【Original Article】

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

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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[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-internal use), 3, 7, 14, and 44, morning urine samples were collected. The urinary 8-OHdG was measured using high-performance liquid chromatography (HPLC). 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 radicals[1)]. 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 oxidization-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 damage[2)]. Recently, clinical and epide-

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miological research has shown a causal relationship between 8-OHdG and various disease states[3].

Chemoprevention with antioxidant products may plays an important role in reducing risks of oxidization-related diseases, including tobacco-related. Particular attention was paid to the roles of Vitamin E and Pycnogenol® 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®) is a standardized extract composed of a mixture of flavonoids, mainly procyandins and phenolic acids[5,6]. Pycnogenol® has strong free radical-scavenging activity against reactive oxygen and nitrogen species. Moreover, Pycnogenol® 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® 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 preceeding 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® (Pycnogenol®; 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°C until analyses were performed. The last sample (day 44) was collected one month after last capsule consumption to allow for PICACE® 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.

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.

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

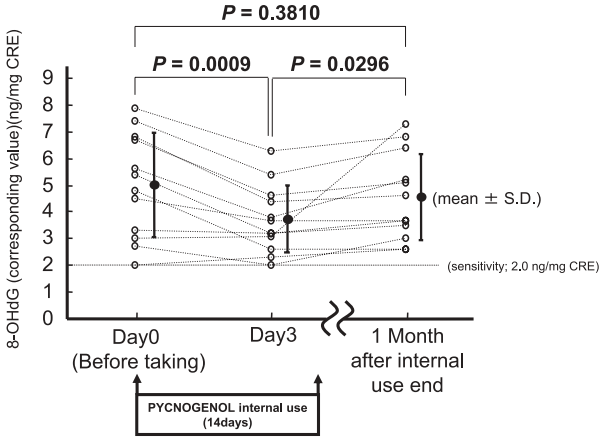

Fig. 1 Change in urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) on day 0, 3, and 44 for individual subjects. The closed circles and bars indicate the mean and standard deviation, respectively.

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[8]. Moreover, Erhola et al[9] 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[10]. 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[11]. 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[5,6]. Other anti-oxidant effects of Pycnogenol® involve a role in the regeneration and protection of vitamin E[7]. 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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REFERENCES

1) Hecht SS. Cigarette smoking and lung cancer: chemical mechanisms and approaches to prevention. Lancet Oncol 2002; 3(8): 461–469.
2) Lunec J, Holloway KA, Cooke MS, et al. Urinary 8-oxo-2'-deoxyguanosine: redox regulation of DNA repair in vivo? Free Radic Biol Med 2002; 33(7): 875–885.
3) Wu LL, Chiou CC, Chang PY, et al. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta 2004; 339(1-2): 1–9.
4) Azzi A, Gysin R, Kempna P, et al. The role of alpha-tocopherol in preventing disease: from epidemiology to molecular events. Mol Aspects Med 2003; 24(6): 325–336.
5) Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther 2002; 40(4): 158–168.
6) Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. Free Radic Biol Med 1999; 27(5-6): 704–724.
7) Virgili F, Kim D, Packer L. Procyanidins extracted from pine bark protect alpha-tocopherol in ECV 304 endothelial cells chal-

lenged by activated RAW 264.7 macrophages: role of nitric oxide and peroxynitrite. FEBS Lett 1998; 431(3): 315–318.
8) Santella RM. DNA damage as an intermediate biomarker in intervention studies. Proc Soc Exp Biol Med 1997; 216(2): 166–171.
9) Erhola M, Toyokuni S, Okada K, et al. Biomarker evidence of DNA oxidation in lung cancer patients: association of urinary 8-hydroxy-2'-deoxyguanosine excretion with radiotherapy, chemotherapy, and response to treatment. FEBS Lett 1997; 409(2): 287–291.
10) Zhang D, Okada S, Yu Y, et al. Vitamin E nhibits apoptosis, DNA modification, and cancer incidence induced by iron-mediated peroxidation in Wistar rat kidney. Cancer Res 1997; 57(12): 2410–2414.
11) Miller ER 3rd, Pastor-Barriuso R, Dalal D, et al. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann Intern Med 2005; 142(1): 37–46.

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