

Pycnogenol Supplementation Retards Immune Dysfunction in Murine AIDS (MAIDS) After LP-BM5 Leukemia Virus Infection by Modulating Cytokine Secretion

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Abstract

We investigated the effect of pycnogenol (PYC) supplementation on retarding the immune dysfunction of C57BL/6 mice after murine AIDS (MAIDS) development. Dysfunction of T and B cell mitogenesis from primary cultured splenocytes has been observed with retrovirus infection and PYC supplementation partially recovered the dysfunction of T and B cells. There was an abnormal shift of cytokine pattern with retrovirus infection, which was designated by the decreased secretion of Th1 cytokines and increased secretion of Th2 cytokines. PYC supplementation increased IL-2 and IFN- γ secretion and decreased IL-4, IL-6, and TNF- α secretion, but it was not sufficient enough to maintain the normal level of these cytokines. Hepatic vitamin E level was significantly decreased by retrovirus infection, in accordance with increased hepatic lipid peroxidation level, whereas PYC supplementation normalized the hepatic level of vitamin E and lipid peroxidation. This study suggests that PYC supplementation may partially help retard the incidence of symptoms during MAIDS.

Key words: pycnogenol, murine AIDS, cytokines, mitogenesis, lipid peroxidation

INTRODUCTION

The infection with HIV has substantial impact on nutritional status which influences the course of HIV disease progression and survival. The people with HIV/AIDS often suffer from malnutrition due to increased nutritional requirement, inadequate intake, and poor absorption (1). The resulting nutritional deficits further compromise the immune system associated with development of opportunistic infection. Among nutritional elements deficient in HIV/AIDS, the body of work includes a variety of micronutrients and antioxidants. Decreased consumption of micronutrients and antioxidants leads to immune dysfunction with impairment of defense mechanisms and lowers tissue levels of antioxidants. These effects should exacerbate immune deficiencies induced by retrovirus infection. Increased production of cellular oxidants and decreased concentration of antioxidants or antioxidant enzymes occur during HIV infection. Thus antioxidant supplementation should retard development of nutritional deficiencies and their accentuation of retrovirus-induced immune dysfunction.

Pycnogenol (PYC), a standardized extract from the French maritime pine bark (*Pinus pinaster* Ait.), consists of a concentrate of polyphenols whose main constituents are procyanidins, pharmacologically active biopolymers composed from units of catechin and epicatechin. In addition, PYC contains the bioflavonoids catechin and taxifolin and a number of phenolic acids (2). It has been known that PYC exhibits efficient antioxidant activity (3,4) and it may act as a modulator of metabolic and antioxidant enzymes (3) and other cellular functions. Several studies have investigated that PYC inhibits LDL oxidation, lipid peroxidation in phospholipid liposomes, lipid peroxidation caused by t-butylhydroperoxide, and inflammation in capillary tissues (4,5).

LP-BM5 murine retrovirus infection in mice produces immune dysfunction, antioxidant nutrient deficiencies, and increased oxidation much as HIV does in humans. Non-nutritive dietary antioxidants like bioflavonoids could play a role in prevention of oxidation and its effects on immune dysfunction, but are unstudied in retrovirus-infected people or animals. Therefore, supplementation with PYC as an antioxidant was assessed for

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immune modulation during oxidation and immune dysfunction produced by a LP-BM5 murine retrovirus.

MATERIALS AND METHODS

Animals and treatments

Female C57BL/6 mice, 4-weeks-old, were obtained from Charles River Laboratories (Wilmington, DE). They were housed in transparent plastic cages with stainless wire lids (4 mice per cage) in the animal facility of the Arizona Health Science Center. The animal use was approved by the University of Arizona Committee on Animal Research. The housing facility was maintained at 20–22°C and 60–80% relative humidity with a 12-hour light:dark cycle.

Mice were randomly assigned to one of uninfected or infected groups with PYC supplementation or non-supplementation by oral administration. PYC was generously donated from Horphag Research Ltd. (Guernsey, FRA). Feeding began at 2 weeks post-infection with LP-BM5 murine leukemia retrovirus and continued for 8 weeks for immunological analysis.

LP-BM5 murine leukemia retrovirus was administered intraperitoneally in 0.1 mL of minimum essential medium with an esotropic titer (XC) of 4.5 log₁₀ plaque forming units $\times 10^{-3}$ /L, which induces disease with a time course comparable with that previously published (6). Infection of female C57BL/6 mice with LP-BM5 retrovirus leads to the rapid induction of clinical symptoms with virtually no latent phase. The mice were infected two weeks prior to initiation of diet supplementation as done previously in numerous studies (6,7). The infection and treatment period for immunological analysis was 10 weeks for all groups.

When MAIDS had developed, all mice were sacrificed while ethyl ether anesthesia. Spleens and lymph nodes were dissected and kept at 4°C. Livers for vitamin E and lipid peroxidation analysis were collected and stored at -70°C until assayed.

ELISA assay for cytokines

Production of IL-2, IL-4, IL-6, IFN- γ , and TNF- α from mitogen-stimulated splenocytes was determined as described previously (8). Briefly, spleens were gently teased with forceps in culture medium (RPMI 1640 CM containing 10% fetal bovine serum, 2 mmol/L glutamine, 1×10^5 units/L of penicillin and streptomycin), producing suspension of spleen cells. Red blood cells were eliminated by the addition of a lysis buffer (0.16 mol/L ammonium chloride tris buffer, pH 7.2) at 37°C for 3 min. Then the primary cells were washed twice with culture medium. Cell concentrations were counted and adjusted to 1×10^7 cells/mL. The cell viability was >95% as de-

termined by trypan blue exclusion. The primary splenocytes [0.1 mL/well (1×10^7 cells/mL)] were cultured in triplicate on 96-well flat-bottom culture plates (Falcon 3072, Lincoln Park, NJ), and were then stimulated with concanavalin A (Con A, 1×10^{-2} g/L, 0.1 mL/well, Sigma, St. Louis, MO) to determine their production of IL-2 and IL-4 after 24 hour incubation and IFN- γ after 72 hour incubation in a 37°C, 5% CO₂ incubator. Splenocytes were also simultaneously incubated for 24 hour after the addition of lipopolysaccharide (LPS, 1×10^{-2} g/L, 0.1 mL/well, GibcoBRL, Grand Island, NY) to induce IL-6 and TNF- α production. After incubation, supernatants were collected and stored at -70°C until analysis. The cytokines were determined by sandwich ELISA.

Mitogenesis of splenocytes

Splenic T and B cell proliferation was determined by ³H-thymidine incorporation as described previously (8). Briefly, splenocytes in 0.1 mL of culture medium (1×10^7 cells/mL) were cultured in 96-well flat-bottom culture plates (Falcon) with Con A (10 μ g/mL) and LPS (10 μ g/mL), were incubated at 37°C, 5% CO₂ incubator for 24 hour, and then pulsed with ³H-thymidine (0.5 μ Ci/well, New England Nuclear, Boston, MA). After 24-hour incubation, they were harvested by a cell sample harvester (Cambridge Technology, Cambridge, MA). Radioactivity was determined by a liquid scintillation counter (Tri-Carb, 2200 CA, Packard, Laguna Hills, CA). Data were collected as counts per minute (cpm).

Measurement of lipid peroxidation

Lipid peroxidation (LPO) in liver was measured by K-Assay LPO-CC Assay Kit obtained from Kamiya Biomedical Company (Seattle, WA). This method has previously been shown to be a more sensitive lipid peroxide measurement (assay range: 2–300 nmol/mL) than conventional chemical analysis (9). Briefly, ~0.2 g of liver tissue was homogenized in 3 mL of CHCl₃/methanol (2:1, v/v). 0.6 mL of 0.9% NaCl was added to clarify and the mixture was centrifuged at $3,000 \times g$ for 10 min. The supernatant was discarded while bottom layer that includes CHCl₃ was evaporated under N₂ gas. 100 μ L of isopropanol was added to dissolve lipid residue and 20 μ L of sample was used to measure lipid peroxides with the LPO kit. In the presence of hemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols) and the MCDP (10-N-methylcarbamoyl-3, 7-dimethylamino-10 H-phenolthiazine) chromagen is oxidatively cleaved to form methylene blue in an equal molar reaction. Lipid peroxides are quantitated by colorimetrically measuring the methylene blue at 675 nm. Lipid peroxidation values were calculated by the equa-

tion given from the manual. LPO value was converted to the percent unit for illustration.

Determination of vitamin E

Hepatic vitamin E levels were measured by HPLC as described previously (10). Briefly, ~0.2 g of tissues was homogenized in 1 mL of water. Butylated hydroxytoluene (BHT) was added to prevent oxidation of α -tocopherol. Pentane, ethanol, and sodium dodecyl sulfate were used to extract α -tocopherol from the homogenate. Extracts were evaporated under steady flow of nitrogen gas at 20°C and then redissolved in 0.5 mL of methanol injection onto a C₁₈ column (3.9×150-mm NovaPak, Millipore, Bedford, MA). A mobile phase composed of methanol and 1 mol/L sodium acetate in the ratio of 98:2 (v/v) at a flow rate of 1.5 mL/min was used. α -Tocopherol with retention time at about 5 min, was monitored by a fluorescence detector (Millipore, Bedford, MA) at 290-nm excitation and 320-nm emission wavelength.

Statistics

Mean differences between treatment groups of PYC diets and the control groups were tested with two-tailed t-test within retrovirus category (positive or negative). Test statistics were considered significant at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Body weight and spleen weight

There was significant difference in body weight between retrovirus-infected group and uninfected control. Spleen and lymph node weights (8 weeks post infection) were significantly ($p < 0.05$) elevated in infected mice (data not shown). Such changes indicated that infection had progressed to severe immunodeficiency and aberrant cell accumulation or proliferation (6).

Mitogenesis of primary splenocytes

For uninfected groups, *in vitro* B cell proliferation stimulated by LPS showed no significant difference by PYC supplementation (Fig. 1A). In contrast, retrovirus-infected group represented the significantly impaired ability of B cell to proliferate as compared to uninfected groups with same diet ($p < 0.05$). PYC supplementation in infected group inhibited impairment in B cell proliferation, indicating that PYC may influence on the ability of B cell to proliferate after MAIDS development. Similar results have been obtained on T cell proliferation in both uninfected and retrovirus-infected group with PYC supplementation (Fig. 1B). In fact, a similar study by Cheshier et al. (11) has been performed to demonstrate the effect of PYC supplementation on murine retrovirus-infected and ethanol-fed mice. The difference compared

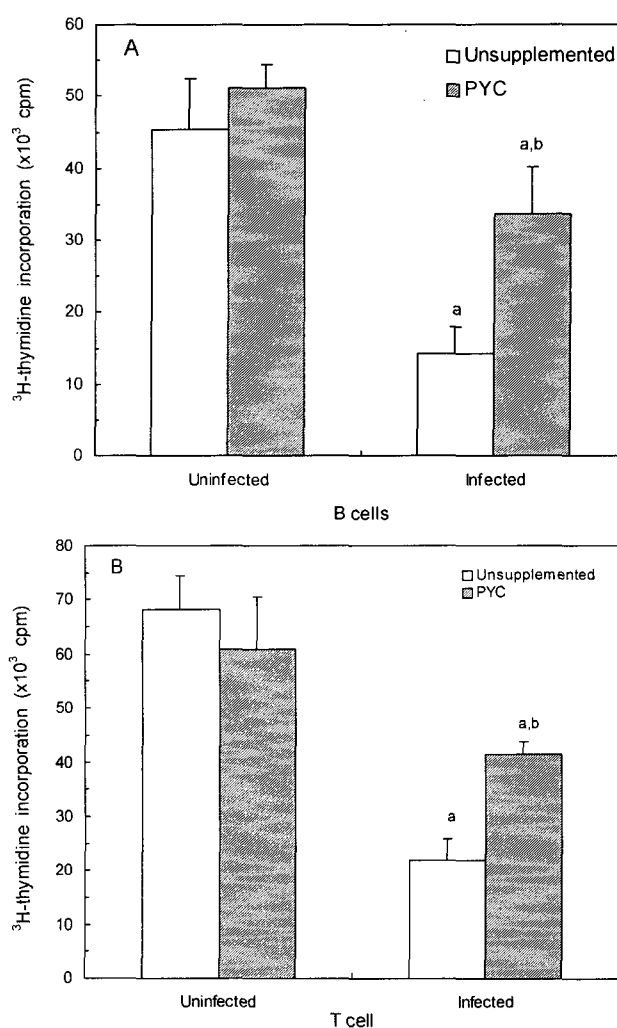


Fig. 1. Effect of pycnogenol supplementation on T and B cell mitogenesis *in vitro*. Data are presented as mean \pm SD of triplicate wells. ^a $p < 0.05$ in comparison with uninfected control mice, ^b $p < 0.05$ in comparison with infected controls.

to our study is that an author began to feed mice simultaneously with retrovirus infection, which could state how PYC supplementation affect the progression to murine AIDS. In our study, PYC supplementation started feeding mice at 2 weeks after murine retrovirus infection when symptoms of MAIDS already developed. This study would inform of how PYC supplementation affects immune system in MAIDS. The function of T and B cell was more active than that of unsupplemented group. LP-BM5 retrovirus infection mainly leads to murine AIDS by two possible mechanisms, immune dysregulation and excessive free radical production. The immune dysregulation involves the loss of T and B cell mitogenesis. Liu et al. (12) suggested that oral feeding with PYC for 2 months significantly improved T- and B-cell function in senescence-accelerated mice (SAM). In addition, the author found that PYC augmented the proliferative capacity of haemopoietic progenitors of bone

marrow in SAM. These results indirectly support our results because LP-BM5-infected mice take a similar course of ageing due to excessive oxidative burden.

Cytokine production by splenocytes

In vitro production of IL-2 by Con A-stimulated splenocytes was significantly ($p < 0.05$) reduced by retrovirus infection and increased by PYC supplementation (Table 1). While PYC supplementation did not affect IFN- γ secretion in uninfected group, it inhibited reduction of IFN- γ secretion ($p < 0.05$). Release of TNF- α , IL-4, and IL-6, by LPS-stimulated primary splenocytes was significantly ($p < 0.05$) increased by retrovirus infection (Table 2). Intake of PYC reduced IL-4, IL-6, and TNF- α production post MAIDS development but did not affect on uninfected group.

Cytokines are regulatory polypeptides secreted during the generation of an immune response by lymphocytes, macrophages, endothelial cells, as well as a variety of other cell types (13). There is a shift from balanced Th1/Th2 cell secretion of cytokines into increased Th2 (IL-4, IL-6, and TNF- α) and decreased Th1 (IL-2 and IFN- γ) cell cytokine production during development of MAIDS (14). It was demonstrated by the study of blocking Th2 cell activation and its excessive cytokine production that should retard development of MAIDS. In the study of IL-4 gene knockout mouse with suppressed Th2 cytokine production after LP-BM5 retrovirus infection, the usual lethality and the development of T-cell abnormalities were delayed (15). In another study, administration of

anti-IL-4 monoclonal antibody in LP-BM5 retrovirus-infected mice also maintains balance in Th1 and Th2 responses, preventing retrovirus-induced suppression of immune responses (16). Our data show that PYC treatment significantly reduced Th2 type cytokine production by splenocytes from retrovirus-infected mice compared to elevated levels in untreated retrovirus-infected mice. This finding is consistent with the observation by Cheshier et al. (11), but the extent of reduction was much greater than that of our data, suggesting that PYC treatment may be less effective in mice already developed MAIDS than mice in progression to MAIDS.

Decreased cell division was expected due to reduced release of IL-2, a major T-cell growth factor. Increased immunoglobulin production during murine retrovirus infection is due to increased number of B cells stimulated by heightened IL-4 secretion. However, our data indicate increased B cell proliferation by PYC supplementation that decreased IL-4 level. It is clear that B-cell proliferation was determined by stimulation of LPS, while IL-4 secretion was induced by Con A. Thus, IL-4 production did not affect the B-cell proliferation in our *in vitro* study.

Hepatic lipid peroxidation and vitamin E levels

Murine retrovirus infection significantly ($p < 0.05$) increased hepatic lipid peroxidation (Fig. 2). PYC supplementation inhibited hepatic lipid peroxidation in both LP-BM5 retrovirus-infected and uninfected group by 68% and 24%, respectively. While hepatic vitamin E level was increased with PYC supplementation in uninfected group, it was decreased during murine retrovirus

Table 1. Effect of PYC supplementation on Th1 type cytokine production by primary cultured splenocytes

Treatment		Cytokines (ng/mL)	
LP-BM5	Pycnogenol	IL-2	IFN- γ
-	-	12.1 \pm 2.3 ¹⁾	21.6 \pm 2.8
-	+	10.6 \pm 1.8	24.9 \pm 3.7
+	-	1.56 \pm 0.5*	4.8 \pm 1.3*
+	+	5.23 \pm 1.1* [#]	14.4 \pm 3.4* [#]

¹⁾Data are presented as mean \pm SD of triplicate wells.

* $p < 0.05$ in comparison with uninfected control mice.

[#] $p < 0.05$ in comparison with infected controls.

Table 2. Effect of PYC supplementation on Th2 type cytokine production by primary cultured splenocytes

Treatment		Cytokines (ng/mL)		
LP-BM5	Pycnogenol	IL-4	IL-6	TNF- α
-	-	2.08 \pm 0.4 ¹⁾	4.21 \pm 1.7	4.65 \pm 0.7
-	+	2.58 \pm 1.2*	4.45 \pm 0.9	5.44 \pm 1.2*
+	-	10.4 \pm 2.7*	18.2 \pm 3.4*	20.3 \pm 4.3
+	+	6.77 \pm 1.9* [#]	13.7 \pm 3.1* [#]	14.1 \pm 3.4* [#]

Data are presented as mean \pm SD of triplicate wells.

* $p < 0.05$ in comparison with uninfected control mice.

[#] $p < 0.05$ in comparison with infected controls.

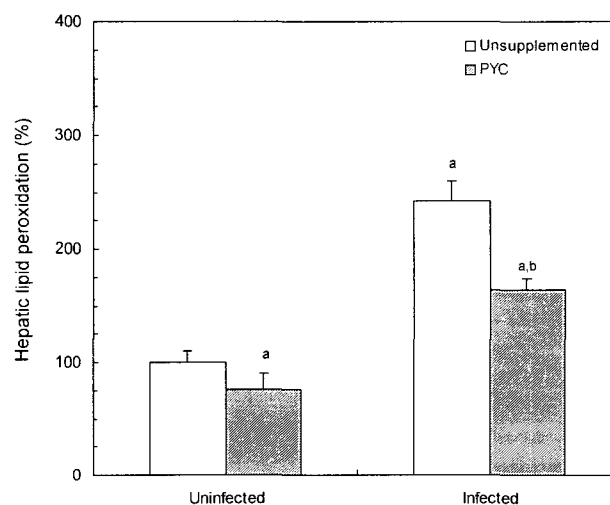


Fig. 2. Effect of pycnogenol supplementation on hepatic lipid peroxidation. Data are converted to percent unit (% = Value of treatment group/value of control group \times 100). ^a $p < 0.05$ in comparison with uninfected control mice, ^b $p < 0.05$ in comparison with infected controls.

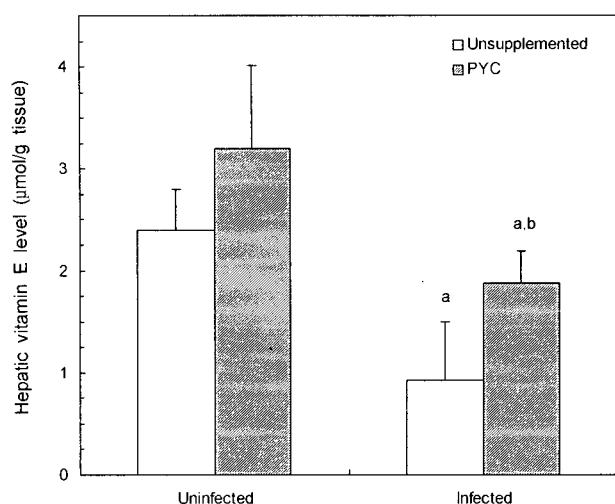


Fig. 3. Effect of pycnogenol supplementation on vitamin E levels in liver. Data are presented by mean \pm SD of triplicate wells. ^a $p < 0.05$ in comparison with uninfected control mice, ^b $p < 0.05$ in comparison with infected controls.

infection due to increased free radicals, resulting in increased tissue lipid peroxidation (Fig. 3). PYC supplementation in retrovirus-infected group significantly ($p < 0.05$) increased hepatic vitamin E level.

Oxidative stress due to reduced levels of antioxidants may be a potent inducer of murine retrovirus replication in addition to DNA damage in virus-infected cells, producing one of the long-term consequences of retrovirus infection such as immunosuppression (17,18). Increased free radicals produced during MAIDS could stimulate utilization of antioxidants including membrane vitamin E for increased lipid peroxidation (19). Pro-inflammatory cytokines (IL-1, IL-6, and TNF- γ) and reactive oxygen species (ROS) are mutually stimulatory (20). The stimulation of cytokine production by ROS involves activation of nuclear factor kappa B (NF- κ B) that induces retrovirus replication. Attack by ROS results in the detachment of the inhibitory component (i κ B) from the NF- κ B complex, which results in transcription of genes for synthesis of pro-inflammatory cytokines. PYC has been well known to have a potent antioxidative capacity that is caused by flavonoid components. Bito et al. (21) reported that PYC inhibited TNF- α production by blocking NF- κ B expression, which supports that reduced lipid peroxidation with PYC supplementation might be correlated to reduced ROS level and Th2 cytokines in this study.

ACKNOWLEDGEMENT

We would give many thanks to Dr. Ronald R. Watson at Arizona Prevention Center in University of Arizona, Tucson for supporting this research and animal facilities.

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(Received March 10, 2005; Accepted May 17, 2005)