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A Pilot Study on Effects of Thai Orange Juice on Oxidative Stress, Neutrophil Function and Cardiovascular Disease Risk Factors in Patients with Type 2 Diabetes

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Abstract

This study aimed to investigate the effects of orange juice on oxidative stress, neutrophil functions and cardiovascular disease risk factors in patients with type 2 diabetes. Fifteen patients with type 2 diabetes with haemoglobin A1c ≥ 8.5% ingested 500 ml of orange juice every day for 4 weeks. Only plasma vitamin C was significantly increased (0.783 ± 0.09 vs 0.844 ± 0.09 mg/dL, $p<0.05$) and superoxide dismutase was tended to increase (46.8 ± 1.44 vs 48 ± 1.45 U/mL, $p=0.06$) after the orange juice intake. The slope of correlation between phagocytic activities and percentages of oxidative burst in patients who had taken orange juice was higher than before the ingestion. The findings suggest that the ingestion of orange juice increased vitamin C although it did not change the level of oxidant, neutrophil function and cardiovascular disease risk factors in patients with type 2 diabetes. This is a pilot study which needs to be further investigated.

Keywords: Immune function, Lipid profile; Malondialdehyde; Orange; Superoxide dismutase; Vitamin C

Introduction

Type 2 diabetes mellitus has become a critical health problem that has been increasing worldwide. It is expected to rise from 366 million in 2011 to 552 million by 2030 [1]. Chronic hyperglycaemia is a well-known consequence contributing in both micro- and macrovascular complications, such as retinopathy, nephropathy, and neuropathy, as well as in increasing risk of cardiovascular diseases [2,3]. Increased oxidative stress [4] and decreased antioxidant activity [5] resulting from uncontrolled hyperglycaemia were shown to be one of the mechanisms leading to impaired immune function [6] and cardiovascular disease risk in patients with type 2 Diabetes. In addition, orange juice was reported to improve anti-oxidative stress [7-13] and reduce cardiovascular disease risks [14,15]. This may be due to vitamin C [16] and other antioxidants [17] in orange juice which were demonstrated to improve immune function [18] and reduce cardiovascular disease risks.

However, no study has investigated effects of commercial fresh-squeezed Thai orange juice on these parameters in patients with type 2 diabetes. Therefore, we aimed to examine the effects of orange juice on oxidative stress indicated by the level of malondialdehyde (MDA), antioxidants determined by the levels of vitamin C and superoxide dismutase (SOD) activity, immune function determined by neutrophil functions as well as cardiovascular disease risk factors determined by the levels of blood glucose and lipid profiles. Moreover, cardiovascular disease risk was determined by high sensitive C reactive protein (hsCRP) [19,20]. The effect of orange juice ingestion on neutrophil functions, which were usually impaired in uncontrolled patients with type 2 diabetes, was also investigated. We hypothesize that ingestion of Thai orange juice could increase antioxidant; improve neutrophil functions and the risk factors for cardiovascular diseases.

Materials and Methods

Experimental Design and Protocol

All subjects ingested 500 ml of Thai orange juice every day for 4 weeks. Blood samples were collected from antecubetal vein two times; immediately before orange juice intake (T1) and four weeks after orange juice intake (T2) to measure blood glucose, insulin, lipid profiles, MDA, vitamin C, SOD and hsCRP. HbA1c was used to determine glycaemic control because it is stable throughout 2-3 months. In addition, patients with type 2 diabetes who had HbA1c > 8.5% were found to have impaired neutrophil functions.

Subjects

Fifteen patients with type 2 diabetes aged 56 ± 3.1 years, in the urban area of Khon Kaen province, Thailand were recruited in this study. They were physically inactive and consumed fewer than 2 glasses of alcoholic beverages daily. They were classified as physically inactive if they did not perform vigorous activities more than 2 times per week for the prior period of 6 months. None of the subjects smoked and had cardiovascular, renal, neuromuscular, orthopedic and liver diseases. All subjects were informed verbally and in writing

before signing the consent form approved by the Ethical Committee of Khon Kaen University (HE521055) in accordance with the 1964 Declaration of Helsinki. No subject was attempting to lose weight during the study period.

Orange juice

The orange juice in this study is commercial fresh-squeezed product distributed in markets of Thailand in amount that has been safely used [21]. It was always stored in subject's refrigerator before the ingestion. There was 41.8 mg vitamin C in 500 mL orange juice used in this study.

Power calculation

A change in vitamin C after the ingestion of orange juice for 4 weeks was used to calculate sample size of this study [21]. It was decided to require 80% power at a significance level of 0.05. Thus, the effect sample size was 15 subjects.

Anthropometric and Body Composition Measurements

Skinfold measurement was used to indirectly measure fat mass. The sum of skinfolds taken at four sites (triceps, biceps, subscapular and supra-iliac crest) was used for evaluating body fat using the equations of Durnin and Womersley (1974) [22].

Dietary, Pharmacology and Physical Activity Control Procedure

Conventional diabetes therapies, such as pharmacology, dietary and exercise treatment, were not modified during the study period. The records of 3 days were averaged to estimate daily energy intake and expenditure. Subjects were also asked to give details of the taken medications at the beginning of the study and whenever the medications were modified.

Blood Sampling and Analytical Procedure

Blood samples were collected into tubes containing fluoride-oxalate for the subsequent determination of whole blood glucose, tubes containing 50 mM EDTA for the subsequent determination of plasma MDA and SOD, and tubes containing clotting activator for serum insulin determination. Heparinized blood samples were used for isolation and functional tests of neutrophils as well as the level of vitamin C. Blood glucose concentration was measured shortly after collection using the glucose oxidase methods. Plasma MDA was estimated by thiobarbituric acid reactive substances according to the method of Draper et al. (1993) [23] using supernatants after centrifuging blood samples at 2500 g for 15 min at 4°C. Plasma SOD was determined by SOD Assay Kit-WST (Sigma-Aldrich Chemie GmbH Industriestrasse, 25 Postfach CH-9471, Buchs/Switzerland). The level of vitamin C was determined by potassium ferricyanide as spectroscopic probe reagent according to the method of Zhang et al. (2009)[24] using supernatants after centrifuging blood sample at 300g for 10 min at 4°C. Serum was analyzed for insulin concentration using radioimmunoassay (Diagnostics Products Corporation, Llanberis, Wales, UK) and for lipid profiles using enzymatic method. Plasma cholesterol, high density lipoprotein (HDL), triglycerides (TG) was analyzed by enzymatic method. Low density lipoprotein (LDL) concentration was calculated from the formula; total cholesterol-TG/5)-HDL. hsC-RP was measured by automated Behringe. Isolation of neutrophils and testing for phagocytosis and oxidative burst activities were performed as previously described [6]. Briefly, neutrophils were isolated by 3.0% Dextran T-500 sedimentation and Ficoll-PaquePLUS centrifugation (Amersham Biosciences, UK). The purity of neutrophils was > 95% and viability of > 98%, as determined by Giemsa and trypan blue staining respectively. Phagocytosis and oxidative burst were determined by flow cytometry using fluorescein isothiocyanate (FITC) labeled bacteria (E. coli) at multiplicity of infection (MOI) of 3 for 15, 30 and 60 minutes or 800 ng/ml phorbol 12-myristate 13-acetate 25 (PMA; Sigma, USA) for 15, 30 and 60 minutes at 37°C as positive controls to stimulate neutrophils for oxidative burst with the inclusion of 25 µl of 2800 ng/mL hydroethidine (HE; (BD Biosciences, USA). The analysis of two-color flow cytometry was performed without the fixation step. Phagocytic activities and oxidative burst were demonstrated as percentages of cells having ingested FITC labeled bacteria and simultaneous oxidation of hydroethidine (HE) to ethidium bromide (EB).

Insulin sensitivity and β cell function

Insulin sensitivity and β cell function were calculated by the following formula [25];

HOMA-IR formula = (FPI x FPG) / 22.5

HOMA-β formula (%) = (20 x FPI) / (FPG – 3.5)

FPI = Fasting plasma insulin (mU/L)

FPG = Fasting plasma glucose (mmol/L)

Statistical Analyses

All dependent variables were analyzed using a paired t-test by Sigma Stat version 2 program (Systat Software Inc. California, USA.) [26]. Results are presented as mean ±test and paired t test) was performed by Graphpad PRISM statistical software (GraphPad, San Diego, 5 USA). A probability of $p<0.05$ was taken to indicate significance.

Results

Anthropometric and Physical Data, Daily Dietary Intake and Energy Expenditure

Three male and 12 female patients with type 2 diabetes mellitus were completely tested in the study. Anthropometric and physiological characteristics of the subjects were summarized in (Table 1).

Age (year)	56 ± 3.1
Height (cm)	155.8 ± 1.9
Body mass (kg)	67.1 ± 3.7
Body mass index (kg/m2)	27.8 ± 1.7
Heart rate (/min)	79 ± 9.5
Blood pressure (mmHg)	129 ± 17.3/81 ± 10.4
Fasting blood glucose (mg/dL)	161.92 ± 9.6
HbA1c (%)	9.38 ± 0.2
Insulin (uIU/mL)	26.5 ± 4.0

Insulin sensitivity (HOMA-IR)	8.07 ± 7.8
HOMA-β (%)	3.03 ± 2.1
Total cholesterol (mg/dL)	186.4 ± 8.6
Triglycerides (mg/dL)	214.4 ± 28.3
High density lipoprotein (mg/dL)	45.0 ± 3.2
Low density lipoprotein (mg/dL)	98.6 ± 9.4
Vitamin C (mg/dL)	0.753 ± 0.12
MDA (mM)	5 ± 2.03
SOD (U/mL)	49.2 ± 5.91
hsCRP (mg/L)	5.24 ± 1.22

Table 1: Anthropometric and physiological parameters of subjects

Data are expressed as mean ± SD. n = 15 (3 men, 12 women). HbA1c- Haemoglobin A1c; HOMA-IR- Homeostatic Model Assessment-Insulin Resistance; HOMA-β- Homeostatic Model Assessment–β cell; MDA- Malondialdehyde; SOD- Superoxide Dismutase; hsCRP- high sensitive C Reactive Protein

At the beginning of the experiment, subjects had high cardiovascular disease risk factors such as hyperglycaemia, dyslipedemia and obesity (based on criteria of World Health Organization Western Pacific region, 2000) [27] (Table 1). All patients had the same doses of hypoglycaemic drug (including metformin and glipizide) and anti-hyperlipidemic drug (including simvastatin, atorvastatin and Gemfibrozil) throughout the experiment. Mean daily dietary, energy intakes and energy expenditure were similar between the two phases (Table 2).

Carbohydrate (kJ/day)	2,337.6 ± 257.4
Fat (kJ/day)	1,698.1 ± 275.5
Protein (kJ/day)	1,271.9 ±241.1
Vitamin C (mg/day)	20.5 ± 19.1
Energy intake (kJ/day)	5,643.9 ± 626.2
Energy expenditure (kJ/day)	5,890.5 ± 679.7

Table 2: Daily dietary intake and energy expenditure. Data are expressed as mean ± SD. n = 15 (3 men, 12 women)

Clinical Chemistry and Oxidant status

Plasma vitamin C was increased ($p<0.05$) and SOD activity tended to increase ($p=0.06$) after the ingestion of orange juice when compared with before the ingestion (Table 3).

	Before	After	P value
Fasting blood glucose) (mg/dL)	158.2 ± 6.7	179.8 ± 9.7	NS
Insulin (uIU/mL)	26.5 ± 4.6	30.8 ± 4.6	NS
Insulin resistance (HOMA-IR)	7.08 ± 1.6	10.75 ± 2.5	NS
HOMA-β (%)	2.89 ± 2.7	3.17 ± 0.6	NS
Total cholesterol (mg/dL)	187.6 ± 8.0	189.4 ± 5.8	NS
Triglycerides (mg/dL)	183.4 ± 22.8	210.4 ± 30.4	NS
High density lipoprotein (mg/dL)	45.4 ± 3.0	44.2 ± 3.8	NS
Low density lipoprotein (mg/dL)	105.4 ± 7.6	103.1 ± 5.4	NS
Vitamin C (mg/dL)	0.783 ± 0.09	0.844 ±0.09	*<0.05
MDA (mM)	6 ± 0.95	5 ± 0.59	NS
SOD (U/mL)	46.8 ± 1.44	48 ± 1.45	0.06
hsCRP (mg/L)	4.30 ± 0.90	4.91 ± 0.85	NS

Table 3: Changes in blood parameters

Data are expressed as mean ± SE. n = 15 (3 men, 12 women). HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; HOMA-β: Homeostatic model Assessment–β cell; MDA: Malondialdehyde; SOD: Superoxide Dismutase; hsCRP, high sensitive C Reactive Protein. *Significatly different from before 4-week ingestion of the orange juice

There were no significant differences in the level of blood glucose, insulin, MDA, lipid profiles and hsCRP after the ingestion (Table 3). Insulin sensitivity and β cell function were also not different after the ingestion (Table 3).

Phagocytic and Oxidative Burst Activities of Neutrophil

There was no statistical significance of phagocytic and oxidative burst percentages of neutrophils before and after the orange juice phase (Figure 1).

However, the slope of correlation between phagocytic activities and oxidative burst in patients who had orange juice intake for four weeks was higher than that before the ingestion (Figure 2).

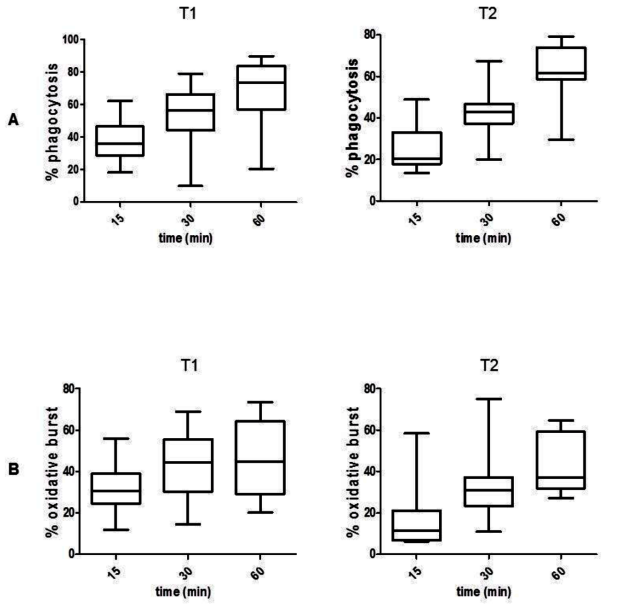

Figure 1: Percentages of FITC labeled E. coli ingested neutrophils (MOI = 3) between different incubation times (A) and the oxidative burst (B). T1 = before 4-week ingestion of the orange juice; T2 = after 4-week ingestion of the orange juice

Discussion

The findings in this preliminary study demonstrated that daily ingestion of 500 mL orange juice for 4 weeks increased plasma vitamin C in patients with type 2 diabetes.

The results supported the hypothesis of this study on benefit effect of orange juice on antioxidant i.e. plasma vitamin C. This was consistent with previous studies investigating effects of acute and long-term ingestion of orange juice in healthy [7,8,11,21] and subjects with hypercholesterolemia on oxidative stress and other related parameters [14]. A previous study in healthy men aged 25-26 years reported that within 30 minutes after a single consumption of 150 mL of orange juice, damage from oxidative stress was reduced and maintained for up to 90 minutes post-consumption. This may be due to the increased antioxidant activity measured by using dichlorofluorescein (DCF) fluorescence.8 The antioxidant may be vitamin C which was found to be significantly increased as a result of orange juice ingestion [8,14,21]. Riso and colleagues found 21-day ingestion of orange juice in healthy female subjects had increased plasma vitamin C level. Moreover, long-term ingestion of orange juice (750 but not of 250 or 500 mL/day) for 4 weeks improved blood lipid profiles in hypercholesterolemic subjects i.e. increased HDL-cholesterol concentrations by 21%, decreased triacylglycerol concentration by 30% and decreased the LDL-HDL cholesterol ratio by 16% [14]. However, the ingestion of 500 mL/day of orange juice in healthy subjects by the study of Sánchez-Moreno et al, 2003 found increased plasma vitamin C. Data from big examination survey with above 8000 participants participating in the National Health and Nutrition Examination Survey 2003-2006 confirmed that orange juice intake contributes to increase in plasma vitamin C concentration [13]. Although the amount of vitamin C in the orange juice intake per day in this study seemed to be too small to increase plasma vitamin C concentration, vitamin C intake of less than 200 mg (41.8 mg in this study) increased the absorption rate to 98% [28]. This may contribute to the higher vitamin C concentration after the ingestion period in this study. Another reason that explain the increased plasma vitamin C concentration in this study is that citrus juice like orange juice contains many bioactive substances (not only vitamin C but also flavonoids), which influence on oxidant/antioxidant status [17]. Although flavonoids in participants‟ blood were not measured in this study, it may play antioxidant role during the ingestion period. The other reason is that anti-hyperlipidemic drug such as simvastatin and atorvastatin in this study has been shown by previous studies to have antioxidant enzymes activity [29,30]. Flavonoids and statin may spare the antioxidant activity of vitamin C resulting in increased plasma vitamin C after the ingestion.

Unfortunately, this study did not show effects of orange juice on other parameters including SOD activity, neutrophil function and cardiovascular disease risk factors (including lipid profile, insulin sensitivity and β cell function). The high blood glucose and HbA1c concentrations with high oxidative stress in patients with type 2 diabetes in this study may attenuate the beneficial effects of orange juice on these parameters. Although, the increased fasting blood glucose and triacylglycerol in patients after 4-week of orange juice ingestion in this study were not significantly different, the results of a long-term cohort study, led by Harvard School of Public Heath scientists [31], imply that orange juice may make diabetes worse. The previous study showed that greater consumption of specific whole fruits e.g. blueberries, grapes, and apples, is significantly related to a lower risk of type 2 diabetes than the fruit juice. The findings of the previous study support recommendations on promoting ingestion of a variety of whole fruits for diabetes prevention. Alternatively, we suggest the manufacturer to modify the orange juice product by adding much more orange paste in the juice in order to reduce the risk of type 2 diabetes.

It was reported that patients with HbA1c > 8.5% had impaired neutrophil functions [6]. We did not find the effect of orange juice intake on significantly improving these functions. However, the correlation of phagocytosis and oxidative burst was higher after 4 weeks of Thai orange juice intakes suggesting a better neutrophil function. This observation should be confirmed. Many explanations are possible for the absence of the significant difference. First, the amount of vitamin C in orange juice in this study may be inadequate to yield the beneficial effects [21]. Although this study used daily 500 mL orange juice which was similar to that used by the study of Sánchez-Moreno and collegues, the amount of vitamin C was much lower (41.8 mg/500 mL) than that (250 mg/500 mL) in the previous study. Adding more antioxidants such as vitamin C, anthocyanins and carotenoids into orange juice, may reveal significant beneficial effects of orange juice since it was reported to increase antioxidant activity [11].

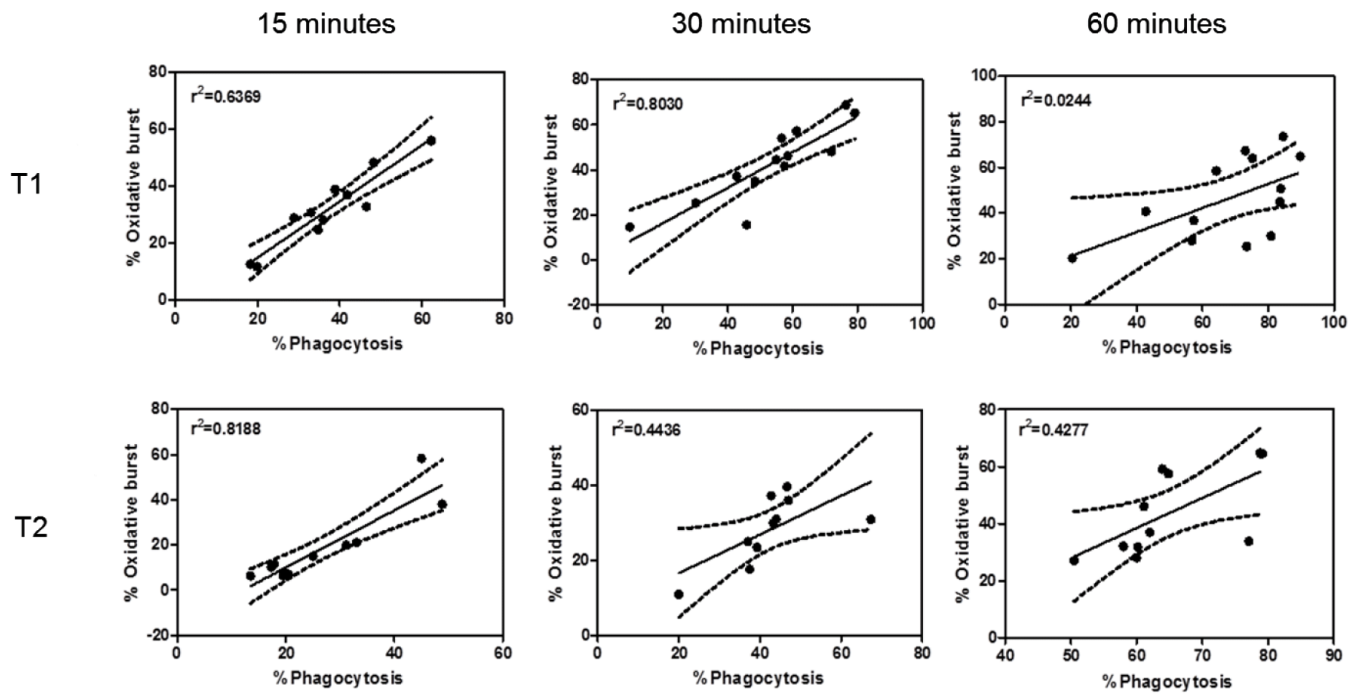

Figure 2: Correlation of % phagocytosis and oxidative burst of neutrophils in type 2 diabetes patients. T1 = before 4-week ingestion of the orange juice; T2 = after 4-week ingestion of the orange juice

The other reason for the non-significant changes in many cardiovascular disease risk factors in this study is due to the loss of ascorbic acid in refrigerated storage juice during the experiment. Therefore, it is not known whether the longer time for the ingestion, greater vitamin C or other antioxidants added or providing a better method for vitamin C preservation may be required to take the effect. This should be further investigated in healthy individual or patients with diabetes type 2. Moreover, according to the positive effect of vitamin C on endothelial function [32] cardiovascular diseases may be reduced [33]. Further study investigating endothelial function is also needed. One might argue that it is proper to evaluate the functions of macrophage and T cell, not neutrophil, to understand the effect of orange juice on the immunity of patients with diabetes type 2. Since the functions of macrophage and T cell are also impaired in diabetes patients [6] whereas increasing number of neutrophil is critical to systemic inflammation-related severity of diabetes [34]. Therefore, further study investigating the effect of ingestion of orange juice paste on macrophage and T cell functions should be investigated.

It is noted that this is a preliminary study examining the effect of orange juice on the immune function. Thus the lack of a placebo resulting in an unclear effect of the orange juice is a limitation of this study. Therefore, to provide good knowledge for prevention and treatment for diabetic patients a further study examining the effects of the orange juice on the above parameters with a placebo control or with control healthy participants is needed. Moreover, it is interesting to perform a research determining how long this effect is maintained in diabetic patients and control healthy participants.

In conclusion, the findings suggest that the 4-week ingestion of Thai orange juice increased plasma vitamin C in patients with type 2 diabetes without any changes in neutrophil functions and cardiovascular disease risk factors. The longer time for the ingestion, greater vitamin C or other antioxidants added or providing a better method for vitamin C preservation may be required to take the effect.

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Conflict of interest

The authors declare that they have no conflict of interest.

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