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Research Article

Pycnogenol enhances immune and haemopoietic functions in senescence-accelerated mice

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Abstract. Pycnogenol (procyanidin extracted from *Pinus maritima*) has been shown to be a potent free radical scavenger and an antioxidant phytochemical. The effects of pycnogenol on immune and haemopoietic dysfunction in senescence-accelerated mice (SAM), as a murine model of accelerated ageing, were determined. SAMP8, a strain of senile-prone mice, exhibit learning and memory deficits, immunodeficiency and dysfunction of the haemopoietic system. Oral feeding with pycnogenol for 2 months significantly improved their T- and B-cell function. Pycnogenol also augmented the proliferative capacity of haemopoietic progenitors of bone marrow in SAMP8. These data suggest that pycnogenol may be useful for either retardation or restoration of parameters associated with ageing.

Key words. Pycnogenol; immunodeficiency; haemopoietic system; senescence-accelerated mouse; antiageing.

Introduction

Pycnogenol is a blend of oligomeric and monomeric procyanidins isolated from the bark of pine (*Pinus maritima*) [1]. 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 (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 free radical-scavenging activity [2, 3]. It has been shown to lower histamine levels and to protect against increased vascular permeability [4]. Pycnogenol has been shown to modulate the immune responses of retrovirus-infected or ethanol-fed mice in that it reduced the elevated levels of both interleukin-6 and interleukin-10; it also increased the cytotoxicity of natural killer cells [5]. In our laboratory, we demonstrated that pycnogenol can protect vascular endothelial cells from injury induced by an organic oxidant, *t*-butyl hydroperoxide [6]. It can increase the levels of intracellular glutathione and enhance the activities of antioxidant enzymes [7]. More recently we showed that pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation and hydroxyl radical-induced DNA damage [8].

Senescence-accelerated mouse (SAM) was established as a model of accelerated ageing by Prof. T. Taketa [9], including strains that are prone to accelerated senes-

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cence designated as SAMP (senile-prone) and others which are resistant to early ageing, designated as SAMR (senile-resistant). SAMP strains have been documented to exhibit systematic ageing syndromes with advancing age [10, 11]. It was reported that the proliferative activities of haemopoietic progenitors in the bone marrow of SAMP mice declined significantly from 7 months onwards, compared with age-matched SAMR mice [12, 13]. SAMP mice also show age-related immunodeficiency in addition to learning and memory deficits [14, 15]. In this study, the effects of pycnogenol on several immune and haemopoietic functions in SAMP mice were determined.

Materials and methods

Chemicals and reagents. Pycnogenol was provided by Joseph Weiss of Hankel Corporation, La Grange, IL, USA. [^{3}H]thymidine (41 Ci/mmol) was obtained from the Shanghai Institute of Nuclear Research, China. Newborn calf serum and horse serum were purchased from the Beijing Institute of Radiomedicine, China. Concanavalin (ConA), lipopolysaccharide (LPS), RPMI 1640 medium and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Mice. SAMP8 (senile-prone) and SAMR1 (senile-resistant) were provided by Prof. T. Taketa of Kyoto University, Japan. The age of mice used in this study was 7 months. SAMP8 mice were randomly divided into three groups of 10 mice each. Two groups were given pycnogenol daily (5 mg/kg or 10 mg/kg body weight) by intragastric perfusion for 2 months. The third group was given saline daily as controls. Ten age-matched SAMR1 mice were given saline and also served as controls.

Lymphocyte proliferation assay. Proliferation of splenic lymphocytes in response to mitogen, ConA or LPS was performed using the method of [^{3}H]thymidine incorporation as previously described [16]. Briefly, mice were killed, spleens were removed and gently teased with forceps in RPMI 1640 medium supplemented with 10% calf serum, Hepes buffer 10 mmol/l, penicillin 100 units/l, streptomycin 100 mg/l, and L-glutamine 2 mmol/l. Red blood cells were lysed. Nonadherent lymphocytes were harvested, counted, and adjusted to 1×10^7 cells/ml. Cells (0.1 ml/well) were cultured in triplicate in 96-well flat-bottomed culture plates. ConA or LPS diluted with the culture medium was added to each well to yield a final concentration of 5 mg/l and 20 mg/l, respectively. The culture was incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 54 h, and [^{3}H]thymidine (0.5 μCi/well) was then added and incubated for another 16 h. Cells were harvested onto glass fiber filters, and the uptake of radioactivity was determined in a liquid scintillation counter.

Plaque-(antibody) forming cell (PFC) assay. The assay was carried out as previously described [17]. Mice were immunized by intraperitoneal injection of 10% (v/v in saline) sheep red blood cells (SRBC), 0.2 ml/mouse, 4 days before the PFC assay. On the testing day, the spleen was removed, and splenocyte suspension was prepared in complete RPMI 1640 medium. The cell suspension (0.2 ml) was mixed with 0.025 ml of complement (1:1 v/v fresh guisa pig serum) and 0.025 ml of 10% SRBC. The mixture was added to Cunningham's chamber and incubated at 37 °C for 1 h. The haemolytic plaques formed in the chamber were counted under light background, and the result was expressed as the number of PFC/10^6 splenic lymphocytes.

Preparation of bone marrow cells (BMC). Mice were killed, and the femurs were aseptically removed and flushed with complete RPMI 1640 medium. The cells were dispersed by passing through needles and syringes with small pores, and the numbers of cells were counted with a haemocytometer.

Assay of colony-forming unit of granulocytes/macrophages (CFU-GM). For in vitro haemopoietic colony-forming assay, the technique of Pike and Robinson [18], modified by O'Hara et al. [19], was used. Semisolid-agar colony cultures were set up using 35-mm tissue culture dishes. One millilitre of the culture in each dish contained 1×10^5 BMCs, complete RPMI 1640 medium, 25% horse serum and 0.3% agar. Colony formation was stimulated by the addition of 0.2 ml of mouse lung conditioned medium (prepared according to Burgess et al. [20] and used as a source of granulocyte-macrophage colony-stimulating factor (GM-CSF)). Cultures were incubated at 37 °C in a 5% CO_2 incubator. Dishes were removed after 7 days and counted. Colonies, defined as 50 or more cells, were counted under a dissecting microscope.

Assays of erythroid colony-forming unit (CFU-E) and erythroid burst-forming unit (BFU-E). The microvolume methyl cellulose culture method was employed [21, 22]. Briefly, BMCs were seeded at 5×10^4 cells per well in 1% methyl cellulose medium containing 30% newborn calf serum, 0.9% deionized bovine serum albumin (fraction V), 10% phytohaemagglutinin and 20 μM 2-mercaptoethanol. BMCs were plated in a 24-well flat-bottomed microplate, incubated in a humidified atmosphere of 5% CO_2 at 37 °C for either 3 days (for CFU-E) or 8 days (for BFU-E), when erythroid colonies or bursts, respectively, were scored under a dissecting microscope. The colonies and bursts were stained in situ with benzidine according to the method of Ogama et al. [23]. Groups of eight or more cells exhibiting red or gold colour, which were benzidine-positive, were counted as colonies derived from CFU-E. After cultures were incubated for 8 days, groups of 50 or more red-coloured cells were counted as bursts derived from BFU-E.

Table 1. Effects of pycnogenol on proliferation of splenic lymphocytes.

Animal groups	Pycnogenol (mg/kg)	[^{3}H]Thymidine incorporation (cpm)		
		No mitogen	ConA (5 μg/ml)	LPS (20 μg/ml)
SAMR1	0	3846 ± 701	63079 ± 5203	57957 ± 2315
SAMP8	0	2940 ± 351*	32693 ± 2782*	25651 ± 814*
SAMP8	5	4988 ± 425†	45425 ± 3671†	50171 ± 4925†
SAMP8	10	5695 ± 245†	51685 ± 1696†	52205 ± 4780†

Data are expressed as means ± SEM. * $P < 0.01$ compared with SAMR1. † $P < 0.01$ compared with SAMP8 without pycnogenol treatment.

Statistical analysis. All parameters were compared using two sample t tests assuming unequal variances between any two groups. The probability level, $P < 0.05$, was considered a statistically significant difference between two groups.

Results

The splenocyte proliferation stimulated by ConA (for T cells) or LPS (for B cells) in SAMP8 mice was declined significantly ($P < 0.01$), compared with age-matched SAMR1 (table 1). Feeding pycnogenol at 5 mg/kg and 10 mg/kg significantly enhanced the proliferation of T cells and B cells, stimulated by ConA and LPS, respectively, in SAMP8 mice.

Figure 1 shows the effects of pycnogenol on the numbers of PFCs in SAM mice. Compared with the SAMR1 mice, the SAMP8 mice had significantly reduced numbers of PFCs. The numbers of PFCs in SAMP8 mice fed with either 5 mg/kg or 10 mg/kg of pycnogenol were significantly increased, compared with the SAMP8 control group without pycnogenol treatment. The effects of pycnogenol on the proliferative capacity of granulocyte/macrophage progenitors in bone marrow are shown in figure 2. The number of CFU-GMs was measured by using colony culture techniques in vitro, which reflected the proliferative capacity of granulocyte/macrophage progenitors in the bone marrow. The number of CFU-GMs in the SAMP8 group was significantly lower than that of SAMR1. The number of CFU-GMs in SAMP8 mice fed with either 5 or 10 mg/kg of pycnogenol was significantly elevated

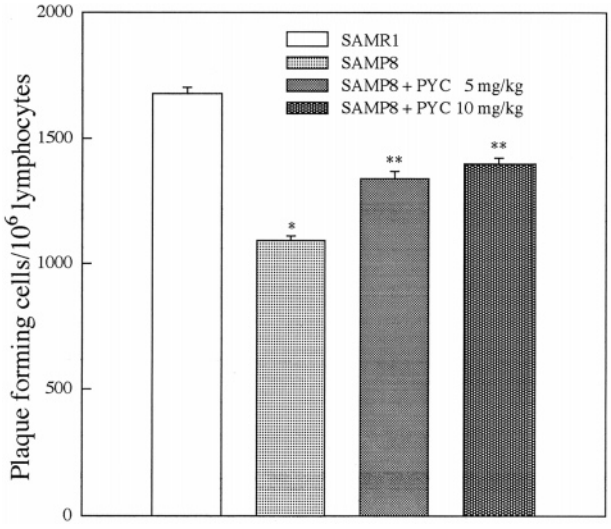

Figure 1. Effects of pycnogenol on PFCs in SAM mice. Pycnogenol (5 mg/kg or 10 mg/kg) or saline was administered daily for 2 months. Four days before the end of 2 months, mice were immunized with sheep erythrocytes. Assays were performed 4 days later. Data are expressed as means ± SEM ($n = 10$). *$P <$ 0.01 compared with SAMR1. **$P < 0.01$ compared with SAMP8 without pycnogenol treatment.

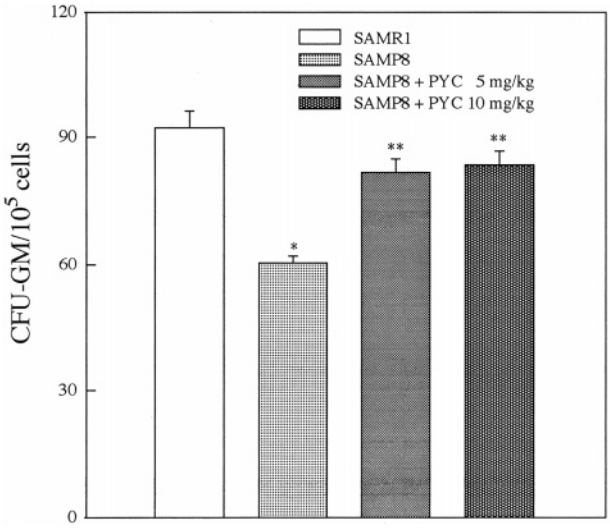

Figure 2. Effects of pycngenol on CFU-GM in SAM mice. Pycnogenol (5 mg/kg or 10 mg/kg) or saline was administered daily for 2 months. Data are expressed as means ± SEM ($n = 10$). *$P < 0.01$ compared with SAMR1. **$P < 0.01$ compared with SAMP8 without pycnogenol treatment.

above those of the SAMP8 control group without pycnogenol treatment.

Figure 3 shows the effects of pycnogenol on late erythroid progenitors measured by testing for CFU-E. The number of CFU-Es was significantly reduced in SAMP8 ($P < 0.01$), compared with age-matched SAMR1. Feeding SAMP8 mice with pycnogenol restored the CFU-E level to that of SAMR1.

Figure 4 shows the effects of pycnogenol on early erythroid progenitors of bone marrow, measured by testing for BFU-E. The number of BFU-Es was significantly reduced in SAMP8 ($P < 0.01$) compared with age-matched SAMR1. Pycnogenol treatment significantly augmented the number of BFU-Es in SAMP8 mice. These increases were significant compared with the SAMP8 control group without pycnogenol treatment.

Discussion

Ageing is a general phenomenon that occurs in all individuals of the population. The ageing mechanism is very complicated, and still not completely elucidated. Various theories have been proposed to account for the ageing process – such as molecular cross-linking [24], changes in immunological function [25], damage by free radical reactions [26] and expression of senescence genes [27]. Harman [26] first proposed the free radical theory of ageing, and stated that the ageing process may be simply the sum of random changes produced by free radical reactions. A great deal of research has since accumulated to support this theory [28]. Oxidative damage to DNA, proteins and other macromolecules has been shown to accumulate with age [29–31]. Free radicals also appear to play a major role in various disorders such as heart disease, cancer, decline of immune functions and neurodegenerative diseases [32–34].

Pycnogenol used in this study is a patented product consisting of proanthocyanidins extracted from french maritime pine (*Pinus maritima*). Studies have shown that pycnogenol has potent radical-scavenging activity [1, 7, 8]. Cheshier et al. reported that pycnogenol can modulate immune dysfunction in mice fed with ethanol or infected with the LP-BM5 murine retrovirus [5]. Our present experiment, using a murine model of accelerated senescence, demonstrated that pycnogenol can restore depressed immune and haemopoietic functions associated with ageing.

Our data show that B- and T-lymphocyte response to mitogens is severely depressed in SAMP8 mice. Feeding these mice with as little as 5 mg/kg body weight of pycnogenol extract for 2 months significantly improved this depression. SAMP8 mice also have a reduced number of antibody-forming cells. Pycnogenol feeding significantly increased the number of antibody-forming cells, further confirming its ability to augment B-lymphocyte function in aged animals.

In this study, we used in vitro colony culture techniques to measure the number and proliferative capacity of

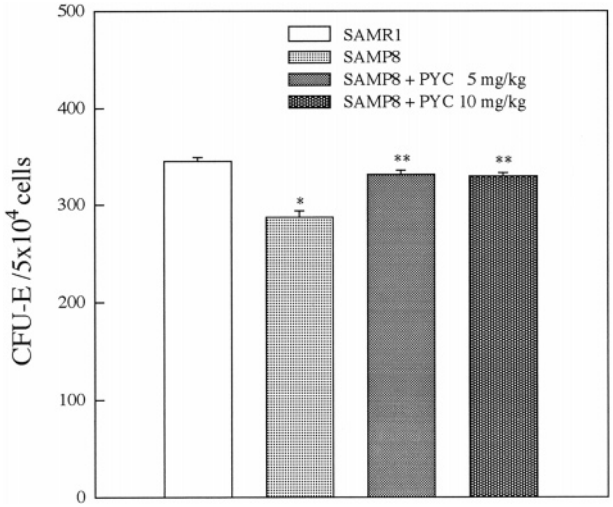

Figure 3. Effects of pycnogenol on CFU-E in SAM mice. Pycnogenol (5 mg/kg or 10 mg/kg) or saline was administered daily for 2 months. Data are expressed as means ± SEM ($n = 10$). *$P < 0.01$ compared with SAMR1. **$P < 0.01$ compared with SAMP8 without pycnogenol treatment.

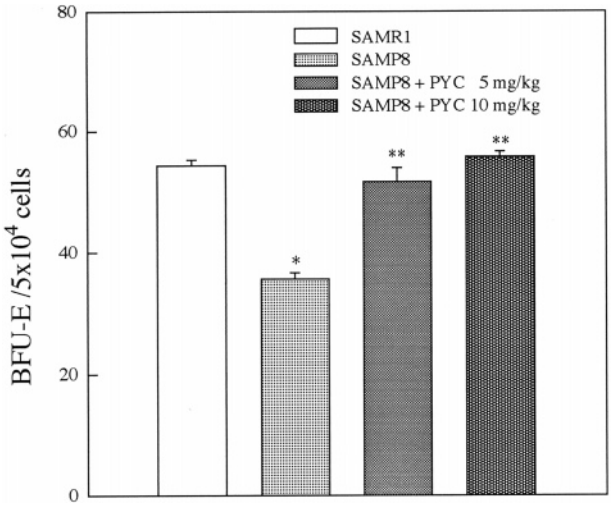

Figure 4. Effects of pycnogenol on BFU-E in SAM mice. Pycnogenol (5 mg/kg or 10 mg/kg) or saline was administered daily for 2 months. Data are expressed as means ± SEM ($n = 10$). *$P < 0.01$ compared with SAMR1. **$P < 0.01$ compared with SAMP8 without pycnogenol treatment.

haemopoietic progenitors in the bone marrow. As noted in figures 2, 3 and 4, there is a significant decrease in the numbers of CFU-GM, CFU-E and BFU-E in SAMP8 mice. Feeding with pycnogenol significantly restored these parameters, indicating that this phytochemical can improve the haemopoietic functions of bone marrow in aged mice by enhancing the proliferative capacity of their progenitors.

In conclusion, this study demonstrated a potent modulating effect of pycnogenol on immune and haemopoietic systems in SAMP8, senile-prone mice as a model of ageing. In addition to its antioxidant activity previously reported, this study suggests that pycnogenol may play an important role in the retardation or restoration of depressed immune and haemopoietic functions associated with ageing. Whether or not this antiageing property also occurs in human subjects taking pycnogenol supplements awaits further clinical study.

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