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Effect of Propolis Supplementation on the Redox State of Human Serum Albumin during High-Intensity *Kendo* Training

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Abstract

IMAI, H., ERA, S., HAYASHI, T., NEGAWA, T., MATSUYAMA, Y., OKIHARA, K., NAKATSUMA, A., and YAMADA, H., Effect of Propolis Supplementation on the Redox State of Human Serum Albumin during High-Intensity *Kendo* Training. Adv. Exerc. Sports Physiol., Vol.11, No.3 pp.109-113, 2005. Human serum albumin (HSA) is a mixture of human mercapt- (HMA, reduced form) and nonmercaptalbumin (HNA, oxidized form), and is known to be one of the major *extra*cellular scavengers for reactive oxygen species. Using a high-performance liquid chromatographic (HPLC) system with an ES-502N column, we studied the redox state of HSA for male Japanese fencing ("*kendo*") athletes (n=11) before and after an intense *kendo* training camp for 4 days. In order to clarify the antioxidative effect of propolis supplementation on the HSA redox state during camp, subjects were divided into two groups (placebo group, n=5; propolis group, n=6). A double blind test was utilized. The propolis used in this study was a product of Brazil (daily amount of propolis was 787.5 mg). During camp, the observed decrease in the mean values of [HMA/(HMA+HNA)] ($\bar{f}$(HMA)) in the placebo group was significant (76.8±1.66% before and 63.0±2.29% after camp; $P<0.05$). Similarly, those values for the propolis group were also significant (77.0± 0.94% before and 70.5±1.51% after camp; $P<0.05$). For the propolis group, however, the degree of the decrease in the $\bar{f}$(HMA) value was significantly small compared with that of the placebo group ($P<0.05$). In addition, the change in the $\bar{f}$(HMA) value of the propolis group during the training camp was roughly within the normal range of that of healthy male subjects previously reported (73.2±2.34%). These findings suggest that propolis may be an effective supplement to improve the redox state of HSA for *kendo* athletes in repetitive and highly intensive training camps.

Keywords: antioxidant, propolis, redox state, serum albumin (human), training camp

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Introduction

Human serum albumin (HSA) is the most abundant protein in the circulatory system and it has a number of functions. It is well known that it plays an important role in the osmotic regulation of the circulating fluid within the vascular system and is able to transport a wide variety of endogenous and exogenous substances through the body (14). Another functional role of this molecule seems to be the maintenance of the redox potential in the *extra*cellular fluid, because it is a mixture of mercaptalbumin (reduced form; in humans, HMA) and non-mercaptalbumin (oxidized form; in humans, HNA), i.e., a major part of the redox couple in plasma (20-22). Namely, HMA has one highly reactive sulfhydryl (SH) group in position 34 (Cys-34) and is responsible for the largest fraction of free SH in the *extra*cellular fluid. On the other hand, HNA, the oxidized form of albumin, is composed of at least three kinds of compounds; the major HNA compound is the mixed disulfide with cystine or oxidized glutathione (HNA(Cys) or HNA(Glut)), and the other is an oxidation product higher than mixed disulfide, such as the sulfenic (−SOH), sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) state (HNA(Oxi)) as a very minor component in the *extra*cellular fluid (5).

We have developed a convenient high-performance liquid chromatographic (HPLC) system for the clear separation of HSA into HMA and HNA, using a Shodex-Asahipak GS-520H or ES-502N column, and extensively studied the dynamic change in the redox state of HSA in various pathophysiological states (8,9,11,19,24,25). From the re-

sults obtained, the fraction of HMA (f(HMA)) has been shown to be markedly decreased in various diseases compared with that in healthy subjects, whereas there has been almost no change in the f(HMA) value for the healthy subjects under normal circumstances. However, even in healthy subjects, we observed that strenuous physical exercise, such as intensive *kendo* training camps, significantly decreased their reduced serum albumin level (10).

Propolis, a natural product derived from plant resins collected by honeybees, has been considered to play a beneficial role as a supplemental antioxidant (2), and in our preliminary observations, approximately 2 months of oral supplementation of propolis, especially that obtained from Brazil, seemed to improve the redox state of HSA from cancer patients under severe oxidative stress, such as that caused by cancer therapy (6). We therefore tried to evaluate systematically *in vivo* the antioxidative effect of propolis on the redox state of HSA from male *kendo* athletes in the highly intensive *kendo* training camp.

Material and Methods

Eleven male students, who were members of the *kendo* club at Gifu University (mean age, 20.1±1.2 years old; average *kendo* experience, 12.4±2.0 years), participated in this intense *kendo* training camp, which was held from October 31 to November 4, 2000. The subjects were divided into two groups and ingested either placebo (group A: n=5) or propolis (group B: n=6). A double blind test was utilized. The dose recommended by the sales company was used in the clinical study described in the Introduction (6), in which the subjects were elderly. Generally, antioxidative activity is higher in the young than in the elderly (1). The active oxygen production due to repeated exercise loads during the intensive *kendo* training camp was expected to exceed antioxidative activity in the young subjects (10). Thus, the clinical dose of propolis for the elderly was not employed, and the dose was increased in this experiment. During the camp, subjects in group B took 15 propolis tablets per day (daily amount of propolis was 787.5 mg of Brazilian propolis, extracted by ethanol), and subjects in group A (control group) took 15 placebo tablets per day (propolis extract was completely replaced with rapeseed oil). Brazilian propolis glue obtained from the virgin forests of Alecrim in the Southern part of Minas Gerais State in Brazil, and placebo tablets were supplied by Yamada Apiculture Center, Inc., Okayama, Japan. Every subject gave his informed consent, and all procedures were performed in accordance with the Helsinki Declaration.

Practice during the training camp consisted of 2 h in the afternoon on the first day, and 2 h in the morning and 3 h in the afternoon on the 2nd through 4th days, for a total of 7 practices. Detailed daily practice at the camp was constructed as follows: about 60 min of *kihon-keiko* (practicing to acquire the basic movements); 90 min of *gokaku-keiko* (*keiko* practiced by persons who are almost equal in their skills, also *keiko* in which the participants treat each other with equal respect even if their skills differ); 30 min of *kakari-keiko* (the *keiko* method where the trainee for a short period practices striking the *motodachi* (person acting as instructor) with all his/her might, using all *waza* (motor skill) he/she has learned, and without thinking of being struck or dodging); and approximately 120 min of *shiai-keiko* (a method of *keiko* performed with referees as in a match). The $\dot{V}O_{2max}$ percentages for *kihon-keiko*, *gokaku-keiko*, and *kakari-keiko* were approximately 40, 55 and 70%, respectively, and *shiai-keiko* at its maximum value was nearly the same as the rate for *kakari-keiko* (10). The environment was controlled throughout the camp period, with an average temperature of 19.1±1.0℃ and an average humidity of 49.6±5.4%. Blood samples were taken from the first day of camp (October 31, 8:00 a.m.) to the day after camp (November 4, 5:00 p.m.), for a total of 10 samples. After each sampling, specimens underwent immediate pressure filtration. Serum specimens were stored at −80℃ until HPLC analysis.

The HPLC system consisted of a Model AS-8010 autosampler, a Model CCPM double-plunger pump and a Model FS-8000 fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 340 nm) in conjunction with a Model SC-8020 system controller (all from Tosoh Co., Tokyo, Japan). A Shodex-Asahipak ES-502N column (10 x 0.76 cm I.D., DEAE-form for ion-exchange HPLC, Showa Denko., Co., Tokyo, Japan; column temperature, 35.0±0.5℃) was used. Elution was carried out with a linear gradient of increasing ethanol concentration from 0 to 5%, in 0.05 M sodium acetate-0.40 M sodium sulfate (pH 4.85) (acetate-sulfate buffer) at a flow rate of 1.0 ml/min. Serum samples were injected by means of an autosampler with a fixed volume of 2 μl. All chemicals and reagents were of an analytical grade. All solvents and solutions were filtered through a filter unit (0.22 μm, Sterivex-GS, Millipore, MA, USA) prior to use.

To determine the value for each fraction of serum albumin, i.e., f(HMA)=[HMA/(HMA+HNA)] and f(HNA) =[HNA/(HMA+HNA)], the obtained HPLC profiles were subjected to numerical curve fitting and each albumin peak shape was approximated by a Gaussian function so that the area underneath the peak could be calculated (Peak Fit, SPSS Science, IL, USA). For the purpose of the statistical analyses, comparisons between the propolis group and the placebo group utilized the Mann-Whitney *U* test, while the Wilcoxon signed-ranks test was used for comparisons within the group. Values are expressed as the mean±SE.

Results

HSA is known to be a mixture of the reduced form (HMA) and oxidized form (HNA), i.e., a major part of the redox couple in *extra*cellular fluid (20, 21). Moreover,

there are several kinds of HNA, i.e., HNA(Cys), HNA(Glut) and HNA(Oxi). We have previously reported that our HPLC system, with its fluorescence detection, was able to separate the two kinds of HNA, i.e., [HNA(Cys) & HNA(Glut)] (tentatively called HNA-1 in this study) and HNA(Oxi) (called HNA-2) (9-11,25).

Fig. 1 shows a typical HPLC profile of serum from a healthy young male *kendo* athlete (K.N.: 19 years old) obtained just before the training camp. In this profile, the value for the fraction of HMA (f(HMA)) was 74.7%, and those for HNA-1 and HNA-2 were 23.9 and 1.4%, respectively. These values were within the normal range for young male subjects, as previously reported (73.2±2.34% for $\bar{f}$(HMA), n=20) (10).

Fig. 2 shows the chronological change in the $\bar{f}$(HMA) values (%) for the placebo group (n=5, dotted line) and the propolis group (n=6, solid line) derived from blood samples taken 10 times during the training camp. In the samples taken on the morning of the 1st day before the start of the training camp (①), the first evening before the afternoon practice (②) and on the morning of the 2nd day (③), the $\bar{f}$(HMA) levels for both placebo and propolis groups were basically the same (74.8, 76.8 and 75.9% for the placebo group; 74.6, 77.0 and 75.3% for the propolis group, respectively). However, from the 2nd day, afternoon training, especially in the 3rd and 4th days of the training (④, ⑤, ⑥, ⑦, ⑧), both groups showed a decrease in their $\bar{f}$(HMA) values before and after each practice (63.4, 71.2, 66.1, 69.7 and 63.0% for the placebo group; 69.5, 74.0, 70.5, 73.1 and 70.5% for the propolis group, respectively) and these dynamic changes in the redox state of HSA from *kendo* athletes were in accordance with that reported previously (10). However, it is worth noting that the degree of decrease in the $\bar{f}$(HMA) value for the placebo group was significantly much larger than that for the propolis group (⑥, ⑧; $P<0.05$). In addition, in the present study, just after the training camp (⑨, ⑩; the recovery period), the $\bar{f}$(HMA) value in both groups increased compared to that just before the start of camp, indicating that their oxidative damage caused by the strenuous exercise might have immediately recovered to the normal state and further approached a more reduced state (72.6 and 80.3% for the placebo group; 76.1 and 83.2% for the propolis group, respectively).

Discussion

Our previous studies have demonstrated that the HSA redox state can be used as a biomarker of oxidative stress both in the physiological (5,10) and pathophysiological states (8,9,11,19,22,24,25). Therefore, before starting the

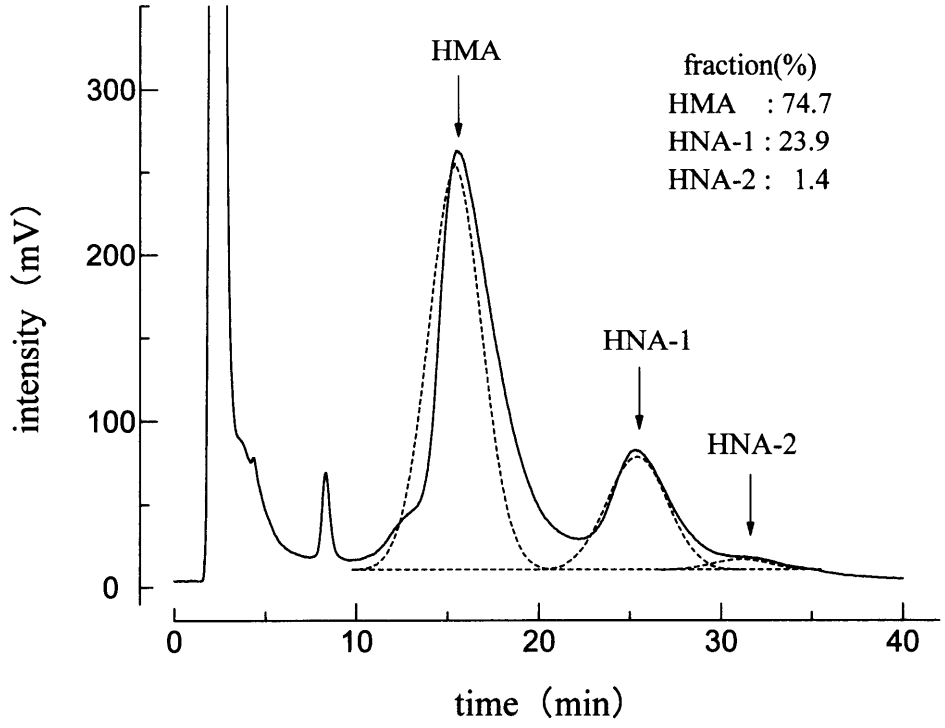

Fig. 1 Representative HPLC profile of serum from a healthy young male subject (N.K.: 19 years old) eluted from an ES-502N column with an increasing ethanol concentration from 0 to 5% in acetate-sulfate buffer (pH 4.85). Peaks 1, 2 and 3 correspond to HMA, HNA-1 and HNA-2, respectively. The profile was subjected to numerical curve fitting (dashed line) and the obtained values for each fraction are indicated in the upper right part of the figure.

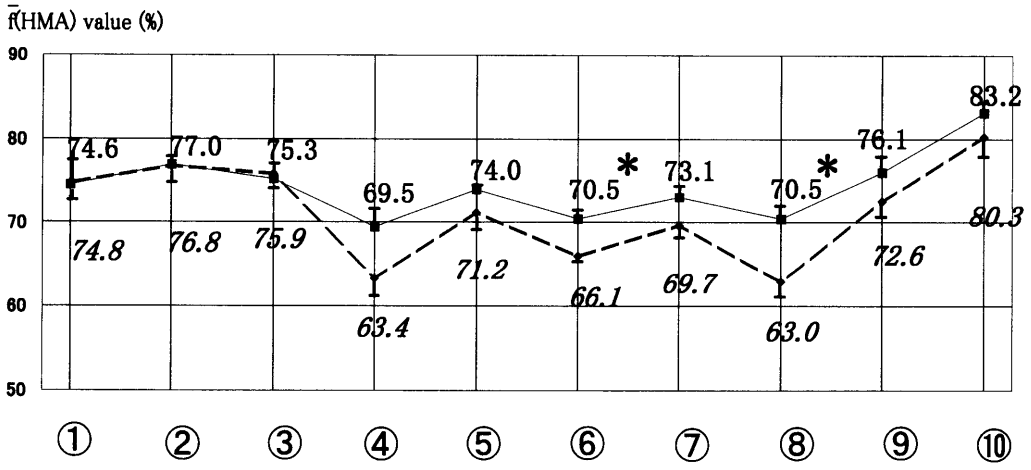

Fig. 2 Change in the f̄(HMA) value of HSA from the placebo group (n=5, dotted line) and propolis group (n=6, solid line) during the *kendo* training camp. Numbers indicate the blood sampling points (①, 1st day in the morning; ②, 1st day before the afternoon training; ③, 2nd day before the morning training; ④, 2nd day after the afternoon training; ⑤, 3rd day before the morning training; ⑥, 3rd day after the afternoon training; ⑦, 4th day before the morning training; ⑧, 4th day after the afternoon training; ⑨, 5th day in the morning (recovery period); ⑩, 5th day in the evening (recovery period)). Closed points and bars represent the mean value and SE, respectively. *, $P<0.05$ (vs. placebo group).

main discussion, it is appropriate to note briefly our preliminary observations, i.e., the influence of Brazilian propolis supplementation on the redox state of HSA from cancer patients (6).

In the case of a patient with liver cirrhosis and esophageal cancer (male, 68 years old), the f(HMA) value before ^{60}Co radiation therapy with no propolis supplementation was 66.6%. During the radiation therapy for 2 weeks, he took Brazilian propolis extract every day (525 mg per day) and continued to take them for 4 more weeks. Values for f(HMA) just before, immediately after, and 4 weeks after radiation therapy, were 63.5, 67.7 and 74.1%, respectively, and moreover, his poor appetite was improved. Radiation therapy with ^{60}Co radiation, even though it is the therapeutic dose for humans, has been known to produce a large number of oxygen radicals, and they are generally believed to be responsible for numerous findings of oxidative stress (4). However, our findings on the change in the f(HMA) value from a cancer patient demonstrates that his oxidative status due to the radiation therapy might have been attenuated by continuous oral propolis supplementation (6).

It is widely accepted today that strenuous physical exercise can induce oxidative stress in animals (15,17) and in humans (15,16,23). As already reported, concentric physical practice in the *kendo* training camp was highly intense, suggesting that this large amount of exercise produced a large increase in oxidative stress (10). As shown in Fig. 2, a significant decrease in the reduced albumin level was observed in both groups during camp, suggesting that repetitive practice might also have increased the oxidative stress in their bodies. By the 3rd and 4th day of training, however, the degree of decrease in the f̄(HMA) value for the propolis group was much smaller than that for the placebo group (⑥, ⑧; $P<0.05$). These results suggest that the antioxidant activity of propolis may moderate the decrease in the f̄(HMA) value and the antioxidative property of propolis may be similar to that of other low molecular mass antioxidants, such as vitamins C and E, glutathione and others, as has been well demonstrated (15-17,23).

Propolis is a resinous hive product collected by honeybees from various plant sources, and has been demonstrated to possess several physiological and pharmacological activities, such as antiviral, antibacterial, anti-inflammatory and antioxidative effects. Burdock (2) reviewed the biological properties and toxicity of propolis. Even in his review, little information is available on the active components of propolis, because the precise composition of raw propolis varies with the source. However, it is reported that, in general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris (3). In the present study, we used an ethanol extract of Brazilian propolis, which was supplied by Yamada Apiculture Center, Co., Okayama, Japan. More recently, their group has reported the new chemical composition of Brazilian propolis, i.e., terpenoids and aromatic compounds (12), long-chain alkanoic acid esters (7) by spectroscopic methods, and pharmacologically active phenolic compounds such as cinnamic acid derivatives, artepillin C and capillartemisin by an inclusion technique (13). Among them, with regard to their radical scavenging

ability, Shimizu *et al.* (18) most recently reported that artepillin C could prevent oxidative damage dose-dependently in Caco-2 and hepatic HepG2 cells exposed to reactive oxygen species (ROS).

A large amount of albumin is synthesized only in the liver every day and circulates through the body. As already mentioned, albumin forms a major part of the redox potential in the body, because a large portion of the SH groups in the human *extra*cellular fluid is primarily derived from the Cys-34 in the albumin molecule. In the present study, although a possibly active substance in the Brazilian propolis composition could not be determined, we successfully observed the *in vivo* antioxidative effect of propolis monitored by changes in the redox state of HSA from *kendo* athletes during a highly intensive training camp, as shown in Fig. 2.

In summary, this study presents the first evidence as to how propolis supplementation may affect the HSA redox status during strenuous exercise-induced oxidative stress, such as a *kendo* training camp. Findings of this study also suggest that exercise-associated perturbation of the redox status in the body might be circumvented by supplementation with propolis, and therefore, it is possible that the amount of propolis administered in the present study was enough to decrease oxidative damage following the exhaustive physical exercise for the young athletes.

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