Original Article

The effect of astaxanthin on vascular endothelial growth factor (VEGF) levels and peroxidation reactions in the aqueous humor

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We explored the effect of astaxanthin on vascular endothelial growth factor in the aqueous humor, by measuring vascular endothelial growth factor levels and oxidation-related parameters, including $O_2^{\bullet-}$ scavenging activity, H_2O_2 level, and total hydroperoxide level in the aqueous humor, obtained from 35 patients before and after astaxanthin administration. We evaluated the relationship between vascular endothelial growth factor and the oxidation-related parameters as well as the patient's diabetic status, age, and sex. Vascular endothelial growth factor levels did not change significantly but $O_2^{\bullet-}$ scavenging activity and total hydroperoxide level significantly ($p<0.05$) increased and decreased, respectively. Both pre- and post- astaxanthin intake, vascular endothelial growth factor and total hydroperoxide levels were positively correlated (Pearson: r = 0.42, $p<0.05$; r = 0.55, $p<0.01$, respectively). Analysis of vascular endothelial growth factor levels and $O_2^{\bullet-}$ scavenging activities gave a negative correlation but only pre-astaxanthin intake (r = –0.37, $p<0.05$). Differences in levels pre- and post-astaxanthin only showed association between vascular endothelial growth factor and total hydroperoxide (r = 0.49, $p<0.01$) analyzed by multiple linear regression. Using multivariate analysis, pre-astaxanthin vascular endothelial growth factor level was associated with two factors of total hydroperoxide and $O_2^{\bullet-}$ scavenging activity (r = 0.49, $p<0.05$), and post-astaxanthin vascular endothelial growth factor level with two factors of total hydroperoxide and sex (r = 0.60, $p<0.01$). Astaxanthin intake may have affected vascular endothelial growth factor level through its antioxidant effects by increasing $O_2^{\bullet-}$ scavenging activity and suppressing peroxide production.

***Key Words*:** **astaxanthin, aqueous humor, vascular endothelial growth factor, oxidation, superoxide**

Oxidation reactions are involved in various pathologies including brain and heart ischemia, reperfusion injury after such ischemic events and tumor growth.(1) This has led to increased leading research on antioxidant agents. In the field of ophthalmology, oxidation has been implicated(2,3) in such pathologies as cataracts, diabetic retinopathy, uveitis and age-related macular degeneration (AMD) and the benefits of antioxidant treatments have been considered.

Lutein, a carotenoid, has been recommended as a supplement based on its reported effects in prevention of AMD.(4) Similarly, astaxanthin (AX) (Fig. 1), another kind of carotenoid, has recently attracted attention and a number of studies have focused on its potent antioxidant activity and its safety.(5,6)

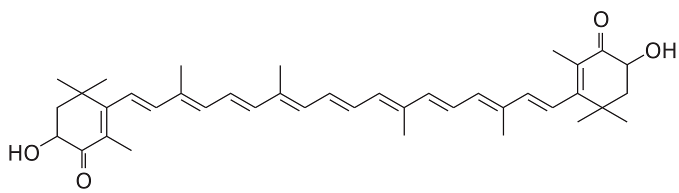

Fig. 1. Structural formula of astaxanthin.

Our laboratory has conducted research on use of an AX supplement in ophthalmology, and has reported the following findings: (i) AX intake suppressed inflammation after cataract surgery;(7) (ii) AX intake increased superoxide ($O_2^{\bullet-}$) scavenging activity in the aqueous humor of diabetic patients;(8) (iii) AX intake changed hydrogen peroxide (H_2O_2) levels in the aqueous humor;(9) and (iv) AX intake decreased total hydroperoxide (TH) levels in the aqueous humor, indicating overall suppression of peroxidation reactions.(10) More recently, we reported on the relationship between AX administration and three parameters relevant to oxidative reactions, $O_2^{\bullet-}$, H_2O_2, and TH.(11)

Intravitreal injections of anti-vascular endothelial growth factor (VEGF) formulations are increasingly used to treat AMD, with the goal of suppressing macular angiogenesis.(12,13) In human retinal pigment epithelial cells, VEGF mRNA levels increased in response to superoxide.(14) We hypothesized that, if AX, through its antioxidant effects, would decrease VEGF levels, it might attenuate pathologies associated with intraocular angiogenesis.

In this study, we evaluated effects of AX treatment on VEGF levels in the aqueous humor of patients, also analyzing levels of substances related to oxidation ($O_2^{\bullet-}$, H_2O_2, and TH). Our analysis also included consideration of sex, age and diabetic status of the subjects.

Materials and Methods

The study subjects were 35 patients who underwent bilateral cataract surgery (intraocular lens implantation) at Tsukuba Hashimoto Optical Clinic after giving an informed consent based on a detailed explanation of the purpose of the study (Table 1). Patients with inflammatory diseases such as uveitis, with a high degree of refractive error of 8 diopters or above and who had been

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Table 1. Sex and diabetes status of the subjects. Error bars represent standard deviations

	Diabetic	Non diabetic	Male	Female
Patients (n)	16	19	16	19
Average age (years)	70.3 ± 6.2	71.5 ± 7.6	71.3 ± 6.4	70.6 ± 7.4

taking other supplements were excluded. The study was approved by the Bioethics Committee, Dokkyo Medical University Koshigaya Hospital (approval number: 22025).

Patients began AX (6 mg/day) intake immediately after receiving surgery in one eye, then underwent surgery in the other eye after 2 weeks. The AX supplement used in this study was Astavita® (Fuji Chemical Industry, Toyama, Japan) derived from algae. Aqueous humor was taken from each of the eyes during surgery for analysis of $O_2^{\bullet-}$ scavenging activity and levels of H_2O_2, TH and VEGF.[15–19]

We measured VEGF levels before and after AX intake and calculated the difference between these levels, known as "change" or ΔVEGF. Relationships between these VEGF-related parameters and factors including $O_2^{\bullet-}$ scavenging capacity, levels of H_2O_2 and TH, diabetic status, age, and sex were analyzed by multivariate analysis. In the analysis, presence and absence of diabetes were scored as 1 and 0, respectively, and male and female were scored as 1 and 0, respectively.

Wilcoxon's signed rank sum test was used for quantitative comparisons of values before and after AX treatment, stepwise multiple linear regression was used for multivariate analysis and Pearson's correlation coefficient was used for analysis of the correlations among the factors. For each statistical test, values of $p<0.05$ were considered significant.

Nitro blue tetrazolium (NBT) reduction[16] was used to measure $O_2^{\bullet-}$ scavenging activity and a titanium colorimetric method[17] was used to measure H_2O_2. The NBT assay was performed with a SOD test kit, "SOD Test Wako R" (Wako Pure Chem. Ind., Ltd., Osaka, Japan). This method measures $O_2^{\bullet-}$ scavenging activities by various $O_2^{\bullet-}$ scavengers such as reduced glutathione (GSH) or L-ascorbic acid (L-AsA) and is not limited to only detecting SOD.

TH was measured by a microassay using the Free d-ROMs reagent (Diacron Srl, Grosseto, Italy).[18] *N*,*N*-diethylparaphenylenediamine, the chromogen pigment in the Free d-ROMs reagent, reacts with H_2O_2, lipid peroxides, peroxidized nucleic acids and nucleotides, as well as peroxides of proteins, peptides and amino acids. Thus measured TH levels indicate the total amount of these peroxidized (-OOH modified) substances.[19]

VEGF levels were measured with an enzyme-linked immunosorbent assay (ELISA) using Quantikine (R&D Systems, Minneapolis, MN) and detecting $VEGF_{165}$ at a limit of detection of 5.0 pg/ml.[15]

For each parameter measured, the "change" caused by AX administration was expressed by subtracting the value measured in the aqueous humor sample collected after AX administration (from the second eye surgery) from that in the sample collected before AX administration (from the first eye surgery).

Results

Levels before and after AX intake (Fig. 2 and Table 2). There was no statistical difference in VEGF levels in samples from subjects before and after AX intake; however, a significant positive correlation was seen between values before AX intake (r = 0.68, $p<0.01$). As compared with samples taken before AX intake, those taken after AX intake showed significantly higher $O_2^{\bullet-}$ scavenging activities ($p<0.05$) and lower TH levels ($p<0.05$).

Factors associated with VEGF levels determined by multiple linear regression analysis (Tables 3A, B and C).

- Before AX intake: Two factors of 1) TH level and 2) $O_2^{\bullet-}$ scavenging activity (r = 0.49, $p<0.05$)
- After AX intake: Two factors of 1) TH level and 2) sex (r = 0.60, $p<0.01$)
- ΔVEGF level: One factor of 1) ΔTH (r = 0.49, $p<0.01$)

Correlation between VEGF levels and each factor, before and after AX intake (Fig. 3, 4 and 5, and Table 4, 5 and 6). Before AX intake, the VEGF level was negatively correlated with $O_2^{\bullet-}$ scavenging capacity (r = –0.37, $p<0.05$). TH level was the only factor showing a positive correlation both before and after AX intake (before AX intake: r = 0.42, $p<0.05$, after AX intake: r = 0.55, $p<0.01$). Regarding the "change", ΔVEGF showed a significant positive correlation only with ΔTH (r = 0.492, $p<0.01$).

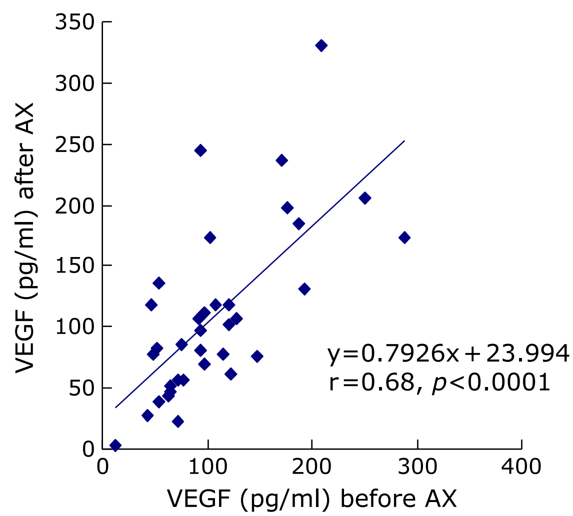

Fig. 2. Relationship between VEGF levels in the aqueous humor before and after AX intake. VEGF levels were measured by ELISA in aqueous humor samples collected from patients before and after AX intake, plotted as shown. Each symbol represents data from one patient.

Table 2. Measurements of each parameter in aqueous humor samples from patients before and after AX intake

Parameters	Before AX intake	After AX intake
$O_2^{\bullet-}$ scavenging activity (U/ml)	18.2 ± 4.1	19.9 ± 3.6*
H_2O_2 level (nmol/ml)	109.8 ± 81.4	134.1 ± 81.1
Total hydroperoxides (U CARR)	1.16 ± 0.18	1.04 ± 0.31*
VEGF (pg/ml)	108.7 ± 61.4	110.2 ± 71.2

Values are means ± SD. *$p<0.05$.

Table 3. Results of multivariate analyses of relationships between VEGF measurements (levels before and after AX intake and change) and various other parameters, as shown (multiple correlations)

A: Factors affecting VEGF level in subjects before AX intake

Rank	Item	Standardized partial regression coefficient (β)
1	TH level before AX intake	0.3339
2	$O_2^{\bullet-}$ scavenging activity before AX intake	−0.271

Multiple regression equation: (VEGF level before AX intake) = 115.3557 × (TH level before AX intake) − 4.0149 × ($O_2^{\bullet-}$ scavenging activity before AX intake) + 48.5403
Multiple correlation coefficient: r = 0.49 (p = 0.0123)

B: Factors affecting VEGF level in subjects after AX intake

Rank	Item	Standardized partial regression coefficient (β)
1	TH level after AX intake	0.6026
2	sex	0.2381

Multiple regression equation: (VEGF level after AX intake) = 136.5143 × (TH level after AX intake) + 33.5714 × (sex) + 47.1114
Multiple correlation coefficient: r = 0.60 (p = 0.0008)

C: Factors affecting change in VEGF levels (difference between values after and before AX intake in the same subject)

Rank	Item	Standardized partial regression coefficient (β)
1	Change in TH levels	0.4923

Multiple regression equation: (Change in VEGF level) = 96.5404 × (Change in TH level) + 12.7185
Multiple correlation coefficient: r = 0.49 (p = 0.0027)

Note: All parameters were entered into the stepwise analysis, but change in VEGF level was associated with only one factor, change in TH level, thus ended up with a simple correlation.

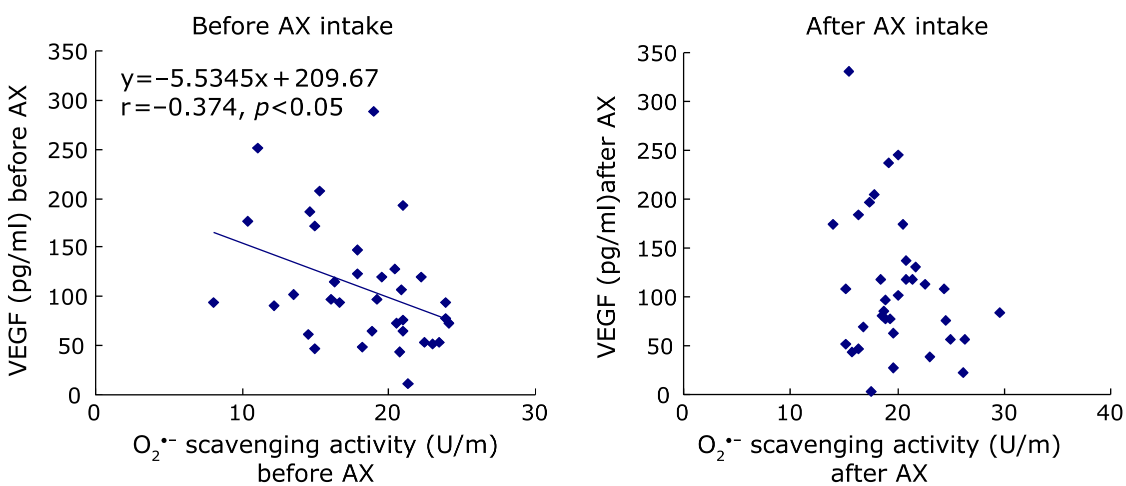

Fig. 3. Relationships between VEGF levels and $O_2^{\bullet-}$ scavenging activities in the aqueous humor, before and after AX intake. VEGF levels and $O_2^{\bullet-}$ scavenging activity were measured in aqueous humor samples collected from patients before (left panel) and after (right panel) AX intake. Each symbol represents data from one patient.

In nearly half the cases (48.6%), both VEGF and TH levels had decreased in samples taken after AX intake (Table 6).

Discussion

In diabetic retinopathy and AMD, VEGF levels increased with aggravation of symptoms[20] and many studies in ophthalmology have shown efficacy of anti-VEGF drugs in the treatment of these diseases.[12,13] These findings give an impression that VEGF is a "villain" in the field of ophthalmology, but it is also a cytokine indispensable in the human body and is produced under normal circumstances. In contrast to anti-VEGF therapy, treatments administering VEGF to patients with lower limb ischemia or ischemic heart diseases, aiming to increase circulation, have been studied.[21,22] Since VEGF is an essential cytokine, a substantial decrease would be of concern, potentially inducing infarction of the myocardium or brain. Also, the intravitreal administration of anti-VEGF agent in an attempt to suppress the development of oxygen-induced retinopathy of prematurity has been studied, but there are some concerns of systemic effect of anti-VEGF agent which escaped from the eye into the systemic circulation.[23] In this study, VEGF levels in the aqueous humor did not change after 2-week administration of AX, at 6 mg/day, as a supplement. This dose corresponds the amount of AX in, for example, a 300 g salmon fillet, within the expected range obtained through a normal daily diet.

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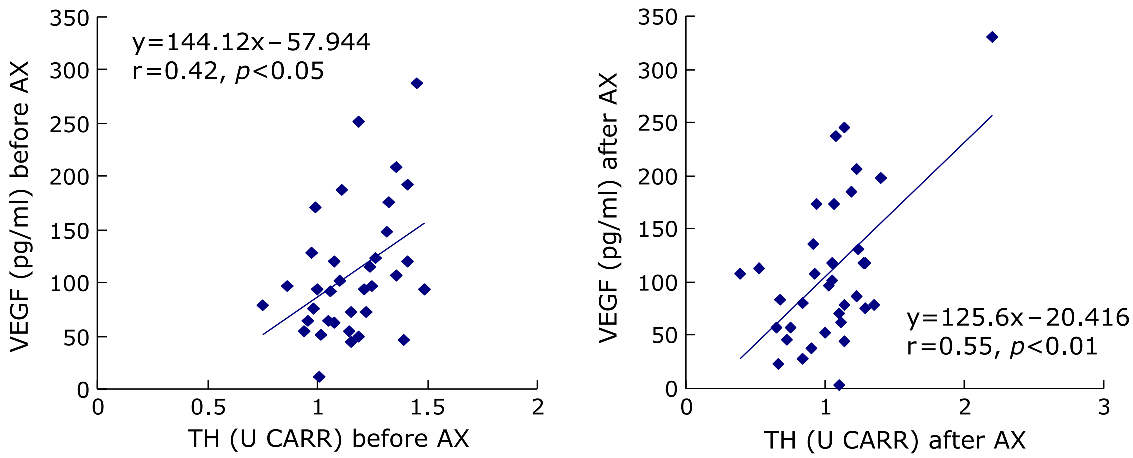

Fig. 4. Relationships between VEGF and TH levels in the aqueous humor, before and after AX intake. VEGF and TH levels were measured in aqueous humor samples collected from patients before (left panel) and after (right panel) AX intake. Each symbol represents data from one patient.

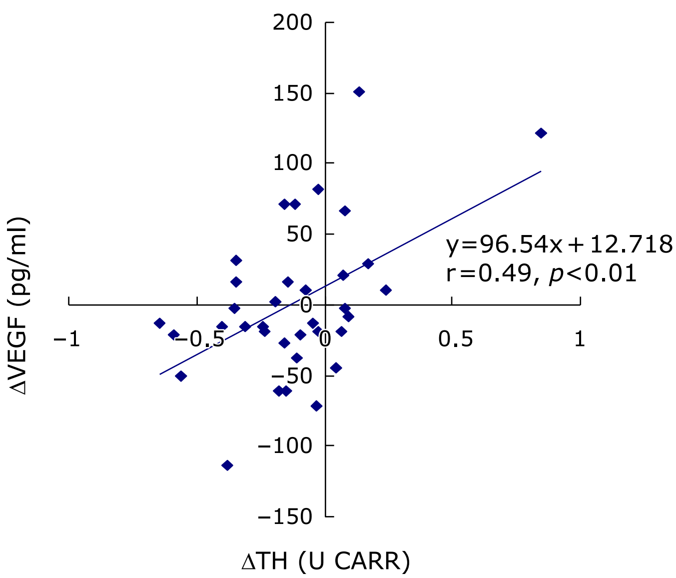

Fig. 5. Relationship between changes (with respect to AX intake) in VEGF and TH levels in the aqueous humor. Values of each parameter after AX intake were subtracted from corresponding values before AX intake for each patient. Each resulting difference is expressed as ΔVEGF or ΔTH, respectively.

Table 5. Relationship between ΔVEGF and various factors (simple correlation)

	Change in VEGF level
$\Delta O_2^{\bullet -}$ scavenging activity	r = –0.038, *p* = 0.830
ΔH_2O_2 level	r = –0.026, *p* = 0.883
ΔTH level	r = 0.492**, *p* = 0.0027
Diabetic status	r = –0.143, *p* = 0.412
Age	r = 0.256, *p* = 0.137
Sex	r = 0.001, *p* = 0.957

**$p<0.01$

Table 6. The number of samples that showed following changes in VEGF and TH levels after AX intake

Change	TH level decreased	TH level increased
VEGF level increased	8	6
VEGF level decreased	17	4

Table 4. Relationship between VEGF level and various factors (simple correlation)

	VEGF level	
	Before AX intake	After AX intake
$O_2^{\bullet -}$ scavenging activity before AX intake	r = –0.374*, *p* = 0.027	r = –0.0282, *p* = 0.100
H_2O_2 level before AX intake	r = 0.094, *p* = 0.592	r = –0.007, *p* = 0.968
TH level before AX intake	r = 0.417*, *p* = 0.013	r = 0.554**, *p* = 0.0005
Diabetic status	r = 0.247, *p* = 0.152	r = 0.105, *p* = 0.547
Age	r = 0.041, *p* = 0.814	r = 0.228, *p* = 0.187
Sex	r = 0.127, *p* = 0.469	r = 0.116, *p* = 0.506

*$p<0.05$, **$p<0.01$

In this study, AX intake did not cause significant quantitative changes in VEGF levels in the aqueous humor. However, VEGF levels before and after AX intake as well as ΔVEGF showed significant positive correlations with those of TH. In nearly half of the cases (48.6%), levels of both VEGF and TH decreased after AX intake, indicating that levels of TH and VEGF in the aqueous humor are likely be linked.

VEGF levels in the aqueous humor or vitreous body fluctuate according to the severity of the pathology in diabetic retinopathy,[15,20] suggesting that VEGF is secreted locally in the eye irrespective of its serum levels. VEGF, with a molecular weight of about 20,000 Da, is unlikely to cross the blood–ocular barrier readily. A significant rise in $O_2^{\bullet-}$ scavenging capacity and decrease in TH levels in the aqueous humor after AX intake would indicate an overall suppression of oxidative stress. In this study, VEGF levels were significantly correlated with those of TH. This was true in samples from subjects before and after AX intake and the changes of TH and VEGF were also correlated. These findings indicate that VEGF production is affected by oxidative stress. In addition, because only levels of TH, among various factors, showed a correlation with those of VEGF both before and after AX intake, we infer that overall oxidation levels played a role in VEGF production by surrounding tissues and, therefore, its levels in the aqueous humor.

TH includes lipid peroxides. In rabbit corneal parenchymal cell model, administration of lipid peroxides (peroxidized linoleic acid) induced VEGF release.[24] Similar effects are possible in the aqueous humor.

In an experimental mouse macular degeneration model induced by laser irradiation, intraperitoneal injection of AX suppressed VEGF levels in the retinal pigment epithelium (RPE).[6] In contrast, we found that VEGF levels did not decrease significantly after AX intake. This discrepancy might be explained by our use of aqueous humor, which is behind the blood-aqueous barrier, as the specimen. It is also possible that the total AX concentration, on a per tissue weight basis, was far lower in our study than in the mouse model, where up to 100 mg/kg body weight was reported. Administering such large doses of VEGF and harvesting RPE specimens for analysis would be ethically unfeasible in humans. Any studies on the effects of supplement intake in humans should use the range of recommended supplement doses. Using HPLC, we could detect a small peak corresponding to AX in the aqueous humor of patients after oral AX administration (data not shown), indicating that this dosing did result in AX being present in the aqueous humor.

Relevant to the relationship between peroxidation processes and VEGF levels, one study reported dose dependent NO release in cultured vascular endothelial cells treated with VEGF.[25] NO is believed to immediately react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), resulting in loss of the vasodilating effects of NO, also known as endothelium-derived relaxing factor (EDRF). This would lead to poor blood perfusion.

In our study, before AX intake, $O_2^{\bullet-}$ scavenging capacity showed a negative correlation with VEGF levels, indicating that $O_2^{\bullet-}$ affects VEGF levels in the aqueous humor normally, even without AX intake. A relationship between $O_2^{\bullet-}$ and VEGF levels may exist not only in the RPE cells but also in the aqueous humor. Our multivariate analysis showed that, while TH levels were consistently and most strongly associated with VEGF levels in the aqueous humor, both before and after AX intake, $O_2^{\bullet-}$ scavenging activity showed the next strongest association with VEGF levels only before the AX intake. No such association was observed after AX intake. We propose that AX affects not only $O_2^{\bullet-}$ levels but also those of other unknown agents influencing oxidation reactions.

We showed that VEGF levels in the aqueous humor did not change significantly with AX intake, on average, in our patient population. However, VEGF levels did show a tendency to change in association with those of TH and TH levels were significantly lower with AX intake.

Based on our findings, we believe that AX led to suppression of oxidative reactions in the aqueous humor and this could have had some influence on VEGF production in surrounding tissues and the level in the aqueous humor. This implies that AX may be of value in the prevention and treatment of AMD and diabetic retinopathy through suppression of neoangiogenesis.

Abbreviations

AMD	age-related macular degeneration
AX	astaxanthin
EDRF	endothelium-derived relaxing factor
ELISA	enzyme-linked immunosorbent assay
Gpx	glutathione peroxidase
GSH	glutathione
H_2O_2	hydrogen peroxide
L-AsA	L-ascorbic acid
NBT	nitro blue tetrazolium
NO	nitric oxide
$O_2^{\bullet-}$	superoxide
-OOH	peroxidated substances
$ONOO^-$	peroxynitrite
ROMs	reactive oxygen metabolites
RPE	retinal pigment epithelium
SOD	superoxide dismutase
TH	total hydroperoxide
VEGF	vascular endothelial growth factor

Conflict of Interest

No potential conflicts of interest were disclosed.

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