의학석사 학위논문

망막색소상피세포주의 산화스트레스 유발 모델에서 아스코르브산과 아스타잔틴의 항산화효과에 관한 연구

Antioxidative effect of ascorbic acid and astaxanthin on ARPE-19 as an oxidative stress model

2019년 8월

서울대학교 대학원 의학과

줄기세포생물학 전공

오 상 현

의학석사 학위논문

Antioxidative Effect of Ascorbic Acid and Astaxanthin on ARPE-19 as an Oxidative Stress Model

망막색소상피세포주의 산화스트레스 유발 모델에서 아스코르브산과 아스타잔틴의 항산화효과에 관한 연구

2019년 8월

Seoul National University

The Graduate School of College of Medicine

Interdisciplinary Program in Stem Cell Biology

오 상 현

망막색소상피세포주의 산화스트레스 유발 모델에서

아스코르브산과 아스타잔틴의 항산화효과에 관한 연구

Antioxidative effect of ascorbic acid and astaxanthin on ARPE-19 as an oxidative stress model

지도교수 유 형 곤

이 논문을 의학석사 학위논문으로 제출함

2019년 4월

서울대학교 대학원 의학과

줄기세포생물학 전공

오 상 현

오 상 현의 석사 학위논문을 인준함

2019년 7월

위 원 장 구 승 엽 (인)

부 위 원 장 유 형 곤 (인)

위 원 김 미 금 (인)

Abstract

Antioxidative effect of ascorbic acid and astaxanthin on ARPE-19 as an oxidative stress model

Sanghyeon Oh
Interdisciplinary Program in Stem Cell Biology
The Graduate School of College of Medicine
Seoul National University

Purpose: This study is to research antioxidative effect of ascorbic acid (vitamin C) and astaxanthin on ARPE-19 cells within an oxidative stress model induced by endogenous (hydrogen peroxide) and exogenous (ultraviolet B) sources of reactive oxygen species (ROS).

Methods: 0, 0.1, 0.2, 0.4, 0.6, 0.8mM of hydrogen peroxide and 0, 20, 40, 60, 80, 100mJ/cm^2 of UVB were treated to ARPE-19 cells to find sublethal and lethal dose of each source. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay were done to examine cell viability and intracellular reactive oxygen species level change. With the sublethal and lethal dose of each inducers, 0-750uM of ascorbic acid and 0-40uM of astaxanthin were treated to examine their antioxidative effect on the oxidative stress induced ARPE-19.

Results: Ascorbic acid and astaxanthin had antioxidative effects on ARPE-19 cells by significantly increasing cell viability and reducing intracellular reactive oxygen species level after oxidative stress induction. 500uM ascorbic acid increased the cell viability 27% and 14% respectively for 0.2mM and 0.4mM hydrogen peroxide and 17% and 12% respectively for 20mJ/cm^2 and 100mJ/cm^2 of UVB. The

increment was due to its antioxidative effect considering decreased intracellular ROS level. Astaxanthin also showed a antioxidative effect but extended time period of time was needed for it to act. 30 hours of culture with 90uM ascorbic acid and 20uM astaxanthin increased the cell viability from 75% to 129% when exposed to 0.2mM hydrogen peroxide.

Conclusion: Our data suggest that hydrogen peroxide and UVB-induced oxidative stress model of ARPE-19 could be used to examine antioxidative effect of compounds of interest. Ascorbic acid and astaxanthin can be strong antioxidants and the mixture of the two compounds can have synergistic effect.

……

keywords: Antioxidant; Ascorbic Acid; Astaxanthin; Oxidative Stress; Retinal Pigment Epithelium; Retinal Disease.
Student Number: 2017-27541

국문초록

망막색소상피세포주의 산화스트레스 유발 모델에서 아스코르브산과 아스타잔틴의 항산화효과에 관한 연구

오 상 현
협동과정 줄기세포생물학 전공
의과대학 대학원
서울대학교

목적: 과산화수소로 세포 내부에서 발생한 산화스트레스를 재현하고 중파장 자외선으로 세포 외부의 산화스트레스를 재현한 모델을 사용하여 아스코르브산과 아스타잔틴이 망막색소상피세포에 항산화 효과가 있는지 알아보고자 하였다.

방법: 0, 0.1, 0.2, 0.4, 0.6, 0.8mM의 농도의 과산화수소와 0, 20, 40, 60, 80, 100mJ/cm^2 세기의 중파장 자외선을 처리하여 세포의 생존율과 세포 내의 활성산종의 농도 변화를 측정하였다. 세포의 생존률은 MTT assay, 세포 내의 활성산소종의 농도는 DCFH-DA assay를 통해서 측정하였다. 두 산화스트레스 유발인자의 치사량, 준치사량의 조건에서 0-750uM 농도의 아스코르브산과 0-40uM 농도의 아스타잔틴을 처리하고 세포의 생존율과 세포내 활성산소종의 농도 변화를 측정하였다.

결과: 아스코르브산과 아스타잔틴을 처리했을 때 과산화수소와 중파장 자외선으로 인한 세포의 손상이 유의하게 감소하였다. 0.2mM와 0.4mM의 과산화수소를 처리했을 때 세포는 각각 80%, 69%의 생존율을 보였다. 이 조건에서 500uM의 아스코르브산을 처리했을 때 각각의 조건에서 생존율이 27% 14% 증가했다. 20mJ/cm^2, 40mJ/cm^2 의 중파장을 세포에 처리했을 때 85%, 65%의 생존율을 보였고 이는 500uM 농도의 아스코르브산을 처리했을 때 각각 17%, 12% 증가하였다. 각 조건에서 세포 내부의 활성산소종의 농도가 감소한 것을 토대로 세포 생존율의 증가는 아스코르브산의 항산화 효과에 기인한 것으로 판단된다. 아스타잔틴도 마찬가지로 항산화 효과를 나타내어 세포의 생존율을 증가시켰지만, 아스코르브산보다 긴 처리 시간이 필요했다. 세포에 0.2mM의 과산화수소로 산화스트레스를 유발하고 90uM의 아스코르브산과 20uM의 아스타잔틴을 30 시간 동안 같이 처리했을 때 각 약물의 단독 처리보다 향상된 효과를 보이며 75%에서 129%로 세포의 생존율을 증가시켰다.

결론: 과산화수소와 중파장 자외선으로 유도된 망막색소상피세포주의 산화스트레스 모델이 항산화 후보 물질의 효과를 실험하는데 타당한 것을 밝혔다. 아스코르브산과 아스타잔틴이 이 모델에서 항산화 효과를 나타냈고 아스타잔틴은 항산화 물질로 작용하는데 수용성 환경에서 아스코르브산보다 긴 처리시간이 필요한 것을 밝혔고 아스코르브산과 아스타잔틴을 혼합하여 처리하면 각 약물의 단독처리보다 뛰어난 항산화 효과를 나타내는 것을 확인하였다.

.....

주요어: 항산화물질; 아스코르브산; 아스타잔틴; 산화스트레스; 망막색소상피세포; 망막질환.
학번: 2017-27541

CONTENTS

LIST OF TABLES AND FIGURES

1. Introduction

There have been studies regarding the effect of oxidative stress on retinal diseases. Age-related macular degeneration (AMD), known to be the leading cause of blindness among the elderly population throughout the world [1], is closely related to increased reactive oxidative species (ROS) [2-8]. Oxidative damage accumulation, decreases of antioxidants capacity, and efficacy of the repair system occur as people age and this results in the dysfunction of retinal cells which eventually leads to various types of cell loss [6]. In addition to AMD, glaucoma pathogenesis is also susceptible to ROS [9-13]. In vitro and in vivo studies with hydrogen peroxides revealed that it plays a pathogenic role in glaucoma including alteration in cell adhesion, integrity, ganglion cell death, and damage to DNA [14-16].

As the relationship between ROS and various retinal pathogenesis have been studied, defense mechanisms against ROS have been also studied [17-22]. Organisms have defense mechanisms against oxygen metabolites and the mechanism includes removal of free radical by enzymes, proteins, and pro-oxidant metal reactions, and reduction of free radicals by antioxidants (vitamin C, vitamin E, GSH) [17]. Autophagy has been studied to be also part of antioxidative processes. Autophagy can directly remove ROS and reactive nitric species out of the cells, and it can also selectively remove oxidized molecules decreasing their toxicity to the cells [23]. Vitamin C, vitamin E, and thiols like Glutathione interact synergistically

with each other and form a complex antioxidant cycle which helps to keep the steady-state of antioxidants and prevents the deprivation of them [24].

Studies have found that with aging, endogenous antioxidants level [25], and antioxidant enzyme activity along with its gene expression and protein level decrease [26]. This alteration in the antioxidative defense system worsens the imbalance between ROS production and its removal. As a consequence, oxidatively damaged macromolecules including lipids, DNA and proteins accumulate accelerating the aging process with oxidative-stress induced aging [27].

For this reason, it becomes more important to maintain the antioxidant defense system and one way is to supplement antioxidants from an outer source. Supplements actively studied for their antioxidative effect are ascorbic acid (vitamin C), glutathione, alpha-tocopherol (vitamin E), and other carotenoids (i.e. astaxanthin, lutein, beta-carotene) [28-30]. One frequently used way to evaluate their antioxidant activity is by studying their reactivity with free radicals and metal ions (DPPH, ABTS, FRAP, CUPRAC, ORAC, HORAC, TRAP) [31-34]. However, giving them enough credence for their antioxidant capacity assumption is often controversial since one same antioxidant can have a different relative capacity to other antioxidants when measured with different methods [35-40].

For this reason, it is necessary to study potential antioxidants' capacities and properties based on a solid oxidative stress model. A solid oxidative stress model portrays the biological environment well so that a more accurate assumption is possible, and the result is reproducible. Hydrogen peroxide [41-43] and ultraviolet B irradiation [44-46] have been studied to establish an oxidative stress model within cells. Hydrogen peroxide represents endogenous reactive oxygen species production

and ultraviolet B represents an outer source of oxidative stress to retinal cells. In this study, both hydrogen peroxide-induced oxidative stress model and UVB-induced oxidative stress model will be used to evaluate the antioxidative potential of ascorbic acid and astaxanthin.

Ascorbic acid and astaxanthin are known to have antioxidative properties [47, 48] and they are studied under AREDS (Age-Related Eye Disease Study) and CARMIS (The Carotenoids in Age-Related Maculopathy in Italians Study) respectively for their effect on AMD patients. However, there have been controversies regarding their effect on cells under oxidative stress. In this study, their antioxidative potential and properties to be concerned when they are used on cells will be studied based on a solid antioxidative stress model using both hydrogen peroxide and UVB.

2. Materials and methods

2.1 ARPE-19 cell culture

ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were cultured and maintained as a monolayer in 1:1 mixture of Dulbecco's Modified Eagle's Medium and Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Gibco, Carlsbad, CA, USA) and 1X penicillin-streptomycin (Invitrogen, Gibco, Carlsbad, CA, USA). Cells were incubated at 37°C in a humidified 5% CO_2 incubator in the complete medium with 2-3 times a week change until they reach 80% confluency. Then the medium was changed to a serum-free medium and cells were either passaged with 0.25% trypsin-EDTA (Invitrogen, Gibco, Carlsbad, CA, USA) or frozen and stored in -80°C deep freezer. Cells used for this study were in a passage between 25 to 30.

2.2 Hydrogen peroxide exposure procedure

Cells were seeded in a 96-well plate with a density of $2.5x10^4$ cells/well and allowed to attach to the bottom of the well and to become confluent overnight. The next day, the medium was changed to serum-free medium and cells were maintained in it up to 7 days until the day of the procedure. 30% (w/w) hydrogen peroxide in H_2O containing stabilizer (Sigma Aldrich, St. Louis, MO, USA) was used to make medium with intended H_2O_2 concentration. For the exposure, the used medium of

the cells was changed to serum-free DMEM/F-12 without phenol red (Invitrogen, Gibco, Carlsbad, CA, USA) with the desired concentration of H_2O_2.

2.3 Ultraviolet B irradiation procedure

Cells were seeded in a 96-well plate with a density of $2.5x10^4$ cells/well and allowed to attach to the bottom of the well and to become confluent overnight. The next day, the medium was changed to serum-free medium and cells were maintained in it up to 7 days until the day of the procedure. At UVB irradiation, the medium was changed to DMEM/F-12 without phenol red without serum. As a UVB source, () lamp was used. Its irradiation intensity was $0.2mW/cm^2$ when measured () cm below the lamp where the plates were put. The intensity was measured with a UVB meter (). Cells were irradiated with intended doses of UVB and for the control group and differential dose of UVB irradiation, remaining wells in the same plate were thoroughly masked.

2.4 DPPH ROS scavenging assay

Total antioxidative capacities of ascorbic acid and astaxanthin were estimated using DPPH (2,2-Diphenyl-1-picrylhydrazyl) ROS scavenging assay as previously described[41]. DPPH solution was made by dissolving DPPH in methanol to 0.16mM. Ascorbic acid and astaxanthin were dissolved to various concentrations in either dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) or phosphate-buffered saline (PBS). 20ul of ascorbic acid or astaxanthin solution was mixed with 100ul DPPH solution for 30 minutes or 30 hours with vigorous shaking at room temperature. After the reaction absorbance at 517nm was measured and the relative amount of scavenged DPPH was calculated using the following equation.

$$Scavenged\ DPPH\ fraction\ (\%) = \frac{Ab_{Control} - Ab_{AO}}{Ab_{Control}} \times 100$$

$Ab_{Control}$ is the absorbance of the groups with only DPPH and Ab_{AO} is the absorbance of the groups of the mixture of DPPH and various concentrations of antioxidants.

2.5 Antioxidant treatment

Cells were treated with either ascorbic acid (Sigma Aldrich, St. Louis, MO, USA) or astaxanthin (Sigma Aldrich, St. Louis, MO, USA) in DMEM/F-12 without phenol red to study their antioxidative effect on ARPE-19 cells. Ascorbic acid containing medium was made from ascorbic acid stock (0.5M in PBS) and astaxanthin containing medium was made from astaxanthin stock (1mg/ml in DMSO). Cells were pretreated with ascorbic acid or astaxanthin for 6 hours and then they were irradiated by UVB or exposed to hydrogen peroxide. For UVB irradiation group, after pretreatment, used medium was changed to the fresh medium containing the same concentrations of compounds and followed the UVB irradiation (20mJ/cm^2 or 100mJ/cm^2) procedure. For the H_2O_2 exposure group, after pretreatment, the used medium was changed to the fresh medium containing the same concentrations of the compounds with a sublethal or lethal dose of H_2O_2 (0.2mM or 0.4mM).

2.6 MTT assay

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) was used to determine cell viability. MTT is enzymatically turned into purple formazan crystals by mitochondrial respiration

activity. The procedure was done following the manufacturer's instructions. Briefly, after antioxidants, UVB, or H_2O_2 treatment to the cells, the medium was removed and MTT (0.5mg/ml) was added diluted in serum-free medium. After 3 hours of incubation at 37°C in a humidified 5% CO_2 incubator, MTT containing medium was carefully aspirated from the well and DMSO was added to each well to solubilize formazan crystals. Absorbance at 570nm was measured using a microplate reader (EPOCH 2, BioTek Instruments Inc. Winoosky, VT, USA) with a reference wavelength of 630nm. Cells untreated or treated with the only vehicle were set to be 100% cell viability for the normalization of the absorbance and experiments had more than three replicates for each condition.

2.7 Crystal violet assay

The relative number of cells attached to the bottom of the well was measured by crystal violet uptake assay. The procedure was done as previously described [49]. Briefly, after UVB, or H_2O_2 treatment to the cells, the medium was removed, and cells were fixed with 4% paraformaldehyde in 4°C. After they were washed 3 times and 0.1% crystal violet (Sigma Aldrich, St. Louis, MO, USA) in 10% ethanol was added to each well for 5 minutes. After washing 3 times, the remaining stain was dissolved in 10% acetic acid and absorbance at 540nm was measured.

2.8 DCFH-DA intracellular ROS level assay

Intracellular ROS level was measured by 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay. DCFH-DA is cell-permeable and is not fluorescent which enters cells to be de-esterified to 2′,7′-Dichlorodihydrofluorescein (DCFH)

and become impermeable to the cell membrane. It then reacts with ROS to be highly fluorescent 2',7'-dichlorofluorescein (DCF). Before UVB irradiation or H_2O_2 exposure, cells were cultured with 10uM DCFH-DA (Sigma Aldrich, St. Louis, MO, USA) in DMEM/F-12 without phenol red for 30 minutes at 37°C in a humidified 5% CO_2 incubator. After incubation, they were washed 2 times in PBS and antioxidant treatment, UVB irradiation or H_2O_2 exposure was done following measurement of fluorescence of DCF at excitation and emission wavelength of 495nm and 529nm, respectively with a microplate reader (Synergy Mix, BioTek Instruments Inc. Winoosky, VT, USA). Cells untreated or treated with the only vehicle were set to be 100% intracellular ROS level for the normalization of the fluorescence intensity and experiments had more than three replicates for each condition.

3. Results

3.1 H_2O_2 decreases viability of ARPE-19 cells and increases their intracellular ROS

To establish the H_2O_2-induced oxidative stress model in ARPE-19 cells, different concentrations of H_2O_2 were treated to the cells and their viability and intracellular ROS level were measured. Viability measured with MTT assay decreased as the concentration of treated H_2O_2 increased. When cells were treated with 0.4mM H_2O_2, they showed the viability of 66% and the viability change was the greatest between 0.2mM and 0.6mM. (Fig. 1A) Crystal violet assay resulted in a similar aspect of viability change as MTT assay with 69% of viability at 0.4mM. (Fig. 1B) This trend was confirmed in bright field imaging. (Fig. 1D) Intracellular ROS level increased dependently to the concentration of H_2O_2. (Fig. 1C) The mean value of the ROS level measured in 0.8mM H_2O_2 increased to 176% compared to the control group. As cell viability changed rapidly at 0.4mM H_2O_2, 0.4mM was set to be a lethal dose of H_2O_2 and 0.2mM was set to be sublethal dose.

3.2 UVB irradiation decreases the viability of ARPE-19 cells and increases their intracellular ROS

To establish the UVB-induced oxidative stress model in ARPE-19 cells, different doses of UVB were exposed to the cells and their viability and intracellular ROS level were measured. Viability measured with MTT assay decreased as the

dose of UVB irradiation increased. When cells were exposed to 20mJ/cm^2 UVB, they showed the viability of 80% and with 100mJ/cm^2 UVB, the viability was 60%. (Fig. 2a) In a crystal violet assay with the same range of UVB dose, the viability dropped to 78% at 20mJ/cm^2 UVB and to 72% at 100mJ/cm^2 UVB. (Fig. 2B) Morphological change of the cells was observed in bright field imaging. Cells became rounder and holes in the monolayer were observed as UVB dose increased. (Fig. 2D) Intracellular ROS level increased dependently to the UVB dose. (Fig. 2C) The mean value of ROS level measured at 20mJ/cm^2 UVB increased to 140% and 270% at 100mJ/cm^2 UVB compared to the control group. 20mJ/cm^2 UVB where the cells show 80% of viability without significant morphological change was set to be the sublethal dose of UVB and 100mJ/cm^2 UVB where the cells show 60% of viability with morphological change was set to be the lethal dose of UVB.

3.3 Ascorbic acid and astaxanthin show antioxidative effect by scavenging DPPH

DPPH scavenging assay was performed with ascorbic acid and astaxanthin. (Fig. 3) 0.025mM, 0.1mM, 0.4mM, and 1.6mM of ascorbic acid dissolved in DMSO were mixed with DPPH solution and each concentration scavenged 33%, 52%, 57%, 73% of DPPH respectively after 30 minutes of reaction. (Fig. 3A) When 75uM, 85uM, 95uM, and 105uM astaxanthin dissolved in DMSO were reacted with DPPH solution for 30 minutes, 44%, 50%, 64%, and 69% of DPPH were scavenged respectively. (Fig. 3B) Both ascorbic acid and astaxanthin showed antioxidative effect.

3.4 Ascorbic acid has an antioxidative effect on ARPE-19 cells under H_2O_2-induced oxidative stress

ARPE-19 cells were pretreated with various concentrations of ascorbic acid or astaxanthin for 6 hours and then they were treated together with H_2O_2 and the same concentrations of antioxidants for another 3 hours. Viability after the treatment was assessed with MTT assay. When groups treated together with ascorbic acid and H_2O_2 they showed increased viability compared to controls. Cells treated only with 0.2mM H_2O_2 showed the viability of 80% and groups treated together with ascorbic acid showed 81%, 107%, and 126% of viability respectively for 250uM, 500uM, and 750uM of the drug concentration. (Fig. 4A) For 0.4mM H_2O_2 treatment, the control group showed 58% of viability while 250uM, 500uM, and 750uM of ascorbic acid increased the viability to 64%, 72%, and 95% respectively. (Fig. 4B) On the other hand, astaxanthin did not show any significant effect on the viability of ARPE-19 with H_2O_2 induced oxidative stress.

3.5 Ascorbic acid and astaxanthin have an antioxidative effect on ARPE-19 cells under UVB-induced oxidative stress

ARPE-19 cells were pretreated with various concentrations of ascorbic acid or astaxanthin for 6 hours and then they were irradiated with UVB. Viability 24 hours after the irradiation was assessed with MTT assay. When cells were pretreated with ascorbic acid and then UVB irradiated with it, the cell viability increased compared to the UVB irradiation-only group. Cells irradiated only with 20mJ/cm^2 UVB showed the viability of 85% and groups treated together with ascorbic acid

showed 92%, 102%, and 130% of viability respectively for 250uM, 500uM, and 750uM of the drug concentration. (Fig. 5A) For 100mJ/cm^2 UVB irradiation, the control group showed 66% of viability while 250uM, 500uM, and 750uM of ascorbic acid increased the viability to 68%, 78%, and 109% respectively. (Fig. 5B) Astaxanthin treated cells also showed increased viability compared to the control group. 10uM, 20uM, and 40uM astaxanthin group showed 95%, 101%, and 102% respectively after 20mJ/cm^2 UVB irradiation and 67%, 74%, and 83% after 100mJ/cm^2 UVB irradiation.

3.6 Ascorbic acid reduces the intracellular ROS level of ARPE-19

The effect of ascorbic acid on the intracellular ROS level of ARPE-19 cells was studied with DCFH-DA assay. (Fig. 6) The intracellular ROS level was measured after cells were treated with H_2O_2 or UVB with or without 500uM ascorbic acid. H_2O_2 of 0.2mM and 0.4mM increased the intracellular ROS level to 123%, and 135% compared to the non-treated group while ascorbic acid treated group showed reduced ROS level of 33%, and 34% respectively. UVB of 20mJ/cm^2 and 100mJ/cm^2 UVB increased the intracellular ROS level to 123%, and 234% respectively and 500uM ascorbic acid treatment reduced the ROS level to 105%, and 115%. This fluorescence difference between groups were confirmed with fluorescence microscopy. (Fig. 6C)

3.7 Astaxanthin and ascorbic acid show antioxidative effect by reducing intracellular ROS in ARPE-19 cells

Because of astaxanthin's poor solubility in an aqueous environment, its antioxidative capacity in aqueous solution was tested using DPPH assay with PBS as a diluting solvent instead of DMSO. In an aqueous environment, unlike DPPH assay done in DMSO, astaxanthin showed poor antioxidative capacity with 60uM astaxanthin scavenging only 12% DPPH when reacted for 30 minutes while after 30 hours of reaction, 40uM of astaxanthin scavenged 52% of DPPH and 60uM astaxanthin scavenged 95% of DPPH. Based on this result that astaxanthin needs an extended period of reaction time to show antioxidative capacity in an aqueous environment, its effect on cell viability under H_2O_2 – induced oxidative stress was assessed with longer treatment time.

ARPE-19 cells were pretreated with either 20uM astaxanthin, 90uM ascorbic acid, or a mixture of 20uM astaxanthin and 90uM ascorbic acid. Then the cells were exposed to 0.2mM H_2O_2 together with the same drug condition but for 24 hours based on the DPPH assay result that astaxanthin needs extended reaction in an aqueous environment to have an antioxidative effect. When cells were exposed to 0.2mM H_2O_2 for 24 hours, the viability decreased to 75%. 20uM astaxanthin and 90uM ascorbic acid treatment could increase the viability to 97% and 93% respectively. The mixture of 20uM astaxanthin and 90uM ascorbic acid increased the viability to 129%. (Fig. 7B) Each drug could also decrease the intracellular ROS level. (Fig. 7C) When cells were treated with 0.2mM H_2O_2 for 24 hours, the intracellular ROS level increased to 200%. 20uM astaxanthin and 90uM ascorbic

acid treatment reduced the ROS level to 169%, and 135% respectively. The mixture of 20uM astaxanthin and 90uM ascorbic acid decreased the ROS level to 104%.

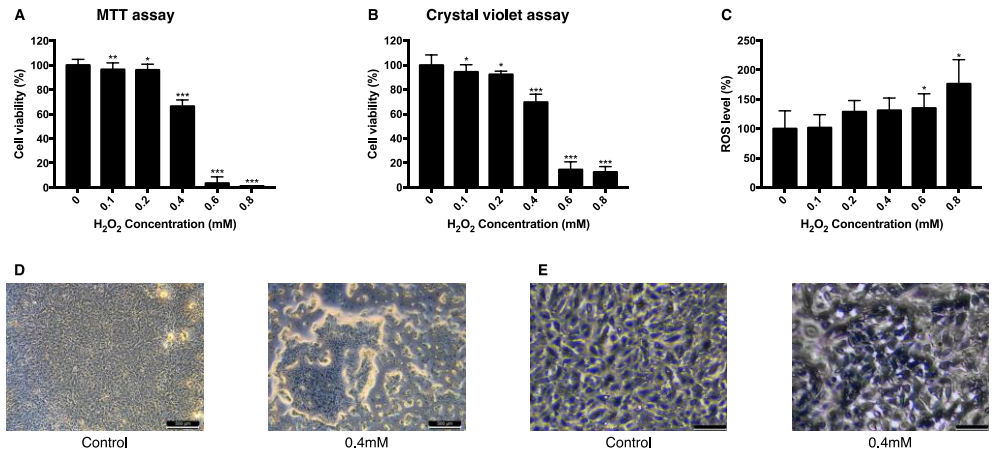

Fig. 1. Change of viability and intracellular ROS level in ARPE-19 cells after exposure to hydrogen peroxide

H_2O_2 decreases viability of ARPE-19 cells and increases their intracellular ROS

The response of ARPE-19 cells to 0-0.8mM H_2O_2 exposure for 3 hours for MTT assay (A), and crystal violet assay (B) to determine cell viability. For intracellular ROS level, DCFH-DA assay was done 30 minutes after the H_2O_2 exposure. (C) Exposure to H_2O_2 reduced the cell viability (A and B) and increased the intracellular ROS level. (C) The cell morphology was observed with bright field microscopy (Scale bar 500um) (D) higher magnification (Scale bar 100um). (E)

Note: Asterisks indicate a significant reduction in cell viability or increment in ROS level compared with untreated cells (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate.

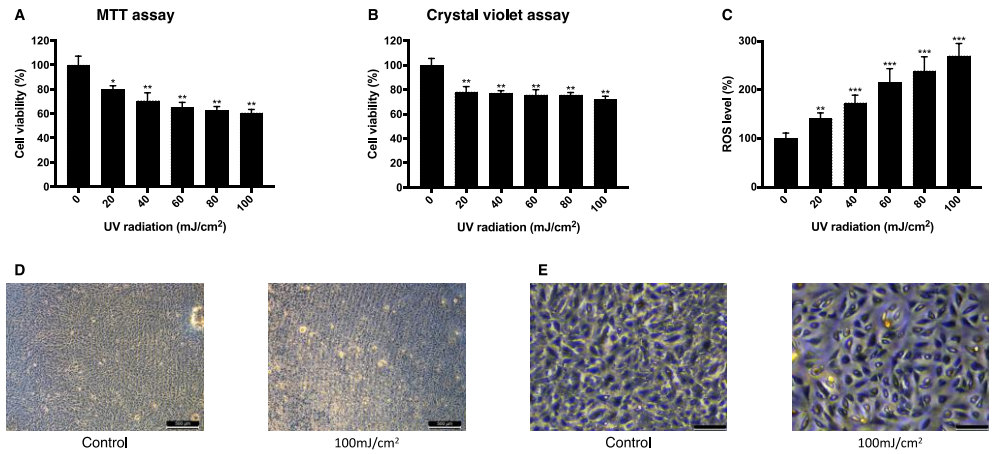

Fig. 2. Change of viability and intracellular ROS level in ARPE-19 cells after UVB irradiation

UVB irradiation decreases the viability of ARPE-19 cells and increases their intracellular ROS

The response of ARPE-19 cells 24 after 0-100mJ/cm^2 UVB irradiation with MTT assay (A), and crystal violet assay (B) to determine cell viability. For intracellular ROS level, DCFH-DA assay was done 30 minutes after the UVB irradiation. (C) Irradiation by UVB reduced the cell viability (A and B) and increased the intracellular ROS level. (C) The cell morphology was observed with bright field microscopy (Scale bar 500um) (D) higher magnification (Scale bar 100um). (E)

Note: Asterisks indicate a significant reduction in cell viability or increment in ROS level compared with untreated cells (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Abbreviations: UVB, ultraviolet B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate.

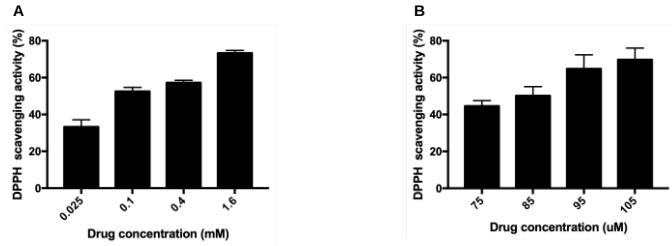

Fig. 3. DPPH scavenging activity of ascorbic acid and astaxanthin

Ascorbic acid and astaxanthin show antioxidative effect by scavenging DPPH

The antioxidative capacities of ascorbic acid and astaxanthin were determined by their capabilities to scavenge DPPH. 0.025-1.6mM of ascorbic acid was reacted with DPPH (A), and 75-105uM of astaxanthin was reacted with DPPH. (B) The compounds were diluted in DMSO. Both compounds scavenged DPPH in dose-dependent way in 30 minutes of reaction time.

Abbreviations: DPPH, 2,2-Diphenyl-1-picrylhydrazyl; DMSO, Dimethyl sulfoxide.

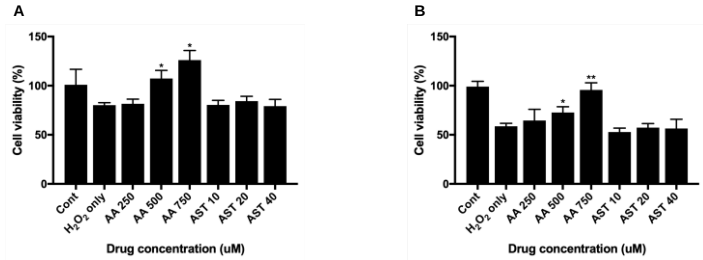

Fig. 4. Effect of ascorbic acid and astaxanthin on hydrogen peroxide-induced oxidative stress model of ARPE-19

Ascorbic acid has an antioxidative effect on ARPE-19 cells under H_2O_2-induced oxidative stress.

The effect of various concentration of ascorbic acid or astaxanthin (pretreated for 6 hours and co-treated with H_2O_2 for 3 hours) on the response of ARPE-19 cells to sublethal or lethal dose of H_2O_2 (0.2mM and 0.4mM). The cell viability was determined by MTT assay. Treatment of ascorbic acid (500-750uM) significantly increased ARPE-19 cell viability following 0.2mM H_2O_2 exposure. However, astaxanthin (10-40uM) did not significantly affect the cell viability. (A) Ascorbic acid (500-750uM) also significantly increased the cell viability under 0.4mM H_2O_2 but astaxanthin (10-40uM) did not have significant effect on the viability. (B)

Note: Asterisks indicate a significant increment in cell viability compared with cells treated with H_2O_2 only (* $P < 0.05$, ** $P < 0.01$).

Abbreviations: AA, ascorbic acid; AST, astaxanthin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

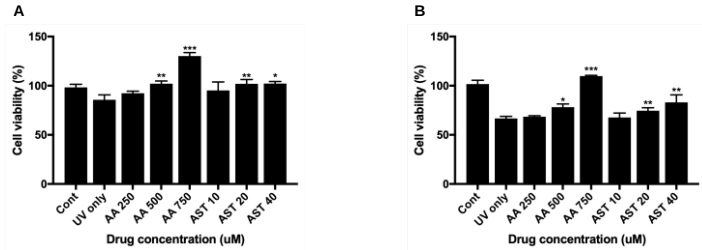

Fig. 5. Effect of ascorbic acid and astaxanthin on UVB-induced oxidative stress model of ARPE-19

Ascorbic acid and astaxanthin have an antioxidative effect on ARPE-19 cells under UVB-induced oxidative stress

The effect of various concentration of ascorbic acid and astaxanthin (pretreated for 6 hours and additional 24 hours after UVB irradiation) on the response of ARPE-19 cells to sublethal or lethal dose of UVB (20mJ/cm^2 and 100mJ/cm^2). The cell viability was determined by MTT assay 24 hours after the irradiation. Treatment of ascorbic acid (500-750uM) and astaxanthin (20-40uM) significantly increased ARPE-19 cell viability following 20mJ/cm^2 UVB irradiation. (A) Ascorbic acid (500-750uM) and astaxanthin (20-40uM) also significantly increased the cell viability after 100mJ/cm^2 UVB irradiation.

Note: Asterisks indicate a significant increment in cell viability compared with cells treated with UVB only (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Abbreviations: UVB, ultraviolet B; AA, ascorbic acid; AST, astaxanthin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

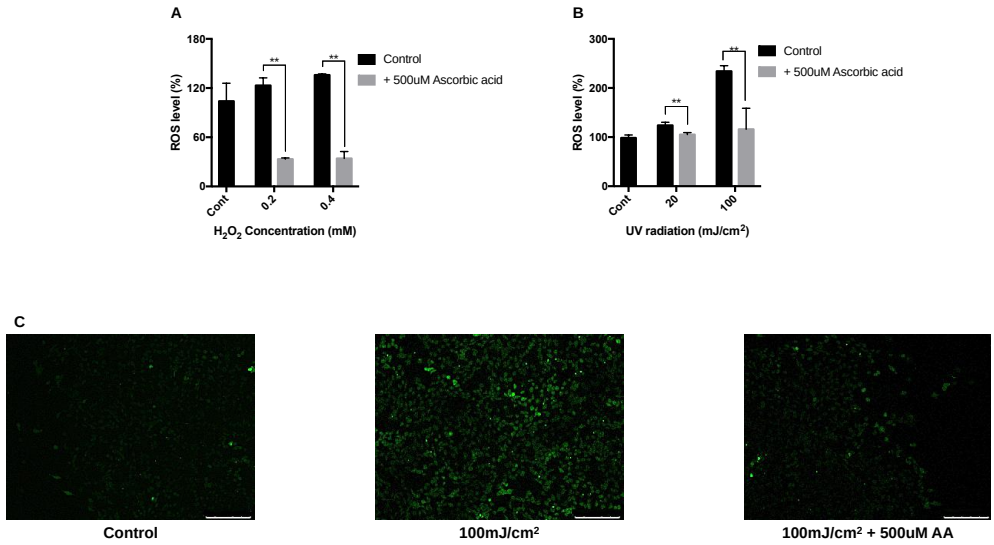

Fig. 6. Intracellular ROS level of ARPE-19 after hydrogen peroxide and UVB treatment with ascorbic acid

Ascorbic acid reduces the intracellular ROS level of ARPE-19

The effects of ascorbic acid on the intracellular ROS level of ARPE-19 under H_2O_2 or UVB-induced oxidative stress were examined by DCFH-DA assay. 500uM of ascorbic acid significantly reduced the intracellular ROS level under sublethal and lethal dose of H2O2 (0.2-0.4mM) compared to the control group without ascorbic acid treatment. (A) and 500uM of ascorbic acid also significantly reduced the ROS level after UVB irradiation (20-100mJ/cm^2) compared to groups with UVB irradiation only. (B) The fluorescence was observed with fluorescence microscopy. (Scale bar 250um) (C)

Note: Asterisks indicate a significant reduction in ROS level compared with control cells only with H_2O_2 or UVB exposure without ascorbic acid treatment (** $P < 0.01$).

Abbreviations: ROS, reactive oxygen species; UVB, ultraviolet B; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; AA, ascorbic acid.

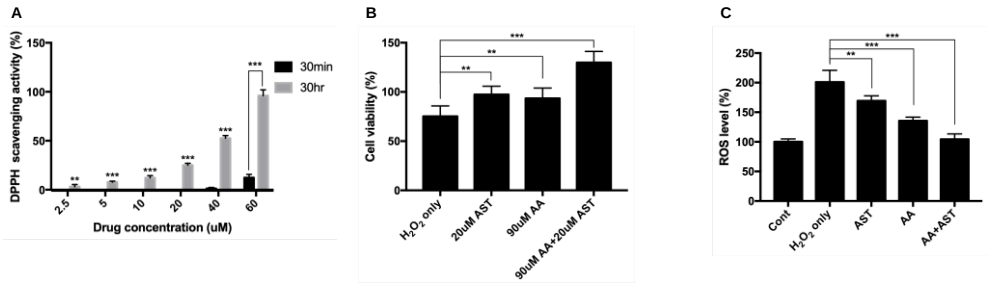

Fig. 7. DPPH assay of astaxanthin in aqueous environment and effects of the mixture of ascorbic acid and astaxanthin

Astaxanthin and ascorbic acid show antioxidative effect by reducing intracellular ROS in ARPE-19 cells

Antioxidative capacity of astaxanthin in aqueous environment was examined with DPPH assay and based on the result, modification was made to astaxanthin treatment scheme under H_2O_2-induced oxidative stress model. When astaxanthin was diluted in PBS and reacted with DPPH for 30 minutes, it showed poor antioxidative capacity while when it was reacted for 30 hours, DPPH scavenging activity was significantly increased. (A) The effect of 20uM astaxanthin, 90uM ascorbic acid and the mixture of the two compounds on the cell viability of ARPE-19 under H_2O_2-induced oxidative stress was examined by MTT assay. Cell viability was significantly increased when the cells were pretreated with 20uM astaxanthin, 90uM ascorbic acid, and the mixture of the two compounds for 6 hours and with 0.2mM H_2O_2 for 24 hours, compared to H_2O_2 only

Note: Asterisks indicate a significant difference between DPPH scavenging activity, increment in cell viability, or reduction in intracellular ROS level compared to control cells only with H_2O_2 exposure without antioxidant treatment (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Abbreviations: DPPH, 2,2-Diphenyl-1-picrylhydrazyl; AST, astaxanthin; AA, ascorbic acid; ROS, reactive oxygen species; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate.

4. Discussion

In this study, antioxidative properties of ascorbic acid and astaxanthin were evaluated based on hydrogen peroxide-induced and UVB-induced oxidative stress models within ARPE-19 cells. Studies have found that hydrogen peroxide and UVB have different effects on cells regarding oxidative stress. First, even directly adding hydrogen peroxide in the cell culture medium results in a short-term exposure because its concentration decreases rapidly in the presence of the cells. Hydrogen peroxide can penetrate the cell easily, but it is also reduced rapidly by the antioxidative mechanism. [41] On the other hand, UVB has a lingering effect on the cells by directly damaging DNA, causing gene mutation, and modifying gene expression, and enzyme activity along with increasing ROS level. [50] UVB-induces damage is mediated by two different pathways. One is by ROS generated immediately after the irradiation and the other is reactive nitrogen species in the later time point. [51] As a result, even with a single and momentary exposure to UVB, the viability of the exposed cells decreases in the course of time. [50]

Based on the precedent research, viability change of ARPE-19 cells was evaluated after 3 hours and 24 hours respectively for hydrogen peroxide model and UVB model. 0-0.8mM of H_2O_2 was exposed to ARPE-19 cells and their viability was dose-dependently reduced and intracellular ROS level was increased. UVB also reduced the cell viability and increased the intracellular ROS level but the H_2O_2 seemed to decrease the viability exponentially. One explanation can be that because hydrogen peroxide not only produces ROS, but it also affects junctional integrity of

RPE cell [52], weakening the cell adhesion to the bottom of the well, the cell viability assay result may have been affected. This can also explain the lower cell viability at 0.4mM of H_2O_2 than 40mJ/cm^2 UVB even though cells with H_2O_2-induced oxidative stress have lower ROS level.

Within the condition of sublethal and lethal doses of hydrogen peroxide and UVB, antioxidative potencies of ascorbic acid and astaxanthin were evaluated. Although their antioxidative properties had been studied and have moved on to patients with age-related retinal diseases (AREDS, CARMIS), there are controversies about whether they have a protective effect on cellular oxidative stress model. In one study, ascorbic acid did not have a protective effect on Fenton-reaction-mediated oxidative stress model of ARPE-19 but it rather decreased the cell survival ratio at a low concentration (0.1-1mM) compared to the group without ascorbic acid. [53] This was also the case for *tert*-butyl hydroperoxide (t-BOOH)-induced oxidative stress model. In a study by Kagan et al, ascorbic acid (0.02-0.2mM) also decreased the cell viability of ARPE-19 with oxidative stress induced by t-BOOH. [54] The effect of t-BOOH in porcine RPE also could not be diminished by ascorbic acid. [55] In our study, however, ascorbic acid increased the viability of the cells even at a low concentration where studies mentioned above suggest it decrease the viability and this was confirmed within two different oxidative stress models mediated by hydrogen peroxide and UVB. While the central mechanism of t-BOOH to induce oxidative stress is by generating alkyl radicals, [56] hydrogen peroxide is the central redox signaling molecule in general [42] forming hydroxy radicals, [57] which can react intracellularly to generate various radicals including alkyl radical. [58] Considering hydrogen peroxide model reproduces more general situation of

oxidative stress, and UVB model mediates hydrogen peroxide as the central signaling molecule, [59] our result based on both models is more convincing.

Ascorbic acid neutralized the effect of the oxidative stress inducer in both H_2O_2 and UVB model but astaxanthin only did so in UVB-induced stress model. Astaxanthin is known to have poor solubility in water [60]. Despite its low solubility in water, its hydrophilic property made it possible to be introduced to tissues and cells solubilized in fat. [61] Then it enters the cell via passive diffusion through the cell membrane and the absorption increases in a time course. [62] This led to an idea that the treatment period is possibly a reason. DPPH assay with PBS as a solvent was done to study ROS scavenging ability of astaxanthin in aqueous solution. As expected, astaxanthin showed poor antioxidative capacity which presumably because of its poor solubility in aqueous solution but it increased after a longer period of the reaction time of 30 hours. Based on this result, the antioxidative effect of astaxanthin was evaluated again in H_2O_2 induced oxidative stress model with longer treatment time and it increased the cell viability and decreased the intracellular ROS level. With the finding that extended time is needed for astaxanthin to act as an antioxidant, the synergistic effect of ascorbic acid and astaxanthin was evaluated. The combination of ascorbic acid and astaxanthin showed better antioxidative effect compared to each drug alone.

In summary, the antioxidative effect of ascorbic acid and astaxanthin was evaluated in this study using two different oxidative stress models achieved by hydrogen peroxide and UVB. Despite controversies questioning the antioxidative property of ascorbic acid, it was shown in this study that ascorbic acid diminishes the oxidative damage within human RPE oxidative stress models of hydrogen

peroxide and UVB which reflect general circumstance of oxidatively stressed environment. This study also compared the ROS scavenging capacity of astaxanthin in aqueous and organic environment concluding that in aqueous solution, astaxanthin takes more time to show its antioxidative property which is also applied to the cultured cell system. Synergistic effect of ascorbic acid and astaxanthin was also shown when the cells were treated in a longer period of time.

5. References

1. Congdon, N., et al., *Causes and prevalence of visual impairment among adults in the United States.* Archives of Ophthalmology (Chicago, Ill.: 1960), 2004. **122**(4): p. 477-485.
2. Masuda, T., M. Shimazawa, and H. Hara, *Retinal diseases associated with oxidative stress and the effects of a free radical scavenger (Edaravone).* Oxidative medicine and cellular longevity, 2017. **2017**.
3. Chiras, D., et al., *Oxidative stress in dry age-related macular degeneration and exfoliation syndrome.* Critical reviews in clinical laboratory sciences, 2015. **52**(1): p. 12-27.
4. Lewin, A., et al., *Modeling early stages of age-related macular degeneration (amd) by inducing oxidative stress in the rpe.* Investigative Ophthalmology & Visual Science, 2007. **48**(13): p. 5055-5055.
5. Bellezza, I., *Oxidative Stress in Age-Related Macular Degeneration: Nrf2 as Therapeutic Target.* Frontiers in pharmacology, 2018. **9**.
6. Jarrett, S.G. and M.E. Boulton, *Consequences of oxidative stress in age-related macular degeneration.* Molecular aspects of medicine, 2012. **33**(4): p. 399-417.

7. Shen, J., et al., *Oxidative damage in age-related macular degeneration.* Histology and histopathology, 2007. **22**(10-12): p. 1301-1308.

8. Khandhadia, S. and A. Lotery, *Oxidation and age-related macular degeneration: insights from molecular biology*. Expert reviews in molecular medicine, 2010. **12**.

9. Izzotti, A., A. Bagnis, and S.C. Saccà, *The role of oxidative stress in glaucoma.* Mutation Research/Reviews in Mutation Research, 2006. **612**(2): p. 105-114.

10. Chrysostomou, V., et al., *Oxidative stress and mitochondrial dysfunction in glaucoma*. Current opinion in pharmacology, 2013. **13**(1): p. 12-15.

11. Aslan, M., A. Cort, and I. Yucel, *Oxidative and nitrative stress markers in glaucoma.* Free Radical Biology and Medicine, 2008. **45**(4): p. 367-376.

12. Nita, M. and A. Grzybowski, *The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults*. Oxidative Medicine and Cellular Longevity, 2016. **2016**.

13. Seki, M. and S.A. Lipton, *Targeting excitotoxic/free radical signaling pathways for therapeutic intervention in glaucoma.* Progress in brain research, 2008. **173**: p. 495-510.

14. Zhou, L., Y. Li, and B.Y. Yue, *Oxidative stress affects cytoskeletal structure and cell‐matrix interactions in cells from an ocular tissue: The trabecular meshwork.* Journal of cellular physiology, 1999. **180**(2): p. 182-189.

15. Ko, M.L., et al., *The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma.* Investigative ophthalmology & visual science, 2000. **41**(10): p. 2967-2971.

16. Saccà, S.C., et al., *Oxidative DNA damage in the human trabecular meshwork: clinical correlation in patients with primary open-angle glaucoma.* Archives of Ophthalmology, 2005. **123**(4): p. 458-463.

17. Limón-Pacheco, J. and M.E. Gonsebatt, *The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2009. **674**(1-2): p. 137-147.

18. Valle, I., et al., *PGC-1α regulates the mitochondrial antioxidant defense system in vascular endothelial cells.* Cardiovascular research, 2005. **66**(3): p. 562-573.

19. de Vries, H.E., et al., *Nrf2-induced antioxidant protection: a promising target to counteract ROS-mediated damage in neurodegenerative disease?* Free Radical Biology and Medicine, 2008. **45**(10): p. 1375-1383.

20. Slemmer, J.E., et al., *Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging.* Current medicinal chemistry, 2008. **15**(4): p. 404-414.

21. Moradas- Ferreira, P., et al., *The molecular defences against reactive oxygen species in yeast.* Molecular microbiology, 1996. **19**(4): p. 651-658.

22. Gultekin, F., et al., *In vivo changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats.* Archives of Toxicology, 2001. **75**(2): p. 88-96.

23. Filomeni, G., D. De Zio, and F. Cecconi, *Oxidative stress and autophagy: the clash between damage and metabolic needs.* Cell death and differentiation, 2015. **22**(3): p. 377.

24. Packer, L., S.U. Weber, and G. Rimbach, *Molecular aspects of α-tocotrienol antioxidant action and cell signalling.* The Journal of nutrition, 2001. **131**(2): p. 369S-373S.

25. Okoduwa, S.I., et al., *Age-dependent alteration of antioxidant defense system in hypertensive and type-2 diabetes patients.* Journal of Diabetes & Metabolic Disorders, 2015. **14**(1): p. 32.

26. Luo, L., et al., *Aging and the brown Norway rat leydig cell antioxidant defense system.* Journal of andrology, 2006. **27**(2): p. 240-247.

27. Liguori, I., et al., *Oxidative stress, aging, and diseases.* Clinical interventions in aging, 2018. **13**: p. 757.

28. Halliwell, B., *Antioxidants in human health and disease.* Annual review of nutrition, 1996. **16**(1): p. 33-50.

29. Noctor, G. and C.H. Foyer, *Ascorbate and glutathione: keeping active oxygen under control.* Annual review of plant biology, 1998. **49**(1): p. 249-279.

30. Naguib, Y.M., *Antioxidant activities of astaxanthin and related carotenoids.* Journal of agricultural and food chemistry, 2000. **48**(4): p. 1150-1154.

31. Gil, M.I., et al., *Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from California.* Journal of Agricultural and Food Chemistry, 2002. **50**(17): p. 4976-4982.

32. Marc, F., et al., *Studies of several analytical methods for antioxidant potential evaluation in food.* Medecine sciences: M/S, 2004. **20**(4): p. 458-463.

33. Ou, B., et al., *Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe.* Journal of agricultural and food chemistry, 2002. **50**(10): p. 2772-2777.

34. Denev, P., et al., *Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties.* Food Chemistry, 2010. **123**(4): p. 1055-1061.

35. Cao, G. and R.L. Prior, *Comparison of different analytical methods for assessing total antioxidant capacity of human serum.* Clinical chemistry, 1998. **44**(6): p. 1309-1315.

36. Ozcan, T., et al., *Assessment of antioxidant capacity by method comparison and amino acid characterisation in buffalo milk kefir.* International Journal of Dairy Technology, 2019. **72**(1): p. 65-73.

37. Rao, P.S., et al., *Comparison of two analytical methods used for the measurement of total antioxidant status.* Journal of antioxidant activity, 2015. **1**(1): p. 22.

38. Roy, M.K., et al., *ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: relationship between total polyphenol and individual catechin content.* International journal of food sciences and nutrition, 2010. **61**(2): p. 109-124.

39. Valverde Malaver, C.L., A.J. Colmenares Dulcey, and J.H. Isaza Martínez, *Comparison of DPPH free radical scavenging, ferric reducing antioxidant power (FRAP), and total phenolic content of two*

meriania species (Melastomataceae). Revista de Ciencias, 2015. **19**(2): p. 117-124.

40. Rácz, A., et al., *Comparison of antioxidant capacity assays with chemometric methods.* Analytical Methods, 2015. **7**(10): p. 4216-4224.

41. Gille, J. and H. Joenje, *Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia.* Mutation Research/DNAging, 1992. **275**(3-6): p. 405-414.

42. Sies, H., *Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress.* Redox biology, 2017. **11**: p. 613-619.

43. Wijeratne, S.S., S.L. Cuppett, and V. Schlegel, *Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells.* Journal of agricultural and food chemistry, 2005. **53**(22): p. 8768-8774.

44. Glickman, R.D., *Ultraviolet phototoxicity to the retina.* Eye & contact lens, 2011. **37**(4): p. 196-205.

45. Podda, M., et al., *UV-irradiation depletes antioxidants and causes oxidative damage in a model of human skin.* Free Radical Biology and Medicine, 1998. **24**(1): p. 55-65.

46. Wenk, J., et al., *UV-induced oxidative stress and photoaging.* CURRENT PROBLEMS IN DERMATOLOGY-BASEL-, 2001. **29**: p. 83-94.

47. Meister, A., *On the antioxidant effects of ascorbic acid and glutathione.* Biochemical pharmacology, 1992. **44**(10): p. 1905-1915.

48. Olugbami, J.O., M.A. Gbadegesin, and O.A. Odunola, *In vitro free radical scavenging and antioxidant properties of ethanol extract of Terminalia glaucescens.* Pharmacognosy research, 2015. **7**(1): p. 49.

49. Cai, J., et al., *β- Secretase (BACE1) inhibition causes retinal pathology by vascular dysregulation and accumulation of age pigment.* EMBO molecular medicine, 2012. **4**(9): p. 980-991.

50. Zhao, L., Y. Man, and S. Liu, *Long non-coding RNA HULC promotes UVB-induced injury by up-regulation of BNIP3 in keratinocytes.* Biomedicine & Pharmacotherapy, 2018. **104**: p. 672-678.

51. Terra, V., et al., *Time-dependent reactive species formation and oxidative stress damage in the skin after UVB irradiation.* Journal of Photochemistry and Photobiology B: Biology, 2012. **109**: p. 34-41.

52. Bailey, T.A., et al., *Oxidative stress affects the junctional integrity of retinal pigment epithelial cells.* Investigative ophthalmology & visual science, 2004. **45**(2): p. 675-684.

53. Zeitz, O., et al., *Lack of antioxidative properties of vitamin C and pyruvate in cultured retinal pigment epithelial cells.* Graefe's Archive

for Clinical and Experimental Ophthalmology, 2007. **245**(2): p. 276-281.

54. Kagan, D.B., H. Liu, and C.M. Hutnik, *Efficacy of various antioxidants in the protection of the retinal pigment epithelium from oxidative stress.* Clinical ophthalmology (Auckland, NZ), 2012. **6**: p. 1471.

55. Woo, K.I. and J. Lee, *The effects of ascorbic acid on free radical injury in cultured retinal pigment epithelial cells.* Korean Journal of Ophthalmology, 1995. **9**(1): p. 19-25.

56. Korte, C.S., *Tert-Butyl Hydroperoxide Stimulates Parturition-Associated Pathways in a Human Placental Cell Line.* 2013.

57. Wang, Y., et al., *Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling.* J Cell Biol, 2018. **217**(6): p. 1915-1928.

58. McKettaJr, J., *Encyclopedia of Chemical Processing and Design : Volume 65 -- Waste: Nuclear Reprocessing and Treatment Technologies to Wastewater Treatment: Multilateral Approach.* 2017.

59. Masaki, H., T. Atsumi, and H. Sakurai, *Detection of hydrogen peroxide and hydroxyl radicals in murine skin fibroblasts under UVB irradiation.* Biochemical and biophysical research communications, 1995. **206**(2): p. 474-479.

60. Boesch-Saadatmandi, C., et al., *Comparison of tetrahydrofuran, fetal calf serum, and Tween 40 for the delivery of astaxanthin and canthaxanthin to HepG2 cells.* Cytotechnology, 2011. **63**(1): p. 89-97.

61. Wu, H., et al., *Astaxanthin as a potential neuroprotective agent for neurological diseases.* Marine drugs, 2015. **13**(9): p. 5750-5766.

62. Mimoun-Benarroch, M., et al., *The bioavailability of astaxanthin is dependent on both the source and the isomeric variants of the molecule.* BULLETIN OF UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE CLUJ-NAPOCA-FOOD SCIENCE AND TECHNOLOGY, 2016. **73**(2): p. 61-69.