Bulletin UASVM, Veterinary Medicine 66(1)/2009
ISSN 1843-5270; Electronic ISSN 1843-5378

Chlorogenic Acid Reduces Oxidative Stress in Rpe Cells

PINTEA Adela, RUGINĂ Dumitrița, PÂRLOG Raluca, VARGA Andreea

University of Agricultural Sciences and Veterinary Medicine, Mănăştur Street, No. 3-5, Cluj-Napoca, România, e-mail: apintea@usamvcluj.ro

Abstract. Our purpose was to determine whether chlorogenic acid (CGA) influence the levels of antioxidant defense status by monitoring the activity of catalase (CAT), of superoxide dismutase (SOD), reduced glutathione (GSH) content and the generation of intracellular reactive oxygen species (ROS). Confluent RPE cells (D407) were preincubated with l00 µM chlorogenic acid and after phenolic acid removal, cells were oxidatively challenged with 500µM H_2O_2.

The dose of l00µM chlorogenic acid did not induce morphological changes in RPE cells, indicating a viability of 88% comparing with control after 24h of treatment. CGA treatment has not cytotoxic effect and reduces the cytotoxic effect of hydrogen peroxide in RPE cells. Chlorogenic acid treatment (1) up regulates the antioxidant enzymes activities CAT and SOD in RPE, especially in oxidatively stressed cells; (2) determine an increase of reduced glutathione concentration and, (3) decrease intracellular ROS generation in RPE cells subjected to oxidative stress.

Chlorogenic acid could be a potential antioxidant agent for RPE cells, protecting them by oxidative stress.

INTRODUCTION

Chlorogenic acid is the main polyphenolic component in many fruits and beverages, in sunflower seeds and particularly in coffee, making up 5–10% of the weight of coffee beans. Chlorogenic acid exerts actions linked to antioxidant activities associated with free radical scavenging ability (Clifford, M. N., 1999).

Although chlorogenic acid has various physiological actions, its in vivo absorption remains controversial. It was previously reported that the urinary excretion of chlorogenic acid and its in vivo metabolites such as caffeic and *m*-coumaric acids after coffee ingestion was significantly higher than that of the control group in a human study (Ito, H. et al., 2005). More recently it was proved that CGA from green coffee are highly absorbed and metabolized in humans. More than 30 % of ingested cinnamic acid moieties were recovered in plasma of patients treated with a green coffee extract rich in chlorogenic acid (Farah, A. et al., 2008).Cheng et al. found that caffeic acid and chlorogenic acid are equally effective antioxidants for in vitro peroxidation of human low density lipoproteins (Cheng, J. C. et al., 2007). By chemical kinetics techniques was demonstrated that CGA is an efficient antioxidant in protecting erythrocytes against hemin-induced hemolysis. CGA was also able to trap radicals, as proved by ABTS and DPPH assays (Tang, Y. Z. and Liu, Z. Q., 2008).

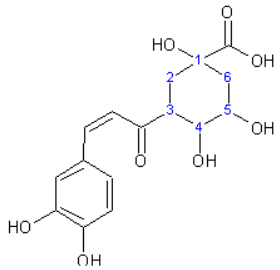

Fig.1. Chemical structure of chlorogenic acid

RPE cells have evolved sophisticated mechanisms to combat the potential deleterious effects of reactive oxygen species, resulting from the process of phagocytosis and other metabolic processes, in the form of a defense system consisting of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). In addition, reducing agents and free radical scavengers such as glutathione (GSH) and phenolic acids provide protection against reactive oxygen species through nonenzymatic mechanisms (Singhal, S. S. et al., 1999). Inducing enzymes involved in glutathione (GSH) biosynthesis also enhances cellular antioxidant defenses (Hayes, J. D. and McLellan, L. I., 1999). Age related macular degeneration is associated with decline of GSH (Samiec, P. S. et al., 1998).

Our previous results show that exposure to peroxide hydrogen (500µM) affects antioxidant defense system. Since oxidative stress induced cellular changes involves ROS, antioxidants might be helpful to minimize oxidative stress-induced pathologies, like age related macular degeneration.

MATERIAL AND METHODS

Cell culture and culture conditions

Human adult retina epithelial cells D407 were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at 37°C, 5% CO_2, and 95% relative humidity. The cells were seeded in 25 cm^3 flask at a concentration of 6 x 10^5 upon reaching the 90 % confluence; growth medium was removed and replaced with medium containing chlorogenic acid (Sigma, USA) in concentration of 100 µM in culture medium. After 24 hours of chlorogenic acid treatment, the cells were exposed to 500 µM H_2O_2 at 37 °C for 1hour in serum free DMEM medium.

Cell viability assay

RPE cells were plated (10,000 cells per well) in 96-well plates and, after the cells attached, were incubated for 24h with chlorogenic acid and for 1h with H_2O_2. The number of viable cells at each time point was determined with the thiazolyl blue tetrazolium bromide (MTT) cell proliferation reagent. This method uses the property of viable cells to reduce MTT into a colored formazan, product which is detected by absorbance at 570 nm with a 96-well plate reader. Cell viability was expressed as a percentage of control (cells incubated in normal medium only).

LDH activity assay

Method based on LDH activity is used for monitoring the *in vitro* cytotoxicity of substances tested in cell cultures. LDH is an enzyme which can be released in cytosol only after cell lysis. To evaluate the quantity of LDH released in cell culture medium after 24h of treatment with chlorogenic acid and/or peroxide hydrogen, we used the colorimetric LDH assay kit instructions (Sigma-Aldrich).

Assay for evaluation of antioxidant enzymatic and non-enzymatic defense system

Protein concentrations for each sample were determined using the bicinchoninic acid assay kit instructions (Sigma, USA). Activity of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) content were measured using commercial kits (Cayman Chem).

Measurement of reactive oxygen species

The determination of intracellular reactive oxygen species 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 microplate reader (BioTek). Cells were incubated with dichlorofluorescein diacetate (DCFDA) (stock solution 20 mM) using dilution 1:1000 in PBS. Fluorescence was monitored for 4h at 37 ºC at excitation 485/10 nm and emission 528/20 nm.

Statistical analysis

Statistical analysis were done using GraphPad Prism version 5.00. The points or bars represent the mean ± SEM, calculated from three experimental values.

RESULTS AND DISCUSSIONS

Viability of pigmented epithelial cells from human retina (D407) treated with chlorogenic acid. Human cells were grown to confluence and exposed to different concentrations of chlorogenic acid (0-250μM) for 24 h. There were not significant changes of cells viability in this concentration range. At a concentration of 100 μM CGA, the cells viability was 88%. This concentration was further used for all experiments.

Cytotoxicity of chlorogenic acid in RPE cells. To assess the cytotoxicity effect induced by chlorogenic acid in RPE cells we used an LDH colorimetric assay (Sigma-Aldrich). LDH release in cell culture medium revealed an higher cytotoxicity for peroxide hydrogen treated cells, comparing to untreated RPE cell. In the case of treatment with both hydrogen peroxide and chlorogenic acid, the LDH leakage was 20% lower comparing to cell treated only with H_2O_2, with a *p* value of 0.0026. This result may reflect that the chlorogenic acid could reduce the cytotoxicity induced by hydrogen peroxide exposure in RPE cells (Fig. 2).

Evaluation of antioxidant enzymes in RPE cells treated with chlorogenic acid. The activity of two key antioxidant enzymes - superoxide dismutase and catalase - were monitored after 24 hours of cells treatment with 100 μM chlorogenic acid and one hour exposure to 500 μM hydrogen peroxide (after removing the media containing phenolic acid). Treatment with hydrogen peroxide causes a decrease of antioxidant enzyme activity. An increase activity of both enzymes was observed in cells treated with CGA.

Cells pretreated with CGA and exposed to hydrogen peroxide showed enhanced activity of catalase and superoxide dismutase, compared with untreated cells exposed only to H_2O_2. Activity of superoxide dismutase and catalase in cells treated with CGA and H_2O_2 was practically restored at the level of control cells (Fig. 3, Fig. 4).

Effect of chlorogenic acid on reduced glutathione concentration. GSH content in RPE cells increased significantly after treatment with CGA, in both exposed/not exposed to hydrogen peroxide cells (Fig. 5). It is supposed that phenolcarboxylic acids acts as antioxidants in vivo together with reduced glutathione, forming an intricate antioxidant network (Kadoma, Y. and Fujisawa, S., 2008). Studies realized on HepG2 cell line demonstrated that CGA treatment had a slight effect on cell viability and ROS formation but increased glutathione levels (Granado-Serrano, A. B. et al., 2007).

Chlorogenic acid induces generation of intracellular reactive oxygen species. Hydrogen peroxide treatment induced an increase in fluorescence compared with untreated cells and those treated only with CGA. In cells pretreated with CGA and exposed to hydrogen peroxide can be observed a decrease in DCF fluorescence, comparing to cells treated with hydrogen peroxide (Fig. 6). It demonstrates that CGA is also able to scavenge directly the intracellular ROS. An apple extract rich in catechin and chlorogenic acid, as well as the pure compounds, reduced by 50 % the oxidative injuries in gastric epithelial adenocarcinoma cells MKN 28 exposed to xanthine/xanthine oxidase and determined a four fold increase of intracellular antioxidant activity (Graziani, G. et al., 2005)

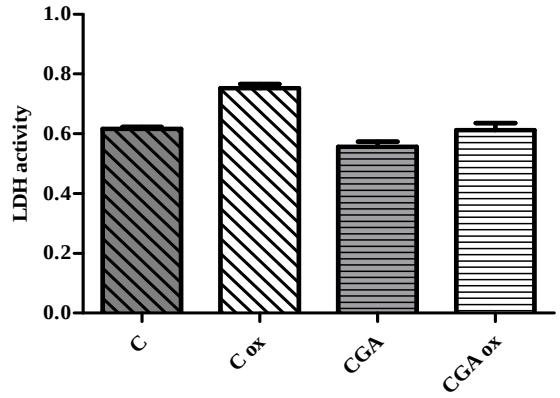

Fig. 2. LDH cytoxicity assay for epithelial cells from human retina (D407) treated with acid chlorogenic

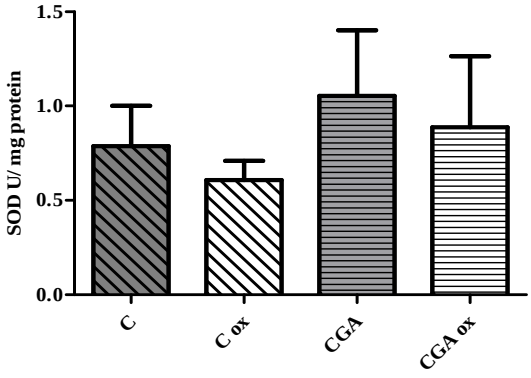

Fig. **3.** Superoxide dismutase activity in RPE cells after treatment with 100 μM chlorogenic acid

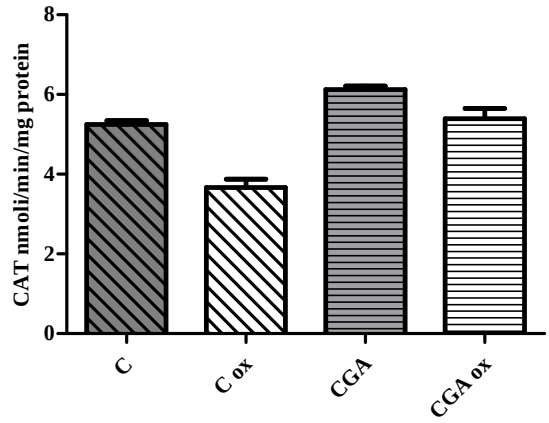

Fig. 4. Catalase activity in RPE cells after treatment with 100 μM chlorogenic acid

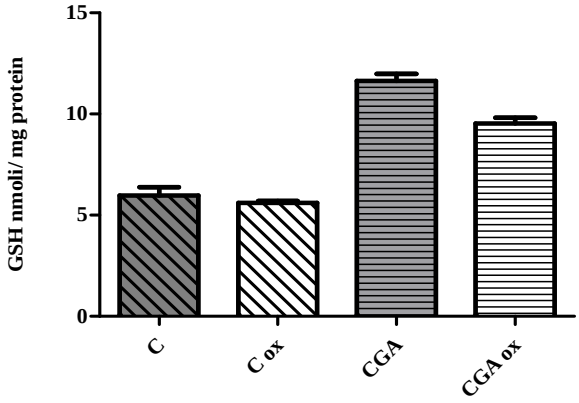

Fig. 5. GSH content in RPE cells after treatment with 100 μM acid chlorogenic

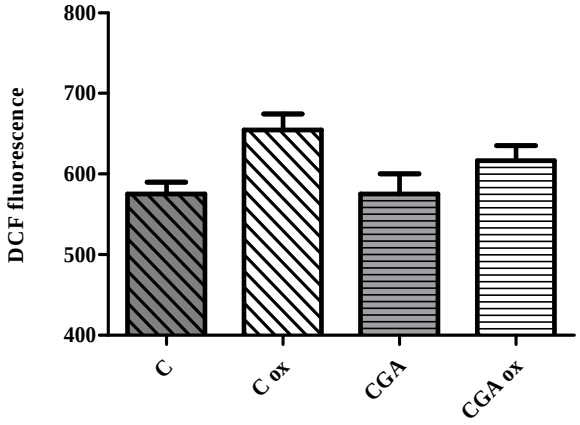

Fig. **6.** Determining the level of intracellular reactive oxygen species in pigmented epithelial cells of human retina
C = control; Cox = control+ H_2O_2; CGA = chlorogenic acid; CGA ox = chlorogenic acid + H_2O_2

CONCLUSIONS

During this work were investigated antioxidant properties of chlorogenic acid, in pigmented epithelial cells from human retina, in both normal and induced oxidative stress conditions.

Chlorogenic acid is not cytotoxic for RPE cells on concentration range 0-250μM and reduces the cytotoxic effect of hydrogen peroxide in RPE cells. Treatment of RPE cells with chlorogenic acid causes an increase in CAT and SOD activity in control cells and in those treated with hydrogen peroxide. CAT and SOD may be important to the protection mechanisms against H_2O_2. Chlorogenic acid determines an increase of reduced glutathione concentration and a decrease of intracellular ROS generation in RPE cells subjected to oxidative stress.

Chlorogenic acid could be a potential antioxidant agent for RPE cells, protecting them by oxidative stress, the major causes of retinal diseases such as age related macular degeneration disease.

ACKNOWLEDGEMENTS

This work was supported by PN II ID-854 research grant.

REFERENCES

1. Cheng, J. C., Dai, F., Zhou, B., Yang, L. and Liu, Z.-L., 2007. Antioxidant activity of hydroxycinnamic acid derivative in human low density lipoprotein: Mechanism and structure–activity relationship, Food Chemistry, 104(1): 132–139.

2. Clifford, M. N., 1999. Chlorogenic acids and other cinnamates-Nature, occurence and dietary burden, J. Sci. Food Agric., 79: 362–372.

3. Farah, A., Monteiro, M., Donangelo, C. M. and Lafay, S., 2008. Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans, Journal of Nutrition, 138(12): 2309-2315.

4. Granado-Serrano, A. B., Martín, M. A., Izquierdo-Pulido, M., Goya, L., Bravo, L. and Ramos, S., 2007. Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line, J. Agric. Food Chem., 55(5): 2020-2027

5. Graziani, G., D'argenio, G., Tuccillo, C., Loguercio, C., Ritieni, A., Morisco, F., Del Vecchio Blanco, C., Fogliano, V. and Romano, M., 2005. Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo, Gut, 54: 193–200.

6. Hayes, J. D. and Mclellan, L. I., 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress, Free Radic Res, 31(4): 273-300.

7. Ito, H., Gonthier, M. P., Manach, C., Morand, C., Mennen, L., Remesy, C. and Scalbert, A., 2005. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages, Br J Nutr, 94(4): 500-509.

8. Kadoma, Y. and Fujisawa, S., 2008. A comparative study of the radical-scavenging activity of the phenolcarboxylic acids caffeic acid, p-coumaric acid, chlorogenic acid and ferulic acid, with or without 2-mercaptoethanol, a thiol, using the induction period Molecules, 13(10): 2488-2499.

9. Samiec, P. S., Drews-Botsch, C., Flagg, E. W., Kurtz, J. C., Sternberg, P., Jr., Reed, R. L. and Jones, D. P., 1998. Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes, Free Radic Biol Med, 24(5): 699-704.

10. Singhal, S. S., Godley, B. F., Chandra, A., Pandya, U., Jin, G. F., Saini, M. K., Awasthi, S. and Awasthi, Y. C., 1999. Induction of glutathione S-transferase hGST 5.8 is an early response to oxidative stress in RPE cells, Invest Ophthalmol Vis Sci, 40(11): 2652-2659.

11. Tang, Y. Z. and Liu, Z. Q., 2008. Chemical Kinetic Behavior of Chlorogenic Acid in Protecting Erythrocyte and DNA against Radical-Induced Oxidation, Journal of agricultural and food chemistry, 56(22): 11025-11029.