Protective effect of lutein on ARPE-19 cells upon H$_2$O$_2$-induced G$_2$/M arrest

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Abstract. Oxidative damage is a key factor for the pathogenesis of age-related macular degeneration (AMD), therefore, anti-oxidative stress is a valuable method for the prevention or treatment of AMD. The aim of the present study was to reveal the protective mechanism of lutein on retinal pigment epithelium (RPE) cells subjected to oxidative stress. Acute retinal pigment epithelial 19 (ARPE-19) cells were exposed to oxidative stress induced by H$_2$O$_2$ following lutein pretreatment. The activities of caspases, level of intracellular reactive oxygen species (ROS) and cell cycle were analyzed using flow cytometry. The expression levels of cell cycle regulatory proteins and inflammation-associated genes were detected using western blot and reverse transcription-polymerase chain reaction analyses, respectively. The data showed that oxidative stress reduced cell viability, and increased total apoptosis and ROS generation, however, lutein prevented cells from oxidative stress-induced damage. In addition, oxidative damage triggered G$_2$/M phase arrest of the ARPE-19 cells, which was reversed by lutein in a concentration-dependent manner, through the activation of cyclin-dependent kinase 1 and cell division cycle 25C, and degradation of cyclin B1. These results demonstrated that lutein may be an effective antioxidant, which can be applied in the prevention of AMD, or other age-related diseases associated with oxidative damage.

Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss among individuals >65 years of age in developed countries (1). The most common type of AMD, also termed the ‘dry-type’, is initiated by the death of retinal pigment epithelium (RPE) cells and eventually results in the degeneration of photoreceptors, which leads to visual loss (2,3). AMD is a multifactorial disease; aging, genetic background, cigarette smoking, oxidative damage and chronic inflammation are all factors, which contribute to its onset and progression (4-6).

It is well established that oxidative stress is important in the pathogenesis of AMD (7,8). The retina requires a higher oxygen concentration, compared with other organs, in order to maintain the high metabolic rate of photoreceptors. The higher the level of oxygen consumed, the more reactive oxygen species (ROS) is produced. In addition, the daily phagocytosis of shed photoreceptor outer segments leads to the generation of free radicals and toxic oxidized materials in RPE cells. Therefore, RPE cells are susceptible to long-term oxidative stress, and oxidative stress induces the dysfunction of RPE cells, contributing to the development of AMD (2,9). There remains no effective treatment for the dominant type of AMD, and current interventions are commonly focused on prevention rather than treatment. Antioxidant supplements have been used to reduce the risk of AMD, and dietary lutein is considered to act as a protector against visual impairment from AMD (10).

Lutein is a type of carotenoid, which forms human macular pigments with zeaxanthin in the retina, inhibiting noxious blue light into retina and contributing to strengthening of the anti-oxidant defense of RPE cells (11,12). The human body cannot synthesize lutein. The sources of lutein are primarily dietary in origin, for example, green leafy vegetables, including spinach and cabbage; fruits, including grapes and kiwis; egg yolks, and corn (13). It is reported that the risks of the onset and progression of AMD are negatively correlated with lutein concentration in the macula (5,14).

Lutein has already been used in the healthcare setting (15), however, the exact molecular mechanism underlying the protective effect of lutein against stress remains to be fully
elucidated. To better understand the function of lutein, the present study aimed to examine its underlying mechanism and widen its areas of application.

Materials and methods

Cell culture. The acute retinal pigment epithelial 19 (ARPE-19) human RPE cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (50 µg/ml) in a 5% CO₂-humidified environment at 37°C.

Lutein and H₂O₂ treatment. The cells were seeded at a density of 4x10⁴ per well in 96-well plates and 8x10³ per dish in 60 mm dishes, and then cultured with lutein (Aladdin Chemical Co., Ltd., Shanghai, China) at concentrations of 0, 1, 5, 10 and 15 µM for 12 h at 37°C. Lutein was dissolved in dimethyl sulfoxide (DMSO; MP Biomedicals, Illkirch, France) with a stock concentration of 1 mM and maintained in the dark. Following washing once with PBS, the RPE cells were incubated in culture media containing 0, 200, 400, 600, 800, 1,000, 1,200, 1,600 and 2,000 µM H₂O₂ (Guangzhou Chemical Reagent Factory, Guangzhou, China) for 12 or 24 h at 37°C prior to the specific assays.

Measurement of apoptosis, ROS levels and cell cycle. The RPE cells pretreated with lutein for 24 h were incubated with 800 µM H₂O₂ for another 24 h. The cell apoptosis, ROS levels and cell cycle were detected using a multicaspase kit, oxidative stress kit and cell cycle kit, respectively (Muse™; Merck Millipore, Darmstadt, Germany). All procedures were performed according to the manufacturer’s protocols. The Muse™ Cell Analyzer software (version 1.3) was used for accurate statistical analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted with TRIzol reagent (Takara Bio, Inc., Shiga, Japan). A 1 µg sample of RNA was reverse transcribed using a PrimeScript™ RT reagent kit (Takara Bio, Inc.) of RPE cells cultured without treatment, the cell viability and proliferation of the RPE cells remained unchanged with concentrations of lutein up to 15 µM (Fig. 1A).

The RPE cells were treated with different concentrations of H₂O₂ (0-2,000 µM) for 24 h. Cell viability was also
Lutein increases cell viability, and decreases apoptosis and ROS in RPE cells exposed to \( \text{H}_2\text{O}_2 \) stress. In the experiments, \( \text{H}_2\text{O}_2 \) reduced the cell viability of the RPE cells to 43.66% of the control. Lutein reversed the reduction in cell viability in a dose-dependent manner. When pretreated with lutein at concentrations of 5 and 15 \( \mu \text{M} \), the cell viability of the RPE cells was increased to 49.95, 65.39 and 74.32 of the control, respectively (Fig. 2A).

The expression levels of total caspases in the RPE cells increased to 66.3% when the cells were exposed to \( \text{H}_2\text{O}_2 \), compared with 11.1% in the control group. Lutein inhibited the increased expression of total caspases in a concentration-dependent manner. Following retreatment with lutein at concentrations of 5 and 10 \( \mu \text{M} \), the expression of total caspases in RPE cells reduced to 49.3 and 26.9%, respectively (Fig. 2B).

In the RPE cells treated with \( \text{H}_2\text{O}_2 \), the ROS levels increased to 65.21%, compared with 10.76% in the control group. Lutein reversed the elevation in ROS levels. The ROS levels reduced to 52.8 and 42.4% when the RPE cells were pretreated with 5 and 10 \( \mu \text{M} \) lutein, respectively (Fig. 2C).

Lutein increases the expression of IL-6, IL-8 and TNF-\( \alpha \) inflammatory cytokines in RPE cells treated with \( \text{H}_2\text{O}_2 \). In the present study, \( \text{H}_2\text{O}_2 \) markedly increased the expression levels of the IL-6, IL-8 and TNF-\( \alpha \) inflammatory cytokines in the RPE cells (Fig. 3). When the RPE cells were pretreated with lutein at a concentration of 10 \( \mu \text{M} \), the transcription levels of these inflammatory cytokines were also elevated, although pretreatment with lutein at a concentration of 5 \( \mu \text{M} \) did not alter the expression of these inflammatory cytokines.

Lutein reduces RPE G2/M phase arrest induced by \( \text{H}_2\text{O}_2 \). When the concentration of \( \text{H}_2\text{O}_2 \) reached 400 \( \mu \text{M} \), cell cycle arrest of the RPE cells was observed in the G2/M phase (Fig. 4A). It was found that, in the RPE cells treated with 600 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), the proportion of cells in the G2/M phase was 47.3%, compared with 35.9% in the control group. Lutein reversed the increased proportion of cells in the G2/M phase in a concentration-dependent manner. When the cells were pretreated with 5 and 10 \( \mu \text{M} \) lutein, the proportions of RPE cells in the G2/M phase were reduced to 40.8 and 33.4%, respectively (Figs. 4B and 5A).

Lutein attenuates RPE cell cycle arrest in the G2/M phase by activating CDK1 and CDC25C, and decreasing cyclin B1. When the RPE cells were treated with \( \text{H}_2\text{O}_2 \), the expression levels of CDK1 and CDC25C were inhibited, and the protein expression of cyclin B1 was increased in the cells. However, the inactivation of CDK1 and CDC25C, and increase of cyclin B1 were attenuated when lutein was added to the cells (Fig. 5B and C).

Discussion

In the present study, it was demonstrated that the oxidative stress triggered by \( \text{H}_2\text{O}_2 \) decreased cell viability, increased intracellular ROS and increased apoptosis in RPE cells. It was noted that marked G2/M phase arrest occurred in the RPE cells when subjected to \( \text{H}_2\text{O}_2 \) and for the first time, to the best

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### Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>F:CCCCGTTTCGCTCCTGCCTCC R:ACCAAGGCCCAATACGACCA</td>
</tr>
<tr>
<td>IL-6</td>
<td>F:ACAGCCACTCACCTCCTCCAG R:GAAGCATCACCCTTTTTCAGCCA</td>
</tr>
<tr>
<td>IL-8</td>
<td>F:GAGCTCTGTCCTGAAACCTCA R:CTTCCACTATGTTCTGGATACCA</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>F:GGGACCTCTCTCACCAGCC R:GGTTTCGAAAGTGTTGGTCTTG</td>
</tr>
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F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \).
of our knowledge, it was found that lutein attenuated this G\textsubscript{2}/M arrest in a concentration-dependent manner.

Lutein is present at a high concentration in the macula of the eye (17). It contains several double bonds, which react with ROS to scavenge free radicals (1). Lutein functions as a cytoprotective antioxidant in a direct anti-apoptotic or indirect anti-oxidation manner (12,18). In addition, the reversal of G\textsubscript{2}/M phase arrest observed in oxidative stressed cells induced by lutein contribute to its role in cell protection.

When DNA is damaged, the G\textsubscript{2} checkpoint inhibits cells entering mitosis. The cell cycle arrest provides an opportunity for repair and inhibits proliferation of the damaged cells (19). In H1299 cells, DNA damage and G\textsubscript{2}/M phase arrest were found to be induced by oxidative damage, whereas an antioxidant in red seaweed *Gracilaria tenuistipitata* protected the cells from DNA damage and G\textsubscript{2}/M arrest (20). The results of the
Present study demonstrated that lutein protected cell viability and reversed G2/M arrest of RPE cells under oxidative stress.

Cell cycle progression is regulated by various factors, including CDKs and cyclins. The cyclin B1/CDK1 complex regulates cell cycle progression from the G2 to M phase, and cyclins accumulate steadily during the G2 phase and are rapidly eliminated as cells exit mitosis. The activation of CDK1 kinase is an ordered process, which triggers the

Figure 4. Cell cycle phases of RPE cells exposed to H2O2 with or without lutein pretreatment. (A) RPE cells were subjected to 0, 100, 200, 400 and 800 μM of H2O2. Flow cytometry results of cell cycle phases are shown. (B) RPE cells pretreated with lutein (5, 10 and 15 μM) for 24 h were exposed to H2O2 (600 μM) for 24 h. Flow cytometry results of cell cycle phases are shown. All the experiments were repeated three times. RPE, retinal pigment epithelium.

Figure 5. Lutein attenuates the G2/M phase arrest induced by oxidative stress. (A) RPE cells were pretreated with lutein (0, 5, 10 and 15 μM) for 24 h and then challenged with or without H2O2 for 24 h. A histogram of the cell cycle phases of the RPE cells is shown. (B) Expression levels of CDK1, CDC25C and cyclin B1 were determined using western blot analysis; α-tubulin and GAPDH were used as internal controls. (C) Densitometric analyses of the protein expression levels of CDK1, cyclin B1 and CDC25C from the western blots are shown.*P<0.05, vs. control; #P<0.05 and ##P<0.01, vs. cells treated with H2O2 only. Analysis was repeated at least three times. RPE, retinal pigment epithelium; CDK2, cyclin-dependent kinase 1; CDC25C, cell division cycle 25C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
initiation of mitosis. CDC25 is also a key regulator, which activates CDK1 and drives cell cycle progression (21). In the present study, when the RPE cells were subjected to oxidative stress, a significant increase in cyclin B1 and decreases in CDK1 and CDC25C were observed, which suggested that cell cycle progression was inhibited prior to entering the mitosis phase. This suggestion was confirmed by the analysis of cell cycle using flow cytometry, as RPE cells in the G2/M phase increased when exposed to H2O2. However, lutein protected the RPE cells from G2/M phase arrest by degrading the cyclin B1 protein, and increasing the activities of CDK1 and CDC25C in a concentration-dependent manner. As the results of the flow cytometry indicated, fewer RPE cells were arrested in the G2/M phase when treated with lutein.

Increasing evidence has indicated the role of inflammation in the pathogenesis of AMD. Inflammatory proteins make up the composition of drusen in AMD, and RPE cells are a rich resource of inflammatory cytokines (11,22,23). Lutein prevents the proteasome from inactivation by photo-oxidative damage and alters the expression of the inflammatory-associated genes, monocyte chemoattractant protein-1, IL-8 and complement factor H in RPE cells (11). Lutein also exerts an anti-inflammatory effect in the ischemic/hypoxic retina by reducing the expression of IL-1β and cyclooxygenase 2 in mRC-1 cells (24). The present study demonstrated that H2O2 treatment upregulated the expression of the inflammation-associated genes, IL-6, IL-8 and TNF-α. At concentrations >10 μM, lutein increased the expression levels of IL-6, IL-8 and TNF-α. These results improve current understanding of the effect of lutein on inflammation and indicated the potential cytotoxic effect of lutein; therefore, the use of large concentrations of lutein requires caution (25).

In conclusion, the present study demonstrated that lutein protected RPE cells from oxidative damage, and reversed G2/M phase arrest through activating CDK1 and CDC25C, and degrading the protein expression of cyclin B1. As AMD is a disease prevailing worldwide and a socioeconomic burden requiring resolution, dietary lutein supplementation may offer a suitable measure for preventing AMD.

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References