Original

**Inhibitory Effects of Tea Catechins on Lipid Peroxidation
Induced by Rose Bengal and Blue Light Exposure
in Porcine Retinal Homogenate**

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Abstract: The effect of fractions extracted from green tea and catechins on lipid peroxidation induced by rose bengal and blue light exposure were investigated using porcine retinal homogenate. Thiobarbituric acid reactive substances (TBARS) were measured as lipid peroxide and expressed as nmol malondialdehyde (MDA) per milligram protein. In the presence of 10 μ M rose bengal and after 30-minute exposure to blue light, the levels of TBARS in 5 % porcine retinal homogenates was 72.1 ± 5.7 nmol MDA /mg protein. Addition of fractions (25 μ g /mL) extracted from green tea significantly decreased the production of TBARS to 32.2 ± 2.7 nmol MDA /mg protein. The inhibitory rates were directly proportional to the concentrations of epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) in the fraction extracted from green tea, but were not proportional to the concentrations of (+) - catechin, epicatechin (EC) or caffeine. The 50 % inhibitory concentration (IC_{50}) of the purified ECG, EGCG, EC and (+) - catechin were 3.8, 5.3, 39.0 and 98 μ M, respectively. These results confirm that ECG and EGCG extracted from green tea are mainly responsible for the inhibition of the lipid peroxidation in this system.

Key words: lipid peroxidation, blue light, rose bengal, catechin, retinal homogenate

Introduction

Light in the spectral region between 400-1400 nm can reach the retina¹⁾. Light with a wavelength of less than 400 nm or greater than 1400 nm is absorbed by the mammalian ocular media, such as the cornea, aqueous humor, lens, and vitreous humor. Light damages the retina, and it is generally accepted that short wavelengths are significantly more hazardous than longer wavelengths²⁾. Walbarst evaluated the light emitted from ophthalmic instruments for potential ocular hazards from ultraviolet, visible and near-infrared radiation. The Threshold Limit Values listed are now widely used as safety guidelines for ophthalmic light equipments³⁾. Recent publications report that blue (460 nm peak) light-emitting diode

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Fig. 1. Scheme of photosensitized lipid peroxidation.

This reaction is initiated by electron or hydrogen atom transfer from a substrate to ^1S . Blue light is 470 nm LED light. ^1RB and ^3RB are singlet and triplet rose bengal. LH: lipid (unsaturated lipid), $\text{L}\cdot$: lipid alkoxy radical, $\text{LOO}\cdot$: lipid peroxy radical, LOOH: lipid hydroperoxide.

(LED) light exposure causes retinal damage in rhesus monkeys^{3,4}. Retinal damages, recorded as the resulting lesion scores, was measured from the color fundus photographs, scanning laser tomographs and fluorescein angiogram responses. Lesion scores were evident at 2 and 30 days after exposure greater than 30 J/cm^2 ^{2,4}, and a marked disruption of photoreceptor cells discs, damaged retinal pigmented epithelial cells (RPE) and loss of RPE melanin were reported with exposure over 60 J/cm^2 ^{2,5}. Retinal light damage occurs via a photochemical reaction^{6,7}. Retinal tissues contain a high amount of polyunsaturated fatty acid (PUFA) compared to other tissues⁸. PUFA is easily oxidized and leads to retinal damage. Molecular oxygen, a sensitizing agent and exciting light initiate the photochemical reaction responsible for retinal lipid peroxidation⁹ (Fig. 1). Environmental light is a possible cause of age-related macular degeneration (AMD)¹⁰ a leading cause of blindness, and the resulting retinal photochemical damage is attributed to oxidative stress¹¹.

Green tea is widely consumed in the eastern world. Studies have shown that green tea possesses diverse pharmacological properties that include antioxidative, anti-inflammatory, anticarcinogenic, antiarteriosclerotic and antibacterial effect¹²⁻¹⁴. Green tea may prevent retinal light damage through its antioxidant activity, and may be an economical and safe treatment for AMD. However, green tea contains several unknown substances, such as caffeine which have adverse effects at high concentrations. The preventive effects might be a result of integral components. In other words, it is possible that the preventive effects would not appear with merely one compound. Thus, it is important to investigate the effect of fractions extracted from green tea as well as the polyphenols in green tea.

In this study, we determined the concentration of catechins in fractions extracted from green tea. The purpose of this study was to determine which green tea components inhibit lipid peroxidation. For this purpose, we investigated the effect of green tea fractions containing different amounts of catechins on lipid peroxidation induced by blue LED light

exposure and 10 μ M of the rose bengal as sensitizer in 5 % porcine retinal homogenate.

Materials and Methods

Retinal homogenate preparation

The retinas from 50 porcine eyes were obtained from a local slaughterhouse, placed on ice in a dark container and transported within 1 hour to the laboratory for sample preparation. The anterior segment and vitreous humor were removed. The retinas were collected with fine forceps, diced, homogenized with 20 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.

Extraction and fractionation of green tea

Greentea leaves (the leaves of *Camellia sinensis* L.), from Yuukisaibai-cha (Yabukita 80 %, Kurasawa 10 %, others 10 %) purchased from Nakai-hotoen Co. Ltd. (Kyoto, Japan) was used. The purposes of this fractionation were: 1) to remove caffeine, and 2) to retain the catechins efficiently. The dried tea leaves (500 g) were extracted with 5 L of hot methanol (Wako Pure Chemical Co, Osaka, Japan) for 2 hours. The methanolic extract was then dissolved in water (1.7 L) and extracted with 2 L of *n*-hexane (Wako Pure Chemical Co., Osaka, Japan) 3 times, followed by 2 L of *n*-butanol (Wako Pure Chemical Co., Osaka, Japan) 3 times. The water layers were evaporated to obtain the water-soluble fraction (M-W, 29.9 g). The *n*-butanol layer was evaporated to obtain a residue (M-B, 81.5 g). M-B (20.0 g) was partitioned between 400 mL of chloroform (Wako Pure Chemical Co., Osaka, Japan) and 400 mL of water. The water layer was concentrated and subjected to Sephadex LH-20 column chromatography and eluted with water followed by 50 % acetone (Wako Pure Chemical Co., Osaka, Japan). These fraction were evaporated to obtain the water eluate (DM-W, 5.6 g) and the 50 % acetone eluate (DM-A50, 12.3 g), respectively.

Analysis of catechins, gallic acid and caffeine in each fraction

(+)-Catechin, (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-) epigallocatechin gallate (EGCG), caffeine and other chemicals were purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

The HPLC analyses were performed using an LC-10ADvp system with an SPD-10Avp UV-VIS detector and LC solution software (Shimazu, Kyoto, Japan). Chromatographic conditions were as follows: column, Shiseido Capcell pad C₁₈ (4.6 mm i.d. $\times$ 100 mm, 3 μ M); mobile phase, (A) 0.1 % H₃PO₄ containing 0.1 % CH₃CN and 5 % N,N-dimethylformamide, (B) CH₃CN; at 0 min, A : B was 99 : 1, after 40 min, A : B was 83 : 17; flow rate, 1.0 mL / min; detector, 280 nm; column temperature, 40°C.

Incubation conditions

One hundred μ L of 10 μ M rose bengal (Wako Pure Chemical Co., Osaka, Japan) as sensitizer and 1.8 mL of 5 % porcine retinal homogenate were placed into a 35 mm cell culture dish (Corning, Grand Island, NY, USA) and irradiated with a blue (470 nm) LED light irradiation device (Oki electric Industry Co., Osaka, Japan). The power density was 4 mW / cm² and the exposure time was 30 min. The concentration of lipid peroxide in the

porcine retinal homogenate was determined using the thiobarbituric acid (TBA) assay. To measure the basal levels of TBA reactive substances (TBARS) in the retinal homogenate, 100 μ L of distilled water was added to the retinal homogenate and analyzed by the TBA assay after 30 min of irradiation at 37°C.

Various concentrations of the green tea fractions (M-B, M-W, DM-W, DM-A50) or catechins ((+)-catechin, EGCG, EC, ECG) were added simultaneously to the retinal homogenates with 10 μ M rose Bengal, and the retinal homogenates were reassayed to determine the extent of lipid peroxidation inhibition. The total volume of the reaction mixture was 2 mL.

Measurements of thiobarbituric acid reactive substances

Lipid peroxide concentrations were determined using a modification of the methods described by Okawa *et al*, which measured the amount of TBARS¹⁵⁾. The reaction mixture contained 0.2 mL of sample, 1.2 mL of 1% phosphoric acid (Wako Pure Chemical Co., Osaka, Japan), and 0.4 mL of 0.6% thiobarbituric acid (Wako Chemical Co., Osaka, Japan). The mixtures were incubated at 100°C for 45 min, and chilled for 15 min in an ice bath. L-butanol (1.5 mL) (Wako Pure Chemical Co., Osaka, Japan) was added and the reaction was mixed for 5 seconds. After centrifugation at 1500 $\times$ g for 10 min, 0.1 mL of the supernatant layer was removed by pipette, and the absorbance measured in a spectrophotometer (Ultrospec 3300 Pro, Amersham Pharmacia Biotech, Uppsalla, Sweden) at 535 nm. Standards were prepared at concentrations ranging from 0.1 to 5 μ M using 1,1,3,3-tetramethoxypropane (Sigma Chemical Co., St. Louis, MO, USA) and determined as for malondialdehyde (MDA).

Total protein was measured using the Lowry method¹⁶⁾ with bovine serum albumin as the standard (Bio-Rad Lab., Hercules, CA, USA). TBARS values were expressed as nanomoles MDA per milligram protein. IC₅₀ (50% inhibitory concentration) values were calculated from the green tea fractions or catechin inhibition curves, expressed as a percentage of control.

Statistical analysis

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

Results

Analysis of green tea fractions

The concentrations of EC, EGCG and ECG were higher in both the DM-A50 and M-B fractions than in the DM-W and MW fractions. The levels of these components in DM-A50 were nearly two-fold higher than those in the M-B fraction. In contrast, EGCG and ECG were not present in the DM-W and M-W fraction, and EC and catechin were present at very low concentrations. High levels of caffeine were found in the DM-W and M-B fractions (Table 1).

Effect of green tea fractions on blue LED light exposure and 10 μ M rose bengal-induced lipid peroxidation in porcine retinal homogenate

The basal levels of TBARS in the porcine retinal homogenate was 19.2 ± 0.3 nmol MDA /

Table 1. Concentrations of catechins and caffeine in the various green tea fractions

	DM-A50	DM-W	M-B	MW
(+)-Catechin	1.1	0.3	0.8	0.04
(-)-EC	6.8	0.6	4.1	0.1
(-)-EGCG	18.9	—	10.7	—
(-)-ECG	10.7	—	6.6	—
Caffeine	0.4	19.6	6.7	1.1

The concentrations of (+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), and caffeine in fractions were determined by HPLC. The data represents the percentages of catechins and caffeine in the total weight of each fraction.

Table 2. TBARS concentration in porcine retinal homogenate after blue LED light exposure for 30 minutes.

	TBARS (nmol MDA / mg protein)
Without 10 μ M rose bengal	19.2 $\pm$ 0.3
With 10 μ M rose bengal	72.1 $\pm$ 5.7**

TBARS values (nmol MDA / mg protein) are expressed as mean $\pm$ SE (n = 4).

**P < 0.01 compared to the value without rose bengal.

Fig. 2. Effect of green tea fractions on blue light and rose bengal-induced lipid peroxidation in porcine retinal homogenate.

(●): M-W; (□): DM-W; (○): M-B; (▲): DM-A50. Data are mean $\pm$ SE from five independent experiments. *P < 0.05, **P < 0.01 compared to the value without green tea fractions (0 μ g / mL).

mg protein after 30 min of blue LED light exposure (control). When 10 μ M rose bengal was added to the retinal homogenate, TABRS formation was initiated immediately and continued to a maximum at 30 min, at which point the TBARS level in the porcine retinal homogenate had increased significantly to 72.1 $\pm$ 5.7 nmol MDA / mg protein (376 % of control, Table 2). Following the addition of 25 μ g / mL DM-A50, and M-B, the blue light and rose bengal-induced TBARS formation significantly decreased to 32.2 $\pm$ 2.7 (n = 5, P < 0.01), and 40.1 $\pm$ 2.2 nmol MDA / mg protein (n = 5, P < 0.01), respectively (Fig. 2).

Fig. 3. Effect of tea catechins on blue light and rose bengal-induced lipid peroxidation in porcine retinal homogenate. (○): (+)-catechin; (■): EC; (▲): ECG; (□): EGCG. Data are mean $\pm$ SE from four independent experiments. * $P < 0.05$, ** $P < 0.01$ compared to the value without catechins (0 μ M).

Table 3. IC_{50} of green tea fractions and catechins

Green tea fractions	IC_{50} (μ g / mL)	Catechins	IC_{50} (μ M)
DM-A50	7.1	ECG	3.8
M-B	15.0	EGCG	5.3
DM-W	460.0	EC	39.0
M-W	3400.0	(+)-catechin	98.0

Effect of catechins on blue LED light exposure and 10 μ M rose bengal-induced lipid peroxidation in porcine retinal homogenate

The blue light and rose bengal-induced TBARS formation was inhibited significantly by 10 μ M EGCG, ECG and EC to 38.9 ± 2.0 ($n = 4$, $P < 0.01$), 39.1 ± 0.9 ($n = 4$, $P < 0.01$) and 51.0 ± 1.7 ($n = 3$, $P < 0.05$) nmol MDA / mg protein, respectively (Fig. 3).

IC_{50} calculations

Table 3 shows the IC_{50} values of green tea fractions and catechins for blue light and rose bengal-induced TBARS formation. DM-A50 possessed the lowest IC_{50} of the green tea fractions. ECG and EGCG had the lowest IC_{50} values of the catechins at 3.8 μ M and 5.3 μ M, respectively (Table 3)

Discussion

In this study, the blue light and rose bengal-induced TBARS formation was inhibited by catechins, especially ECG and EGCG, and was significantly inhibited by the green tea fractions DM-A50 and M-B. The DM-A50 and M-B fractions contains a large amounts of ECG and EGCG (Table 1), therefore these antioxidant activities show higher than those of the M-W or DM-W fractions.

The biological effects of light can be explained by at least three fundamental mechanisms, which include photochemical, thermal, and ablation reactions. A photochemical reaction is responsible for the biological effects of environmental illumination, were a sensitizer receives

a photon from the light and converts it to an excited singlet state. After intersystem crossing and spin inversion to the triplet excited state, triplet state oxygen receives energy from the excited triplet state sensitizer, as this is faster than the rate of internal conversion⁹⁾ (Fig. 1). Biologically active compounds may be more readily produced from blue light than other visible light. According to Foote¹⁷⁾, the absorption of light energy is followed by electron displacement, hydrogen abstraction or energy translation, and the subsequent production of reactive oxygen species (ROS). In the retina, photosensitizing agents that can absorb blue light are porphyrins (cytochrome, hemoglobin), rhodopsin, all-*trans* retinal, flavins and A2E (the potential photosensitizer component of lipofuscin)¹⁸⁾. Lipofuscin is a complex aggregate of material in the RPE formed by ingestion of photoreceptor outer segment, and accumulates with increasing age. The absorbance spectrum of A2E in methanol has a major peak at 435 nm, therefore A2E can initiate blue light-induced apoptosis in RPE cells¹⁹⁾. The adverse effects of blue light on the retina may be due to the existence of blue light absorption molecules, rather than the high energy of blue light. Endogenous and exogenous photosensitizing agents may initiate ROS in the cell upon light exposure and up-regulate signal transduction pathways, and may also start the lipid peroxidation chain reaction in cell membranes (Fig. 1). Quinolone, for example, is converted to an excited state by absorbing blue light energy and produces ROS, and also increases lipid peroxide in porcine retinal homogenate²⁰⁾. This blue light and quinolone-induced lipid peroxidation was suppressed by vitamin E which acts as a chain-breaking antioxidant in the cell membranes. In the current study, blue light and rose bengal-induced lipid peroxidation was suppressed by DM-A50 and M-B, which contain high amounts of ECG, EGCG and EC. Zhao *et al* reported that tea catechins had stronger scavenging effect than vitamin E on oxygen free radicals produced from cell systems²¹⁾. Green tea catechins also have a synergic scavenging effect on the superoxide anion O_2^- and a combination with catechins in the ratio EGCG : ECG : EC = 5 : 2 : 2 : 1 had the strongest effect²²⁾. Therefore, blue light and rose bengal-induced lipid peroxidation in porcine retinal homogenate was suppressed by DM-A50 and M-B. In contrast, EGCG produced H_2O_2 in an aqueous solution containing dissolved oxygen¹⁴⁾. The concentration of TBARS in 5 % porcine retinal homogenate was not increased by 1 mM EGCG or 1 mM H_2O_2 after 30-minute incubation at 37°C. The porcine retinal homogenate contains catalase and glutathione peroxidase. Therefore, these enzymes may metabolize H_2O_2 quickly under the experimental conditions in this study.

The retina contains several antioxidant enzymes and compounds, but their activities and contents decrease with age²³⁾. The occurrence of age-related macular degeneration (AMD) increases with age, and it is a leading cause of blindness²⁴⁾. It is possible that environmental light and oxidative stress might contribute to the development of AMD¹¹⁾, and the condition is closely correlated with an increase in lipid peroxide²⁵⁻²⁹⁾. Therefore, antioxidant treatments may be useful for preventing AMD progression. Lipophilic antioxidants scavenge free radicals in the cell membranes, and are converted into a free radical form. Hydrophilic antioxidants then scavenge the free radicals from the lipophilic antioxidants and are excreted from the cell³⁰⁾. One study has shown that the efficacy of a complex treatment containing zinc and vitamins for age-related eye disease was better than using a single treatment. From these results, it is important to investigate the effects of different fractions and these fractionation methods.

The simple model described in the current study may be employed in the further studies

to determine the protective effects of various therapeutic agents against retinal light damage.

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