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Honey with High Levels of Antioxidants Can Provide Protection to Healthy Human Subjects

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Free radicals and reactive oxygen species (ROS) have been implicated in contributing to the processes of aging and disease. Humans protect themselves from these damaging compounds, in part, by absorbing antioxidants from high-antioxidant foods. This report describes the effects of consuming 1.5 g/kg body weight of corn syrup or buckwheat honey on the antioxidant and reducing capacities of plasma in healthy human adults. The corn syrup treatment contained 0.21 ± 0.06 mg of phenolic antioxidants per gram, and the two buckwheat honey treatments contained 0.79 ± 0.02 and 1.71 ± 0.21 mg of phenolic antioxidants per gram. Following consumption of the two honey treatments, plasma total-phenolic content increased ($P < 0.05$) as did plasma antioxidant and reducing capacities ($P < 0.05$). These data support the concept that phenolic antioxidants from processed honey are bioavailable, and that they increase antioxidant activity of plasma. It can be speculated that these compounds may augment defenses against oxidative stress and that they might be able to protect humans from oxidative stress. Given that the average sweetener intake by humans is estimated to be in excess of 70 kg per year, the substitution of honey in some foods for traditional sweeteners could result in an enhanced antioxidant defense system in healthy adults.

KEYWORDS: Honey; antioxidants; polyphenolics; oxidation; functional food

INTRODUCTION

Antioxidants are nutritive and nonnutritive agents that can retard biologically destructive chemical reactions in foods and living organisms. These compounds are thought to protect humans from disease, in part, through their ability to scavenge oxidants and free radicals, absorbing molecular damage that might otherwise compromise the function of essential lipids, proteins, and nucleic acids (*1*−*2*).

Throughout evolution, plants and animals have been constantly exposed to oxidative stress. In response, organisms have evolved to acquire, synthesize, store, and use antioxidant chemicals and proteins (*3*−*4*). For example, humans synthesize the antioxidant enzymes Cu−Zn superoxide dismutase (SOD), Mn−SOD, glutathione peroxidase, and catalase, and they can incorporate dietary antioxidants such as plant phenolics, vitamin C, and vitamin E into their tissues (*1*−*2*).

In the current investigation, we assessed the impact of honey consumption on the plasma antioxidant defenses of healthy human subjects. To control for potential changes in oxidative defense associated with the acute consumption of the carbohydrate load found in honey, subjects were also evaluated prior to, and following, a feeding of corn syrup. Plasma phenolic content, antioxidant capacity, and reducing capacity were assessed in subjects, prior to, and following the consumption of corn syrup and honey treatments. The results obtained in this work support the concept that honey consumption can have a positive impact on the antioxidant defense system of healthy human subjects.

MATERIALS AND METHODS

Subjects and Intervention. Recruitment of 40 volunteers occurred by flyer at the University of California at Davis with subjects being screened for allergies, health, medications, and dietary habits by questionnaire. Subjects were between the ages of 18 and 54 and were free of known pathology and food allergies to corn syrup, honey, and bread. Subjects abstained from medications, vitamins, and foods rich in phenolic antioxidants (e.g., coffee, honey, tea, wine, fruit juice, fruits, vegetables, cocoa products) for a 24-h period prior to, as well as throughout, the experiment. Subjects were randomized into treatment groups ($n = 10$/treatment).

All subjects were provided a low polyphenolic control meal of bread and water (1.5 g/kg and 10 mL/kg subject body weight, respectively) as described previously (*5*). In group 1 (Control), subjects consumed a control meal of bread and water. In groups 2−4, subjects consumed the control meal plus corn syrup, a low-antioxidant buckwheat honey, or a high antioxidant buckwheat honey and were classified in the CS, Low-A, and High-A honey treatment groups, respectively. The honey was a gift from the Dutch Gold company. The honey was heat-treated

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at 65 °C for 30 min to destroy yeast and prevent undesired fermentation. All experimentation took place at the Ragle Human Nutrition Research Facility at the University of California, Davis. The study protocol was approved and conducted according to guidelines set by the Human Subjects Review Committee of the University of California, Davis.

Plasma Collection. Blood samples were drawn by venipuncture at baseline (immediately prior to meal consumption) and at 1, 2, and 6 h time points after the consumption of the meal. Venous blood samples were drawn into evacuated, heparin coated tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was separated by low-speed centrifugation (1800 g) at 4 °C for 12 min, allocated, and stored at −80 °C until analyzed.

Assessment of Plasma Phenolics Using HPLC. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Sample recovery, extraction, and phenolic detection was performed as previously reported (6-7). Chromatography was carried out using an HP 1100 HPLC system equipped with a quaternary pump, temperature-controlled autosampler, column oven, and diode array detector (Hewlett-Packard, Wilmington, DE) in series with an ESA CoulArray 5600 detector (Chelmsford, MA). Separation was achieved using a reversed phase Alltima C18 column (5 μm, 150 mm × 4.6 mm; Alltech Associates, Deerfield, IL) with a C18 5-μm guard column (Alltech Associates, Deerfield, IL). Coulometric electrochemical array detection was carried out using the following cell settings: −50, +65, +150, +200, +250, +300, +700, and +800 mV. The resulting chromatograms were analyzed using CoulArray for Windows software (ESA, Chelmsford, MA). 4-Hydroxybenzoic acid and 4-hydroxycinnamic acid peak identification on analytical cell 8 was based on coelution with standards and authentic standards (Sigma Chemical Co., St Louis, MO; and Indofine Chemical Co., Somerville, NJ).

Assessment of Plasma Total Phenol Content. The total phenolic content of heparin-treated plasma was determined with Folin-Denis reagent according to the method published by the Association of Official Analytical Chemists (*8*). This method estimates phenolics by absorbance at 760 nm relative to a standard curve for gallic acid.

Plasma Antioxidant and Reducing Capacity. Plasma antioxidant capacity was determined as previously described by Lissi et al. (*9*) with minor modifications. Plasma samples were diluted 1:10 with 1 mmol/L phosphate buffered saline, pH 7.4 (Invitrogen, Life Technologies, Grand Island, NY), and added to a solution containing 20 mmol/L 2′2-azobis-(2-amidinopropane) dihydrochloride and 16.6 μM 5-amino-2,3-dihydro-1,4-pthalazinedione (Luminol) in 1 mmol/L phosphate buffered saline, pH 7.4 (Invitrogen, Life Technologies, Grand Island, NY). Luminescence was recorded for 20 min using a Perkin-Elmer HTS 7000 Series Bio Assay Reader (Perkin-Elmer Ltd., Buckinghamshire, United Kingdom). The plasma antioxidant capacity value was calculated as the lag time before an increase in chemiluminesence was observed. The lag time is proportional to the cumulative amount of antioxidants present in the sample. A reference lag time was obtained by using a known amount of ascorbic acid (Vitamin C) (Aldrich Chemical Co., Milwaukee, WI).

Plasma Reducing Capacity. Plasma reducing capacity was determined by the Prussian blue assay, which is based on a coupled reduction of ferric-cyanide and the formation of Prussian blue (10). Thirty microliters of plasma were reacted with 180 μL of 0.4 mM $K_3Fe(CN)_6$ and twenty μL of 8 mM $FeCl_3$ in 8mM HCl. Reaction occurred at room temperature for 20 min on a rocker plate set to 150 rpm. Values were compared to vitamin C standards.

Analysis. Data are shown as mean ± SD. Data were compared using one way repeated measures analysis of variance with all pairwise multiple comparison procedures (Tukey Test). Statistical significance was accepted at $p < 0.05$.

RESULTS

A total of 37 subjects completed the trial. All of the subjects ($N = 10$ per treatment group) in the Control, low-antioxidant honey (Low-A), and high-antioxidant honey (High-A) treatments completed the experiment. Seven of the 10 subjects recruited for the corn syrup (CS) treatment completed the experiment; three were excluded due to an inability to finish the required amount of corn syrup. Mean age ± standard deviation for subjects in Control, CS, Low-A, and High-A honey treatment groups were 25.1 ± 1.41, 28.3 ± 4.88, 26.1 ± 1.45, and 22.7 ± 1.44, respectively. Mean BMI ± standard deviation for subjects in Control, CS, Low-A, and High-A honey treatment groups were 24.1 ± 1.77, 21.7 ± 2.05, 23.2 ± 1.15, and 25.6 ± 1.12 kg/m^2, respectively.

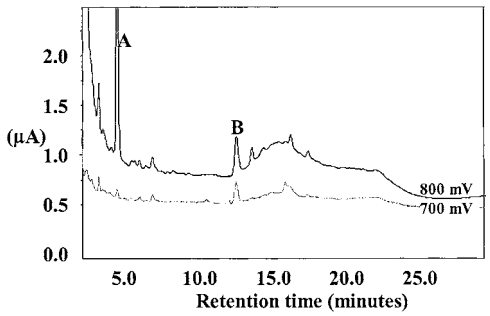

Figure 1. HPLC-derived chromatogram for high-antioxidant buckwheat honey. Peaks A and B mark the two most prominent phenolics identified in both honeys, 4-hydroxybenzoic acid and 4-hydroxycinnamic acid, respectively.

Corn syrup, Low-A, and High-A honey provided subjects with 0.21 ± 0.06, 0.79 ± 0.02, and 1.71 ± 0.21 mg of phenolics/g of treatment, respectively. On the basis of the antioxidant capacity of the treatments, corn syrup, Low-A, and High-A honey provided subjects with 59 ± 6, 107 ± 18, and 211 ± 24 ng vitamin C (ascorbic acid) equivalents of antioxidant/g treatment, respectively.

Figure 1 depicts the chromatogram from High-A buckwheat honey. Chromatograms of Low-A and High-A honeys were similar. Peaks A and B in **Figure 1** mark the two most prominent phenolics identified in both honeys, 4-hydroxybenzoic acid and 4-hydroxycinnamic acid, respectively. High-A and Low-A honeys contained 20 and 2.5 mg 4-hydroxybenzoic acid/kg honey, respectively. In addition, 3 and 2 mg of 4-hydroxycinnamic acid were present per kg of Low-A and High-A honey, respectively. Prior to treatment, Control, Low-A and High-A honey groups averaged plasma 4-hydroxybenzoic acid concentrations of 0.307 ± 0.177, 0.294 ± 0.1, and 0.322 ± 0.186 mg/L, respectively. Plasma 4-hydroxycinnamic acid concentrations were 1.31 ± 0.507, 1.20 ± 0.390, and 0.957 ± 0.291 mg/L in Control, Low-A, and High-A honey groups prior to treatment. Recovery was 86% ± 9% and 81% ± 7% for 4-hydroxybenzoic acid and 4-hydroxycinnamic acid, respectively. No significant changes in plasma 4-hydroxyphenolic or 4-hydroxycinnamic acid concentrations were observed following treatment.

Following the consumption of the bread and water control treatment, plasma antioxidant and reducing capacities decreased over time. These changes are consistent with the observation that food consumption induces free radical formation (*11*−*14*). Relative to the bread and water Control treatment group, the consumption of the Low-A honey was associated with a significant increase in total plasma phenolic concentration 2 h after consumption (**Figure 2**). Following the consumption of the High-A honey, the total plasma phenolic concentration was higher at both the 2 and 6 h time points relative to baseline values (**Figure 2**). In contrast, corn syrup had no significant effect.

In the corn syrup treatment group, a significant increase in total plasma antioxidant capacity 6 h after treatment was observed relative to baseline (**Figure 3**). The consumption of Low-A honey was associated with a significant increase in total plasma antioxidant capacity 2 h after the meal. The consumption

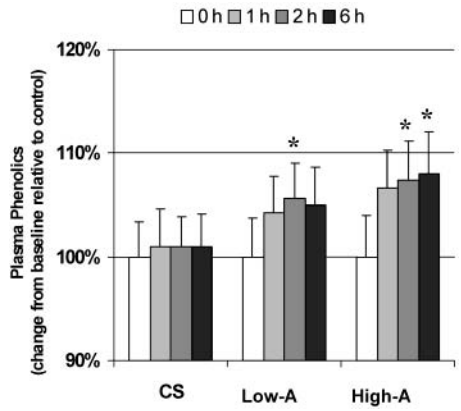

Figure 2. Effect of treatment consumption on plasma total-phenolic content.

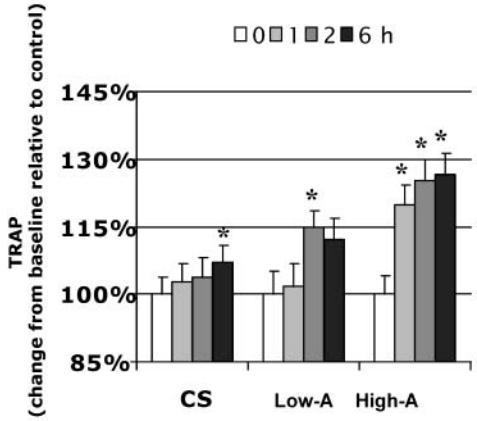

Figure 3. Effect of treatment consumption on plasma antioxidant capacity.

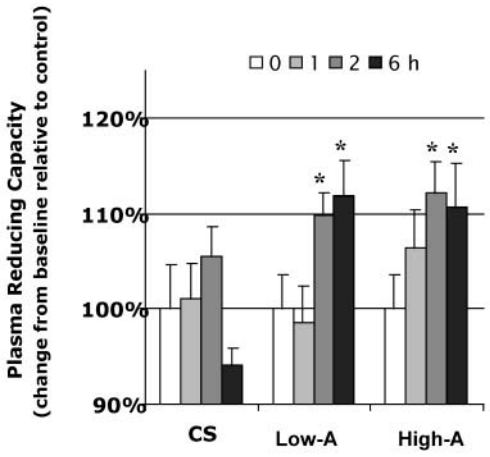

Figure 4. Effect of treatment consumption on plasma reducing capacity.

of High-A honey was associated with elevated total plasma antioxidant capacities at 1, 2, and 6 h time points (**Figure 3**).

Although CS treatment was capable of conveying an increase in plasma antioxidant capacity 6 h after consumption **(Figure 3**) relative to the control treatment, it did not influence plasma reducing capacity at any time point (**Figure 4**). In contrast, both honey treatments were effective in increasing not only total plasma antioxidant capacity, but also the total plasma reducing capacity in subjects.

DISCUSSION

Investigating the ability of phenolic-rich food products to impact plasma oxidant defense systems in human subjects, and the clinical implications of such changes, is a relatively new field. However, intervention studies have been conducted using several phenolic-rich food products. For example, flavonoid-rich chocolate, spinach, strawberries, and red wine have been observed to increase plasma antioxidant capacity in human subjects by 7−25% when consumed in quantities of around 4 g/kg body weight (*5*−*6*, *15*). In the current investigation, honey fed at 1.5 g/kg body weight was observed to convey both phenolic antioxidants and increase plasma antioxidant capacity to healthy human subjects. Future investigations that conduct direct comparisons between honey and other high-antioxidant foods will provide additional insight into the relative efficiency of antioxidant transfer from these various antioxidant-rich foods.

The United States Department of Agriculture estimates that the average US citizen consumes approximately 68 kg of sweetener food additives on an annual basis. Results from the present work suggest that if honey was substituted for the sweeteners currently used, it could make a significant antioxidant contribution to the human diet. However, honey samples can have a high degree of variability in their antioxidant content. This variable composition is dependent on the honey's floral source. For example, when the antioxidant capacity of 19 honeys was assessed in vitro, 20-fold differences in efficacy were observed (*16*−*17*). Test samples represented 14 different primary floral nectars, including clover, fireweed, mesquite, and star thistle. While honeys varied widely in the quantity of water-soluble antioxidants they contained, it was observed that the darker a honey's color, the higher its antioxidant activity. For example, a milliliter of Illinois buckwheat honey, the darkest honey tested, contained $4.32 \times 10-3$ meq, which was 20 times the antioxidant activity in the same quantity of California sage honey, one of the lightest-colored samples (*16*−*17*). Christmasberry, and tupelo honeys, also at the dark end of the color range, were the next richest sources of antioxidants, although their antioxidant content was only 35−45% of that of buckwheat (*16*−*17*). Overall, color predicted more than 60% of the variation in a honey's antioxidant capacity. Therefore, while the substitution of honey for other sweeteners used by food manufacturers could increase the antioxidant content of the human food supply, the impact of honey would be dependent on the type(s) utilized.

The antioxidants present in honey come from a variety of sources such as Vitamin C, monophenolics, flavonoids, and polyphenolics. Although there is a wide spectrum of antioxidant types, monophenolics such as 4-hydroxybenzoic and 4-hydroxycinnamic acids predominate in many honeys, including the buckwheat honey used in this investigation (**Figure 1**).

Volunteers in this investigation consumed 1.5 g of honey/kg of subject body weight. Following honey consumption, but not corn syrup consumption, the total phenolic content of plasma increased (**Figure 2**). Since the honey used in this investigation provided mg quantities of 4-hydroxybenzoic and 4-hydroxycinnamic acids per kg of body weight, the plasma concentration of 4-hydroxybenzoic acid and 4-hydroxycinnamic acids could have been increased by a detectable amount if they were completely absorbed by a subject and the distribution volume of the plasma compartment in our subjects was of 0.04 L/kg body weight. However, changes in the plasma content of these monophenolics could not be verified by HPLC analysis. These data suggest several possibilities, including (a) that less than one-third of the total 4-hydroxybenzoic and 4-hydroxycinnamic acids were absorbed, (b) that these compounds may rapidly distribute into body compartments other then plasma, or (c) that

the monophenols undergo rapid first pass metabolism in the human body.

Further clinical inquiry into the comparative efficacy of honey relative to other high-antioxidant foods could benefit food technologists and public health associates alike by providing a basis on which decisions could be made regarding food recommendations. Although CS treatment was capable of increasing plasma antioxidant capacity 6 h after consumption (**Figure** 3) relative to the control treatment, it did not influence plasma reducing capacity at any time point (**Figure 4**). This observation could be accounted for by the fact that carbohydrates are excellent substrates for the Maillard reaction (*17*). Work by Gheldof et al. (*17*) suggests that carbohydrates and Maillard products can act as antioxidants in vitro. The observation that the CS treatment did not increase the level of phenolics in the plasma of our subjects, but did increase antioxidant capacity of the plasma might indicate that Maillard products can act as weak antioxidants in vivo. However, more work is needed to assess this possibility. When the design of future investigations is considered, it is important to note that the results from this investigation demonstrate that the aqueous portion of the blood (plasma) is protected by honey. This is in agreement with the fact that most of the antioxidant components in processed honey are water soluble, not lipid soluble. However, assessing the ability of honey to improve the antioxidant content of our food supply through the substitution of honey for other sweeteners should also be approached by determining the impact of honey antioxidants on lipid components of the human body. To our knowledge this is the first investigation of honey's effects on oxidant defense in humans. Given the recommended limitations for interpreting these data, we conclude that high antioxidant honey as a dietary component can be a significant source of phenolic antioxidants.

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LITERATURE CITED

(1) Lenaz, G.; Bovina, C.; D'Aurelio, M.; Fato, R.; Formiggini, G.; Genova, M. L.; Giuliano, G.; Pich, M. M.; Paolucci, U.; Castelli, G. P.; Ventura, B. Role of mitochondria in oxidative stress and aging. *Ann. N.Y. Acad. Sci.* **2002**, *959*, 199−213.

(2) Bistrian, B., Ed. Taking stock of antioxidants. *Harv. Health Lett.* **2002**, *4*, 1-3.

(3) Harman, D. Aging: overview. *Ann. N.Y. Acad. Sci.* **2001**, *928*, 1−21.

(4) Hensley, K.; Floyd, R. A. Reactive oxygen species and protein oxidation in aging: a look back, a look ahead. *Arch. Biochem. Biophys.* **2002**, *397*, 377−383.

(5) Wang, J. F.; Schramm, D. D.; Holt, R. R.; Ensunsa, J. L.; Fraga, C. G.; Schmitz, H. H.; Keen, C. L. A dose-response effect from chocolate consumption on plasma epicatechin and indices of oxidative damage. *J. Nutr.* **2000**, *130*, 2109S-2114S.

(6) Holt, R. R.; Schramm, D. D.; Schmitz, H. H.; Keen, C. L. Chocolate as a cardioprotective dietary component. *JAMA* **2002**, *287*, 2212−2213.

(7) Holt, R. R.; Lazarus, S. A.; Schramm, D. D.; Schmitz, H. H.; Keen, C. L. Simultaneous measurement of catechin, epicatechin, and procyanidin dimer in human plasma after cocoa consumption. *Am. J Clin. Nutr.* **2002**, (in press).

(8) Helrick, K. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed.; Association of Official Analytical Chemists: Arlington, VA, 1990; p 703.

(9) Lissi, E.; Salim-Hanna, M.; Pascual, C.; del Castillo, M. D. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Radical Biol. Med.* **1995**, *18*, 153−158.

(10) Thompson, L. U.; Yoon, J. H.; Jenkins, D. J.; Wolever, T. M.; Jenkins, A. L. Relationship between polyphenol intake and blood glucose response of normal and diabetic individuals. *Am. J. Clin. Nutr.* **1984**, *39*, 745−751.

(11) Kwiterovich, P. O., Jr. The effect of dietary fat, antioxidants, and pro-oxidants on blood lipids, lipoproteins, and atherosclerosis. *J. Am. Diet. Assoc.* **1997**, *97*, Suppl, S31−41.

(12) Staprans, I.; Rapp, J. H.; Xian-Mang Pan, K. Y.; Feingold, K. R. Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. *Arterioscler. Thromb.* **1994**, *14*, 1900−1905.

(13) Vine, D. F.; Croft, K. D.; Beilin, L. J.; Mamo, J. C. L. Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomicrons. *Lipids* **1997**, *32*, 877−893.

(14) Ursini, F.; Zamburlini, A.; Cazzolato, G.; Maiorino, M.; Bittolo-Bon, G.; Sevanian, A. Postprandial plasma lipid hydroperoxides: a possible link between diet and atherosclerosis. *Free Radical Biol. Med.* **1998**, *25*, 250−252.

(15) Cao, G.; Russell, R. M.; Lischner, N.; Prior, R. L. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J. Nutr.* **1998**, *128*, 2383−2390.

(16) Gheldof, N.; Engeseth, N. J. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J. Agric. Food Chem.* **2002**, *50*, 3050−3050.

(17) Gheldof, N.; Wang, X.-H.; Engeseth, N. J. Identification and quantification of antioxidant compounds of honeys form various floral sources. *J. Agric. Food Chem.* **2002**, *50*, 5870−5877.

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