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RESEARCH PAPER

Pycnogenol Inhibits Macrophage Oxidative Burst, Lipoprotein Oxidation, and Hydroxyl Radical-Induced DNA Damage

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ABSTRACT

Pycnogenol (procyanidins extracted from Pinus maritima*) has been reputed as a potent free-radical scavenger and an antioxidant phytochemical. We previously reported that pycnogenol prevents vascular endothelial cells from injury induced by an organic oxidant t-butyl hydroperoxide. In this study, we determined the effects of pycnogenol on (a) oxidative burst of macrophages, (b) oxidation of plasma low density lipoprotein (LDL), and (c) hydroxyl radical-induced breakage of plasmid DNA. Pycnogenol was incubated with J774 murine macrophages at 37°C and 5% CO_2 and oxidative burst was triggered by zymosan. The intensity of fluorescence was measured. Pycnogenol exhibited a concentration-dependent inhibition of oxidative burst. $CuSO_4$ was used to oxidize human plasma LDL and the formation of thiobarbituric acid reactive substances (TBARS) was determined. Co-incubation with pycnogenol resulted in a concentration-dependent inhibition of LDL oxidation. Exposure of pBR322 plasmid DNA to iron/ascorbic acid system resulted in cleavage/damage of DNA by hydroxyl radical, measured by agarose gel electrophoresis. Pycnogenol significantly minimized this cleavage. The results indicate that pycnogenol exhibits an extensive antioxidant effect in all three in vitro systems.*

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INTRODUCTION

There is growing evidence that the active oxygen-induced and free-radical-mediated oxidation of biological molecules, membranes, and tissues is closely related to a variety of pathological events such as cancer, atherosclerosis, diabetes, the damage of reperfusion after organ ischemia, liver disease, inflammation, arthritis, and the regressive changes in aging (1,2). Once free radicals are produced, they multiply geometrically in chain reactions unless they are quenched by antioxidants or other free-radical scavengers. Antioxidants are compounds that react easily with free radicals and thus protect neighboring structures from oxidant damage. Common protective antioxidant nutrients include vitamins C, E, and β-carotene.

Pycnogenol is a blend of oligomeric and monomeric procyanidins isolated from the bark of pine (*Pinus maritima*) (3). These compounds are also found in fruits, vegetables, and other plants. About 85% of the compounds in pycnogenol are identified as procyanidins. Of these procyanidins, about 60% are oligomeric procyanidins (dimers and trimers); 20% are oligomers and phenolic acids such as gallic acid, caffeic acid, and ferulic acid. Pycnogenol has been extensively used in European countries as a dietary supplement because of its putative free-radical scavenging activity (4,5). This phytochemical is currently being sold in the health food market in the U.S. as well. Very few scientific studies have, however, been carried out.

In our laboratory, we previously demonstrated that pycnogenol can protect vascular endothelial cells from oxidant injury induced by an organic oxidant *t*-butyl hydroperoxide (6). In the present study, three in vitro systems were used to determine the antioxidant activity of pycnogenol.

MATERIALS AND METHODS

Reagents

Pycnogenol was provided by M. W. International (Hillside, NJ). Agarose, α-tocopherol succinate, ascorbic acid, butylated hydroxytoluene (BHT), ethidium bromide, ethylenediamine tetraacetic acid (EDTA), human low density lipoprotein (LDL), ferric chloride, Hanks' balanced salt solution (HBSS), *N,N'*-dimethyl formamide (DMF), phosphate buffer solution (PBS), sodium dodecyl sulfate (SDS), tetraethoxypropane, 2-thiobarbituric acid (TBA), Tris-borate-EDTA buffer, and zymosan A were purchased from Sigma Chemical Co. (St. Louis, MO). 2',7-dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc. (Eugene, OR). *n*-Butanol and pyridine were from Fisher Chemical (Fair Lawn, NJ). Dulbecco's modified Eagle's medium (DMEM), and penicillin–streptomycin solution were from Mediatech Co. (Washington, DC). Cupric sulfate was purchased from J.T. Baker Co. (Phillipsburg, NJ). Fetal bovine serum (FBS) was obtained from Gemini Bioproducts (Calabasas, CA). Plasmid pBR322 DNA was obtained from Promega (Madison, WI).

α-Tocopherol succinate was dissolved in 95% ethanol and pycnogenol was dispensed in PBS. LDL was dialyzed in PBS for 48 hr at 4°C to remove EDTA. The protein content was measured (7).

Oxidative Burst Assay

The murine macrophage cell line J774 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). J774 cell line has been used as a model for studying oxidative burst (8–10). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 500 U/ml penicillin, and 0.5 mg/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere for 3–4 days. Cells were harvested, washed once, and resuspended in HBSS. Assays were performed using 96-well tissue culture plates (Falcon, 3072) as previously described (11). The cell density was adjusted to 2×10^5/ml and 100 μl of the cell suspension was pipetted into each well. Different concentrations of pycnogenol in HBSS were added to wells. Plates were incubated for 1 hr at 37°C in 5% CO_2. Following the removal of pycnogenol from wells, zymosan and DCFH-DA diluted in HBSS were added. The plate was covered with a lid and incubated for 2 hr in a 37°C humidified incubator with 95% air and 5% CO_2. The fluorescence intensity (relative fluorescence unit, RFU) was determined at 485 nm excitation and 530 nm emission wavelengths using an automated fluorescence reader (Microplate Fluorometer Model 7610, Cambridge Technology, Watertown, MA) interfaced with a PC-compatible computer.

LDL Oxidation

Cu^{2+}-induced oxidation of LDL was performed (12). Several concentrations of pycnogenol (or α-tocopherol succinate as a control) in 0.1 ml and LDL (0.1 ml of 0.2 mg protein/ml) were added to 0.8 ml of 5 μM $CuSO_4$ and incubated at 37°C for 24 hr. After the incubation, the reaction was stopped by adding 0.1 ml of 1 mM EDTA. The extent of lipid oxidation was deter-

mined by measuring thiobarbituric acid reactive substances (TBARS) as previously described (13). Briefly, LDL solution was mixed with SDS, acetic acid solution, BHT-acetic acid solution, TBA, and distilled water in this order. The mixture was boiled at 100°C for 60 min. TBARS were extracted with *n*-butanol-pyridine, the butanol layer was removed, and the absorbance at 532 nm was measured using Bausch & Lomb Spectronic 2000 spectrophotometer. Tetraethoxypropane was used as a reference standard and the results were expressed in nmol/mg protein.

Quantification of Hydroxyl Radical-Induced DNA Damage

Hydroxyl radical-mediated DNA damage was carried out by exposing pBR322 plasmid DNA to various concentrations of ferric chloride/ascorbic acid for 1 hr at room temperature (14). Samples were run with various concentrations of pycnogenol. Determination of different conformations of pBR322 DNA (I, supercoiled; II, circular resulting from single-strand breaks; III, linear resulting from double-strand breaks) was made using 0.8% agarose and 0.5 μg/ml ethidium bromide in a horizontal gel electrophoresis chamber at 30 mA for 2 hr in 90 mM Tris borate/2 mM EDTA buffer at pH 8 (15). Relative intensity of bands was quantified by densitometry scanning using the Bio Image Band Analyzer (Millipore Corporation, Ann Arbor, MI).

Statistical Analyses

The data were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference and the results were expressed as the means ± SE. A p value less than 0.05 was considered significant. All statistical procedures were performed with Statgraphics software version 5.0 (STSC, Inc., Rockville, MD).

RESULTS

To determine the effect of pycnogenol on oxidative burst of macrophages, J774 cells were preincubated with 0, 2.5, 5, 10, 20, 40, 80, and 160 μg/ml of pycnogenol for 1 hr. After removal of pycnogenol, zymosan and DCFH-DA were added to each well. Fluorescence readings reflecting oxidative burst were assessed 2 hr later by an automated micro-fluorometric assay. Pycnogenol exhibited a concentration-dependent suppression of oxidative burst of J774 cells (Fig. 1). Cell viability remained >95% when evaluated with trypan blue exclusion, thus ruling out the possibility that the suppression might be due to pycnogenol toxicity.

When LDL was incubated with $CuSO_4$ for 24 hr, a significant increase of TBARS indicating LDL oxidation was observed, but in the absence of this metallic salt only trace amount of TBARS was detected. A concentration-dependent decrease of TBARS was noted with pycnogenol [Fig. 2(a)]. A known antioxidant, α-tocopherol succinate, also suppressed Cu^{2+}-induced formation of TBARS, although much higher concentrations were required [Fig. 2(b)]. Multiple range statistical analysis shows pycnogenol to be 50–100 times more active than α-tocopherol in inhibiting LDL oxidation.

The iron/ascorbic acid system produces hydroxyl radicals which cause cleavage of DNA manifested as nicking of supercoiled pBR322 plasmid to yield circular form (II) and linear form (III). Figure 3(a) shows that as the concentrations of Fe^{3+}/ascorbic acid increased, there was a decrease of the supercoiled DNA (I) accompanied by an increase of circular/linear forms (II/III). Densitometry scanning of the image showed

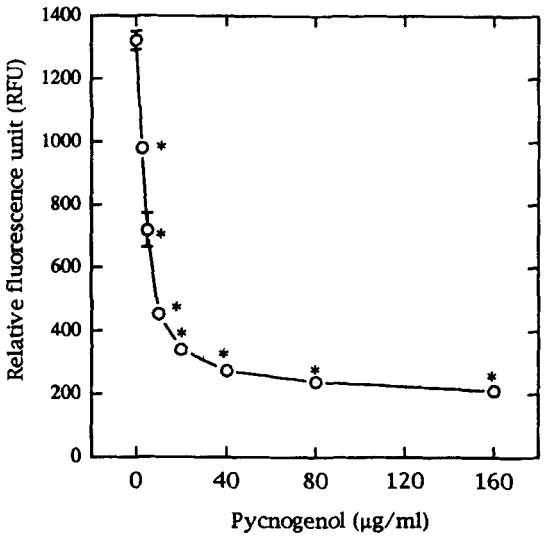

Figure 1. Effect of pycnogenol on oxidative burst of J774 cells. Cells (2×10^5/ml) were preincubated with various concentrations of pycnogenol (0, 2.5, 5, 10, 20, 40, 80, and 160 μg/ml) at 37°C and 5% CO_2 for 1 hr. Following removal of pycnogenol, zymosan and DCFH-DA were added. The fluorescence readings were taken after an additional 2 hr of incubation at 37°C and 5% CO_2. Data represent means ± SE of triplicate samples. Asterisks denote significant difference from control without pycnogenol ($p < 0.05$).

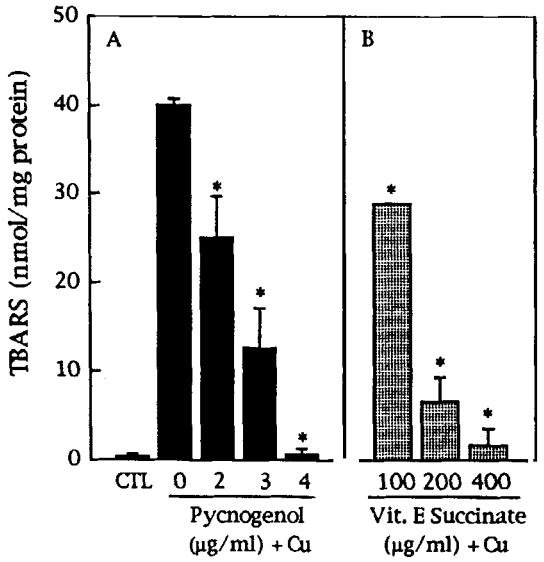

Figure 2. Effects of pycnogenol and vitamin E on Cu^{2+}-induced oxidation of LDL. Data represent means ± SE of triplicate samples. CTL = control not exposed to $CuSO_4$. Asterisks denote significant difference from samples without pycnogenol ($p < 0.05$).

untreated pBR322 to contain 80% form I and 20% forms II/III. Treatment with 40 pM $FeCl_3$/40 mM ascorbic acid resulted in a change of distribution of 10% form I and 90% forms II/III and this concentration of the inducers was used to determine the effects of pycnogenol. As shown in Fig. 3(b), 3.75–6.25 μg/ml of pycnogenol minimized the nicking of supercoiled plasmid DNA; the supercoiled form (I) detected was in the range of 50–60% of the total. Pycnogenol alone had no effect on pBR322 DNA [Fig. 3(c)].

DISCUSSION

The oxidative burst assay employed in this study utilizes dichlorofluorescein diacetate as a fluorescent probe. This assay detects mainly superoxide anion and hydrogen peroxide, and to a lesser extent, other reactive oxygen species such as hydroxyl radicals (11). The inhibition of oxidative burst by pycnogenol would suggest its ability to scavenge superoxide anion and hydrogen peroxide.

Copper-dependent oxidation of LDL has been shown to result from direct reduction of Cu^{2+} to Cu^{+} by a reductant associated with the lipoprotein (16,17). Very low concentrations of pycnogenol were capable of inhibiting Cu^{2+}-induced oxidation of LDL [Fig. 2(a)]. The inhibition of LDL oxidation by pycnogenol would suggest its ability to effectively scavenge Cu^{2+} oxidant. Oxidation of LDL has been recognized as one of the pivotal events in plaque formation in blood vessels leading to the development of atherosclerosis (18–20). The ability of pycnogenol to prevent LDL oxidation may thus have several clinical implications including lowering the risk of heart disease, stroke, and hypertension (20).

In this study, three in vitro systems were employed to determine the antioxidant activity of pycnogenol. J774 macrophages were used to detect its effect at the cellular level. Experiments with this cell line involved preincubation of J774 cells with pycnogenol in the culture medium. Oxidative burst reflecting generation of reactive oxygen species was triggered by zymosan and measured with the fluorescent probe. Since the triggering process was carried out after removal of pycnogenol, the suppression of oxidative burst observed

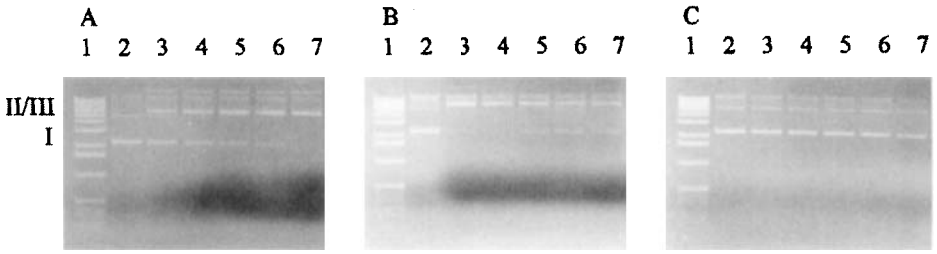

Figure 3. Effects of pycnogenol on iron/ascorbic acid-induced damage of plasmid DNA. (a) Lane 1 = DNA molecular weight marker; lane 2 = untreated pBR322 DNA; lanes 3–7 = pBR322 DNA treated with 5, 10, 20, 30, and 40 pM Fe^{3+}/μM ascorbic acid, respectively. (b) Lane 1 = DNA molecular weight marker; lane 2 = untreated pBR322 DNA; lanes 3–7 = pBR322 DNA treated with 40 pM Fe^{3+}/40 μM ascorbic acid; in addition, they were treated with 1.25, 2.5, 3.75, 5, and 6.25 μg/ml of pycnogenol, respectively. (c) Lane 1 = DNA molecular weight marker; lanes 2–7 = pBR322 DNA treated with 1.25, 2.5, 3.75, 5, 6.25, and 7.5 μg/ml of pycnogenol, respectively, in the absence of Fe^{3+}/ascorbic acid.

in this study would indicate that pycnogenol was able to enter the cells to carry out its protective effect. Among the cellular constituents generally thought to be most sensitive to free radicals are the lipids and proteins in the biomembranes. Oxidation of these macromolecules may be the basis for many toxicological phenomena (21). Previously, we demonstrated that pycnogenol protects lipid oxidation of vascular cell membrane (6). In the present study, we observed that pycnogenol inhibited oxidation of LDL, possibly by direct quenching of Cu^{2+} oxidant.

Another target of free-radical oxidation is the nucleic acid. Relatively low concentrations of pycnogenol (3.75–6.25 μg/ml) minimized the breakage of pBR322 DNA. The iron/ascorbic acid system used in this study generates mainly hydroxyl radicals (14). Our inability to observe a more complete protection of plasmid DNA breakage may suggest that pycnogenol is not as potent a scavenger for hydroxyl radicals as it is for superoxide anion or hydrogen peroxide. Nevertheless, its ability to minimize DNA damage is still of interest.

Pycnogenol used in this study is a patented product consisting of proanthocyanidins extracted from maritime pine (*Pinus maritima*). According to the U.S. patent granted to J. Masquelier (3) of the University of Bordeaux in France, pycnogenol has potent radical scavenging activity. Data from this study confirm this claim. This product has been extensively used in European countries as a dietary supplement because of its value as an antioxidant (4,5). A computer literature search for pycnogenol or procyanidins reveals that only a few research papers have thus far been published. One paper shows that pycnogenol prevents elastin degradation (22) while another paper demonstrates absence of toxicity of procyanidins in a mutagenicity assay (23). Our present study, using three in vitro systems, demonstrates a rather extensive antioxidant activities of pycnogenol at the cellular, lipoprotein, and DNA levels.

In summary, this study demonstrates antioxidant effects of pycnogenol in three in vitro systems. A significant effect was noted in the prevention of oxidative burst of macrophages, oxidation of plasma LDL, and hydroxyl radical-induced breakage of plasmid DNA. The data from this study are encouraging as researchers across the world are searching for ways to prevent the development of free-radical-mediated disorders such as atherosclerosis, arthritis, cancer, and aging. Whether or not pycnogenol may be beneficial for prevention of free-radical-mediated processes awaits further animal and human studies. Such studies are warranted and should be encouraged.

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REFERENCES

1. H. Sies, *Oxidative Stress: Oxidants and Antioxidants*, Academic Press, London, 1991.
2. L. Spatz and A. D. Bloom, *Biological Consequences of Oxidative Stress—Implications for Cardiovascular Disease and Carcinogenesis*, Oxford University Press, New York, 1992.
3. J. Masquelier, Plant extract with a proanthocyanidins content as therapeutic agent having radical scavenging effect and use thereof, United States Patent #4,698,360, 1987.
4. R. A. Passwater, *The New Superantioxidant-Plus*, Keats Publishing Inc., New Canaan, CT 1992, p. 23.
5. R. A. Passwater and C. Kandaswami, *Pycnogenol: The Super "Protector" Nutrient*, Keats Publishing Inc., New Canaan, CT 1994, p. 9.
6. Y. Rong, L. Li, S. Vandana, and B. H. S. Lau, Pycnogenol protects vascular endothelial cells from t-butyl hydroperoxide induced oxidant injury, Biotech Therapeut., 5, 117–126 (1995).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem., 193, 265–275 (1951).
8. B. H. S. Lau, P. Ong, and J. Tosk, Macrophage chemiluminescence modulated by Chinese medicinal herbs *Astragalus membranaceus* and *Ligustrum lucidum*, Phytother. Res., 3, 148–153 (1989).
9. B. H. S. Lau, T. Yamasaki, and D. S. Gridley, Garlic compounds modulate macrophage and T-lymphocyte functions, Mol. Biother., 3, 103–107 (1991).
10. J. R. Rittenhouse, P. D. Lui, and B. H. S. Lau, Chinese medicinal herbs reverse macrophage suppression induced by urological tumors, J. Urol., 146, 486–490 (1991).
11. C. P. Wan, E. Myung, and B. H. S. Lau, An automated micro-fluorometric assay for monitoring oxidative burst activity of phagocytes, J. Immunol. Methods, 159, 131–138 (1993).
12. S. Miura, J. Watanabe, T. Tomita, M. Sato, and I. Tominta, The inhibitory effects of tea polyphenols (flavano-3-ol derivatives) on Cu^{2+} mediated oxidative modification of low density lipoprotein, Biol. Pharm. Bull., 17, 1567–1572 (1994).
13. N. Ide, A. B. Nelson, and B. H. S. Lau, Aged garlic extract and its constituents inhibit Cu^{2+}-induced oxidative modification of low density lipoprotein, Planta Med., 63, 263–264 (1997).
14. J. E. Schneider, M. M. Browning, X. Zhu, K. L.

Eneff, and R. A. Floyd, Characterization of hydroxyl free radical mediated damage to plasmid pBR322 DNA, Mut. Res., 214, 23–31 (1989).
15. J. Onuki, M. H. G. Medeiros, E. J. H. Becharra, and P. Di Mascio, 5-aminolevulinic acid induces single-strand breaks in plasmid pBR322 DNA in the presence of Fe^{2+} ions, Biochim. Biophys. Acta, 1225, 259–263 (1994).
16. S. M. Lynch, and B. Frei, Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein, J. Lipid Res., 34, 1745–1753 (1993).
17. S. M. Lynch and B. Frei, Mechanisms of metal ion-dependent oxidation of human low density lipoprotein, J. Nutr., 126, 1063S–1066S (1996).
18. D. Steinberg, S. Parthasarathy, T. E. Carew, and J. L. Witztum, Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity, N. Engl. J. Med., 320, 915–924 (1989).
19. S. Yla-Herttuala, W. Palinki, M. E. Rosenfeld, D. Steinberg, and J. L. Witztum, Lipoproteins in normal and atherosclerotic aorta, Eur. Heart J., 11, 88–99 (1990).
20. D. Steinberg, Modified forms of low-density lipoprotein and atherosclerosis, J. Int. Med., 233, 227–232 (1993).
21. P. Hochstein, S. K. Jani, and C. Rice-Evans, The physiological significance of oxidative perturbation in erythrocyte membrane lipids and proteins, Prog. Clin. Biol. Res., 55, 449–459 (1981).
22. J. M. Tixier, G. Godeau, A. M. Robert, and W. Hornebeck, Evidence by in vivo and in vitro studies that binding of pycnogenols to elastin affect its rate of degradation by elastases, Biochem. Pharmacol., 33, 3933–3939, 1984.
23. C. L. Yu and B. Swaminathan, Mutagenicity of proanthocyanidins, Food Chem. Toxicol. 25, 135–139 (1987).