Pilot Study of Antioxidant Mixture (Vitamin E, Pycnogenol® and Squalene) in Healthy Smokers: Inhibitory Effect on Oxidative DNA Damage

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[ABSTRACT]
Oxidative stress is considered to contribute to degenerative disease. The urinary excretion of the DNA repair product 8-hydroxy-2'-deoxyguanosine (8-OHdG) is proposed as a noninvasive biomarker of current oxidative stress in vivo. We investigated the effect of an antioxidant mixture on urinary 8-OHdG excretions in 12 otherwise healthy smokers. During the intervention period for 2 weeks, subjects consumed four capsules of PICACE® (Pycnogenol® 15 mg/capsule, Vitamin E; 56.1 mg/capsule, Squalene; 138.9 mg/capsule) per day. On days 0 (pre-intervention), 3, 7, 14, and 44, morning urine samples were collected. The urinary 8-OHdG level on day 3 was significantly reduced compared to day 0. The level of 8-OHdG after a washout period for PICACE® (days 44) returned to day 0 baseline. These preliminary data suggest that PICACE® supplements can protect smokers from oxidative stress and possibly reduce disease risk caused by free radicals associated with smoking.

[Key words] Pycnogenol®, Vitamin E, 8-hydroxy-2'-deoxyguanosine (8-OHdG), smoking, clinical trial

INTRODUCTION
Smoking is associated with significantly increased overall morbidity and mortality. Cigarette smoke contains approximately 3800 chemicals, including many carcinogenic compounds and free radicals1. The resulting process of cellular oxidative damage has been linked to the etiology of several chronic degenerative conditions such as cancer and cardiovascular disease.

The determination of oxidative damage in vivo is considered an important target for assessment and estimation of oxidation-related diseases. Urinary excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG), the repair product from oxidative DNA modification by excision enzyme, is an in vivo measure of overall oxidative DNA damage2. Recently, clinical and epide-
miological research has shown a causal relationship between 8-OHdG and various disease states\(^3\).

Chemoprevention with antioxidant products may play an important role in reducing risks of oxidation-related diseases, including tobacco-related. Particular attention was paid to the roles of Vitamin E and Pycnogenol\(^8\) as antioxidant products in this study. Vitamin E (alpha-tocopherol) is a well-known scavenger of peroxide radicals in cellular lipid membranes\(^4\). French maritime pine bark extract (Pycnogenol\(^8\)) is a standardized extract composed of a mixture of flavonoids, mainly procyanidins and phenolic acids\(^5,6\). Pycnogenol\(^8\) has strong free radical-scavenging activity against reactive oxygen and nitrogen species. Moreover, Pycnogenol\(^8\) participates in the cellular antioxidant network as indicated by its ability to protect endogenous vitamin E from oxidative stress\(^7\).

In this study, we designed a preliminary clinical trial to clarify the chemopreventive effect of an antioxidant mixture of Vitamin E and Pycnogenol\(^8\) in otherwise healthy smokers using urinary 8-OHdG as an outcome measure.

**MATERIAL AND METHODS**

**Subjects**

Study subjects were twelve otherwise healthy smokers who are employees at SRL Inc. in Tokyo. Characteristics of subjects are shown in Table 1. Normal health status was previously established by medical history, biochemical and hematologic screenings, and physical examination. No volunteers had any diseases such as asthma, chronic pulmonary disease, diabetes mellitus, hypertension, cardiac disease, or other symptoms pertaining to the cardiovascular system at the time of the trial. They were of average physical fitness, and had not taken any medication or antioxidant supplements during the 4 weeks preceding entry into the study. The experimental procedure and its purpose were explained thoroughly to all subjects, and written consent was obtained.

**Study Design**

During the 2-week intervention period subjects consumed four capsules of PICACE\(^8\) (Pycnogenol\(^8\); 15 mg/capsule, Vitamin E; 56.1 mg/capsule, Squalene; 138.9 mg/capsule) daily. Subjects were instructed to lead their usual lifestyle pattern, including smoking, drinking, diet, work hours, and sleep during their study participation. On days 0 (pre-internal use), 3, 7, 14 and 44, morning urine samples were collected and stored at \(-20^\circ\)C until analyses were performed. The last sample (day 44) was collected one month after last capsule consumption to allow for PICACE\(^8\) washout. The concentration of 8-OHdG was measured using high-performance liquid chromatography (HPLC) in a laboratory of SRL Inc. and was adjusted for urinary creatinine concentration. Creatinine-adjusted urinary 8-OHdG levels are shown in units of ng/mg CRE.

**Statistical analysis**

Differences between subject means at each point were assessed with Wilcoxon’s signed rank test and Student’s paired t test. As the results were essentially similar, only the latter are presented. The level of significance was \(P<0.05\).

**RESULTS**

All subjects completed the assigned intervention without any adverse effects. Figure 1 shows the 8-OHdG corresponding values of the 12 subjects on days 0 (mean±standard deviation; 5.01±1.96 ng/mg CRE), 3 (3.72±1.28 ng/mg CRE), and 44 (4.70±0.86 ng/mg CRE). In each subject, mean 8-OHdG corresponding values on day 3 were significantly reduced compared to baseline. Moreover, 8-OHdG after the washout period (day 44) returned to baseline (day 0). In 7 of 12 subjects on day 7 and 5 of 12 subjects on day 14 8-OHdG in urine was less than

**Table 1** Baseline characteristics of subjects

| Age (years) | 38.0±6.7* |
| Gender (Male/Female) | 6/6 |
| Brinkmann index | 264.2±166.6* |

* Values are mean±SD.
the detection sensitivity of HPLC. Therefore this data is not displayed in Figure 1.

**DISCUSSION**

We demonstrated that an antioxidant mixture of Vitamin E and Pycnogenol® appears to remarkably reduce urinary 8-OHdG excretions in otherwise healthy smokers. In the present study, urinary excretion levels of 8-OHdG were used to determine the efficacy of a mixture of antioxidant products in decreasing the harmful effects of cigarette smoking. The measurement of DNA damage via urinary 8-OHdG is an intermediate or surrogate end point during carcinogenesis. Although the validity of surrogate end point markers depends on the extent to which the marker is a necessary event in the causal pathway to cancer, DNA damage including 8-OHdG is generally considered a necessary step in cancer initiation and is being used extensively in intervention studies. Moreover, Erhola et al have suggested that urinary 8-OHdG might serve as a useful tool in evaluating response to chemotherapy and radiotherapy in lung cancer patients.

Vitamin E is considered one of the most important lipid-soluble micronutrient antioxidants. *In vitro* studies suggest that vitamin E may protect against oxidative damage to DNA as measured by 8-OHdG. Epidemiologic studies and small-sized clinical trials have also revealed that ingestion of vitamin E correlated closely with decreasing risk of oxidization-related diseases and the level of oxidative-stress biomarkers. Subsequent results of large-scale randomized controlled trials of vitamin E supplementation however are controversial. We believe these inconsistent findings may be due to the level of vitamin E intake in trials: according to the results of a meta-analysis on vitamin E supplementation trials, Miller et al have recently recommended that compared with placebo or no treatment, intake of high-dosage (≥400 IU/day=267 mg/day) vitamin E supplements may increase all-cause mortality and should be avoided. In our study, the amount of vitamin E supplementation was 224.4 mg/day. Therefore, PICACE® may be considered a safe and effective orally-ingested supplement.

Pycnogenol®, an extract of French maritime pine bark, is a standardized potent antioxidant with potential health benefits. Pycnogenol® contains a wide variety of procyanidins that are biopolymers of catechin and epicatechin subunits, which are recognized as important constituents in human nutrition. Pycnogenol® protects against oxidative stress in several cell systems by doubling the intracellular synthesis of anti-oxidative enzymes and by acting as a potent scavenger of free radicals. Other anti-oxidant effects of Pycnogenol® involve a role in the regeneration and protection of vitamin E. Since PICACE® is a mixture of Pycnogenol® and vitamin E, it may possess synergistic as well as antioxidant effects.

These preliminary data suggest that supplementation with antioxidants (PICACE®) may protect smokers from oxidative damages and possibly reduce cancer risk or other diseases caused by free radicals associated with smoking.

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要  旨

健常喫煙者における抗酸化天然成分によるパイロット試験：DNA酸化傷害抑制効果の検討

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【目的】喫煙と発癌との因果関係は数多く報告されている。今回、我々は喫煙による有害作用として、活性酸素によるDNA酸化修飾物質であり発癌との因果関係も指摘されている8-ヒドロキシデオキシグアノシン (8-OHdG) を指標に抗酸化成分による介入試験を行った。
【対象と方法】健康な喫煙者（男性6名、女性6名、平均年齢38歳、平均Brinkmann指数264）を対象に行った。抗酸化成分としてPICACE®(Pycnogenol® 15 mg/capsule, Vitamin E; 56.1 mg/capsule, Squalene; 138.9 mg/capsule) 4カプセル/日を14日間服用し、服用前、服用後3、7、14日目、服用終了後1ヶ月目の尿中8-OHdGを高速液体クロマトグラフィー(HPLC)法で測定した。
【結果】服用3日目の尿中8-OHdGは、クレアチニン換算値で3.7±1.3 ng/mg CRE (平均±標準偏差)で、服用前の5.0±2.0 ng/mg CREより有意に低下していた(p<0.01)。服用終了後1ヶ月目の尿中8-OHdGは、4.7±0.9 ng/mg CREであり、投与中止によって再上昇した。
【結論】抗酸化成分複合物であるPICACE®は、喫煙によるDNA酸化傷害を抑制することが示唆された。

キーワード：ピクノジェノール, ビタミンE, 8-OHdG, 喫煙, 臨床試験