Protective effect of lutein on ARPE-19 cells upon H_2O_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_2O_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.

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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 U/ml) in a 5% CO_2-humidified environment at 37˚C.

Lutein and H_2O_2 treatment. The cells were seeded at a density of $4x10^3$ per well in 96-well plates and $8x10^5$ 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 H_2O_2 (Guangzhou Chemical Reagent Factory, Guangzhou, China) for 12 or 24 h at 37˚C prior to the specific assays.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium-bromide (MTT) assay. Following treatment with lutein or H_2O_2, the RPE cells were washed in PBS and incubated at 37˚C in DMEM containing 0.25 mg/ml MTT. After 4 h, the MTT solution was removed and 150 μl DMSO was added to each well. The optical densities at 490 nm were read on a microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA).

Measurement of apoptosis, ROS levels and cell cycle. The RPE cells pretreated with lutein for 24 h were incubated with 800 μM H_2O_2 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.), and the mixtures were incubated at 37˚C for 15 min and 85˚C for 5 sec. Subsequently, 1 μl (10 ng/μl) DNA was added to 5 μl SYBR Green I, 0.5 μl (10 μM) forward primer, 0.5 μl (10 μM) reverse primer and 3 μl ddH_2O, using the Universal qPCR kit (Kapa Biosystems, Wilmington, MA, USA). The qPCR was performed on a LightCycler® 96 sequence detection system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The LightCycler® 96 application software version 1.1 was used for data collection and analysis. Relative quantitative analysis of interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α) mRNAs were performed using the $2^{-\Delta\Delta Cq}$ method with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (16). The system settings were as follows: Preincubation at 95˚C for 60 sec and amplification at 72˚C for 10 sec for 45 cycles, and melting at 97˚C for 1 sec. The primer sets were designed as shown in Table I.

Western blot analysis. The cell proteins were extracted on ice using lysis buffer solution (20 mM Tris HCl, 150 mM NaCl, 10% glycerol, 1% NP-40, 2.1 g NaF, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aptotin, 2 μg/ml leupeptin and 420 ml H_2O) containing phosphatase inhibitor (PhosSTOP; Merck Millipore). Protein concentrations were measured using a bicinchoninic acid assay kit (catalog no. C503021, Sangon Biotech Co., Ltd., Shanghai, China). A total of 20 μg protein from each sample was loaded onto 10% gels and subjected to SDS-PAGE, prior to transfer onto a nitrocellulose membrane for 75 min. The membrane was sealed with 5% defatted milk for 1 h, incubated with primary antibodies at 4˚C overnight, and washed with 1X TBST for 10 min three times. The membrane was then incubated with secondary antibodies for 1 h at room temperature and washed with 1X TBST for 10 min three times. The ECL reagent Immobilon™ Western (EMD Millipore, Billerica, MA, USA) was added to the membranes for 1-3 min, and the immunofluorescence reaction was observed using a western blot luminescence imaging system (Tanon-5200; Tanon, Shanghai, China) and image analysis software (Gel Image System Ver. 4.2.5; Tonon). The antibodies used included tubulin α (cat. no. AF7010; Affinity Biotech, Kansas, MO, USA, GAPDH (cat. no. 60004-1-Ig; Proteintech Group, Inc., Wuhan, China), cyclin-dependent kinase 1 (CDK1; cat. no. BM1028; Boster Biotech, Wuhan, China), cyclin B1 (cat. no. BM0766; Boster Biotech) and cell division cycle 25C (CDC25C; cat. no. BM2728; Boster Biotech). The primary antibodies were diluted 1:500 and an anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (catalog. no. 7076S) or an anti-rabbit IgG Alexa Fluor® 555-conjugated secondary antibody (catalog no. 4413) from Cell Signaling Technology, Inc., Danvers, MA, USA, were diluted 1:2,000.

Statistical analysis. Each experiment was performed in triplicate. All analyses were performed using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA). The data are expressed as the mean ± standard deviation and were statistically compared using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability of RPE cells following treatment with lutein and H_2O_2. The cytotoxicity of lutein was assessed using an MTT assay for 3 days. Different concentrations of lutein were added to the cultural media of RPE cells. Compared with the control group 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 H_2O_2 (0-2,000 μM) for 24 h. Cell viability was also

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

Primer	Sequence (5'→3')
GAPDH	F:CCCGCTTCGCTCTCTGCTCC
	R:ACCAGGCGCCAATACGACC
IL-6	F:ACAGCCACTCACCTCTTCAG
	R:GAAGCATCCATCTTTTTCAGCCA
IL-8	F:GAGCTCTGTCTGGACCCCA
	R:TCTTCACTGATTCTTGGATACCA
TNF-α	F:GGGACCTCTCTCTAATCAGCC
	R:GGTTTCGAAGTGGTGGTCTTG

F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor-α.

evaluated using an MTT assay. The results revealed that the cell viability reduced to ~50% of that in the control when the concentration of H_2O_2 reached 800 μM (Fig. 1B). Based on this result, 800 μM was selected as the concentration for inducing apoptosis and the production of ROS.

Lutein increases cell viability, and decreases apoptosis and ROS in RPE cells exposed to H_2O_2 stress. In the experiments, H_2O_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, 10 and 15 μM, 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 H_2O_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 μM, the expression of total caspases in RPE cells reduced to 49.3 and 26.9%, respectively. (Fig. 2B).

In the RPE cells treated with H_2O_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 μM lutein, respectively (Fig. 2C).

Lutein increases the expression of IL-6, IL-8 and TNF-α inflammatory cytokines in RPE cells treated with H_2O_2. In the present study, H_2O_2 markedly increased the expression levels of the IL-6, IL-8 and TNF-α inflammatory cytokines in the RPE cells (Fig. 3). When the RPE cells were pretreated with lutein at a concentration of 10 μM, the transcription levels of these inflammatory cytokines were also elevated, although pretreatment with lutein at a concentration of 5 μM did not alter the expression of these inflammatory cytokines.

Lutein reduces RPE G_2/M phase arrest induced by H_2O_2. When the concentration of H_2O_2 reached 400 μM, cell cycle arrest of the RPE cells was observed in the G_2/M phase. (Fig. 4A). It was found that, in the RPE cells treated with 600 μM H_2O_2,

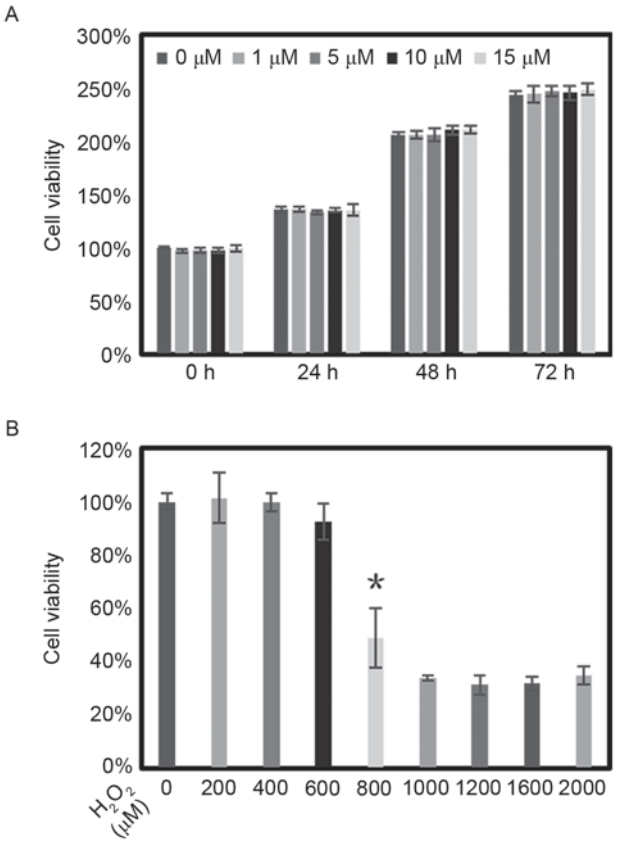

Figure 1. Viabilities of RPE cells following lutein and H_2O_2 treatment. (A) RPE cells were treated with lutein (0, 1, 5, 10 and 15 μM) for 3 days and cell viability was assessed using an MTT assay. (B) Dose-responses of RPE cells to treatment with H_2O_2 (0-2,000 μM) were detected using an MTT assay, with viabilities expressed as a percentage of the control. *P<0.05 for cell viability reduction by 50%, compared with the control at 24 h. The data are presented as the mean ± standard deviation of results from six samples in each group. RPE, retinal pigment epithelium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium-bromide.

the proportion of cells in the G_2/M phase was 47.3%, compared with 35.9% in the control group. Lutein reversed the increased proportion of cells in the G_2/M phase in a concentration-dependent manner. When the cells were pretreated with 5 and 10 μM lutein, the proportions of RPE cells in the G_2/M phase were reduced to 40.8 and 33.4%, respectively (Figs. 4B and 5A).

Lutein attenuates RPE cell cycle arrest in the G_2/M phase by activating CDK1 and CDC25C, and decreasing cyclin B1. When the RPE cells were treated with H_2O_2, the expression levels of CDK1 and CDC25C were inhibited, and the protein expression of 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 H_2O_2 decreased cell viability, increased intracellular ROS and increased apoptosis in RPE cells. It was noted that marked G_2/M phase arrest occurred in the RPE cells when subjected to H_2O_2 and for the first time, to the best

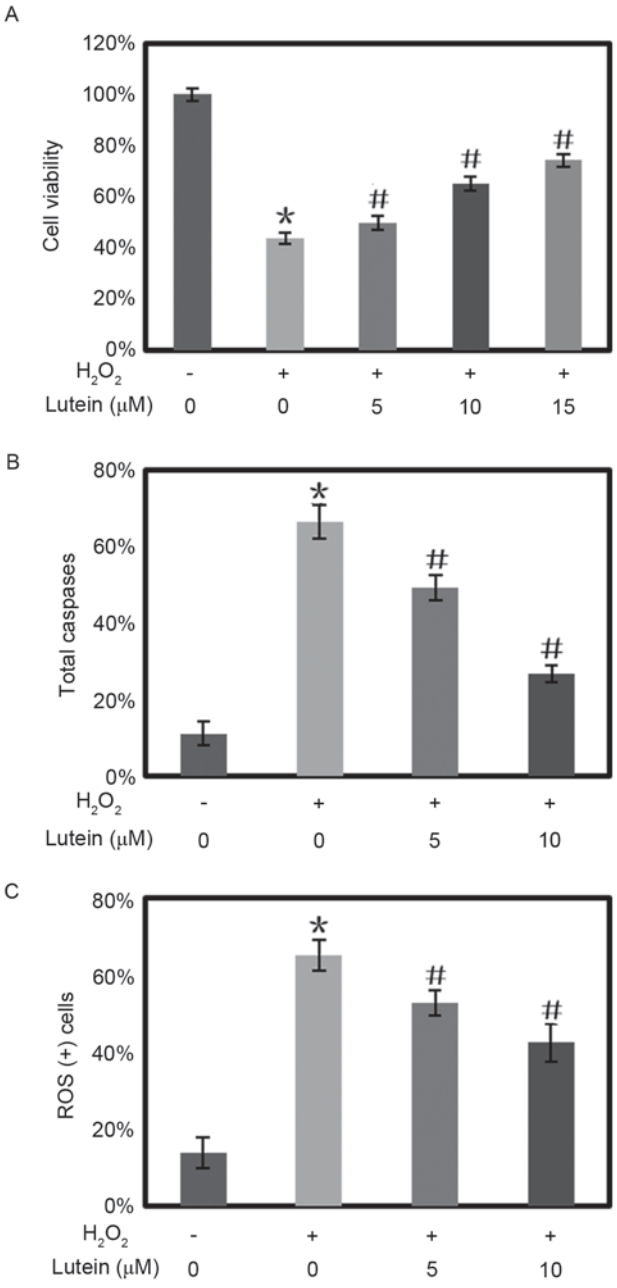

Figure 2. Lutein protects RPE cells from cell toxicity, cell apoptosis and intracellular ROS elevation induced by H_2O_2. (A) Different doses of lutein were added 12 h prior to treating the RPE cells with H_2O_2. After 24 h, the cell viability was quantified using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium-bromide assay. RPE cells were treated with lutein (0, 5, 10 and 15 μM) for 12 h and then challenged with H_2O_2 (800 μM). The levels of (B) caspases and (C) ROS were quantified using flow cytometry. *P<0.05, vs. control; #P<0.05, vs. cells treated with H_2O_2 only. The experiments were repeated at least three times. RPE, retinal pigment epithelium; ROS, reactive oxygen species.

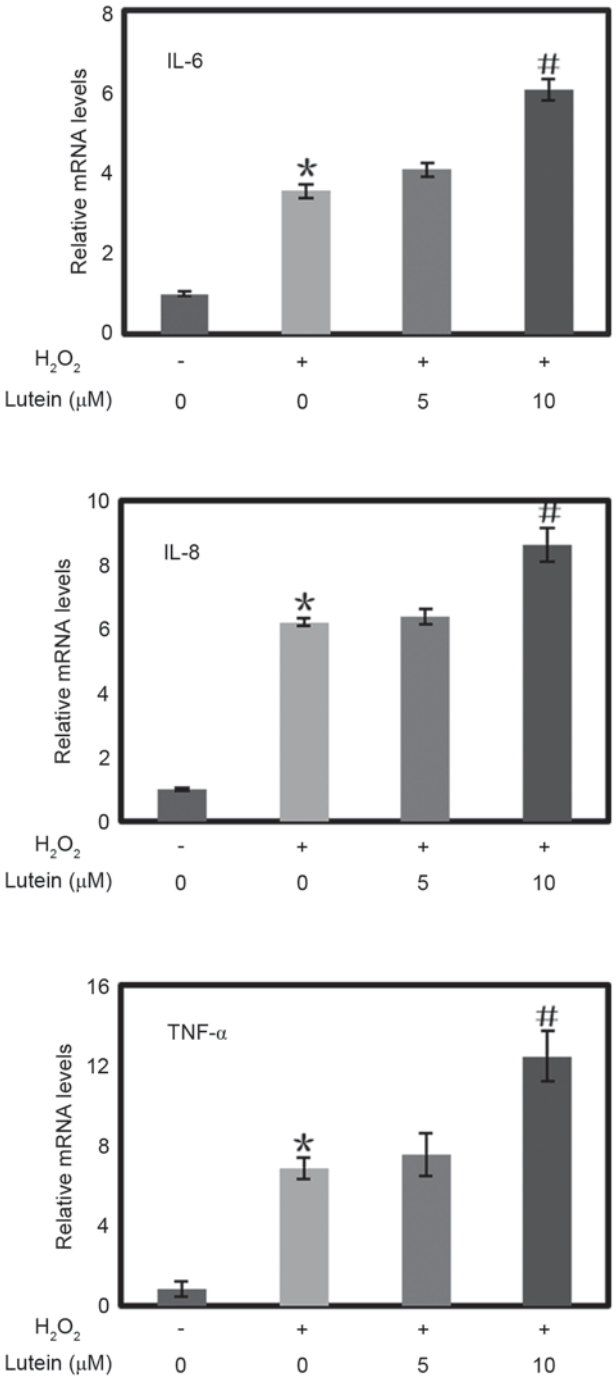

Figure 3. Lutein increases the expression of inflammatory cytokines in RPE cells exposed to oxidative damage by H_2O_2. The RPE cells were pretreated with different doses of lutein for 24 h and then exposed to H_2O_2 for another 24 h. The mRNA levels for IL-6, IL-8 and TNF-α in the treated cells were determined as fold of the control. The data are presented as the mean ± standard deviation of four repeated experiments in each group. *P<0.05, vs. control; #P<0.05, vs. cells treated with H_2O_2 only. RPE, retinal pigment epithelium; IL, interleukin; TNF-α, tumor necrosis factor-α.

of our knowledge, it was found that lutein attenuated this G_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_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_2 checkpoint inhibits cells entering mitosis. The cell cycle arrest provides an opportunity for repair and inhibits proliferation of the damaged cells (19). In Hl299 cells, DNA damage and G_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_2/M arrest (20). The results of the

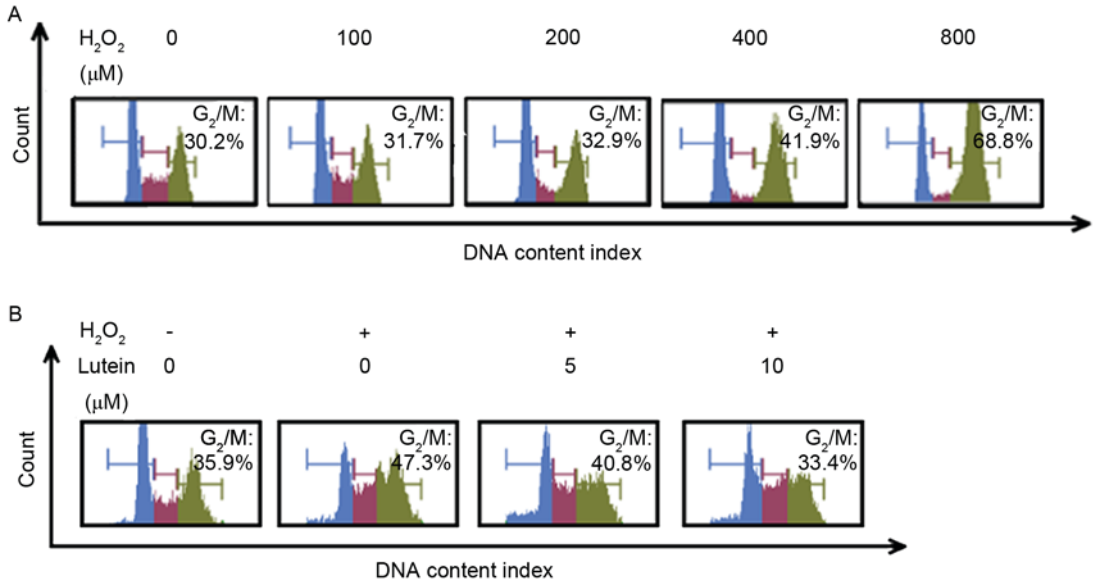

Figure 4. Cell cycle phases of RPE cells exposed to H_2O_2 with or without lutein pretreatment. (A) RPE cells were subjected to 0, 100, 200, 400 and 800 μM of H_2O_2. Flow cytometry results of cell cycle phases are shown. (B) RPE cells pretreated with lutein (5, 10 and 15 μM) for 24 h were exposed to H_2O_2 (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.

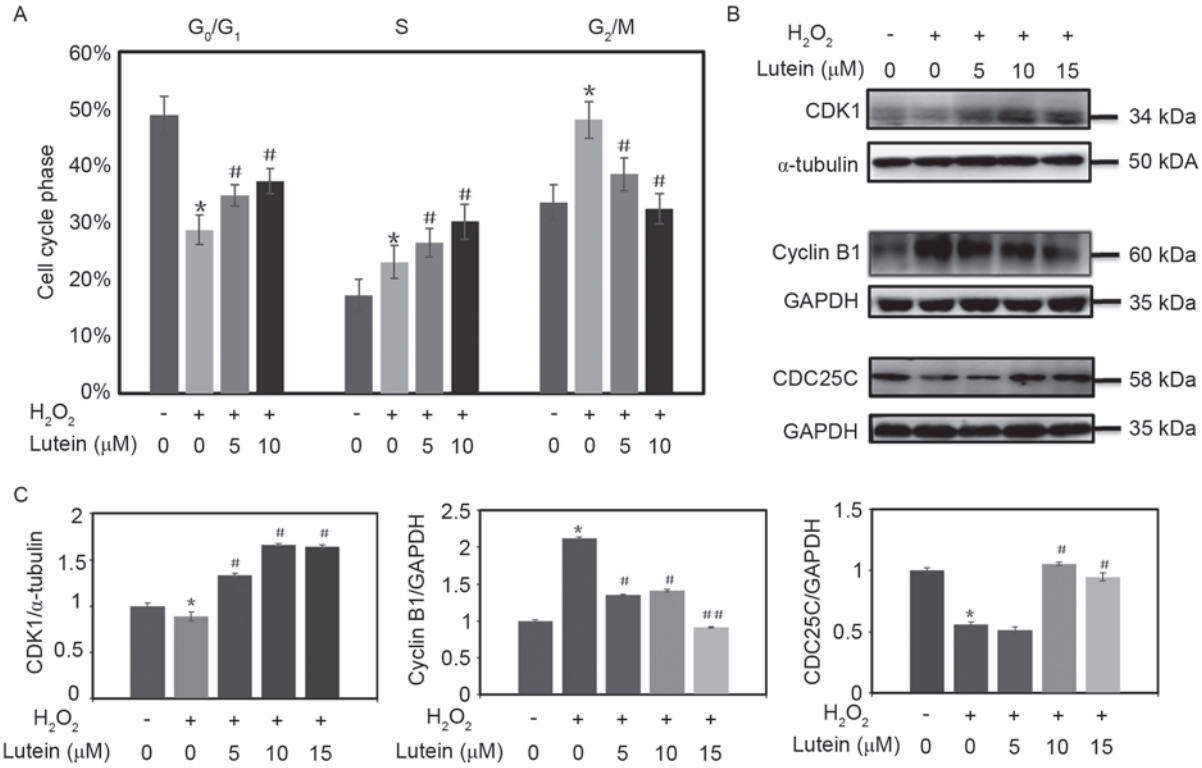

Figure 5. Lutein attenuates the G_2/M phase arrest induced by oxidative stress. (A) RPE cells were pretreated with lutein (0, 5, 10 and 15 μM) for 24 h and then challenged with or without H_2O_2 for 24 h. A histogram of the cell cycle phases of the RPE cells is shown. (B) Expression levels of CDK1, CDC25C 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 H_2O_2 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.

present study demonstrated that lutein protected cell viability and reversed G_2/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 G_2 to M phase, and cyclins accumulate steadily during the G_2 phase and are rapidly eliminated as cells exit mitosis. The activation of CDK1 kinase is an ordered process, which triggers the

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 deceases 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 G_2/M phase increased when exposed to H_2O_2. However, lutein protected the RPE cells from G_2/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 G_2/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 rMC-1 cells (24). The present study demonstrated that H_2O_2 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 G_2/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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