

Catechin-rich green tea extract modulates the oxidative status of human retinal pigment epithelial cells

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Abstract

Oxidative damages to the retinal pigment epithelium (RPE) have been suggested to play a key role in the pathogenesis of age related macular degeneration (AMD). Our purpose was to evaluate the effects of a catechin rich green tea extract Polyphenon E against induced oxidative stress in cultured human RPE cells. Cell viability (MTT assay) and level of intracellular reactive oxygen species (ROS) generated after treatments with Polyphenon E +/- hydrogen peroxide were measured. The activities of superoxid dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as well as the glutathione GSH concentration were determined. Polyphenon E has no cytotoxic effect up to concentrations of 200 microgram/ml in culture media and showed a protective effect against hydrogen peroxide induced toxicity. Administration of the catechin-rich extract induced an increase of SOD activity in normal and oxidative stress conditions and an increase of catalase only in induced oxidative stress condition. Polyphenon E determined a decrease of glutathione peroxidase activity and of glutathione level. However, the significant inhibition of intracellular ROS generation supports the hypothesis that catechin can contribute to antioxidant defense by direct scavenge of ROS in RPE cells.

Keywords: RPE cells, catechins, antioxidant enzymes, reduced glutathione, intracellular reactive oxygen species

Abbreviations: AMD, age-related macular degeneration; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SOD, superoxididismutase

Introduction

The retinal pigment epithelium (RPE) represents the barrier between the photoreceptors and the choriocapillaris. RPE have the essential role of providing oxygen and nutrients to the photoreceptors but also to remove their debris and metabolites, a turnover which is vital in the functioning of retina. RPE play also the essential role in phagocytosis and degradation of the photoreceptors outer segments (POS). The RPE is essential for photoreceptor survival and maintenance of vision. The loss of RPE cells is related to several eye diseases, including age related macular degeneration (AMD).

Retina and retinal pigment epithelium (RPE) represent an ideal environment for the generation of reactive oxygen species (ROS) and oxidative damages. There are several sources of ROS generation in the RPE: high metabolic rate and oxygen consumption, high level of irradiation, phagocytosis of POS and the presence of photosensitizers (lipofuscin) [1-4]. The photoreceptor membranes contain highly unsaturated lipids, with large amounts of docosahexaenoic acid, exceptionally sensitive to peroxidation, thus the phagocytosis of

membranes leads to the formation of reactive oxygen species and toxic lipid peroxidation products [5]. Oxidative stress is the most likely primary event in AMD pathogenesis, the most common cause of visual impairment and vision loss in individuals over the age of 60 years in developed countries. The disease occurs in two forms: atrophic (dry) and exudative (wet) AMD and is characterized by accumulation of a yellow deposit called drusen. The vision loss results from photoreceptor damages in the central retina, and it is accepted that degeneration of RPE is involved in first stages of AMD [6].

RPE cells are an excellent model to study the molecular mechanisms of the adaptive response to oxidative stress since several antioxidant enzymes were found to be involved in the primary or secondary antioxidant defense of RPE cells: catalase, glutathione peroxidase, glutathione S-transferase, CuZnSOD and MnSOD, heat shock protein 27 and 90, heme oxygenase [7-11]. The activity of RPE antioxidant enzymes, mainly catalase, decreases with aging [7] but the expression of SOD, catalase and heat shock proteins increased in RPE from human donor with AMD [11]. It was found that antioxidant up-regulation and increased nuclear DNA protection play key roles in adaptation to oxidative stress in retinal epithelial cells [9]. All these studies support the involvement of oxidative stress in the pathogenesis of AMD. Since there is no efficient treatment for AMD, dietary strategies that enhance antioxidants uptake might be important for the prevention of this disease. AREDS study demonstrated that common antioxidants (dietary carotenoids, vitamin C and vitamin E) and zinc are associated with a decreased risk of AMD [12, 13]. Green tea is known for the medicinal properties partially ascribed to the antioxidant properties of the tea flavonoids [14, 15] and its ability to modulate many cellular enzyme functions. The tea flavonoids may reduce oxidative stress through one of several mechanisms that relate to their structural chemistry. For example, the tea flavonoids directly scavenge free radical species through hydrogen/electron donation. The structure-activity relationships of the flavonoids as a group suggest that the presence of a catechol group and the hydroxyl group at position 3 of the B-ring are absolutely essential to their ability to scavenge free radicals [15, 16].

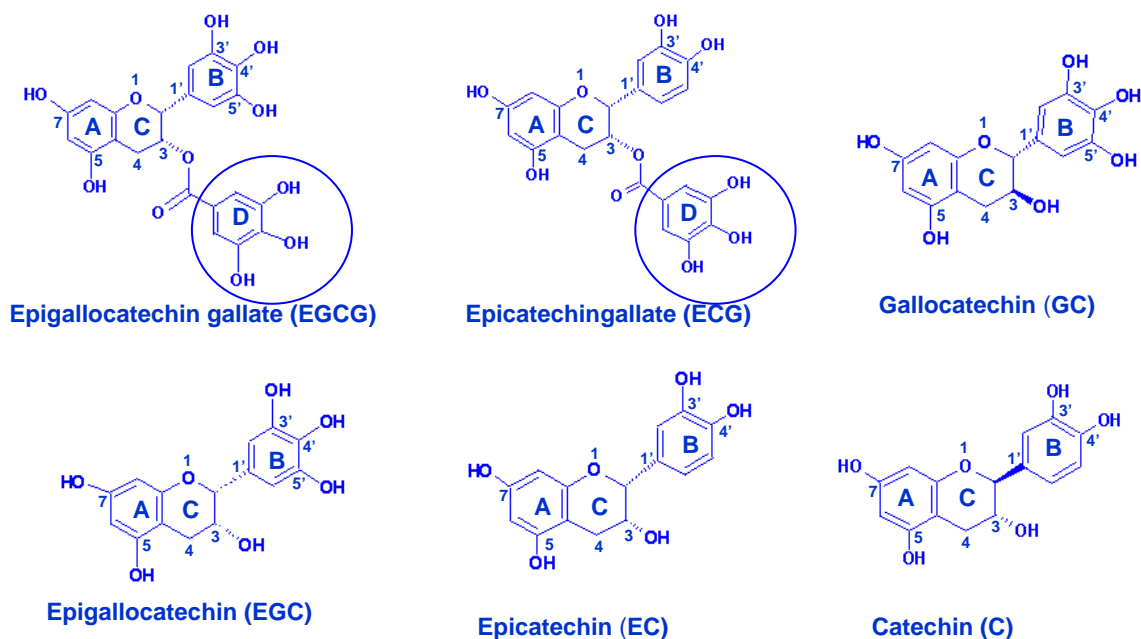


Figure 1: Representative structures of major catechins (the gallate moiety is rounded off).

A number of flavonoids, including (-)-epigallocatechin gallate (EGCG), were shown to protect human RPE cells from oxidative-stress induced death (with hydrogen peroxide and *t*-butyl hydroperoxide), acting through an intracellular route to block the accumulation of reactive oxygen species and by induction of transcription factor Nrf2 and heme-oxygenase 1 (HO-1) expressions [17]. EGCG provides protective effects against UVA [18] and UVB [19] damage in cultured human retinal pigment epithelial cells, attenuates lipid peroxidation induced by sodium nitroprusside (SNP) in brain membranes (*in vitro*) and counteracts the detrimental effects caused by injection of SNP into the retina [20]. EGCG also attenuates the effect of ischemia/reperfusion insult to the intact retina, where oxidative stress is implicated [21].

The goal of this study was to evaluate the effects of a catechin-rich green tea extract on the antioxidant enzymes (SOD, GPx and catalase) activities, reduced glutathione concentration and intracellular ROS generation in cultured human RPE cells in normal and oxidative stress conditions.

Materials and Methods

Chemicals

GIBCO[®] Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, California, USA). Fetal bovine serum, penicillin, streptomycin, amphotericin B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and catechin standards were purchase from Sigma (St. Louis, USA). Methanol, acetic acid, sodium pyruvate, hydrogen peroxide and all the others chemicals used were of analytical grade and supplied from Merck (Darmstadt, Germany).

HPLC analysis of green tea extracts

HPLC analyses were performed on a Shimadzu system, equipped with a binary pump delivery system LC20 AT, a SCL 10A system controller and SPD-M 20A photodiode array detector (Shimadzu Corp, Kyoto, Japan). A SUPELCOSIL[™] LC-18 column, 5 μ m, 25 cm x 4.6 mm (Merck, Darmstadt, Germany) at 25 °C was used. The mobile phase consisted of mixtures of: methanol: acetic acid: double distilled water (10:2:88 v/v/v) (A) and methanol: acetic acid: double distilled water (90:3:7 v/v/v) (B). The linear gradient elution system was: 100% A from 0 to 10 minutes, followed by a decrease to 50% A for 20 min. The flow rate was 1 ml/min and all solvents were HPLC grade, filtered through a 0.45 μ m membrane (Millipore, U.S.A.) and degassed before use. The chromatograms were monitored at 280 nm. The quantification of the catechin was made using calibration curves with catechin standards in the range of concentrations 0.008-1 mg/ml. The linearity coefficient for the all calibration curves ranged between 0.9950-0.9999.

Sample preparation

Catechin standards (EC, ECG, C, EGC, EGCG, GCG, GC, and CG) were purchased from Sigma-Aldrich (St. Louis, USA). The stock solutions were prepared dissolving each standard compound in 1 ml of double distilled water. Each stock solution was then used for the preparation of the diluted solutions, for the calibration curves. Polyphenon E is a highly purified and decaffeinated catechin extract, obtained from green tea leaves. Polyphenon E powder (0.2 g) was dissolved in 10 ml hot water, sonicated for 15 min and centrifuged for 5 minutes at 4000 rpm. Samples were microfiltered before injection.

Cell culture and treatment

Human adult retinal pigment epithelial cells line D407 were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B, at 37°C, 5% CO₂, and 95% relative humidity. The cells were seeded in 25 cm³ flask at a concentration of 6 x 10⁵. After reaching 90% confluence, growth medium was removed and replaced with medium containing Polyphenon E. Polyphenon E was solubilized in water until the final concentration of 100 µg/ml in medium (equivalent of about 130 µM EGCG).

Exposure of cells to H₂O₂

After 24 hours from Polyphenon E treatment, the culture medium was removed and, after washing with PBS, the cells were exposed to 500 µM H₂O₂ for 1h in DMEM medium. Thereafter, the cells were washed twice with cold PBS, and then specifically lysed for each enzyme determination.

MTT assay

D407 cells were plated (10,000 cells per well) in 96-well plates and, after the cells had attached, they were incubated for 24h with Polyphenon E and for 1h with H₂O₂. The number of viable cells at each time point was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation reagent. This method uses the property of viable cells to reduce MTT reagent into a colored formazan which is detected by reading the absorbance at 550 nm [22]. Briefly, the cells were washed with PBS and 200 µl MTT solution in HBSS buffer were added to each well. After 2h of incubation the MTT reagent was removed and the formazan particles were solubilized with 200 µl DMSO. The absorbance was read at 550nm, respectively at 630 nm (for background) with a microplate reader HT BioTek Synergy (BioTek Instruments, USA). Cell viability was expressed as a percentage of control (cells incubated in normal medium only).

Total protein extract

The cells were centrifuged at 1000 rpm, the PBS was removed and the pellet was resuspended in Triton 0.2%. Then the homogenate was centrifuged at 12000 rpm, for 25 minutes. The supernatant was used to determine protein concentration using the bicinchoninic acid assay kit instructions (Sigma, St. Louis, USA).

Glutathione assay

The GSH assay was performed using an optimized enzymatic recycling method with glutathione reductase (Cayman Chemical Company, Michigan, USA). The sulfhydryl group of GSH reacts with DTNB (5, 5'-dithio-bis-2-(nitrobenzoic acid) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. The cell pellet was homogenized with 700 µl of cold phosphate buffer 50 mM containing 1mM EDTA and centrifuged at 13.000 rpm for 15 minutes at 4°C. The supernatant was deproteinated with 700 µl metaphosphoric acid in (5g/50 ml of water), maintained for 5 minutes at room temperature, centrifuged at 2000 g and the supernatant was collected. Before the assay, the supernatant (1 ml) was treated with 50 µl triethanolamine 4M. A volume of 50 µl samples was treated with 150 µl assay cocktail containing MES Buffer, reconstituted Cofactor Mixture (NADP⁺ and glucose-6-phosphate), reconstituted Enzyme Mixture (glutathione reductase and glucose-6-phosphate dehydrogenase), water and reconstituted DTNB (0.45 ml)). The absorbance was read after 30 minutes at 405 nm, using a HT BioTek Synergy microplate reader (BioTek Instruments, USA). Standard curve was made with GSSG

standard, having the equivalent GSH concentration between 0-16 μM . Results are expressed as $\mu\text{moles GSH/mg protein}$ in cell pellet.

Glutathione peroxidase assay

Glutathione peroxidase (GPx, EC 1.11.1.9) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, using reduced glutathione in order to protect the cell from oxidative damage. Cayman Chemical (USA) GPx Assay kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP^+ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is the rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

The cells collected in PBS were homogenized in 100 μl cold buffer (50mM Tris-HCl, pH 7.5, 5 mM EDTA and 1mM DTT) and centrifuged at 1500 g for five minutes at 4°C. A volume of 20 μl of sample was treated with 100 μl of assay buffer, 50 μl of co-substrate mixture (NADPH, glutathione and glutathione reductase). The reaction is initiated by adding 20 μl of cumene hydroperoxide quickly. The plate was shaken few seconds and the absorbance was read at 340 nm. GPx activity was calculated, using the NADPH extinction coefficient of $0.00373 \mu\text{M}^{-1}$ and the rate of $\Delta A_{340}/\text{min}$. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP^+ per minute at 25°C.

Superoxide dismutase assay

Superoxide dismutase (SOD, EC 1.15.1.1) assay kit (Cayman Chemical Company, Michigan, USA) is based on the conversion of a tetrazolium salt to a formazan by superoxide radical generated in xanthine/xanthinoxidase system. This method measures the activity of all three SOD types.

The cells were homogenized in 200 μl cold 20mM HEPES buffer, pH 7.2, containing 1mM EDTA, centrifuged at 1500 g for 5 min at 4°C and the supernatant was used for enzyme assay. A volume of 200 μl buffer solution containing tetrazolium salt and hypoxanthine was mixed with 10 μl of sample. The reaction was initiated by adding 20 μl of diluted xanthine oxidase to all the wells. The plate was incubated on a shaker for 20 minutes at room temperature. The absorbance was monitored at 460 nm using a microplate reader. A standard curve made with bovine erythrocyte SOD was used for determination of enzyme activity. One unit of enzyme is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Catalase assay

The method used in this experiment is based on the reaction of the catalase (CAT, EC 1.11.1.6) enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. Sample preparation and assay procedure were made according kit instructions (Cayman Chemical, USA). Cells pellet was homogenized with 100 μl of cold phosphate buffer 50mM containing 1mM EDTA. The samples were centrifuged at 10.000g for 15 minutes at 4°C and the supernatant was used for enzyme assay. A volume of 20 μl sample was treated with 100 μl of assay buffer and 30 μl of methanol. The reaction is initiated by adding 20 μl of hydrogen peroxide and the plate is incubated on a shaker for 20 minutes at room temperature. The reaction was terminated by adding 30 μl of potassium hydroxide and 30 μl of Purpald (chromogen). The plate was again incubated for 10 minutes on the shaker at room temperature. Then 10 μl of potassium periodate was added and after 5 minutes of incubation on shaker the

absorbance was read at 540 nm. A standard curve made with bovine liver catalase was used for determination of enzyme activity. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of formaldehyde per minute at 25°C.

Intracellular reactive species assay

The determination of intracellular reactive oxygen species (ROS) is based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCHF) by intracellular peroxides, forming the fluorescent compound 2',7'-dichlorofluorescein (DCF), which is measured by a BioTek fluorescence plate reader. Cells were cultured in 96 well black plates and incubated with dichlorofluorescein diacetate (DCFDA). Fluorescence was monitored for 4h at 37°C at excitation 485/10 nm and emission 528/20nm [23].

Statistical analysis was done using Tukey test of Graph Pad Prism version 5.00. The points or bars represent the mean \pm SEM, calculated from three experimental values.

Results

Quantitative analysis of green tea extracts by HPLC

A mixture of eight pure catechin standards was separated over 22 min (Fig. 2). The elution order was: GC, C, EGC, EGCG, EC, GCG, ECG, and CG. The chromatogram corresponding to green tea extract Polyphenon E is presented in Fig. 3. The identification of catechin in the extract samples was carried out by comparing the retention times and the UV absorbance with those of the standards compounds. Content (mg/g) of catechin in Polyphenon E green tea extract, as determined by HPLC and calibrated with pure standards was: GC - 8.33 ± 0.06 ; C - 10.72 ± 0.02 ; EGC - 1.56 ± 0.07 ; EGCG - 196.32 ± 0.72 ; EC - 11.62 ± 0.05 ; GCG - 21.55 ± 0.1 ; ECG - 63.39 ± 0.14 ; CG - 1.89 ± 0.11 . EGCG, ECG and EC are the major catechin in green tea and green tea based dietary supplements, as determined by quantitative HPLC. EGCG accounts for 62 % of Polyphenon E extract.

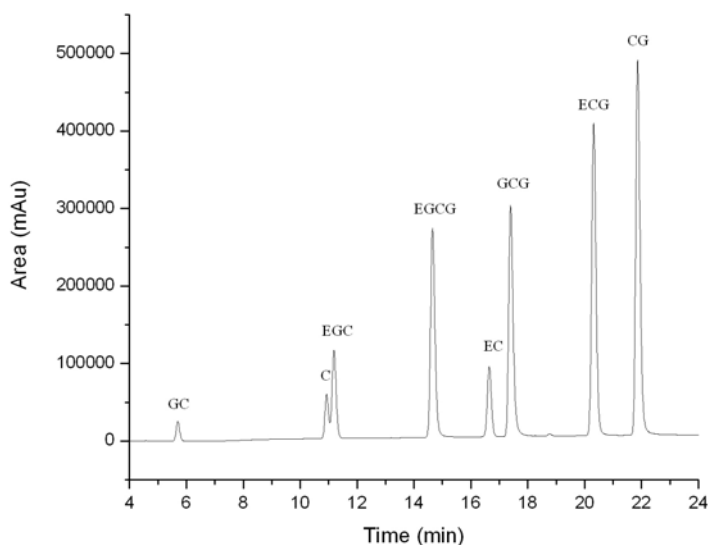


Figure 2: HPLC separation of catechin pure standards: GC, C, EGC, EGCG, EC, GCG, ECG and CG

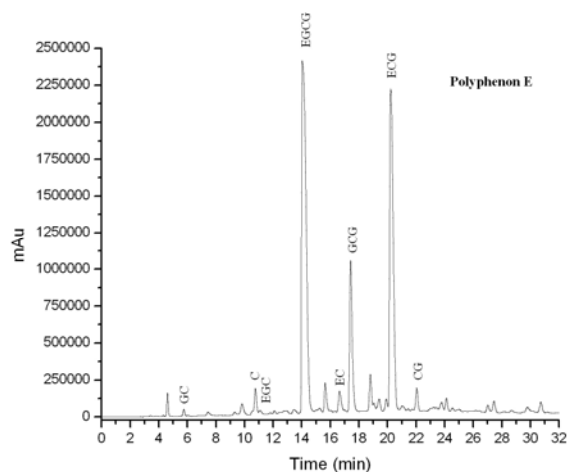


Figure. 3. HPLC separation of catechins from Polyphenon E extract.

Effect of H₂O₂ treatment on morphology and viability of human retinal pigment epithelial cells

Cells were seeded at 10⁴ cells/well in 96 wells microplates, 24h before treatment. The cells were healthy, free of bacterial or fungal contamination. D407 cells were treated with different concentrations of H₂O₂ in DMEM medium for 1h. Each treatment was applied in triplicate, and for each dish three photos of different area were made using Zeiss AxioCam HRC. Cell viability was expressed as a percentage of control (cells incubated in normal medium only), which is considered 100 %.

As can be observed in Fig. 4, D407 cells started to have oval form after treatment with 100 μM H₂O₂, but remained attached on the microplate. After treatment with 500 μM H₂O₂ cells appeared to be more stressed, still attached on the microplate. Cells detachment was observed only at concentrations higher than 2000 μM H₂O₂. Cell viability was expressed as mean percentage of control (100%) and was evaluated after 1h of H₂O₂ treatment with concentrations between 0-2600 μM (Fig. 5). Treatment with hydrogen peroxide at concentration of 500 μM H₂O₂ reduced the cells viability with 50%. Higher concentrations of hydrogen peroxide (1400 – 1800 μM) determined a more evident decrease of viability, up to 80%.

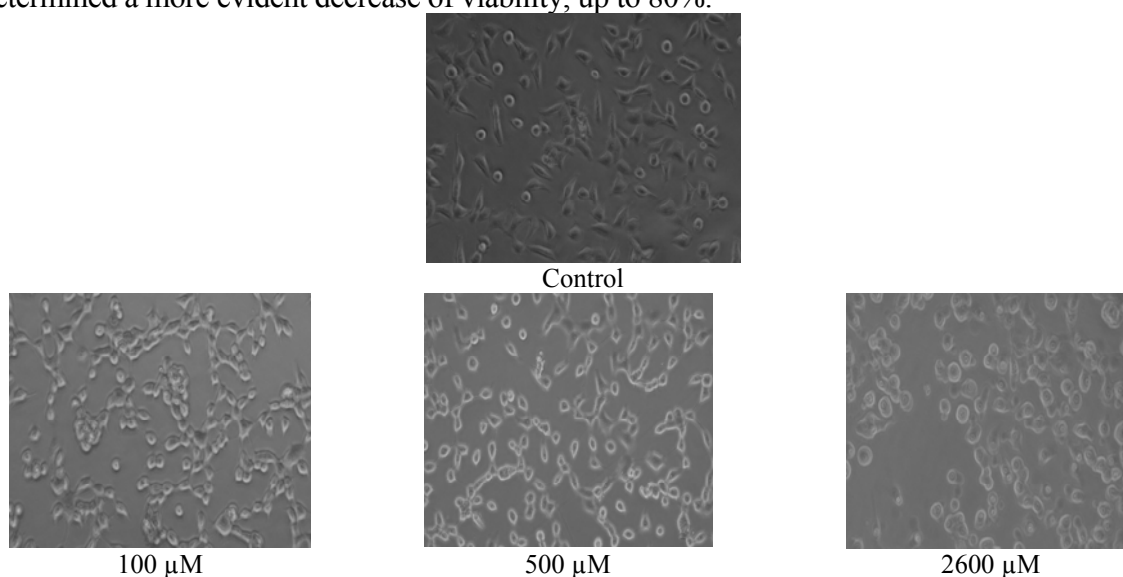


Figure 4: Comparative morphology of D407 cells non-treated and treated with H₂O₂ for 1 h. The morphology of D407 cells was observed under inverted microscopy with magnification X10

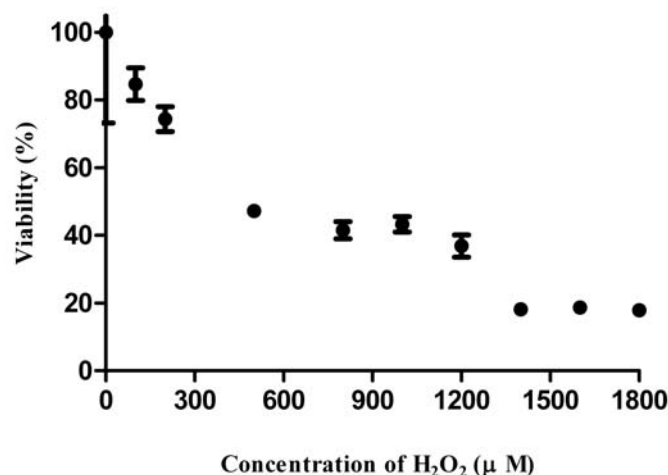


Figure 5: The viability of RPE cells treated with different concentrations of H₂O₂ for 1 hour. Data represent the means \pm SD of three independent experiments.

Effect of Polyphenon E treatment on D407 cell viability

D407 cells were treated with Polyphenon E at concentrations between 20 and 230 µg/ml. Fig. 6 shows that the viability and proliferation of D407 cells is stimulated by Polyphenon E at concentrations from 20-70 µg/ml. A marked decrease of cells viability was recorded for concentrations higher than 200 µg/ml. Starting from these results, we decided to use a concentration of 100 µg/ml Polyphenon E for further experiments.

Viability of D407 cells was also evaluated using Polyphenon E alone and when oxidative stress was induced with 500 µM hydrogen peroxide. Hydrogen peroxide treatment was applied after medium removal and washing cells with PBS, in order to avoid direct contact of H₂O₂ and catechins. MTT proliferation assay was monitored after 24h of incubation with catechins and 1 h H₂O₂ treatment. Treatment of RPE cells with Polyphenon E alone determined a 8 % decrease of cells viability. In oxidative stress induced condition, the addition of Polyphenon E determined a viability increase of 13 % compared with cells treated only with hydrogen peroxide. This result indicates a slightly protective effect of catechin extracts on RPE cells in oxidative stress condition.

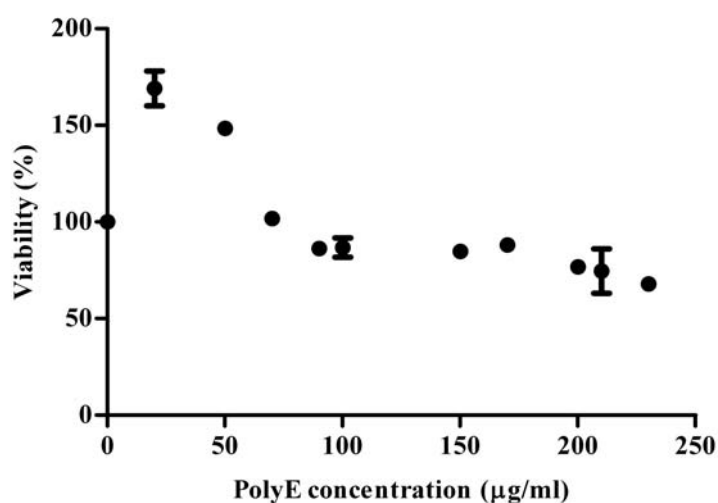
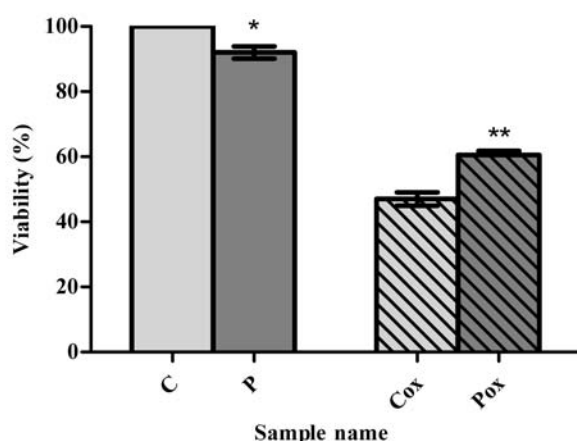


Figure 6: The viability of RPE cells treated with different concentrations of Polyphenon E for 24 h. Data represent the means \pm SD of three independent experiments

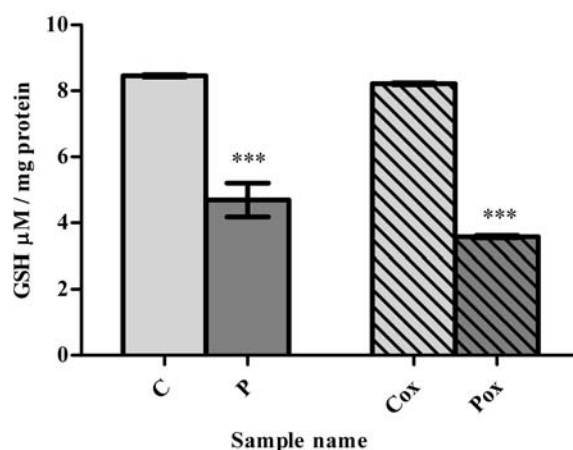


C - control; P - Polyphenon E; Cox - control + H₂O₂; Pox - Polyphenon E+H₂O₂

Figure 7: The viability of RPE cells treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

Glutathione level

The concentration of GSH in D407 cells was calculated using a calibration curve, made from eight concentrations of GSSG standard. The calibration curve was expressed in equivalents of total GSH, because under the assay conditions GSSG is reduced to produced 2 mole equivalents of GSH. Treatment with 500 µM hydrogen peroxide induced a slightly, not significant decrease of GSH levels. The intracellular GSH level decreased significantly (44 %) in cells preincubated with 100 µg/ml Polyphenon E, compared to untreated cells. The GSH level was very slightly decreased in cells treated with both Polyphenon E and H₂O₂, compared to cells exposed to Polyphenon E treatment (Fig. 8).



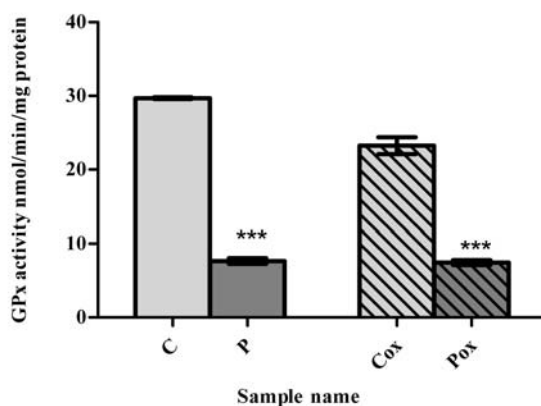
C - control; P - Polyphenon E; Cox - control + H₂O₂; Pox - Polyphenon E+H₂O₂

Figure 8: Levels of GSH concentrations in D407 cells treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

Glutathione peroxidase activity

Figure 9 shows the influence of Polyphenon E with/without hydrogen peroxide on glutathione peroxidase activity. Glutathione peroxidase activity was decreased with 22 % in RPE cells treated with hydrogen peroxide compared with untreated control cells. Cells

treatment with Polyphenon E determines a decrease of 73 % of glutathione peroxidase activity compared to untreated cells. In cells treated with Polyphenon E and hydrogen peroxide the GPx activity had similar values as with those receiving only the catechin extract.

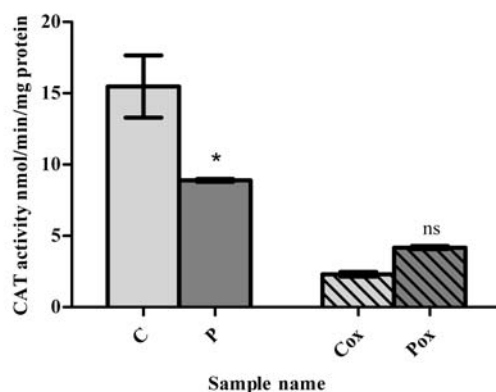


C - control; P - Polyphenon E; Cox - control + H₂O₂; Pox - Polyphenon E + H₂O₂

Figure 9: GPx activity in D407 RPE cells treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

Catalase activity

Catalase activity was decreased by 83 % by addition of hydrogen peroxide, compared with untreated (Fig. 10). Polyphenon E alone decreased catalase activity (with 42 %), compared to untreated cells, but in cells treated with both Polyphenon E and hydrogen peroxide the catalase activity was 1.6 fold higher than for cells treated only with hydrogen peroxide.



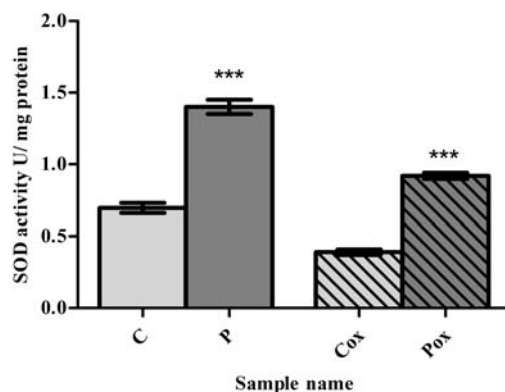
C - control; P - Polyphenon E; Cox - control + H₂O₂; Pox - Polyphenon E + H₂O₂

Figure 10: CAT activity in D407 RPE cells treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

Superoxide dismutase activity

As can be seen in Figure 11, SOD activity was decreased significantly by addition of hydrogen peroxide, compared with untreated control cells 21 % (Fig. 11). The Polyphenon E treatment for 24h, determined a significant increase of SOD activity compared to control cells (51 %). Moreover, in cells treated with both Polyphenon E and hydrogen peroxide, the activity of SOD was also significantly higher (almost 3 fold) than in cells receiving only the

oxidant. In conclusion, the catechin rich extract enhance the activity of SOD in normal and oxidative stress conditions.



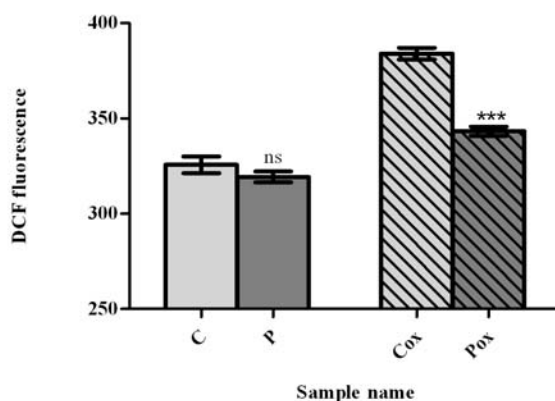
C - control; P - Polyphenon E; Cox - control+H₂O₂; Pox - Polyphenon+H₂O₂

Figure 11: SOD activity in D407 RPE cells Polyphenon E treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

Intracellular reactive species

Therefore, we investigated whether Polyphenon E could influence the generation of intracellular ROS in D407 cells. In order to avoid the direct interaction between the oxidant and the catechins, cells were incubated for 24h either in medium alone or in a medium that contained Polyphenon E. The culture media was removed and cells were washed and treated for 1 h with hydrogen peroxide. In this way, only the intracellular effect of catechins was measured, by monitoring the fluorescence of DCF with a microplate reader.

As shown in Figure 12, there was a significant increase in fluorescence level of D407 cells treated with hydrogen peroxide alone (18 %).



C-control; P-Polyphenon E; Cox-control+H₂O₂; Pox-Polyphenon E + H₂O₂

Figure 12: The level of ROS generation in D407 RPE cells treated with Polyphenon E (100 µg/ml, 24 h) and/or with hydrogen peroxide (500 µM, 1h). Data represent the means ± SD of three independent experiments (* significant $P < 0.05$, ** very significant $P < 0.01$, *** extremely significant $P < 0.001$)

In cells treated only with Polyphenon E a not significant inhibition of ROS generation was observed compared to control. Comparing the samples in which oxidative stress was induced, a strong inhibition of DCF fluorescence can be observed for cells pretreated with Polyphenon E (11 %). This result suggests that Polyphenon E can inhibit the intracellular generation of ROS in D407 RPE cells.

Discussions

Hydrogen peroxide is an ubiquitous oxidant which has been found in ocular tissues *in vivo* [24] and can be produced by the RPE as a reactive oxygen intermediate during photoreceptor outer segment phagocytosis [25]. Induction of oxidative stress by hydrogen peroxide is a common procedure in testing of antioxidant capacities of various substances. In this study we tested several concentrations of hydrogen peroxide and have chosen the 500 μM concentration for the evaluation of catechin effects on the antioxidant status of RPE cells. At this concentration we observed a severe decrease of cell viability (47%), a change in cell morphology (rounding), but not a severe change of cells densities. It was previously reported that treatment of RPE cells with 100 μM hydrogen peroxide for 5 hours determined significant changes in cell morphology, a reduction of cell density, cytopathic effect in 50 % of cells, nuclear condensation and granular cytoplasm [26]. For 500 μM hydrogen peroxide in the culture media the same study mentioned a 32 % decrease of RPE cell viability.

Treatment with Polyphenon E extract showed a protective effect in RPE cells, especially when oxidative stress was induced. Viability test results obtained in our study are consistent with a complex study regarding the flavonoids which showed that major catechins EGCG, EC and C had LD_{50} (the doses of the compounds that cause 50 % of cell death) higher than 50 μM in ARPE-19 line, and higher than 100 μM in primary human RPE 153 cells. The same study mentions a half maximal effective concentration of EGCG at 30 μM on primary cells in milder oxidative stress (250 μM H_2O_2) but no effect in ARPE cells [17].

The role of GSH in maintaining cellular redox state is complex. GSH cooperates with GPx in the detoxification of H_2O_2 . In addition, GSH participates in reactions with glutathione S-transferase (GST) to bind ROS e.g. attachment of NO to form S-nitrosoglutathione adducts. Glutathione reductase (GR) functions to regenerate antioxidant capacity, converting GSSG to GSH [27]. It was found that plasma glutathione reductase is reduced substantially in patients with AMD [28]. In our study, the treatment of RPE cells both with Polyphenon E alone and Polyphenon E + H_2O_2 determined a significant decrease of GSH concentration and of GPx activity, showing not a protecting effect against induced oxidative stress. Even more, the simultaneously decrease of GSH and GPx suggest an increase of oxidative stress level and a serious perturbation of the GSH cell cycle. Treatment of RPE cells with quercetin, another potent antioxidant which belongs to the family of flavonoids, showed that quercetin is able to protect RPE cells from oxidative damage and cellular senescence *in vitro*. Quercetin acts by down-regulation of caveolin-1, by reducing the hydrogen peroxide-induced activation of caspase-3 and attenuation of the increase in β -galactosidase activity. However, quercetin treatment did not have a significant effect on the intracellular level of reduced glutathione [29]. It is possible that catechins, as well as quercetin act as antioxidants by a mechanism which is independent on glutathione and glutathione peroxidase enzyme.

Catalase is an essential enzyme for hydrogen peroxide detoxification in retinal pigment epithelium due to the fact that phagocytosis of rod outer segments leads to a high production of H_2O_2 . It was shown that the treatment of RPE cells with 250 and 500 μM H_2O_2 for 18 hours causes approximately 1.5-fold and 4-fold increases in CAT activity, respectively [25]. Similarly, it has been shown that CAT activity increases by approximately two fold when RPE cells phagocytise bovine rod outer segments [30]. However, in a more recent study a short term exposure (1 h) of non-adapted RPE cells to a higher concentration of H_2O_2 (3mM) determined a very severe decrease in CAT (7 fold), GPx (2.1 fold) and CuZnSOD (37 fold) activities [9]. Absence of any increase in CAT activity after short-term exposure of H_2O_2 during our study suggests that CAT induction may be a later event in the adaptive response of

RPE cells to sustained high levels of oxidative stress. However, the activity of catalase was higher in Polyphenon E + H₂O₂ treated cells versus cells treated with hydrogen peroxide alone.

SOD isoenzymes are present in human eyes, consisting of the extracellular SOD (EC-SOD), the cytosolic copper- and zinc-containing SOD (Cu/Zn-SOD, or SOD1), and the mitochondrial manganese-containing SOD (Mn-SOD) [31]. SOD enzymes work in conjunction with H₂O₂-removing enzymes, such as catalase and glutathione peroxidase [32]. In our study, a significant increase of SOD activity was recorded for cells treated with Polyphenon E +/- H₂O₂. A similar effect was observed when RPE cells were treated with L-carnitine. It determined an increase of SOD activity in a dose dependent manner and enhanced the SOD activity in cells exposed to hydrogen peroxide [26]. Polyphenon E treatment of RPE cells determined an inhibition of intracellular ROS generation in cells treated only with Polyphenon E versus control, but also in cells under induced oxidative stress condition. Hanneken et al. obtained similar results with other flavonoids (quercetin, fisetin, luteolin) when oxidative stress was induced in RPE cells with tert-butyl hydroperoxide [17].

Despite of several evidences that catechins act as antioxidants in vivo and in vitro, the mechanism of their action is not yet elucidated and their antioxidant effect is subject of controversy. Moreover, there are studies showing that catechins exert prooxidant effects especially by generation of hydrogen peroxide in solutions and culture media [33-36]. In our study, administration of the catechin -rich extract induced an increase of SOD activity under normal and oxidative stress conditions and an increase of catalase only in cells in induced oxidative stress. Polyphenon E had a negative effect on glutathione peroxidase activity and glutathione level. The only available literature data show stimulation (only semi-quantitative data +/-) of Nrf2 and heme oxygenase in RPE cells treated with EGCG and EC and a protective effect in primary RPE cells [17]. However, the significant inhibition of intracellular ROS generation supports the hypothesis that catechins can contribute to antioxidant defense by direct scavenge of ROS in RPE cells.

Retinal pigmented epithelium (RPE) is continuously enduring aggressive attack of reactive oxygen species, resulting either from phagocytosis or other metabolic processes. It is considered that oxidative stress at RPE level is one of the major causes of age-related macular degeneration. This study demonstrates that Polyphenon E extract can contribute to the protection of RPE cells in vitro against reactive oxygen species by enhancing SOD activity and acting as ROS scavenger, but without a positive influence on the glutathione cycle. More studies on the expression of antioxidant enzymes are needed in order to understand the mechanism of action of catechins at cellular levels in RPE cells. Taking in account the lack of toxicity even at high concentrations, studies on human subjects are necessary to see if a catechin rich diet can have a preventing effect on age-related macular degeneration.

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