tests.⁽¹¹⁾

Other determinations. Concentrations of selected polyphenols and their metabolites (dihydrocaffeic acid, vanillic acid, caffeic acid, homovanillic acid, hippuric acid, 4-hydroxyhippuric acid, urolithin A) in plasma and urine were determined with combination of solid phase extraction technique with high-performance liquid chromatography with electrochemical or ultraviolet-visible detection as previously described.⁽¹¹⁾ Total phenolic compound concentrations were measured by the Folin-Ciocalteu method⁽¹⁶⁾ and expressed as catechin equivalent in

mmol/L or mmol/24 h for plasma or 24-h urine collection, respectively.

Statistical analysis. Results were expressed as a mean (SD) and median depending on data distribution which was tested with Shapiro-Wilk's *W* test. Analysis of variance (ANOVA) for repeated observations or Friedman's ANOVA was applied for the assessment of changes in variables over time (before, during, and after consumption of strawberries) depending on data distribution. In case of statistically significant ANOVA, the post hoc analyses were done with Scheffe's test or post hoc analysis for Friedman's ANOVA (multiple comparisons at 2 different time points). Comparison of dependent variables at a given time point (fasting vs postprandial) were analyzed with Wilcoxon test (non-normal distributed data) or paired *t* test (normal data distribution). A *p* value < 0.05 was considered significant.

Results

All strawberry consumers and control subjects completed the study. No adverse events were noted. Average consumption (calculated on the basis of subjects' reports) of coffee, tea, fruits, vegetables, alcoholic beverages, meat, eggs, and dairy products did not change in the strawberry or control group over the study period (data not shown). Strawberry consumption increased 24-h urinary excretion of urolithin A and 4-hydroxyhippuric acid which

returned to the baseline after 3 days of washout (Table 1). This is consistent with the 100% compliance with study protocol instructions declared by subjects in the strawberry group. Fasting and postprandial FRAP, non-urate FRAP, DPPH-test and non-urate DPPH-test did not change significantly in healthy controls over 14 days of observation (Table 2). Similarly, no differences were noted between fasting and postprandial measures of plasma antioxidant activity at majority of time-points except for 3rd day for FRAP and 11th day for non-urate DPPH-test (*p* < 0.05) (Table 2). At these time-points mean postprandial FRAP decreased by 4.4%, while mean postprandial non-urate DPPH-test rose by 12.2%. Fasting and postprandial plasma uric acid did not change over the study period. On the other hand, postprandial uric acid concentration revealed tendency to the lower values than the fasting one that reached statistical significance at the 7th and 11th day (Table 2). In strawberry consumers the mean fasting plasma uric acid increased by 19.9% at the 14th day (after 3 day wash-out) vs the baseline value at the day 3rd (Fig. 1). However, 3-h postprandial plasma uric acid concentration did not alter significantly over the study and did not differ from fasting values at any time-point, particularly during strawberry consumption period (Fig. 1).

FRAP in strawberry consumers. Both, fasting and 3-h postprandial FRAP tended to decrease over the whole study period in strawberry consumers. However, only in the case of postprandial values this trend reached statistical significance (Fig. 2). Postpran-

Table 1. Twenty four-hour urinary excretion of total phenolics, 4-hydroxyhippuric acid, urolithin A and creatinine in 11 healthy strawberry consumers

Variable	Day of the study				
	Run-in	Strawberry consumption			Wash-out
	0	3	7	11	14
4-hydroxy-hippuric acid (μmol/24 h)	66.3 ± 69.9 (42.2)	179.0 ± 120.1*	181.1 ± 133.4*	170.1 ± 114.1*	60.9 ± 58.7 (35.5)
Urolithin A (μmol/24 h)	8.9 ± 14.4 (1.5)	18.1 ± 17.3 (20.9)	87.4 ± 86.1* (79.0)	102.9 ± 89.5* [†] (66.1)	10.4 ± 22.6 (1.6)
Total phenolics (mmol/24 h)	8.4 ± 3.2 (7.8)	8.8 ± 4.3 (7.1)	8.2 ± 1.9 (7.9)	8.3 ± 2.5 (7.6)	6.4 ± 1.0 (6.5)
Creatinine (g/24 h)	1.60 ± 0.36 (1.45)	1.46 ± 0.37 (1.49)	1.41 ± 0.36 (1.48)	1.43 ± 0.35 (1.41)	1.41 ± 0.25 (1.50)

Strawberries (500 g a day) were consumed from 3rd till 11th day between 11:00 and 11:30 AM (the first and the last serving of strawberries was eaten at 3rd and 11th day, respectively). During the whole study period all volunteers were on their usual diet. 24-h urine collection started from 11:30 AM till 11:30 AM the next day. Results are expressed as mean ± SD (median). Stable urinary excretion of creatinine confirms accurate and complete collection of 24-h urine samples for phenolics determination. * vs corresponding value at 0th and 14th day, *p* < 0.05. [†] vs 3rd day, *p* < 0.05 (post hoc analysis for Friedman's ANOVA).

Table 2. Plasma antioxidant activity and uric acid levels in the control group over 14 days of study period

Plasma variable	Day of the study				
	0	3	7	11	14
Fasting FRAP (mmol/L)	1.10 ± 0.21	1.13 ± 0.23	1.13 ± 0.22	1.09 ± 0.20	1.05 ± 0.22
Postprandial FRAP (mmol/L)	1.09 ± 0.22	1.08 ± 0.25*	1.11 ± 0.23	1.09 ± 0.25	1.07 ± 0.22
Fasting Nu-FRAP (mmol/L)	0.28 ± 0.05	0.31 ± 0.04	0.30 ± 0.04	0.30 ± 0.05	0.31 ± 0.04
Postprandial Nu-FRAP (mmol/L)	0.29 ± 0.03	0.30 ± 0.06	0.30 ± 0.04	0.34 ± 0.09	0.33 ± 0.06
Fasting DPPH test (%)	22.2 ± 4.2	21.4 ± 4.4	21.9 ± 4.6	21.1 ± 4.0	20.5 ± 4.0
Postprandial DPPH test (%)	21.4 ± 4.1	20.9 ± 4.1	21.2 ± 4.4	20.8 ± 3.8	20.4 ± 4.0
Fasting Nu-DPPH test (%)	4.1 ± 1.8	4.2 ± 1.8	4.2 ± 1.9	4.1 ± 1.6	4.3 ± 1.9
Postprandial Nu-DPPH test (%)	4.1 ± 2.0	3.7 ± 1.6	4.3 ± 1.5	4.6 ± 1.8*	4.4 ± 2.0
Fasting uric acid (mg/dl)	4.9 ± 1.7	4.8 ± 1.6	4.9 ± 1.5	4.8 ± 1.5	4.3 ± 1.2
Postprandial uric acid (mg/dl)	4.7 ± 1.7	4.7 ± 1.6	4.6 ± 1.6*	4.0 ± 1.3*	3.9 ± 0.9

FRAP, ferric reducing ability of plasma; DPPH-test, plasma DPPH radical scavenging activity; Nu, non-urate (plasma samples deprived of uric acid). Fasting venous blood was collected between 8:30 and 9:30 AM. Volunteers had a light snack (roll with butter and cheese and a cup of tea) within 1 h after fasting blood collection. Postprandial blood samples were drawn at 2:30 PM (time corresponding to 3-h postprandial plasma in strawberry consumers). During the whole study period all volunteers were on their usual diet. Results are expressed as mean (SD). No significant differences within any fasting or postprandial variables were noted over the study period. * vs corresponding fasting value, *p* < 0.05 (paired *t* test).

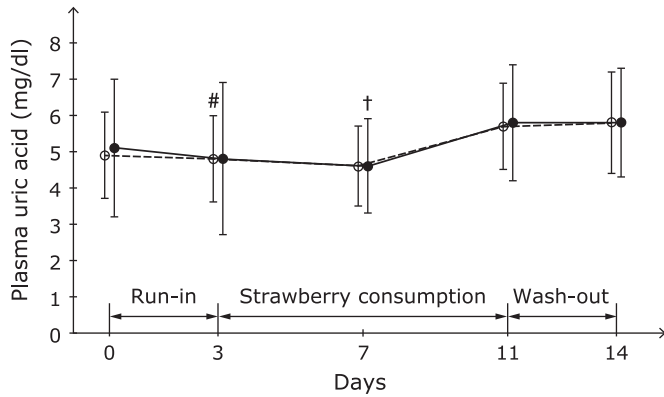


Fig. 1. Effect of strawberry consumption (500 g a day for 9 days) on uric acid levels in fasting (open circles) and 3-h postprandial plasma (closed circles) in 11 healthy subjects. Fasting venous blood was collected between 8:30 and 9:30 AM. Volunteers had a light snack within 1 h after fasting blood collection. Strawberries were consumed from 3rd till 11th day between 11:00 and 11:30 AM (the first and the last serving of strawberries was eaten at 3rd and 11th day, respectively). Postprandial blood samples were drawn at 2:30 PM. During the whole study period all volunteers were on their usual diet. Results are expressed as mean (SD). Postprandial plasma uric acid levels did not alter significantly over the study period. No differences between fasting and postprandial uric acid levels were noted at any time-point. # vs 14th day $p < 0.05$; † vs 11th and 14th day, $p < 0.05$ (ANOVA for repeated observations and post hoc Sheffe's test).

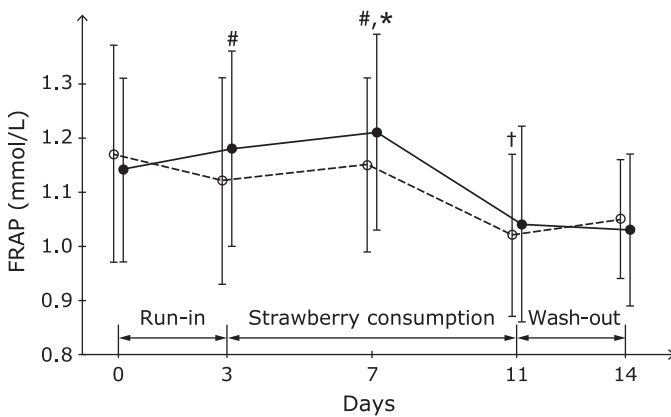


Fig. 2. Effect of strawberry consumption (500 g a day for 9 days) on ferric reducing ability (FRAP) of fasting (open circles) and 3-h postprandial plasma (closed circles) in 11 healthy subjects. Fasting venous blood was collected between 8:30 and 9:30 AM, while postprandial blood samples were drawn at 2:30 PM. Other details as for Fig. 1. Results are expressed as mean (SD). # vs 11th and 14th day, $p < 0.05$; † vs 0th day, $p < 0.05$ (ANOVA for repeated observations and post hoc Sheffe's test). * vs fasting value at the same day, $p < 0.05$ (paired *t* test).

dial FRAP at 11th day (after the last strawberry dose) and at the 14th day (after 3 day wash-out) were lower by about 15% than that noted at 3rd day (after the first strawberry dose) and at 7th day (after the fifth fruits dose). During the period of strawberry consumption (3rd, 7th and 11th day) postprandial FRAP revealed tendency to higher values than the corresponding fasting one, yet statistical significance was noted at the 7th day (1.21 ± 0.18 mmol/L vs 1.15 ± 0.16 mmol/L, $p < 0.05$). When, plasma samples deprived of uric acid were analyzed (non-urate FRAP) similar, but more distinct changes in antioxidant activity were noted than in the case of native plasma (Fig. 3). Fasting and postprandial non-urate FRAP at baseline were higher than those at the end of strawberry

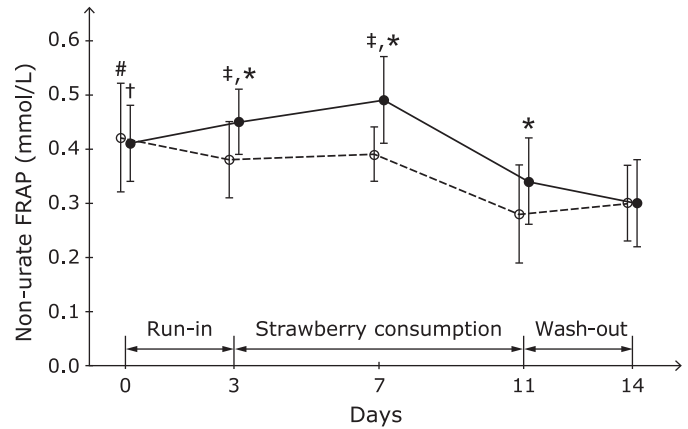


Fig. 3. Effect of strawberry consumption (500 g a day for 9 days) on non-urate ferric reducing ability (non-urate FRAP) of fasting (open circles) and 3-h postprandial plasma (closed circles) in 11 healthy subjects. Plasma samples were treated with uricase and catalase in order to decompose the uric acid. Other details as for Fig. 1. Results are expressed as mean (SD). # vs 11th and 14th day, $p < 0.01$; † vs 14th day, $p < 0.05$; ‡ vs 11th and 14th day, $p < 0.05$ (ANOVA for repeated observations and post hoc Sheffe's test). * vs fasting value at the same day, $p < 0.01$ (paired *t* test).

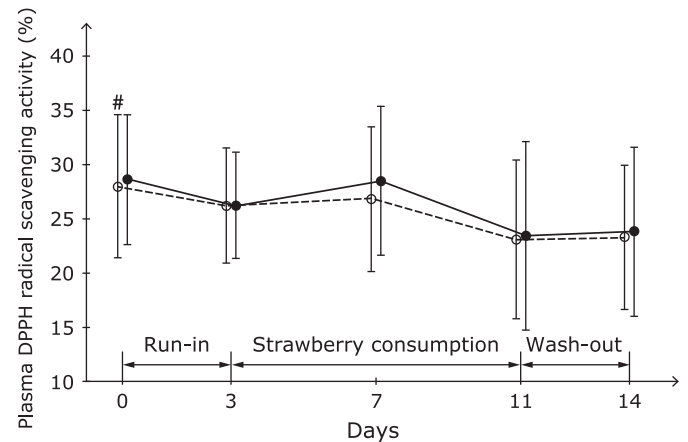


Fig. 4. Effect of strawberry consumption (500 g a day for 9 days) on DPPH radical scavenging activity of fasting (open circles) and 3-h postprandial plasma (closed circles) in 11 healthy subjects. Other details as for Fig. 1. Results (% of decomposed initial amount of DPPH radical) are expressed as mean (SD). 3-h postprandial plasma DPPH radical scavenging activity did not alter over the study period. No differences between fasting and postprandial DPPH radical scavenging activities were noted at any time-point. # vs 11th day, $p < 0.05$ (post hoc analysis for Friedman's ANOVA).

consumption (11th day) and after the wash-out period (14th day). Moreover, mean postprandial non-urate FRAP increased after strawberry intake by 15.5, 25.6 and 21.4% in comparison to fasting values at 3rd, 7th and 11th day, respectively (Fig. 3).

Plasma DPPH-test in strawberry consumers. Fasting and postprandial DPPH-test of native plasma (likewise FRAP) revealed trend to decrease over the study period. However, statistical significance was noted only between fasting DPPH-test values at 0th day (baseline) and 11th (before the last dose of strawberries). Fasting and 3-h postprandial plasma DPPH-tests were almost the same at all time-points, especially at 3rd, 7th and 11th day representing the effect of the 1st, 5th and 9th dose of strawberries, respectively (Fig. 4). On the other hand, mean non-urate DPPH-

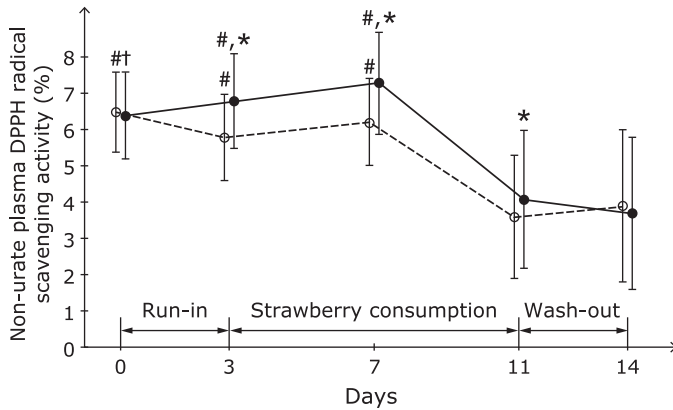


Fig. 5. Effect of strawberry consumption (500 g a day for 9 days) on the non-urate DPPH radical scavenging activity of fasting (open circles) and 3-h postprandial plasma (closed circles) in 11 healthy subjects. Plasma samples were treated with uricase and catalase in order to decompose the uric acid. Other details as for Fig. 1. Results (% of decomposed initial amount of DPPH radical) are expressed as mean (SD). # vs 11th and 14th day, $p < 0.05$; † vs 14th day, $p < 0.05$ (ANOVA for repeated observations and post hoc Sheffe's test). * vs fasting value at the same day, $p < 0.01$ (paired t test).

test increased after 3 h from strawberry ingestion by 17.4, 17.6 and 12.6% at the consecutive time-points of fruits consumption (Fig. 5). As it was observed for FRAP and non-urate FRAP, fasting and postprandial non-urate DPPH-tests at 11th and 14th day were significantly lower than corresponding values at baseline (a decrease by 44.1 and 39.9% for fasting plasma samples and by 35.8 and 42.4% for postprandial plasma samples, respectively) (Fig. 5).

Effect of single 300 mg dose of vitamin C on plasma antioxidant activity. Single dose of 300 mg of ascorbic acid did not alter plasma antioxidant activity in the group of 8 healthy subjects. Fasting and 3-h postprandial values of FRAP and DPPH-test were similar for both, native and non-urate plasma samples (Table 3).

Table 3. Effect of a single 300 mg oral dose of ascorbic acid on plasma antioxidant activity in 8 healthy subjects

Plasma variable	Plasma samples	
	Fasting, before ascorbic acid intake	3 h after ascorbic acid intake
FRAP (mmol/L)	1.11 ± 0.16 (1.17)	1.10 ± 0.16 (1.17)
Nu-FRAP (mmol/L)	0.41 ± 0.10 (0.40)	0.42 ± 0.07 (0.43)
DPPH test (%)	18.5 ± 4.0 (17.6)	19.4 ± 3.8 (17.5)
Nu-DPPH test (%)	4.4 ± 0.6 (4.4)	4.5 ± 0.4 (4.5)

FRAP, ferric reducing ability of plasma; DPPH-test, plasma DPPH radical scavenging activity; Nu, non-urate (plasma samples deprived of uric acid). Fasting venous blood was collected between 8:30 and 9:30 AM. Volunteers had a light snack (roll with butter and cheese and a cup of tea) within one hour after fasting blood collection. 300 mg dose of ascorbic acid was taken at 11:30 AM. Postprandial blood samples were drawn at 2:30 PM (time corresponding to 3-h postprandial plasma in strawberry consumers). Results are expressed as mean ± SD (median). No significant differences were noted between basal and post vitamin C plasma antioxidant activities (paired t test).

Plasma levels of selected polyphenols in strawberry consumers.

Both, fasting and 3-h postprandial plasma concentrations of total phenolics, dihydrocaffeic acid, caffeic acid, homovanillic acid and vanillic acid did not change over the study period (Table 4). Similarly, plasma levels of these phenolic acids (postprandial vs fasting) did not increase in response to strawberry ingestion at any time-points (3rd, 7th and 11th day). However, it should be pointed out that postprandial levels of hippuric acid were lower than fasting ones. It was noted either at the days without strawberry consumption (0th and 14th) or at 3rd and 11th day after intake of fruits. Further, the mean postprandial plasma concentration of hippuric acid at the end of the study (day 14th) was 2.1-times lower ($p < 0.05$) than that at the entry (0th day) (Table 4). On the other hand, plasma 4-hydroxyhippuric acid levels did not change significantly in strawberry consumers over the study (mean fasting and postprandial concentrations ranged from 2.4 ± 0.3 to 2.6 ± 0.4 $\mu\text{mol/L}$ and from 2.5 ± 0.4 to 2.3 ± 0.6 $\mu\text{mol/L}$ at 0th and 14th day, respectively).

Table 4. Concentration of total polyphenols and selected phenolic acids in fasting and 3-h postprandial plasma of 11 healthy strawberry consumers over the study period

Plasma polyphenols ($\mu\text{mol/L}$)		Day of the study				
		Run-in	Strawberry consumption			Wash-out
		0	3	7	11	14
Total polyphenols*	F	0.99 ± 0.11	0.96 ± 0.13	0.96 ± 0.11	1.02 ± 0.10	1.00 ± 0.15
	P	1.01 ± 0.08	0.94 ± 0.13	0.99 ± 0.11	1.00 ± 0.09	1.00 ± 0.15
Hippuric acid	F	9.7 ± 6.2 [†]	9.8 ± 6.9 [†]	6.1 ± 3.6	5.6 ± 3.7 [†]	6.1 ± 7.4 [†]
	P	4.4 ± 3.3	4.9 ± 3.6	6.0 ± 3.6	3.9 ± 2.8	2.1 ± 2.8 [#]
Dihydro-caffeic acid	F	0.06 ± 0.05	0.05 ± 0.06	0.06 ± 0.04	0.07 ± 0.04	0.06 ± 0.06
	P	0.07 ± 0.06	0.07 ± 0.05	0.09 ± 0.08	0.05 ± 0.02	0.06 ± 0.05
Vanillic acid	F	0.01 ± 0.02	0.06 ± 0.09	0.02 ± 0.02	0.03 ± 0.04	0.02 ± 0.02
	P	0.02 ± 0.02	0.05 ± 0.05	0.03 ± 0.03	0.04 ± 0.03	0.03 ± 0.04
Caffeic acid	F	0.03 ± 0.02	0.04 ± 0.05	0.03 ± 0.01	0.03 ± 0.03	0.04 ± 0.04
	P	0.05 ± 0.04	0.04 ± 0.02	0.04 ± 0.04	0.04 ± 0.01	0.04 ± 0.02
Homovanillic acid	F	0.10 ± 0.08	0.10 ± 0.06	0.12 ± 0.09	0.17 ± 0.22	0.10 ± 0.07
	P	0.10 ± 0.07	0.11 ± 0.07	0.10 ± 0.07	0.11 ± 0.08	0.11 ± 0.12

F, fasting; P, 3-h postprandial; * expressed in mmol/L of catechin equivalent. Fasting venous blood was collected between 8:30 and 9:30 AM. Volunteers had a light snack within 1 h after fasting blood collection. Strawberries were consumed from 3rd till 11th day between 11:00 and 11:30 AM (the first and the last portion of strawberries was eaten at 3rd and 11th day, respectively). Three-hour postprandial blood samples were drawn at 2:30 PM. During the whole study period all volunteers were on their usual diet. Results are expressed as mean (SD). † vs corresponding postprandial value, $p < 0.05$ (Wilcoxon test), # vs 0th, 1st, 2nd and 3rd day, $p < 0.05$ (post hoc analysis for Friedman's ANOVA).

Discussion

Uric acid had a strong antioxidant potential and contributed considerably to the total plasma antioxidant activity.^(15,16,19) It was responsible for about 60% and 75% of plasma ability to reduce Fe³⁺ ions (FRAP) and to decompose the DPPH radical (DPPH-test), respectively.^(10,11) Polyphenols can affect urinary excretion of uric acid^(20,21) and fructose contained in ingested fruits or juices can transiently elevate uric acid levels with subsequent increase in plasma antioxidant activity.^(16,22) Therefore, moderate changes in other circulating antioxidants (e.g., phenolics, vitamins, thiols) cannot result in significant rise of plasma antioxidant activity. Hence, it is recommended to analyze native plasma samples and those deprived of uric acid in order to increase the sensitivity and specificity of plasma antioxidant activity monitoring in the studies with dietary interventions.⁽¹⁹⁾ Although, one dose of strawberries contained 18.5 g fructose and 1,320 mg of total polyphenols⁽¹¹⁾ no changes between fasting and postprandial uric acid levels in fruits consumers were noted at any occasion. Thus, any difference between fasting and postprandial plasma antioxidant activities in strawberry consumers cannot be attributed to changes in circulating concentrations of uric acid.

Plasma antioxidant activity in strawberry consumers.

In healthy controls comparison of fasting and postprandial FRAP and DPPH-test measured either in native or non-urate plasma samples did not reveal any significant differences at the majority of time points. Conversely, in strawberry consumers plasma antioxidant activity measured in non-urate samples increased after fruits ingestion. Moreover, at 0th and 14th day when fruits were not consumed no differences between fasting and postprandial non-urate FRAP and DPPH-test were noted. These indicate that non-urate plasma antioxidant activity increased in response to strawberry intake and some phytochemicals contained in this fruits could be responsible for this effect. However, native plasma did not reveal any differences between fasting and postprandial DPPH-test and FRAP (except for that at the 7th day) in response to strawberry consumption what is in concordance with the aforementioned masking effect of uric acid.⁽¹⁹⁾ Both, fasting and postprandial FRAP and DPPH-test were stable over the study period in healthy controls. Surprisingly, plasma antioxidant activity (fasting and postprandial) decreased at the end of study (at the 11th day before the last dose of strawberries and at the 14th day after 3 day washout) in fruits consumers what could be attributed to repeated strawberry intake.

Contribution of ascorbic acid to strawberry intake-induced rise of postprandial plasma antioxidant activity.

A dose of 500 g strawberries contained 271.5 mg of ascorbic acid.⁽¹¹⁾ Ascorbic acid has a strong ability to reduce Fe³⁺ ions⁽¹⁸⁾ that is higher than that of numerous polyphenols and their metabolites.⁽¹⁸⁾ Moreover, it decomposed the DPPH radical *in vitro* and added to human plasma augmented FRAP and DPPH-test results.^(15,18) In clinical studies single rapid (within 10 min) consumption of 1 kg of strawberries increased 3-h postprandial plasma antioxidant activity in healthy subjects.⁽¹⁴⁾ Since, strawberry ingestion was accompanied by 95% rise in circulating ascorbic acid levels this vitamin was recognized as the main factor responsible for 22% augmentation of FRAP.⁽¹⁴⁾ However, TEAC-test, the other measure of plasma antioxidant activity (based on the ability of an antioxidant compound to quench and reduce ABTS⁺ radical cation to the colorless form) did not increase at all.⁽¹⁴⁾ *In vitro*, one molecule of ascorbic acid can reduce 2 molecules of ABTS⁺ radical,⁽²³⁾ thus one may expect any increase in TEAC-test in the case of almost 2-fold rise of plasma ascorbate. These suggest that other compounds and mechanisms can additionally contribute to augmentation of plasma antioxidant activity in the case of rapid ingestion of 1 kg of strawberries. We assumed that strawberry-induced increase in non-urate FRAP and DPPH-test observed in our study could be related to ingestion

of ascorbic acid contained in these fruits. However, our control experiments showing no effect of oral single 300 mg dose of ascorbic acid on native and non-urate plasma antioxidant activity rather excluded this possibility.

Plausible mechanism of strawberry-induced postprandial rise of plasma antioxidant activity.

Postprandial concentrations of total phenolics and six selected phenolic acids in plasma did not increase in comparison to fasting values at any occasion after strawberry consumption. It indicates that the rise of non-urate plasma antioxidant activity cannot be caused by direct antioxidant effect of increased concentrations of circulating polyphenols. Moreover, results of our control experiments with single oral dose of 300 mg of vitamin C (discussed in the previous subsection) excluded the significant contribution of strawberry-derived ascorbate in this phenomenon. However, the rise of urinary concentration of urolithin A and 4-hydroxyhippuric acid indicates that strawberry polyphenols or products of their processing by gut microbiota⁽²⁴⁾ were absorbed and metabolized in liver and enterocytes, or absorptive cells of large intestine.^(25,26) It seems that phenolics concentration in the portal blood could be higher than that in the systemic circulation and thereby they can affect some enzymes present in the liver and the small and large intestine. Xanthine oxidase (XO) and lipoxygenase activities were found in the liver and intestine and their inhibition protected these organs from oxidative stress-induced injury under experimental conditions *in vivo*.⁽²⁷⁻³²⁾ Recently, it was found that extracts from *Aronia melanocarpa* and isolated polyphenols such as quercetin and cyanidin-3 glucoside can inhibit XO and 15-lipoxygenase *in vitro*.⁽³³⁾ XO catalyzes oxidation of hypoxanthine to xanthine with subsequent conversion into uric acid^(27,28) while 15-lipoxygenase participates in arachidonic acid and linoleic acid metabolism.⁽³⁴⁾ Both these processes are accompanied by generation of superoxide radicals with further formation of H₂O₂ and hydroxyl radicals. Since consumed strawberries contained cyanidins and quercetin⁽¹¹⁾ ingestion of these fruits could result in at least transient inhibition of these enzymes with subsequent attenuation of free radicals production. This inhibition can spare to some extent the pool of circulating antioxidants and thus be responsible for the postprandial rise of plasma antioxidant activity. Moreover, consumption of strawberries decreased resting production of reactive oxygen species by circulating polymorphonuclear leukocytes in healthy volunteers⁽¹¹⁾ and inhibited postprandial release of pro-inflammatory and pro-oxidant cytokines in overweight subjects.⁽⁹⁾ These may also contribute to sparing of plasma antioxidants with subsequent increase in postprandial non-urate plasma antioxidant activity. On the other hand, inhibition of XO could result in the decrease in plasma uric acid. We did not observe any significant differences between fasting and postprandial uric acid levels in strawberry consumers. Moreover, uric acid levels raised significantly at the end of study. Therefore, confirmation of this hypothesis requires further studies involving monitoring of blood activity of XO and lipoxygenase along with urinary excretion of uric acid after strawberry ingestion.

Decrease of fasting plasma antioxidant activity over the study period.

Suppression of fasting plasma antioxidant activity at the 11th day after 9 days of strawberry consumption that persisted after 3 days of washout period was the surprising finding. This was specially visible in non-urate plasma samples. Although, circulating uric acid was elevated at the last two time-points (11th and 14th day) the decrease of FRAP and DPPH-test was also noted in native plasma samples. This observation is consistent to some extent with our previous report showing tendency to a decrease in fasting non urate DPPH-test after 9 and 12 days of strawberry consumption (500 g daily) in comparison to the peak value after 3 days of fruit intake in healthy subjects on semi-restrictive low polyphenols diet.⁽¹⁰⁾ On the other hand, strawberry consumption had no effect either on DPPH-test of native plasma or on FRAP determined in both native and non-

urate plasma.⁽¹⁰⁾ Two 30 day courses of strawberry (500 g a day) consumption (separated by 10 days wash-out) did not alter fasting plasma FRAP and DPPH-test and even increased the latter in non-urate plasma of healthy subjects without any dietary restrictions.⁽¹¹⁾ Also other authors did not report any significant changes in fasting plasma antioxidant activity in response to repeated strawberry intake in healthy subjects on their usual diet.⁽³⁵⁾ However, in these studies the time-intervals between consecutive time-points of plasma antioxidant activity measurement were comparable to or even longer than^(11,35) duration of fruits consumption in our present trial. Therefore, some transient plasma antioxidant activity fluctuations including its decrease could have been overlooked in these long-term dietary interventions.

It is possible that total plasma antioxidant activity is not a highly specific and sensitive parameter reflecting effectiveness of the usual diet supplementation with fruits rich in antioxidant and anti-inflammatory polyphenols (e.g., strawberries) in healthy subjects. This hypothesis is supported by the observations that consumption of strawberries 500 g daily for a few weeks had no significant effect on fasting plasma antioxidant activity of healthy subjects on their usual diet while, at the same time, increased: (A) – plasma lipids, erythrocytes and peripheral blood mononuclear cells resistance to oxidative damage;⁽³⁵⁾ (B) – decreased resting generation of ROS by blood phagocytes⁽¹¹⁾ and suppressed plasma paraoxonase PON-1 activity, what maybe a secondary effect to suppression of reactive oxygen species activity⁽³⁶⁾ under *ex vivo* conditions.

Changes of plasma antioxidant activity in response to repeated strawberry polyphenols ingestion could be the net result of several processes including for instance: (A) – elaboration of polyphenols by gut microflora and intestinal absorption;^(24,37,38) (B) – formation of less reactive complexes with plasma proteins and their transportation outside the capillaries;^(39,40) (C) – binding and uptake of polyphenols by blood and endothelial cells;^(41,42) (D) – polyphenols metabolism in liver and their urinary excretion.⁽⁴³⁾ Moreover, some of these processes can be activated or inhibited by polyphenols themselves. In addition, polyphenols can affect metabolic pathways leading to synthesis of antioxidant enzymes and endogenous low molecular weight antioxidants.^(43,44) Therefore, enhanced cellular polyphenols uptake or their binding to plasma proteins or cell surface may decrease free circulating pool of polyphenols and be responsible for paradoxical decrease of plasma antioxidant activity. However, this could be accompanied by the enhancement of antioxidant potential in the intracellular compartment. Hence, decreased fasting FRAP and DPPH-test observed at the end of our dietary intervention does not exclude beneficial antioxidant effect of diet supplementation with strawberries. The rise of postprandial plasma antioxidant activity with its decrease in subsequent fasting samples fits with this hypothesis. Although, total fasting and postprandial plasma polyphenols did not differ from each other

and were stable over the study period, this cannot exclude changes of free and complexed polyphenols pool during the study since the method used for total polyphenols determination measured the sum of these two pools. Relatively low number of studied subjects is the main limitation of our results. Therefore, these findings should be confirmed on the larger group of strawberry consumers with parallel monitoring of antioxidant defense as well as the main strawberry anthocyanins (e.g., pelargonidin, cyanidin) and their metabolites in plasma and intracellular compartment.

We found that strawberry consumption for nine days increased non-urate postprandial plasma antioxidant activity in healthy subjects on their usual diet. This rise observed at the onset, the middle and the end of period of dietary intervention cannot be attributed to direct antioxidant effect of strawberry derived polyphenols and ascorbic acid. Fasting plasma antioxidant activity decreased over the study period indicating the transient nature of strawberry-induced postprandial augmentation of plasma redox potential and that direct antioxidant effect of strawberry phytochemicals is not the crucial mechanism of pro-healthy effect of this fruits consumption. Therefore, acute postprandial rise in plasma antioxidant activity could not be recognized as preventive effect of regular strawberry consumption.

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Abbreviations

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	analysis of variance
BMI	body mass index
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
FRAP	ferric reducing ability of plasma
Sc%	plasma scavenging effect
TEAC	Trolox equivalent antioxidant capacity
TPTZ	ferrous-2,4,6-tripirydyls-triazine
XO	xanthine oxidase

Conflict of Interest

No potential conflicts of interest were disclosed.

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