

Effects of astaxanthin on antioxidant parameters in ARPE-19 cells on oxidative stress model

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Abstract

• **AIM:** To observe the protective effect of astaxanthin (AST) against hydroquinone (HQ) mediated cell death in the apoptotic cascade and evaluate intracellular Ca²⁺ release, caspase-3, and -9 activation, reactive oxygen species (ROS) production in ARPE-19 cells.

• **METHODS:** We cultured ARPE-19 cells in special mediums and performed MTT tests to determine protective effect of AST, before exposing the cells to HQ in an incubator. We analyzed intracellular Ca²⁺ release experiments, mitochondrial membrane depolarization, glutathione (GSH), glutathione peroxidase (GSH-Px) and ROS experiments, and apoptosis assay.

• **RESULTS:** ROS production ranges depend on the amount of cell death. We computed the correlation between ROS ranges and cell death by 20,70-dichlorofluorescein fluorescence, and Ca²⁺ levels by Fura-2-AM. HQ-induced cell death found out to rise ranges of caspase-3 and -9, and mitochondrial depolarization. These three steps were delayed by AST management.

• **CONCLUSION:** ARPE-19 cells are avoided from HQ-induced ROS production and caspase-3 and -9 activation by AST. AST may limit the range of caspase synthesis, Ca²⁺ release and excess production of ROS with antiapoptotic effect. This study proposes a new therapeutic approach for the treatment of age-related macular degeneration.

• **KEYWORDS:** apoptosis; ARPE-19 cell; astaxanthin; oxidative stress

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INTRODUCTION

Age-related macular degeneration (AMD) is the most important reason of irreversible vision loss in aged people in the world and it is also a severe, progressive ophthalmologic problem^[1-2]. There are some risk factors of AMD development and progression like advanced age, Caucasian race, genetic polymorphisms, high body mass index, excess alcohol consumption and smoking history^[3-4]. Today, the pathogenesis of AMD is not known exactly, but oxidative stress play an important role in the pathogenesis. Retina pigment epithelial (RPE) cell degeneration occurred frequently in early stages of the disease, may enlarge in macular region in progress of time^[5-6].

Cigarette smoking enhances the formation of oxidative damage in the body and weakens the antioxidant defense mechanisms with age, may lead to increased inflammation. Some studies report that smoking is one of the major risk factors associated with the prevalence and incidence of AMD. Hydroquinone (HQ) is found not only in cigarettes but also in processed foods, in plastic containers, in the atmosphere, namely that is widely present in nature. HQ present in high concentrations in cigarettes. It is an aromatic organic phenol compound and also known as benzene-1,4-diol or quinol, may return to parabenzoquinone (C₆H₆O₂) by oxidation^[7-14].

Astaxanthin (AST) is an antioxidant molecule found in high amounts in shellfish known as non-provitamin xanthophyll carotenoid^[15]. Recently, AST, for antioxidant effect, is added to the nutritional supplements used in AMD patients^[16]. However, it is not known exactly about the protective activity of AST against oxidative stress generated by cigarettes on RPE cells.

Homeostasis of intracellular calcium concentration ([Ca²⁺]_i) is important for cellular signaling mechanisms because of managing cellular functions like protein synthesis, gene expression, *etc.* The [Ca²⁺]_i may exchange by the cell membranes and releasing from the intracellular calcium stores through specialized calcium channels. By this way cytosolic Ca²⁺ stability is regulated^[5]. On the other hand Ca²⁺ has proapoptotic effects which are mediated by a multifarious level of Ca²⁺-sensitive factors that are separated in several intracellular organelle^[6]. That's why free [Ca²⁺]_i increases after cation channel function degeneration by oxidative stress, physiologic capacities of cells may be lost at the end^[8-9].

Accumulation of Ca^{2+} in the cytosol may lead to stimulate the apoptosis-promoting factors releasing. Some reports indicate that oxidative stress induced dysregulated $[\text{Ca}^{2+}]_i$ stability is accompanied by alterations in the apoptotic activity of diverse cell types.

In this study, our goal was to observe the protective capacity and ability of AST against the HQ-caused oxidative stress on *in vitro* RPE cells. And also we aimed to observe the role of oxidation caused by cigarette smoking in the AMD disease and then to be able to stop or treat the degeneration by using AST.

SUBJECTS AND METHODS

Chemicals All organic solvents were purchased from Merck (Darmstadt, Germany). Buffers and other chemicals; the hydrogen peroxide (H_2O_2), potassium hydroxide, sodium hydroxide, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), 5,5-dithiobis-2 nitrobenzoic acid (DTNB), tris(hydroxymethyl)aminomethane, reduced glutathione (GSH), butylhydroxytoluene, Triton X-100, and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) were obtained from Sigma-Aldrich (St Louis, MO, USA). The fluorescein dye fura-2 acetoxymethyl ester (Fura-2) was purchased from Invitrogen (Carlsbad, CA, USA). All reagents were of analytical grade and were prepared daily and stored at $+4^\circ\text{C}$ except the phosphate buffers. Reagents were equilibrated at room temperature for half an hour before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at $+4^\circ\text{C}$ for 1mo. APOPercentage Assay Kit was purchased from Biocolor (Belfast, Northern Ireland, UK).

Study Groups Group I was the control, the cells were incubated during 24h in its special growth medium. Group II was HQ group, the cells were incubated with $100\ \mu\text{mol/L}$ HQ for 24h. Group III was AST group, the cells were incubated with $200\ \mu\text{mol/L}$ AST for 24h. Group IV was HQ+AST group and the cells were incubated with $100\ \mu\text{mol/L}$ HQ during first 24h, and then incubated with $200\ \mu\text{mol/L}$ AST during second 24h.

Cell Culture The human retinal pigmented epithelial cell line (ARPE-19; ATCC, Manassas, VA, USA) was grown in a special mixture of a growth medium containing 1:1 ratio of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% penicillin-streptomycin combination (Biochrom). Cells were used for the experiments at passages 3-10.

Calcium $[\text{Ca}^{2+}]_i$ Determination by Fluorescent Dye After all incubation processes, cells were loaded with $4\ \mu\text{mol/L}$ Fura-2 acetoxymethyl ester (Carlsbad, CA, USA) dye by incubation for 30min at room temperature according to a procedure published elsewhere^[7]. Then the cells were washed and gently re-suspended in Na-HEPES buffer solution containing

(in mmol/L): NaCl, 140; KCl, 4.7; CaCl_2 , 1.2; MgCl_2 , 1.1; D-glucose, 10; and HEPES, 10 (pH 7.4). The cells in all groups were exposed to H_2O_2 to stimulate $[\text{Ca}^{2+}]_i$ release. Fluorescence signals were recorded from 2 mL aliquots (2×10^6 cells/mL) at 37°C using a spectrofluorometer (Cary Eclipse, Varian Inc., Sydney, Australia) with excitation wavelengths at 340 nm and 380 nm, and emission at 505 nm. The $[\text{Ca}^{2+}]_i$ oscillations were determined by using the 340/380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz *et al*^[12]. Ca^{2+} release was evaluated using the integral of the rise in $[\text{Ca}^{2+}]_i$ for 60s after addition of H_2O_2 . The Ca^{2+} release is expressed as nmol/L, taking a sample every second (nmol/L/s) as described elsewhere^[11].

Measurement of Lipid Peroxidation Level The lipid peroxidation levels in all groups were measured with TBA reaction method by Placer *et al*^[16]. The quantification of TBA-reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane.

Reduced Glutathione, Glutathione Peroxidase, and Protein Assays The GSH content of all groups was measured at 412 nm by using the Sedlak and Lindsay method^[17]. The glutathione peroxidase (GSH-Px) activities of groups were spectrophotometrically measured at 37°C and 412 nm according to the Lawrence and Burk method^[18]. The protein content in all groups was determined by Lowry *et al*'s method^[19] with bovine serum albumin regarded as the standard.

Measurement of Mitochondrial Membrane Potential In order to measurement of mitochondrial membrane potential, all groups were loaded with $1\ \mu\text{mol/L}$ tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for 15min at 37°C as previously described^[20]. The cationic dye JC-1 indicates intermembrane potential-dependent aggregation in mitochondria that shows mitochondrial depolarization by a decrease in the red-to-green fluorescence intensity ratio. After incubation the dye was removed, and the cells were washed with $1 \times$ phosphate buffered saline (PBS). The green JC-1 signal was recorded at 485 nm excitation wavelength and 535 nm emission wavelength. And for the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm were used respectively. Fluorescence changes were screened using a fluorescence microplate reader (Tecan Infinite 200 Pro, Austria). The results are presented as emission ratios (590/535). Changing in the mitochondrial membrane potential was quantified as the integral of the decrease in JC-1 fluorescence ratio.

Apoptosis Assay For the assessment of rational apoptosis, the APOPercentage Assay Kit was purchased by Biocolor Ltd., Belfast, UK. The kit was performed according to the manufacturer's instructions. In brief, the kit content is a dye-

Table 1 Effects of AST, HQ, and their combinations on LP, GSH-Px, GSH mean±SD

Parameters/Groups	Control	HQ	AST	HQ+AST
LP ($\mu\text{mol}/5\times 10^6$ cells)	8.79±0.46	9.82±0.63 ^a	8.45±0.31 ^b	8.60±0.81 ^b
GSH ($\mu\text{mol}/5\times 10^6$ cells)	6.94±0.82	5.25±0.85 ^a	8.34±1.12 ^{a,b}	7.49±0.46 ^{a,b,c}
GSH-Px (IU/ 5×10^6 cells)	5.62±0.67	4.68±0.74 ^a	7.15±0.33 ^{a,b}	6.25±0.93 ^{a,b,c}

AST: Astaxanthin; HQ: Hydroquinone; LP: Lipid peroxidation; GSH-Px: Glutathione peroxidase; GSH: Glutathione. ^a $P<0.05$ vs Control; ^b $P<0.05$ vs HQ; ^c $P<0.05$ vs AST.

uptake assay, which stains only the apoptotic cells with a red color. When the plasma membrane asymmetry lost, the APOPercentage dye is actively entered into cells, stains only apoptotic cells into red and thus allows detection of apoptosis by spectrophotometer^[21].

Assay for Caspase Activities After incubations the cells were sonicated and lysates were incubated with 2 mL of substrate solution [20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L DTT and 8.25 $\mu\text{mol}/\text{L}$ of caspase substrate] for 1h at 37°C, as previously described^[22]. Caspase-3 and -9 were calculated by the cleavage of the specific indicator fluorogenic substrates (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9). The substrate cleavage was measured with a fluorescence spectrophotometer at 360 nm excitation wavelength and at 460 nm emission wavelength. At the presence of inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHD-FMK, respectively, cleaving of substrates were not detected. The data were calculated as fluorescence units per milligram of protein.

Statistical Analysis Data are expressed as means±SEM of the number of determinations. Statistical significance was analysed by using the SPSS packet program (9.05, SPSS, Chicago, IL, USA). To compare the effects of different treatments, statistical significance was calculated by Mann-Whitney *U* test. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Effects of Astaxanthin on Intracellular Ca²⁺ Release in ARPE-19 Cells Intracellular Ca²⁺ levels; there is a significant increase with HQ ($P<0.001$); decrease with AST ($P<0.001$) as shown in Figure 1A and 1B, with a significant additive effect: [Ca²⁺]_i levels were significantly lower in group AST group compared with the other groups ($P<0.05$).

Effects of Astaxanthin on Lipid Peroxidation, Glutathione, and Glutathione Peroxidase Ranges The effects of AST on the balance of [Ca²⁺]_i after HQ implementation correlate with increase in lipid peroxidation as indicated by the increase in MDA, intracellular GSH-Px levels and reduction in GSH (Table 1). However, AST supplementations were associated to an increase in GSH levels compared with other groups, and decreased MDA levels (Table 1).

Effects of Astaxanthin on Mitochondrial Depolarization Levels The effects of AST on mitochondrial depolarization levels

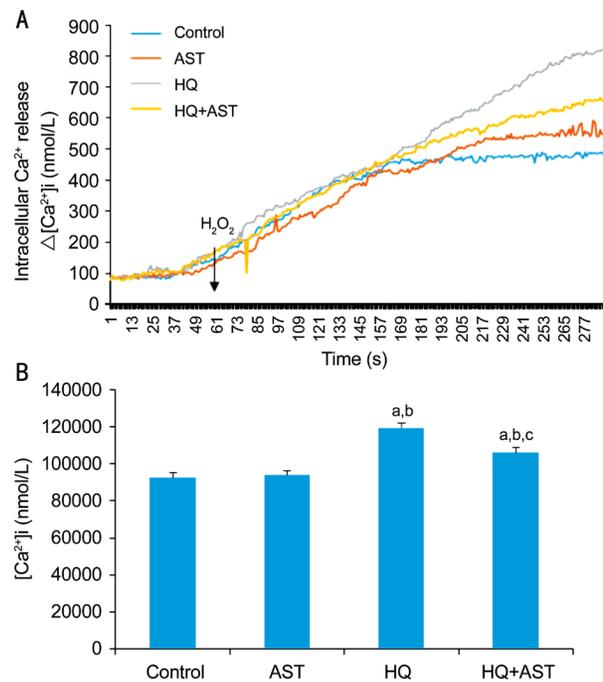


Figure 1 The effects of hydroquinone and astaxanthin on the release of Ca²⁺ in the cytosol in the ARPE-19 cell groups A: Time-flow chart of the Ca²⁺ release in the cytosol in the ARPE-19 cell groups of hydroquinone and astaxanthin. The cells were loaded with Fura-2-AM over 45min in a shaking water bath. The cells were stimulated with H₂O₂. Original time-course chart recordings showing cytosolic Ca²⁺ ([Ca²⁺]_i) transients in ARPE-19 cells; B: Bar charts showing mean±SD data for the cytosolic [Ca²⁺]_i concentration from H₂O₂-stimulated ARPE-19 cells ($n=6$ for each). ^a $P<0.05$ vs Control; ^b $P<0.05$ vs AST; ^c $P<0.001$ vs HQ.

in ARPE-19 cells was shown in Figure 2. AST administration significantly decreased hydroquinone triggered mitochondrial depolarization levels. There was a significant reduction in mitochondrial depolarization levels after AST supplementation according to the other groups ($P<0.05$).

Effects of Astaxanthin on Apoptosis, Caspase- 3, and -9 Levels The effects of AST on apoptosis levels, caspase-3 and -9 levels after HQ implementation were shown in Figures 3, 4, respectively. By itself, AST decreased the apoptosis significantly ($P<0.05$). Caspase-3 and -9 levels were also decreased in a manner relating with apoptosis levels.

DISCUSSION

AMD is one of the main reason of vision loss in those over age 50 in developed nations^[23]. In smokers increased oxidative

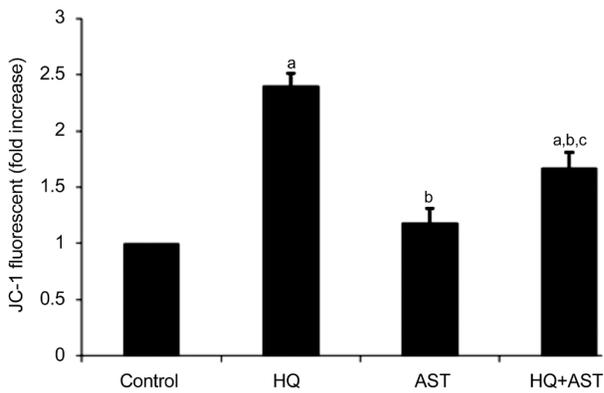


Figure 2 The effects of astaxanthin on mitochondrial depolarization levels in ARPE-19 cells The ARPE-19 cells showed significantly altered mitochondrial membrane depolarization levels after treatment with HQ ($P < 0.05$). The mitochondrial depolarization levels in the astaxanthin group significantly decreased when compared with those in the HQ and the control group. However, astaxanthin has the ability to decrease the increased mitochondrial depolarization levels in the HQ group. ^a $P < 0.05$ vs Control; ^b $P < 0.05$ vs HQ; ^c $P < 0.05$ vs AST.

stress with age is an important reason for the development of AMD. This paves the way for a progressive degeneration of the RPE and photoreceptor cells in the macula^[22,24]. As a result of increased oxidative stress in AMD with cigarette smoking, RPE degeneration occurs and apoptosis in ocular cells are triggered^[25-26].

AST is a red pigment that belongs to carotenoid family and found at high levels in shellfish^[20]. Some studies report that AST is a powerful antioxidant and neutralize the reactive radical products. Also AST can cross the blood-brain barrier by transcytosis^[27-28]. In addition to the immunomodulator activity of AST has some biological protector activity for islet β -cells in the pancreas and liver, hypertension and ischemia-induced amnesia. Animal studies define that AST prevents the lens damage caused by oxidative stress and Ca^{2+} ^[29-32].

Healthy lens contains GSH at high amounts which has antioxidant properties, therefore it is fundamental to retain tissue transparency for clear vision^[33]. Decreasing activity of GSH in old lenses with age, giving rise to oxidation. Zeaxanthin and lutein are the two macular carotenoids which are important for protection from AMD and they are acting on intracellular GSH levels. Moreover, AST has been determined to have ten times higher antioxidant activity than lutein, canthaxanthin, β -carotene and hundred times higher than α -tocopherol^[34-35]. Today, oral intake of zeaxanthin and lutein are recommended for AMD patients^[36]. Wu *et al*^[37] defined that a high intake of bioavailable zeaxanthin and lutein is associated with long-term decreased risk of dry/atrophic AMD in individuals aged 50y or older.

Our study is important because it is the first molecular study evaluating the effects of AST to intracellular Ca^{2+} levels in

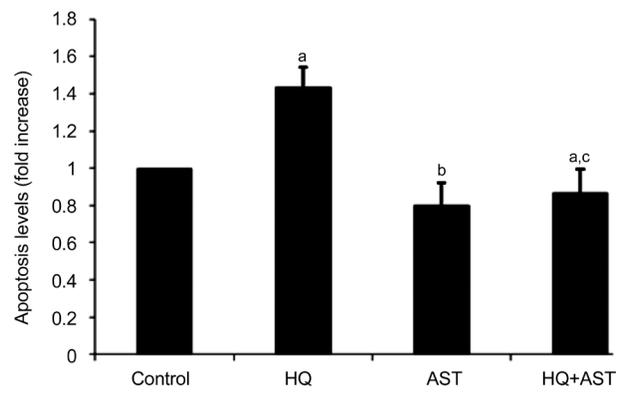


Figure 3 The effects of astaxanthin and hydroquinone on apoptosis levels in ARPE-19 cells The ARPE-19 cells showed significantly increased apoptosis levels after treatment with HQ. AST incubation significantly decreased apoptosis levels. ^a $P < 0.05$ vs Control; ^b $P < 0.05$ vs HQ; ^c $P < 0.05$ vs AST.

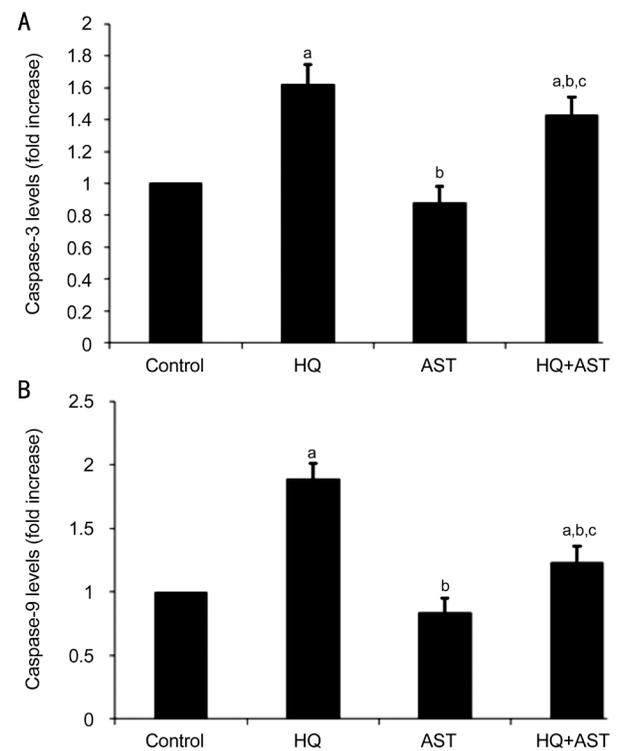


Figure 4 The ARPE-19 cells showed significantly increased caspase-3/-9 (A and B respectively) activity levels after treatment with HQ The caspase-3/-9 levels in the AST group did not show any significant changes when compared to control group. ^a $P < 0.05$ vs Control; ^b $P < 0.05$ vs HQ; ^c $P < 0.05$ vs AST.

ARPE-19 cells. Statistically, AST decreased significantly intracellular Ca^{2+} release in ARPE-19 cells. In the HQ group not incubated by AST, intracellular Ca^{2+} release levels are determined at the maximum amounts. We can easily say that these effects of AST are because of its antioxidant properties. However, due to the limited isomerization and biological activity of AST in humans, astaxanthin is suggested to be useful in the dietary support for AMD patients.

Oxidative stress is a chain of reactions which can occur even in normal physiological processes and reaction products return to

the oxidizing agents. Superoxide radicals, hydrogen peroxide, hydroxyl radicals, peroxynitrite, etc. are the main mediators of oxidising agents.

Mitochondria are the first target of reactive oxygen species and exposed to the greatest damage. Excessive reactive oxygen species (ROS) triggers programmed cell death in cells called apoptosis. As a result of incubation with AST, we found that the statistically significant decrease in ROS levels. Whether buffering systems for increased amount of intracellular Ca^{2+} are insufficient, apoptosis may occur^[38-40].

We observed that, incubation with AST leads significant reduction in amount of apoptosis, caspase-3, caspase-9 and ROS levels when compared with HQ group samples.

The clinical aspects of our findings was supported by different studies previously. Piermarocchi *et al*^[41] determined that AST intake might have a key role in preventing of AMD.

Moreover, in an animal experiment, Izumi-Nagaki *et al*^[42] determined that, AST treatment, suppressed inflammatory processes including subsequent upregulation of inflammatory molecules, macrophage infiltration, and NF-kappa B activation, which significantly suppress choroidal neovascularization (CNV) development.

Similar to this study, Nakajima *et al*^[43] also demonstrated the protective role of AST against oxidative stress model in mice model.

In conclusion, AST's biochemical activities are not yet fully revealed. Our study's aim is to determine the protective effect of AST against oxidative stress in ARPE-19 cells. Hence the AST intake can be used in the treatment of AMD, caused by oxidative stress as the primary factor. AST may reduce or stop the progression of macular degeneration and it may even be that recovery according to the intensity of disease. However, detailed studies may be needed to keep track of more effective treatment.

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