In Vivo Antioxidative Activity of Propolis Evaluated by the Interaction with Vitamins C and E and the Level of Lipid Hydroperoxides in Rats

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In vivo antioxidative activity of propolis was evaluated on the basis of ameliorative effects on the oxidative stress induced by vitamin E deficiency in rats. The control group was fed vitamin E-deficient diet, and the propolis group was fed vitamin E-deficient diet supplemented with 1% of propolis for 4 and 8 weeks. Comparisons were made in tissue concentrations of vitamin C, vitamin E, and lipid hydroperoxides between these groups. No significant difference was observed in tissue vitamin E concentration between these groups after both 4 and 8 weeks. After 4 weeks, the plasma vitamin C concentration of the propolis group was significantly higher than that of the control group. After 8 weeks, the tissue concentrations of vitamin C in the kidney, stomach, small intestine, and large intestine of the propolis group were significantly higher than those of the control group. These results suggest that some components of propolis are absorbed to circulate in the blood and behave as a hydrophilic antioxidant that saves vitamin C. The concentration of lipid hydroperoxides in the large intestine of the propolis group was significantly lower than that of the control group after 8 weeks. These results suggest that propolis exerts its antioxidative effect where it is assumed to accumulate, such as on the kidney, where it is excreted, and on the gastrointestinal tract, where propolis influences these tissues even from the outside of the cell.

Keywords: *Antioxidant; lipid hydroperoxide; propolis; vitamin C; vitamin E*

INTRODUCTION

The propolis of honeybee hives has long been used in folk medicine. Recent studies show that propolis exhibits a broad spectrum of activities such as antibiotic, anti-inflammatory, antifungal, and antitumorigenic properties (Burdock, 1998). Along with these activities, antioxidative effects of propolis and its components have also been studied in vitro (Krol et al., 1990; Pascual et al., 1994; Scheller et al., 1990; Sud'ina et al., 1993; Volpert and Elstner, 1993). Concerning the in vivo antioxidative activity of propolis, protective effects of intraperitoneally administered propolis extract against doxorubicin-induced myocardiopathy (Chopra et al., 1995), carbon tetrachloride-induced liver damage (Merino et al., 1996), galactosamine-induced hepatitis (Rodriguez et al., 1997), and γ-irradiation (Scheller et al., 1989) have been reported.

Although there is considerable interest in the role of propolis as a dietary antioxidant, only limited studies are available on the antioxidative effect of orally administered propolis as well as the bioavailability of propolis (Bourne and Rice-Evans, 1998). El-Ghazaly and Khayyal (1995) reported that orally administered aqueous propolis reduced the damage caused by γ-irradiation, and Basnet et al. (1996) reported that orally given propolis extract ameliorated liver damage induced by carbon tetrachloride, D-galactosamine, and lipopolysaccharide. Although these reports suggest that propolis functions as an antioxidant, no effective method is yet available to evaluate directly the antioxidative activity of dietary propolis in vivo. In the present study, we report the first attempt to evaluate the antioxidant activity of dietary propolis on the basis of ameliorative effects on the oxidative stress induced by vitamin E deficiency in rats.

MATERIALS AND METHODS

Materials. Commercially available propolis (imported from Brazil and processed by Yamada Yohojo Bee Farm Inc., Tomata, Okayama, Japan) was used in this study.

Animals. Guidelines from the Prime Minister's office of Japan (No. 6 of March 27, 1980) for the care and use of laboratory animals were followed. Four-week-old male rats (SLC: Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). The rats were housed in a room with a temperature of 24 ± 2 °C and a 12-h light/dark cycle. Rats were permitted free access to food and water.

Design of the Work. To evaluate the in vivo antioxidant activity of propolis, saving effect of propolis on vitamins C and E was determined during the oxidative stress induced by vitamin E deficiency. Rats were divided into two groups. One group, designated the control group, was fed vitamin E-deficient diet and the other group, defined as the propolis group, received vitamin E-deficient diet supplemented with 1% of propolis. After 4 and 8 weeks, tissue concentrations of vitamins C and E were compared between these two groups. At the same time, tissue concentrations of lipid hydroperoxides as an indicator of oxidative stress (Tokumaru et al., 1995) were also compared. The number of rats used for each treatment was three or four.

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Table 1. Levels of Tissue Vitamin E (Nanomoles per Gram of Tissue) in the Control (Vitamin E-Deficient) and Propolis (Vitamin E-Deficient and Propolis-Supplemented) Groups[a]

	plasma	liver	heart	kidney	stomach	small intestine	large intestine
				After 4 Weeks			
control (3)	2.43 ± 0.41	4.67 ± 0.32	6.75 ± 1.88	3.28 ± 1.44	5.19 ± 0.82	4.38 ± 0.92	5.16 ± 1.00
propolis (3)	2.67 ± 0.23	4.77 ± 0.91	7.87 ± 1.71	4.31 ± 1.14	5.29 ± 0.37	4.00 ± 0.30	6.03 ± 0.74
				After 8 Weeks			
control (4)	1.53 ± 0.22	2.01 ± 0.26	3.19 ± 2.33	1.30 ± 0.07	1.60 ± 0.24	2.02 ± 1.98	1.97 ± 0.31
propolis (4)	1.13 ± 0.72	1.59 ± 1.01	2.38 ± 0.85	1.16 ± 0.80	1.86 ± 0.66	1.85 ± 0.60	2.50 ± 0.88

[a] Rats were fed with the vitamin E-deficient diet or the vitamin E-deficient diet supplemented with 1% of propolis. After 4 and 8 weeks, concentrations of vitamin E in tissues were determined as described in the text. Value is mean ± SD (the number of rats used for each treatment is shown in parentheses), and asterisks shown at the propolis group indicate significant difference from control group (ANOVA Bonferroni/Dunn procedure: *, $P < 0.05$; **, $P < 0.01$).

Table 2. Levels of Tissue Vitamin C (Nanomoles per Gram of Tissue) in the Control (Vitamin E-Deficient) and Propolis (Vitamin E-Deficient and Propolis-Supplemented) Groups[a]

	plasma	liver	heart	kidney	stomach	small intestine	large intestine
				After 4 Weeks			
control (3)	26.9 ± 1.4	1600 ± 310	431 ± 46	705 ± 105	809 ± 79	2229 ± 300	1062 ± 51
propolis (3)	41.5 ± 5.5*	1534 ± 111	431 ± 24	780 ± 67	871 ± 12	2192 ± 180	1180 ± 147
				After 8 Weeks			
control (4)	23.8 ± 3.4	1461 ± 167	402 ± 45	627 ± 13	638 ± 144	1766 ± 42	911 ± 77
propolis (4)	33.9 ± 13.3	1586 ± 242	458 ± 84	840 ± 102**	1018 ± 127**	2306 ± 307*	1162 ± 142*

[a] Rats were fed with the vitamin E-deficient diet or the vitamin E-deficient diet supplemented with 1% of propolis. After 4 and 8 weeks, concentrations of vitamin C in tissues were determined as described in the text. Value is mean ± SD (the number of rats used for each treatment is shown in parentheses), and asterisks shown at the propolis group indicate significant difference from control group (ANOVA Bonferroni/Dunn procedure: *, $P < 0.05$; **, $P < 0.01$).

The diet of the control (vitamin E-deficient) group was prepared by Funahashi Farm according to AIN 76 (American Institute of Nutrition, 1977) using stripped corn oil (5 g/100 g of diet) as the fat and mixed with dextrin (1 g/100 g of diet). The propolis group received the vitamin E-free diet (as described above) containing a 1:1 mixture of propolis and dextrin by 2 g/100 g of diet. The 1:1 mixture of propolis and dextrin was supplied by Yamada Yohojo Bee Farm Inc. (Tomata, Okayama, Japan). Diets were divided into small portions and stored at −80 °C until use. Body weights were recorded daily.

Analytical Methods. Rats were anesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. After perfusion of ice-cooled saline through the portal vein, organs were removed. The excised tissue was homogenized in 5 volumes of 10 mmol/L phosphate-buffered saline (pH 7.4) under cooling in an ice bath. All determinations were made in duplicate. Lipid hydroperoxide was measured as described (Tokumaru et al., 1995). The determination of vitamin C was made according to a specific and sensitive method (Kishida et al., 1992; Tokumaru et al., 1996a) involving chemical derivatization and HPLC. The concentration of vitamin E was determined by using a method of Buttriss and Diplock (1984). The conditions of HPLC and fluorescence detection (type RF-535 manufactured by Shimadzu, Kyoto, Japan) were reported previously (Kishida et al., 1993a,b). Blood was centrifuged at 7000*g* for 5 min at 4 °C to separate plasma.

Protein concentrations were determined according to the method of Lowry et al. (1951) using BSA as the standard.

Data were expressed as means ± standard deviations (SD) and analyzed by ANOVA using StatView software (Abacus Concepts, Berkeley, CA). Differences between group means were considered significant at $P < 0.05$ using the Bonferroni /Dunn procedure generated by this program.

RESULTS AND DISCUSSION

Change in Body Weight. There were no significant differences in body weights between the control and propolis groups (data not shown).

Change in Tissue Concentration of Vitamin E. Vitamin E concentrations in plasma, liver, heart, kidney, stomach, small intestine, and large intestine of the control and propolis groups are shown in Table 1. In the present study, only α-tocopherol was determined and other tocopherols (β, γ, and δ) were not detected. No significant difference was observed between these groups after both 4 and 8 weeks (Table 1). Although propolis contains α-tocopherol [∼400 μg/kg diet, <1% of 50 mg/kg of AIN 76 formula (American Institute of Nutrition, 1977)], this amount of vitamin E did not affect its tissue concentration as compared with the control group fed completely vitamin E-free diet. This result indicates that propolis does not save vitamin E, a hydrophobic antioxidant, during vitamin E deficiency.

Change in Tissue Concentration of Vitamin C. Tissue vitamin C concentrations of the control and propolis groups are shown in Table 2. After 4 weeks, the plasma vitamin C concentration of the propolis group was significantly higher than that of the control group. This result suggests that propolis has a saving effect on vitamin C in plasma, probably by antioxidative mechanism. It is conceivable that some hydrophilic component(s) of propolis save(s) vitamin C, a water-soluble vitamin, different from the case of hydrophobic vitamin E as described above. No significant difference was observed in the liver, heart, kidney, stomach, small intestine, or large intestine after 4 weeks (Table 2).

After 8 weeks, the tissue concentrations of vitamin C in the kidney, stomach, small intestine, and large intestine of the propolis group were significantly higher than those of the control group (Table 2). These differences seem to be caused by the fact that the tissue vitamin C concentration of the propolis group after 8 weeks was maintained at the level of 4 weeks after the vitamin E deficiency. Recently we have reported (Tanaka et al., 1997) that the oxidative stress induced by prolonged vitamin E deficiency causes a decrease in tissue vitamin C concentration using inherently scorbutic rats. Although the present study was done utilizing rats of the Wistar strain, similar results seemed to be observed.

Table 3. Levels of Lipid Hydroperoxides (Picomoles per Milligram of Protein) in the Control (Vitamin E-Deficient) and Propolis (Vitamin E-Deficient and Propolis-Supplemented) Groups after 8 Weeks[a]

	stomach	small intestine	large intestine
control (4)	330.5 ± 90.6	274.4 ± 48.3	263.6 ± 32.2
propolis (4)	210.6 ± 72.1	202.7 ± 83.6	126.4 ± 35.8**

[a] Rats were fed with the vitamin E-deficient diet or the vitamin E-deficient diet supplemented with 1% of propolis. After 8 weeks, concentrations of lipid hydroperoxides in tissues were determined as described in the text. Value is mean ± SD (the number of rats used for each treatment is shown in parentheses), and asterisks shown at the propolis group indicate significant difference from the control group (ANOVA Bonferroni/Dunn procedure: *, $P < 0.05$; **, $P < 0.01$).

These in vivo results indicate that some component(s) of propolis is (are) absorbed and circulate(s) in the blood to affect the concentration of vitamin C in the kidney and plasma. These results also support the view that propolis saves vitamin C in enhanced oxidative stress caused by prolonged vitamin E deficiency. It is conceivable that propolis exerts its antioxidative effect on tissues where it is assumed to accumulate as on the kidney, where it is excreted, and in the gastrointestinal tract, where propolis affects these tissues even from the outside of the cell.

However, no significant difference was observed in the vitamin C concentration of plasma, liver, and heart between these two groups after 8 weeks (Table 2).

Change in Level of Tissue Lipid Hydroperoxides. Although radical reactions receive much attention in relation to pathogenic disorders such as cancer (Halliwell and Gutteridge, 1990), diabetes mellitus (Baynes, 1991), atherosclerosis, and aging (Halliwell and Gutteridge, 1989), the search for a reliable indicator of oxidative stress in animal tissues is still an important activity. Conventional indicators of radical reactions are classified into three main categories, which are *products of lipid peroxidation* such as malondialdehyde (Kishida et al., 1990), thiobarbituric acid reactive substances (TBARS; Buege and Aust, 1978), modified proteins (Stadtman, 1992), and DNA (Shigenaga et al., 1990); *decreased antioxidants* such as vitamin C (Kishida et al., 1992), vitamin E (Kishida et al., 1993a), and glutathione (Meister and Anderson, 1983); and *activity change of antioxidant enzymes* including superoxide dismutase (Stralin and Marklund, 1994) and glutathione peroxidase (Cowan et al., 1993). As another kind of index, the oxidative mediator, rather than the products of peroxidation, may be determined. Lipid hydroperoxide is a probable candidate for such an oxidative mediator because it is formed by radical reactions, has sufficient lifetime to migrate, and finally modifies protein and DNA.

Recently we developed a specific and sensitive method (Tokumaru et al., 1995) to determine the concentration of lipid hydroperoxides in animal tissues. The efficiency of lipid hydroperoxides as an index of oxidative stress has been confirmed by their increase in some typical cases of enhanced oxidative stress such as aged (Tokumaru et al., 1996b), vitamin C-deficient (Tokumaru et al., 1996a), vitamin E-deficient (Tokumaru et al., 1997), iron-overloaded (Ikeda et al., 1998), and streptozotocin-induced diabetic (Sun et al., 1999) animal tissues.

It is reported (Rao et al., 1993, 1995) that dietary propolis inhibits chemical carcinogenesis of rat colon. To investigate whether the antioxidative property of propolis contributes to the inhibition, the lipid hydroperoxide concentrations in the gastrointestinal tract of the control and propolis groups were compared. As shown in Table 3, a significant difference was observed after 8 weeks in the large intestine, where a significant difference in the vitamin C concentration was observed (Table 2). In the stomach and small intestine, the lipid hydroperoxide concentrations of the propolis group were lower than those of the control group, but a statistical significance was not observed (Table 3). These observations indicate that the oxidative stress in the large intestine is attenuated by propolis, although it is not clear whether the antioxidative effect is brought about intracelluarly and/or from outside the cell.

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