Inhibitory Effect of Lutein and Pycnogenol on Lipid Peroxidation in Porcine Retinal Homogenate

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Summary This study aimed to investigate the antioxidant effect of lutein, human macula pigment, and pycnogenol, which contains several flavonoids, on lipid peroxidation induced in 10% porcine retinal homogenate by the addition of 1 mM Ferric chloride (FeCl₃), 50 mM 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), or 25 mM 2,2'-azobis (2,4-dimethyl-valeronitrile) (AMVN). Lipid hydroperoxide concentration was determined from the amount of thiobarbituric acid reactive substances (TBARS) in the sample following treatment. After 60 min of oxidation with FeCl₃, AAPH and AMVN, the TBARS content in the retinal homogenates increased from 28.6 ± 1.6 to 85.4 ± 0.9 , from 27.9 ± 1.2 to 57.2 ± 1.1 , and from 26.0 ± 1.0 to 77.5 ± 2.0 nmol MDA/mg protein, respectively. Lutein did not show remarkable antioxidant activity in this experimental system. However, IC₅₀ of pycnogenol for TBARS formation was decreased by combining 10 μ M lutein in each initiator; from 12 to 5 μ g/mL in FeCl₃, from 2.8 to 0.5 μ g/mL in AAPH, from 465 to 110 μ g/mL in AMVN. These results suggested that a combination treatment of lutein and pycnogenol is more effective for inhibiting lipid peroxidation in porcine retinal homogenate. This synergy might be due to efficient functional antioxidants acting in both hydrophilic and lipophilic cellular environments.

Key Words: lipid peroxide, lutein, pycnogenol, retina

Introduction

Oxydative stress is the cause of several retinal diseases of the eye [1], such as diabetic retinopathy [2], age-related macular degeneration (AMD) [3], and retinopathy of prematurity [4]. All of these conditions are closely correlated with an increase in lipid peroxides [5–9]. Accordingly, ascorbate and glutathione are present at relatively high concentration in the retina of most species and play an

important protective role against oxidative stress [10]. In addition, yellow pigments in the primate macula (*macula lutea*) prevent photochemical-reaction induction by short-wave (blue) visible light in the retina [11]. These macula pigments consist of a mixture of two isomeric dihydroxycarotenes, zeaxanthin and lutein. Lutein also occurs in the epithelium and cortex of the lens, and acts to quench singlet molecular oxygen [12], scavenge oxygen species, and inhibit 2,2'-azobis (2,4-dimethyl-valeronitrile) (AMVN, lipophilic radical initiator)-initiated peroxidation of linoleic acid *in vitro* [13]. Therefore, lutein may play a role in slowing the age-related degeneration of the eye, both directly as an antioxidant and indirectly by absorbing blue light [14].

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The maritime pine-bark extract, pycnogenol has been used in traditional medicine in Europe and North America for treating inflammatory diseases and for wound healing, amongst other uses [15, 16]. Recent studies have revealed that clear that pycnogenol has antioxidant activity [17–19]. The main constituents of pycnogenol are phenolic compounds, monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids (classified as procyanidines/proanthocyanidines) [20, 21]. We previously reported an *in vitro* assay for measuring the antioxidant activity of single compounds against free-radical-induced lipid peroxidation, and observed that pycnogenol decreased this process [22, 23].

The objective of the present study was to identify the efficacy of lutein and pycnogenol as potential therapeutic agents for preventing of oxidative stress in certain retinal pathologies. We evaluated the antioxidant activity of lutein alone, and combination with pycnogenol in a porcine retinal homogenate model. We also compared the IC₅₀ of pycnogenol with or without lutein for TBARS formation induced by ferric chloride, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH, hydrophilic radical initiator), or AMVN (lipophilic radical initiator).

Materials and Methods

Retinal homogenate preparation

One hundred and fifty porcine retina were obtained from a local slaughterhouse, placed on ice in a light-tight container and transported to the laboratory within 1 hr to the laboratory for sample preparation and analysis.

The anterior segment and vitreous were removed. The retinas were collected, cut into dice, homogenized with 10 vol/g of cold distilled water in a glass-teflon homogenizer, and sonicated (Branson Sonifier Cell Disruptor 200, Branson, MI, USA) with 5-second bursts at 20 W in an ice bath.

Incubation conditions

Ten μL of 1 mM ferric chloride (FeCl_3 , Sigma Chemical Co., St. Louis, MO, USA), 50 mM 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH, Wako Pure Chemicals, Tokyo, Japan), or 25 mM 2,2'-azobis (2,4-dimethyl-valeronitrile) (AMVN, Wako Pure Chemicals, Tokyo, Japan) as initiator were added to 170 μL of retinal homogenate and analyzed by the thiobarbituric acid (TBA) assay after 60 min of incubation at 37°C [22–25]. For the basal level of TBA reactive substances (TBARS) in retinal homogenate, 10 μL of distilled water, or ethanol was added to 170 μL of retinal homogenate and analyzed by the TBA assay after 60 min of incubation at 37°C.

Various concentrations of lutein (DSM Nutritional Products, Basel, Switzerland) or/and pycnogenol (Biolandes

Aromes, Le Sen, France) were added simultaneously with the initiators and the retinal homogenate were reassayed to determine the extent of inhibition. The total volume of reaction mixture was 200 μL .

Lutein was resuspended in dimethyl sulfoxide (DMSO, Wako Pure Chemicals, Tokyo, Japan). AMVN was resuspended in ethanol. The other inhibitors were resuspended in distilled water.

Mesurements of thiobarbituric acid reactive substances

Lipid peroxide concentration was determined using a modification of the methods described by Yagi, which measured the amount of thiobarbituric acid reactive substances (TBARS) [26]. The reaction mixture contained 0.2 mL of sample, 1.2 mL of 1% phosphoric acid (Wako Pure Chemicals, Tokyo, Japan) and 0.4 mL of 0.6% thiobarbituric acid (Wako Pure Chemicals, Tokyo, Japan). The mixtures were incubated at 100°C for 1 hr, and cooling for 15 min in an ice bath. L-butanol (1.5 mL) (Wako Pure Chemicals, Tokyo, Japan) was added and mixed for 5 seconds. After centrifugation at 3,000 rpm for 10 min, 0.1 mL of the solvent layer was removed by pipetted off, and the absorbance was measured in a spectrophotometer (Ultrospec 3300 Pro, Amersham Pharmacia Biotech, Uppsala, Sweden) at 535 nm. Standards were prepared at concentrations from 0.1 to 5 μM using 1,1,3,3-tetramethoxypropane (Sigma Chemical Co., St. Louis, MO, USA) and determined as for malondialdehyde (MDA).

Total protein was measured by the Lowry method [27] with bovine serum albumin as the standard (Bio-Rad Laboratories, Heracles, CA, USA). TBARS values are expressed as nanomoles MDA per milligram protein.

IC₅₀ values were calculated from the inhibition curve of lutein and/or pycnogenol for the TBARS levels in retinal homogenate.

Statistical analysis

Samples were analyzed using the Student's t test. Data are expressed as average $\pm$ standard error (SE).

Results

Effect of lutein on FeCl_3 -, AAPH-, or AMVN-induced lipid peroxidation in porcine retinal homogenate

Basal levels of TBARS in porcine retinal homogenate were 28.6 ± 1.6 nmol MDA/mg protein without FeCl_3 , 27.9 ± 1.2 nmol MDA/mg protein without AAPH, and 26.0 ± 1.0 nmol MDA/mg protein without AMVN after 60 min incubation (Table 1). When these initiators were added to retinal homogenate, TBARS was initiated immediately and continued to a maximum at 60 min; values in porcine retinal homogenate were significantly increased to 85.4 ± 0.9 nmol MDA/mg protein with 1 mM FeCl_3 , 57.2 ± 1.1

Table 1. TBARS concentration in porcine retinal homogenate

	FeCl ₃ (1 mM)	AAPH (50 mM)	AMVN (25 mM)
Without Initiator	28.6 ± 1.6	27.9 ± 1.2	26.0 ± 1.0
With Initiator	85.4 ± 0.9**	57.2 ± 1.1**	77.5 ± 2.0**

TBARS values are expressed as nanomoles MDA per milligram protein.

Average ± SE, n = 4, **p < 0.01

Fig. 1. Effect of lutein on FeCl₃- (open circle), AAPH- (open triangle) or AMVN- (open square) induced lipid peroxidation in porcine retinal homogenate. The abscissa shows TBARS levels in porcine retinal homogenate (nmol MDA/mg protein) and the ordinate shows the concentration of lutein (μM). Data show the average ± SE for three independent experiments.

nmol MDA/mg protein with 50 mM AAPH, and 77.5 ± 2.0 nmol MDA/mg protein with 25 mM AMVN. Following the addition of 10 μM lutein, the FeCl₃-induced TBARS formation decreased to 84.9 ± 0.4 nmol MDA/mg protein, while the AAPH-induced TBARS formation decreased to 55.9 ± 5.4 nmol MDA/mg protein, and the AMVN-induced TBARS formation decreased to 70.5 ± 1.8 nmol MDA/mg protein (n = 3, Fig. 1).

Effect of pycnogenol with or without 10 μM lutein on the FeCl₃-induced lipid peroxidation in porcine retinal homogenate

FeCl₃-induced TBARS formation was inhibited by 10 μg/mL pycnogenol to 61.0 ± 6.2 nmol MDA/mg protein (n = 4). When 10 μg/mL pycnogenol plus 10 μM lutein was used, the level was inhibited further to 45.6 ± 3.1 nmol MDA/mg protein (n = 4, p < 0.05 vs without lutein, Fig. 2).

Effect of pycnogenol with or without 10 μM lutein on the AAPH-induced lipid peroxidation in porcine retinal homogenate

The addition of 0.1 μg/mL pycnogenol alone protected the AAPH-induced TBARS formation at 49.9 ± 4.7 nmol MDA/mg protein (n = 3). When 1 μg/mL pycnogenol was used, the level decreased to 46.4 ± 3.1 nmol MDA/mg

Fig. 2. Effect of pycnogenol with or without 10 μM lutein on 1 mM FeCl₃-induced lipid peroxidation in porcine retinal homogenate. The abscissa shows TBARS levels in the homogenate (nmol MDA/mg protein) and the ordinate shows the concentration of pycnogenol (μg/mL), which was added with the FeCl₃. Inhibition curves are shown for conditions without lutein (closed circle) and with 10 μM lutein (closed triangle). Data show the average ± SE for four independent experiments. *p < 0.05 vs. without 10 μM lutein and 10 μg/mL pycnogenol.

protein (n = 3), and in the presence of 1 μg/mL pycnogenol plus 10 μM lutein, it was significantly inhibited to 38.2 ± 1.3 nmol MDA/mg protein (n = 3, p < 0.05 vs without lutein). At 10 μg/mL pycnogenol, the same respective levels were reduced to 39.6 ± 5.4, and 34.9 ± 1.8 nmol MDA/mg protein without and with 10 μM lutein, respectively (n = 3, Fig. 3).

Effect of pycnogenol with or without 10 μM lutein on the AMVN-induced lipid peroxidation in porcine retinal homogenate

AMVN-induced TBARS formation was inhibited by the addition of 10 μg/mL pycnogenol to 61.5 ± 7.6 nmol MDA/mg protein (n = 3), and by 10 μg/mL pycnogenol plus 10 μM lutein to 57.4 ± 12.3 nmol MDA/mg protein (n = 3). When 1 mg/mL pycnogenol was added, the TBARS formation was inhibited to 48.7 ± 2.1, and 45.1 ± 1.0 nmol MDA/mg protein without and with 10 μM lutein, respectively (n = 3, Fig. 4).

IC₅₀ calculations

Table 2 shows the IC₅₀ value of pycnogenol on the FeCl₃-

Fig. 3. Effect of pycnogenol with or without 10 μ M lutein on 50 mM AAPH-induced lipid peroxidation in porcine retinal homogenate. The abscissa shows TBARS levels in the homogenate (nmol MDA/mg protein) and the ordinate shows the concentration of pycnogenol (μ g/mL), which was added with the AAPH. The inhibition curves represent the absence (closed circle) and the presence of 10 μ M lutein (closed triangle). Data show the average $\pm$ SE for three independent experiments. * $p < 0.05$ vs. without 10 μ M lutein and 1 μ g/mL pycnogenol.

Fig. 4. Effect of pycnogenol with or without 10 μ M lutein on 25 mM AMVN-induced lipid peroxidation in porcine retinal homogenate. The abscissa shows TBARS levels in the homogenate (nmol MDA/mg protein) and the ordinate shows the concentration of pycnogenol (μ g/mL), which was added with the AMVN. The inhibition curves represent the absence (closed circle) and presence of 10 μ M lutein (closed triangles). Data show the average $\pm$ SE for three independent experiments.

Table 2. Effect of combining lutein in IC_{50} of pycnogenol for TBARS formation

Initiator	FeCl ₃ (1 mM)	AAPH (50 mM)	AMVN (25 mM)
Without Lutein	12	2.8	465
With Lutein (10 μ M)	5	0.5	110

The data shows IC_{50} of pycnogenol calculations (μ g/mL).

AAPH-, or AMVN-induced TBARS formation, and the combined effect of these agents plus 10 μ M lutein. The control value for each initiator was the difference between TBARS value with initiator and basal level at every experiment. The AAPH-induced TBARS formation yielded the lowest pycnogenol IC_{50} at 2.8 μ g/mL, which was decreased further by the addition of 10 μ M lutein. The highest pycnogenol IC_{50} was observed with the AMVN-induced TBARS formation, and this was also decreased by the addition of 10 μ M lutein, as did the pycnogenol IC_{50} for the FeCl₃-induced TBARS formation.

Discussion

Various cells components are constantly attacked by free radicals and reactive oxygen species generated in both the lipid and aqueous phases [28]. Therefore, two ways are possible to prevent lipid peroxidation *in vivo*. One is the scavenging of radicals in hydrophilic environment before they reached the cell membrane by protective antioxidants, and the other is the scavenging of intermediate radicals in lipophilic region by chain-breaking antioxidants, such as

peroxy and alkoxy radicals. While lipophilic compounds may more easily reach the cell membrane, it is difficult to determine the scavenging effects and concentrations of those compounds in the cell membrane *in vivo*. Therefore, we undertook this comparative *in vitro* study to measure TBARS formation in retinal homogenate (include cell membrane and organelles) to determine the capacity of antioxidants against lipid peroxidation in the cell.

Lutein had no marked antioxidant effect in this experiment system. However, lutein and pycnogenol used in combination greatly enhanced the inhibitory effect on TBARS formation in porcine retinal homogenate. For example, the basal FeCl₃-induced lipid peroxidation was inhibited 1%, and 43% by the addition of 10 μ M lutein and 10 μ g/mL pycnogenol, respectively, while this inhibitory effect was significantly increased to 70% by combining of 10 μ M lutein and 10 μ g/mL pycnogenol. This synergistic effect was also observed with the AAPH-initiated lipid peroxidation. In contrast, the AMVN-induced lipid peroxidation was inhibited 14% with 10 μ M lutein alone. Lutein quenches singlet oxygen [12], and inhibited AMVN-initiated peroxidation of linoleic acid [13]. Pycno-

genol is also an efficient scavenger of superoxide [29], hydroxyl radical [30] and nitrogen monoxide [18]. These results suggest that when aqueous peroxy radicals are formed in porcine retinal homogenate *via* FeCl₃ or AAPH treatment, hydrophilic pycnogenol rapidly scavenges radicals, before the lipid peroxide can increase significantly. The main components of pycnogenol, the phenolic compounds and flavonoids, might be consumed. Subsequently, the lipophilic lutein acts as a chain-breaking antioxidant in the cell membranes, where it might also be consumed and converted to the radical form. This may explain why a combination treatment of lutein and pycnogenol was more effective for inhibiting lipid peroxidation in porcine retinal homogenate in this study than either agent used alone, with efficient and functional antioxidants existing in both the hydrophilic and lipophilic environments, respectively. Age-related macular degeneration (AMD) was recently attributed to oxidative stress [3]. The risk of AMD is higher in aged people with a plasma concentration of lutein lower than 140.9 nmol/L (average in healthy subjects was 177 nmol/L) [31], and a dietary supplement of lutein (30 mg/day for 140 days) increased the levels in serum from 0.15 µmol/L to 1.74 µmol/L, and the macular pigment optic density from 0.756 to 0.939, respectively [32]. Lutein is present in the macular region than the peripheral retina [33]. Together with our data, this evidence suggests that a selective uptake system of lutein exists in the retina.

Pycnogenol also shows a close dose-dependent relationship between dietary intake and bioavailability, with the urinary excretion of ferulic acid was determined in humans [34]. The minimal erythema dose of UV irradiation during supplementation with 1.66 mg/kg/day of pycnogenol for additional 4 weeks was increased when compared to 1.10 mg/kg/day for the first weeks. This may have been due to the increased duration and/or dosage of supplementation [35]. The antioxidant effect of pycnogenol was further demonstrated by a significant increase in oxygen radical absorbance capacity in plasma throughout the supplementation period (150 mg/day for 6 weeks, $p < 0.05$) [36].

There is increasing evidence from epidemiological and clinical trials that an increased intake of the macular pigments, lutein and zeaxanthin is inversely associated with the risk for AMD [14, 37, 38]. Although the direct preventive effect of these agents for AMD remains to be proven in intervention studies [39–41], their physicochemical and biochemical properties make them suitable candidate compounds for photoprotection of the retina.

Ascorbate [42] and glutathione [43] are present at relatively high concentrations in the retina, and probably play an important role in protection against oxidative insults to the retina. Pycnogenol prolongs the half-life of the ascorbate radicals, as demonstrated by ESR studies [44]. In addition, pycnogenol significantly increases glutathione levels *via* an

increase in the activity of the glutathione redox enzymes (glutathione reductase and glutathione peroxidase), and also, the enzyme activities of superoxide dismutase and catalase [45, 46]. The main metabolites of pycnogenol, M1 and M2, also possess antioxidant activity and can inhibit matrix metalloproteinases [47]. We propose then that supplementation of lutein and pycnogenol would provide good antioxidant protection in both the lipophilic and hydrophilic environments of the retina, and that this redox defence would act to prevent AMD.

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