RESEARCH ARTICLE

Protective Effect of Silk Lutein Extract on Hydrogen Peroxide Induced Retinal Pigment Epithelial Cells Damage

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Abstract

Oxidative stress in retinal pigment epithelial (RPE) cells plays a key role in age-related macular degeneration (AMD). There is clinical and cellular evidence that lutein as a dietary supplement can delay the disease due to its antioxidant actions which protects RPE cells. However, there is little data on the efficacy of luteins from different sources, so in this study, the protectant actions of lutein extracted from yellow silk cocoons was compared with two commercial luteins isolated from marigold flowers assessed by H_2O_2-induced oxidative damage in ARPE-19 cells. After 400μM H_2O_2 treatment, cell viability was reduced approximately 60% and intracellular ROS and lipid peroxidation were increased mostly 2 and 3 folds, respectively. The decreased activities of three major antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase were also observed. With different potency, all three tested luteins (at 1, 10 and 50 μM) could dose-dependently diminish such effects of H_2O_2. Silk lutein extract showed the most effective cell protection and anti-cellular oxidative stress by most measurements. Two marigold luteins also differently displayed their cell protection and antioxidant capacity. These data indicated that lutein protected RPE cells from oxidation and sources of lutein determined the potency of their antioxidant properties and/or biological effects that could be the influences of isolation as well as storing procedures.

Keywords: Age-related macular degeneration, retinal pigment epithelial, silk, lutein, hydrogen peroxide, reactive oxygen species

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ฤทธิ์ของสารสกัดลูทีนจากรังไหมในการปกป้องเซลล์เยื่อบุมีสารสีของจอประสาทตาจากไฮโดรเจนเพอร์ออกไซด์

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บทคัดย่อ

สภาวะความเครียดออกซิเดชันของเซลล์เยื่อบุมีสารสีของจอประสาทตา เป็นสาเหตุสำคัญของการเกิดโรคศูนย์กลางจอประสาทตาเสื่อมเนื่องจากอายุ มีข้อมูลจากการศึกษาทั้งที่เป็นการศึกษาทางคลินิกและการศึกษาในระดับเซลล์ ที่แสดงให้เห็นว่าสารลูทีน ซึ่งเป็นสารเสริมอาหารสามารถชะลอการเกิดโรคดังกล่าวนี้ได้ ทั้งนี้เพราะลูทีนมีฤทธิ์ต้านออกซิเดชั่นจึงสามารถปกป้องมิให้อนุมูลอิสระไปทำลายเซลล์เยื่อบุมีสารสีของจอประสาทตา อย่างไรก็ตาม ยังมีข้อมูลจำกัดที่ศึกษาประสิทธิภาพของลูทีนที่สกัดมาจากแหล่งที่แตกต่างกัน ดังนั้น การศึกษานี้จึงทำการศึกษาฤทธิ์ของลูทีนที่สกัดจากรังไหมเหลืองเปรียบเทียบกับลูทีน 2 ตัวอย่างที่จำหน่ายโดยบริษัทสารเคมีซึ่งเป็นลูทีนที่ได้จากดอกดาวเรือง โดยทดสอบในการฤทธิ์ปกป้องเซลล์เยื่อบุมีสารสีของจอประสาทตาชื่อ ARPE-19 จากการถูกทำลายด้วยไฮโดรเจนเพอร์ออกไซด์ ซึ่งเป็นสารที่เหนี่ยวนำให้เกิดกระบวนการออกซิเดชันในเซลล์ ผลการศึกษาพบว่า ไฮโดรเจนเพอร์ออกไซด์ 400 μM ทำให้จำนวนเซลล์ที่มีชีวิตลดลงประมาณร้อยละ 60 และทำให้ระดับอนุมูลอิสระของออกซิเจนภายในเซลล์เพิ่มขึ้นประมาณ 2 เท่า รวมทั้งพบการออกซิเดชันของไขมันเพิ่มขึ้นประมาณ 3 เท่า นอกจากนี้ยังพบการทำงานลดลงของเอนไซม์ต้านอนุมูลอิสระ 3 ชนิด ได้แก่ superoxide dismutase, glutathione peroxidase และ catalase การทดสอบลูทีนทั้ง 3 ตัวอย่าง (ที่ 1, 10 และ 50 μM) พบว่าลูทีนสามารถยับยั้งผลที่เกิดจากไฮโดรเจนเพอร์ออกไซด์ได้ โดยฤทธิ์เป็นไปตามความเข้มข้นของลูทีน โดยในภาพรวมสารสกัดลูทีนจากรังไหมแสดงฤทธิ์ในการปกป้องเซลล์ได้สูงที่สุด สำหรับฤทธิ์ของลูทีน 2 ตัวอย่างจากดอกดาวเรืองก็พบว่ามีความแตกต่างกัน ผลการทดสอบแสดงให้เห็นว่า ลูทีนเป็นสารที่สามารถปกป้องการทำลายเซลล์เยื่อบุมีสารสีของจอประสาทตา ARPE-19 จากกระบวนการออกซิเดชันได้ และแหล่งที่มาของลูทีนยังมีความสำคัญต่อความแรงของสารในการต้านอนุมูลอิสระ รวมทั้งต่อการแสดงฤทธิ์ทางชีวภาพอีกด้วย ซึ่งความแตกต่างนี้อาจเป็นผลมาจากกระบวนการสกัดและการเก็บรักษาของสารที่แตกต่างกัน

คำสำคัญ: โรคศูนย์กลางจอประสาทตาเสื่อมเนื่องจากอายุ, เซลล์เยื่อบุมีสารสีของจอประสาทตา, สารสกัดลูทีนจากรังไหม, ไฮโดรเจนเพอร์ออกไซด์, อนุมูลอิสระของออกซิเจน

Introduction

Age-related macular degeneration (AMD) is a deterioration in the central retina or macula lutea. It is now the principal cause of blindness in elderly people and more than 30 million worldwide having visual impairment from AMD.[1] Risk factors for AMD include age, genetics, gender, smoking, hypertension, and hypercholesterolemia.[2] AMD is a multifactorial disease associated with increased subretinal deposition of lipofuscin and drusen, inflammation and neovascularization.[3] Immune-based therapeutics are currently under intensive investigation, but mostly applicable to neovascular AMD.[1] Oxidative stress caused by reactive oxygen species (ROS) has also been implicated in the pathogenesis of AMD.[2,4] Phagocytosis by retinal pigment epithelial (RPE) cells of fragments of the photoreceptor outer segment and their chronic exposure to UV light adds further to the cellular oxidative stress which then leads to a pathology where the demise of RPE cells is central.[4-6]

Lutein belongs to the xanthophyll family of carotenoids. Lutein and its stereoisomer zeaxanthin are the only carotenoids highly accumulated in the inner retinal layer of the macula lutea.[7] Humans are entirely dependent on dietary sources of these carotenoids especially from dark green leafy plants, egg yolk and some fruits since lutein cannot be elaborated by human metabolism.[8] In cultured RPE cells, lutein and zeaxanthin have been demonstrated to effectively protect RPE cells from various insults.[9-11] These have been borne out by recent clinical trials demonstrating that supplementation of lutein and zeaxanthin alone or in combination with polyunsaturated fatty acids (PUFA) may prevent the progression of AMD.[12,13]

At present, the major source of commercial luteins for these studies and dietary supplements are the marigold flowers. However, the yellow silk cocoon produced by the silk worm *Bombyx mori* is an interesting alternative source of lutein because more than 80% of carotenoids in its yellow pigments were found to be lutein.[14] Thus, it might be a possible useful by-product of the silk industry. Carotenoids extracted from such cocoons exhibit high antioxidant activities[15] and we have shown that silk lutein extract prevents the oxidative damage to RPE cells caused by UVB with similar efficacy to standard lutein.[9]

Therefore, in the present study we aimed to further investigate the protective effects of silk lutein extract and to compare its efficacy with two commercial luteins extracted from marigold; one is an analytical grade ('standard lutein'), and the other, a food grade product ('C-lutein'). The assay model used was H_2O_2-mediated oxidative damage to ARPE-19 cells and the indicators of cell damage were cell cytotoxicity, lipid peroxidation, intracellular ROS generation, and activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. The free radical scavenging and ferric reducing activities of lutein samples were also measured.

Materials and Methods

Chemicals

Dulbecco's modified eagle's medium (DMEM)/F12, 2,2-diphenyl-1-1-picyl-hydrazyl (DPPH), tripyridyl triazine (TPTZ), 2',7'-dichlorfluorescein-diacetate (DCFH-DA), catalase assay kit, A SOD assay kit, and standard lutein were purchased from Sigma-Aldrich (St. Louis, MO). A Caspase-3 assay kit was purchased from Molecular Probes (Eugene, OR). Glutathione peroxidase assay kit was purchased from Enzo Life Sciences (Farmingdale, NY). BCA protein assay kit and M-PER mammalian protein extraction reagent were from Thermo Fisher Scientific (Rockford, IL). Fetal bovine serum (FBS) and trypsin EDTA (0.25%) were purchased from Invitrogen (Ontario, Canada). Penicillin (10,000 U/mL) and streptomycin (10,000 µg/mL) were purchased from Gibco (Auckland, New Zealand). H_2O_2 was purchased from Merck (Darmstadt, Germany). Commercial lutein (C-lutein) was purchased from Kailu Ever Brilliance Biotechnology (Beijing, China).

Preparation of silk lutein extract

The silk lutein extract was partially purified and provided by the Institution of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The extraction procedure of lutein from yellow silk cocoons is described in the patent with international publication number, WO 2012/091683 A1. Briefly, hexane/ethanol/ethyl acetate mixture was used as a solvent for extraction. The extract was then partitioned, evaporated, and filtered. The silk lutein extract contained lutein 5.4% w/w (analyzed by HPLC), the free fatty acid content (C2-C24) was 10.41% w/w (analyzed by GC-MS, 44% saturated, 31% monounsaturated, and 25% polyunsaturated fatty acids), and the remainder was water insoluble waxes. The lutein:zeaxanthin ratio in the silk lutein extract was 98.41 to 1.59. HPLC chromatogram and fatty acids compositions of the silk lutein extract were previously reported.[16] The concentrations of silk lutein extract used in all experiments were calculated based on its lutein content and are stated as such.

Cell culture preparation and treatment

The human retinal pigment epithelial cell line (ARPE-19) was obtained from the American Type Culture Collection, VA, USA and grown in DMEM/F12 containing 10% FBS and 1% penicillin-streptomycin solution. The cells from passage 10 to 25 were used in this study. After seeding ARPE-19 cells for 24 h, culture medium was replaced by serum free medium without phenol red. Cells were treated with silk lutein extract, standard lutein (Std. lutein), or commercial lutein (C-lutein) at 1, 10, and 50 µM for 2 h prior to 24 h H_2O_2 treatment. DMSO at 0.5% v/v final concentration was used as a solvent control of lutein samples.

Cell viability assay

ARPE-19 cells at a density of 2.5×10^4 cells/well were plated into 96-well plates. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added to culture medium (0.5 mg/mL final concentration) 2 h before the end of cell treatment. Succinate dehydrogenase in living cells changes MTT to the insoluble purple formazan. The formazan crystals were dissolved in 200 µL glycine:DMSO (1:9) and the absorbance was then measured at 595 nm.

Measurement of lactate dehydrogenase (LDH) activity

LDH released into the surrounding culture medium is used as a marker of necrotic cells. LDH was assayed by its oxidation of pyruvate to lactate using the reduction of NADH to NAD^+ and H^+. After cell treatment, 50 µL of culture medium was collected and mixed with 150 µL reagent containing 2 mM NADH, 20 mM sodium pyruvate in PBS pH 7.4. The reaction was measured using the decreased absorbance at 340 nm which occurs as the NADH disappears and is directly proportional to the amount of released LDH.

Measurement of caspase-3 activity

Caspase-3 activity was determined by a commercial assay kit using the caspase-3 specific peptide, Ac-DEVD-AMC. The measurement was conducted according to the manufacturer's instructions. Briefly, 25 µL of cell lysate and 25 µL of substrate solution were mixed in a 96-well plate, and incubated for 30 min at 37°C. The fluorescence of the cleavage product was measured using an excitation wavelength of 485 nm and emission 535 nm.

Measurement of intracellular ROS

Intracellular ROS formation was measured using DCFH-DA which permeates the cell membrane and then hydrolyzed by cellular esterases to DCFH which is readily oxidized by intracellular ROS to highly fluorescent DCF. The procedure in these experiments was modified from previous report.[9] ARPE-19 cells were seeded at density 2.5×10^4 cells/well in 96 well black plates. Cells were incubated with 10 µM DCFH-DA in phenol red- and serum-free medium for 30 min, washed once with PBS, and incubated in H_2O_2 for 60 min. The fluorescence was measured at excitation 485 nm and emission 535 nm.

Measurement of lipid peroxidation

The lipid peroxidation was determined by the thiobarbituric acid reactive substances assay (TBARS assay). ARPE-19 cells were seeded at 1.2×10^5 cells/well in 24 well plates. After treatment, TBARS reagent (40% TCA, 1.4% TBA, and 8% HCl (1:2:1)) was added and incubated at 90°C for 1 h. The malondialdehyde-TBA adduct was fluorometrically measured at excitation 535 nm and emission 595 nm.

Superoxide dismutase (SOD) assay

This assay depends on superoxide generation by xanthine oxidase using xanthine as substrate which is converted to uric acid. SOD activity in cell lysate was determined according to the kit instructions. Briefly, 10 µL of cell lysate was mixed with 10 µL of mixed xanthine substrate (0.3 mM xanthine, 150 µM nitroblue tetrazolium, 400 mM Na_2CO_3, and 0.6 mM EDTA) with 100 µL of aqueous tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium). Superoxide converts WST-1 to WST-1 formazan which absorbs at 450 nm and its formation was thus suppressed by the action of SOD.

Measurement of glutathione peroxidase (GPx) activity

GPx activity in cell lysates was determined according to the manufacturer's instructions. Briefly, cells lysate was mixed with the reaction mixture containing glutathione reductase, reconstituted GSH, and NADPH. To initiate the reaction, cumene hydroperoxide was quickly added. GPx activity was estimated by the decrease in NADPH absorbance at 340 nm which was measured at 1 min intervals over a 15 min period in a microplate reader.

Catalase assay

The activity of catalase was determined based on the level of the H_2O_2 substrate remaining after the reaction. A substituted phenol (3,5-dichloro-2 hydroxybenzenesulfonic acid) couples oxidatively to 4-aminoantipyrine in the presence of H_2O_2 and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) that absorbs light at 520 nm in a microplate reader.

Free radical scavenging activity by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH can be reduced by antioxidants such as lutein. Each lutein sample (20 µL) in DMSO was mixed with 180 µL of 0.2 mM DPPH solution in methanol in 96 well plates. The reaction was allowed to reach a steady state at room temperature for 30 min. DPPH (purple) radicals are converted by lutein to the reduced form, DPPH-H (yellow or colorless). Decolorization of DPPH was measured at 510 nm and calculated as percent of free-radical scavenging activity.

Reducing activity by ferric* reducing *antioxidant power (FRAP) assay

To test the reducing activity of lutein, the ability to reduce Fe^{3+} to Fe^{2+} was determined by FRAP assay. The FRAP reagent, containing 300 mM acetate buffer pH 3.6, 10 mM tripyridyltriazine (TPTZ) solution, 20 mM $FeCl_3$ solution (10:1:1), was freshly prepared. Each lutein sample (20 µL) was mixed with 180 µL of FRAP reagent in 96 well plate, incubated for 30 min at room temperature, and absorption measured at 595 nm.

Statistical analysis

All results were expressed as means ± SEM from at least three experiments. Statistical analysis was determined using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test for multiple comparisons. P-values ≤ 0.05 were considered significant.

Results

Lutein reduced oxidative damage to ARPE-19 cells

Exposure to 400 µM H_2O_2 decreased the viability of ARPE-19 cells by approximately 60% (Figure 1A). There was about 9-fold increase in lactate dehydrogenase (LDH) release into the media suggesting that cell attrition was dominated by losing of membrane integrity (Figure 1B). Within the surviving cells, there was also a modest increase in caspase-3 (by about 18%), suggesting that apoptosis made a smaller contribution to H_2O_2 toxicity (Figure 1C).

Each of the three luteins, silk lutein, standard lutein (std. lutein, the analytical grade), and commercial lutein (C-lutein, a food supplement grade) ameliorated these actions of H_2O_2. Their effects were dose-dependent and manifest at every concentration (1–50 μM) (Figure 1). While the luteins failed to completely reverse membrane permeability integrity, the two higher carotenoid concentrations suppressed caspase-3 activity beyond the level observed in untreated cells.

Luteins suppressed H_2O_2 induced oxidative stress in ARPE-19 cells

Hydrogen peroxide (H_2O_2) increased the level of reactive oxygen species (ROS) within the ARPE-19 cells (Figure 2A). It should be noted that the intracellular ROS were detected at 1 h after H_2O_2 treatment indicating the early stage of cell oxidation. The end products of lipid peroxidation were dramatically increased at 24 h of H_2O_2 treatment (Figure 2B). All lutein samples exhibited a dose-dependent reduction on cellular oxidative stress as detected by lipid peroxidation but not by intracellular ROS (Figure 2). Among the three tested luteins, silk lutein extract apparently showed highest ability to suppress H_2O_2 induced oxidative stress in ARPE-19 cells.

Luteins also suppressed endogenous oxidative stress

Lutein at the highest concentration used in this experiment in culture medium without H_2O_2 also suppressed oxidative stress in ARPE-19 cells which results from their normal metabolic activity (Figure 3A, B). This was particularly apparent with all lutein concentrations acting against lipid peroxidation (Figure 3B), and caspase-3, indicating apoptotic death (Figure 3C). These results suggested that endogenous oxidative stress and apoptosis of ARPE-19 cells were effectively inhibited by lutein in the culture medium.

Restoration of antioxidant enzyme activities in H_2O_2

Hydrogen peroxide (H_2O_2) treatment reduced the activities of the three key antioxidant enzymes, SOD, GPx, and catalase, in ARPE-19 cells (Table 1). In the presence of luteins, there were smaller diminutions in enzyme function although for catalase, this effect was weaker. The reduction of SOD activity was also restored but dose-effect of lutein was not obviously seen. In contrast, all the luteins enhanced the activity of GPx above control levels. This appeared not to be artifactual since there was a similar augmentation in cells without H_2O_2 pretreatment (data not shown) which again may reflect the improved basal antioxidant capacity of the culture medium. These results indicated that luteins could enhance and/or restore the activities of antioxidant enzymes following H_2O_2 exposure.

Free radical scavenging and antioxidant activity of luteins

Since there was some evidence that the three lutein samples at the same concentrations showed differences in their ability to suppress oxidative stress in ARPE-19 cells, we sought to assess their reducing activities in cell-free systems using two different oxidizing agents, DPPH and ferric ions. Silk lutein extract at all tested concentrations was found to be superior for the free radical scavenging capacity using DPPH compared to the other luteins (Figure 4A), while the standard lutein exhibited the highest ferric reducing activity (Figure 4B). C-lutein showed the poorest antioxidant ability against both oxidizing agents.

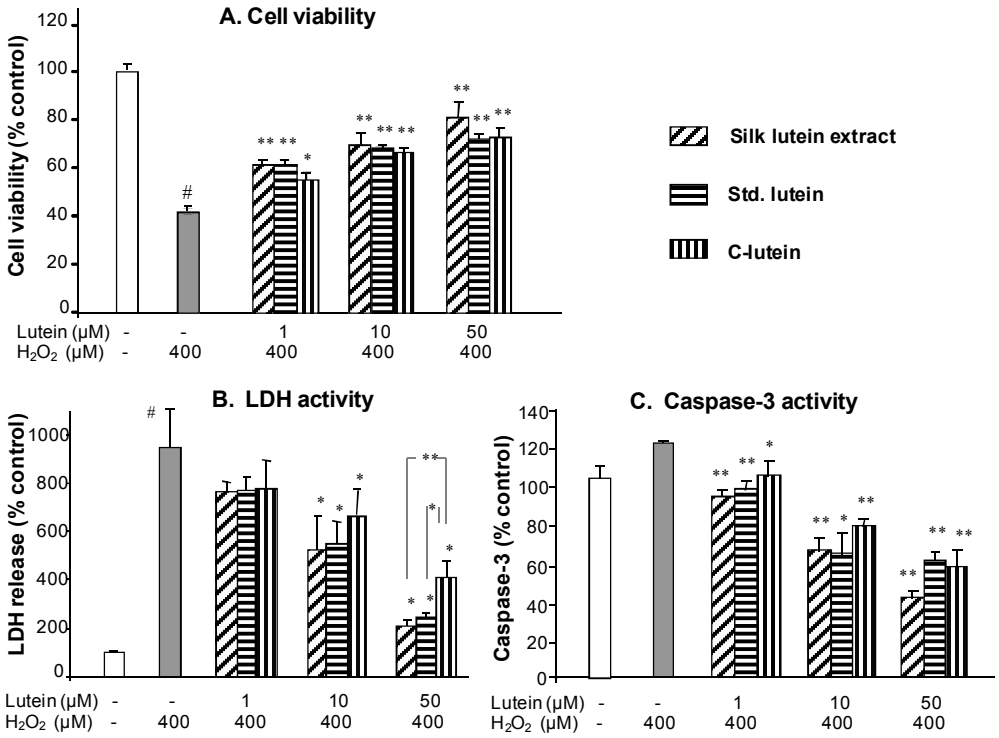

Figure 1. Lutein reduced H_2O_2-induced ARPE-19 cell death.
Cells were pre-treated with each lutein for 2 h prior to adding 400 μM H_2O_2. Twenty-four hours after H_2O_2 treatment, the following determinations were made: (A) Cell viability by MTT assay, (B) LDH release, and (C) caspase-3 activity. Data represent mean ± SEM from at least three experiments. (# $p < 0.05$ *versus* control, * $p < 0.05$, ** $p < 0.01$ *versus* H_2O_2).

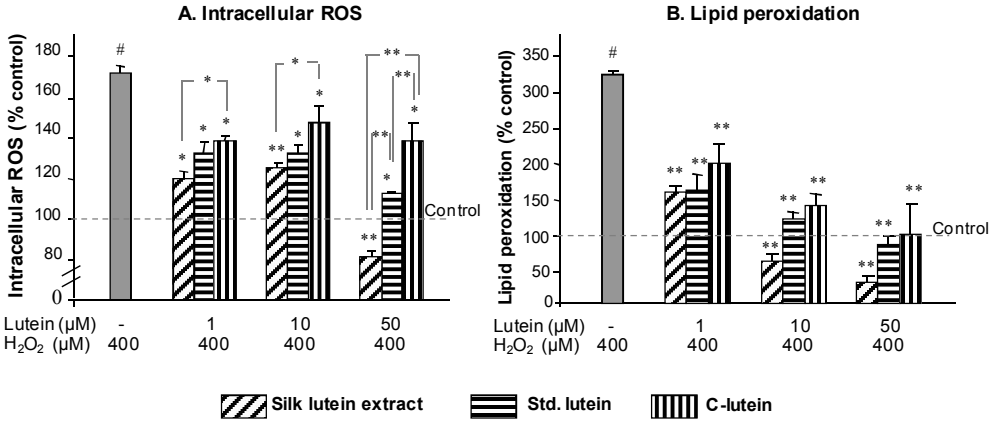

Figure 2. Luteins reduced indicators of oxidative stress in H_2O_2 in ARPE-19 cells.
Cells were pre-treated with each lutein for 2 h prior to 400 μM H_2O_2. (A) Intracellular ROS was measured 1 h after H_2O_2 treatment using the membrane permeant reducing-agent, DCFH. (B) Lipid peroxidation measured as malondialdehyde was determined 24 h after H_2O_2 treatment. Data represent mean ± SEM from at least three experiments (# $p < 0.05$ *versus* control, * $p < 0.05$, ** $p < 0.01$ *versus* H_2O_2) without lutein.

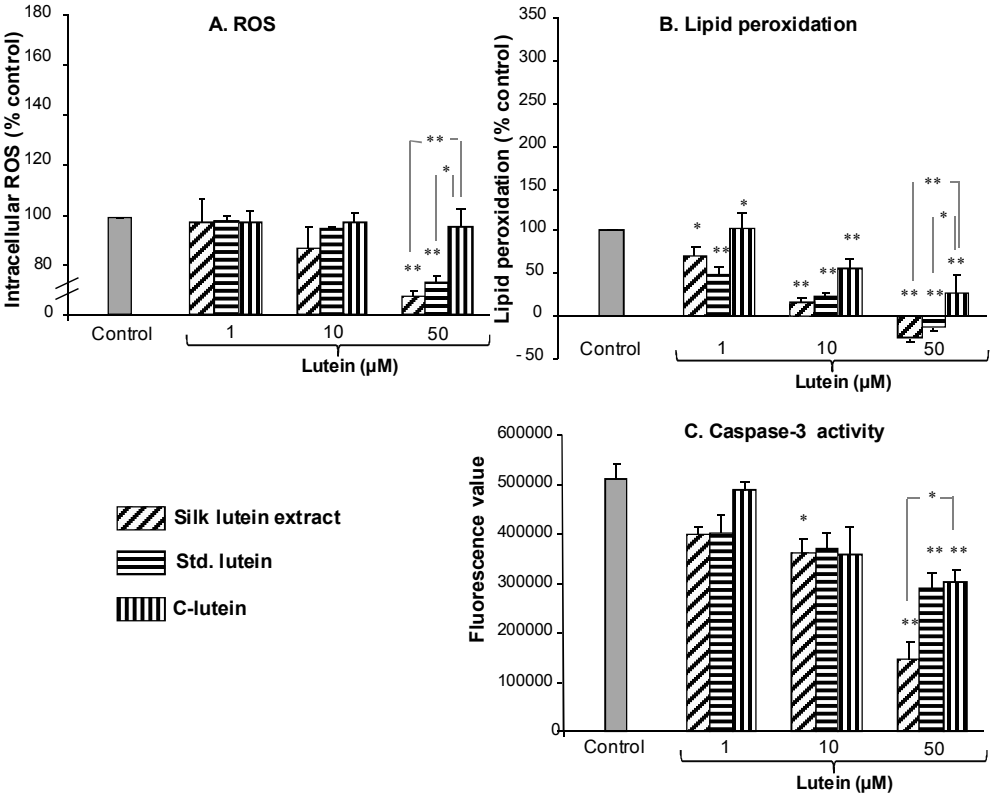

Figure 3. Lutein(s) reduced spontaneous oxidation in cultured ARPE-19 cells. Cells were cultured in the presence of each lutein for 26 h without H_2O_2 prior to determination of intracellular ROS (A), lipid peroxidation (B), and caspase-3 activity. Data are presented as mean ± SEM from at least three experiments. (* $p < 0.05$, ** $p < 0.01$ *versus* control).

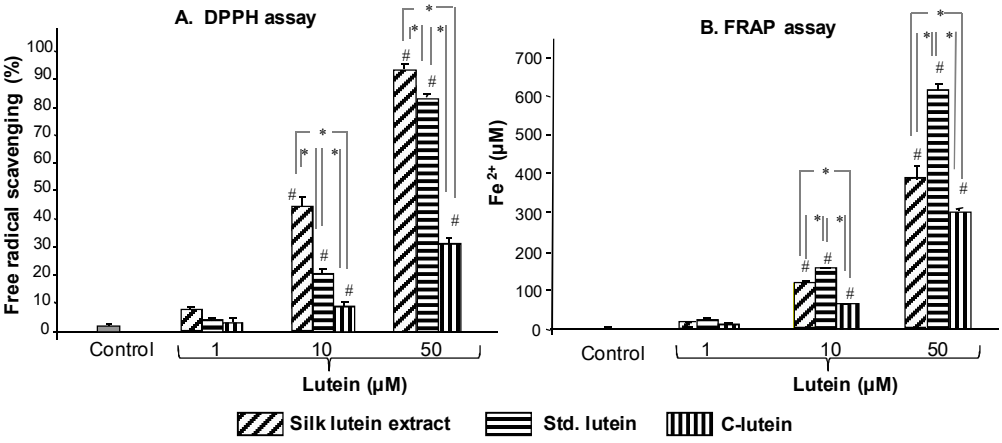

Figure 4. Cell-free antioxidant effects of the luteins.
Anti-oxidative activities were determined by (A) free radical scavenging activity and (B) reducing activity using DPPH and FRAP assays, respectively. Data represent mean ± SEM from at least three experiments. (# $p < 0.05$ *versus* control, * $p < 0.05$ *versus* other lutein sample).

Table 1. Effect of luteins on antioxidant enzyme activity in H_2O_2-treated ARPE-19 cells for 24 h.

Protocol	SOD (% of untreated cells)	GPx (units/mg protein)	Catalase (units/mg protein)
Control (0.5% DMSO)	92.2 ± 4.5	28.7 ± 4.0	12.1 ± 1.3
H_2O_2 (400 µM)	60.0 ± 1.6#	17.6 ± 2.7#	8.5 ± 0.6#
H_2O_2 (400 µM)			
+ Silk lutein extract (1 µM)	70.6 ± 6.5	48.8 ± 4.3*	10.0 ± 1.7
+ Std. lutein (1 µM)	61.9 ± 3.3	41.5± 3.5*	8.8 ± 0.5
+ C-lutein (1 µM)	67.0 ± 3.5	45.5 ± 5.7*	8.9 ± 1.4
H_2O_2 (400 µM)			
+ Silk lutein extract (10 µM)	72.7 ± 4.4*	54.0 ± 8.0*	10.5 ± 1.2
+ Std. lutein (10 µM)	70.1 ± 3.0*	53.8 ± 3.7*	10.8 ± 0.9
+ C-lutein (10 µM)	65.3 ± 5.4	48.6 ± 7.3*	10.1 ± 1.7
H_2O_2 (400 µM)			
+ Silk lutein extract (50 µM)	77.5 ± 5.5*	60.4 ± 6.3*	10.6 ± 0.9
+ Std. lutein (50 µM)	75.1 ± 8.1	55.4 ± 8.1*	12.5 ± 0.6*
+ C-lutein (50 µM)	69.6 ± 6.8	56.4 ± 9.2*	11.1 ± 0.6*

Data represents mean±SEM of at least three experiments. # $p \leq 0.05$ versus control and * $p \leq 0.05$.

Discussion

The present study demonstrated that H_2O_2 induced ARPE-19 cells damage by increasing intracellular ROS formation and lipid peroxidation, and decreasing the activities of key antioxidant enzymes. All three lutein samples protected ARPE-19 cells from such damage in a mostly dose-dependent manner. Silk lutein extract tended to show or in some cases, clearly showed superior protective effects against cell oxidative damage compared to the other two marigold luteins.

Depending on its concentration, H_2O_2 could mediate RPE cells to primarily undergo either apoptotic or necrotic death.[18] Kim and colleagues reported that lower concentrations of H_2O_2 (<600 µM) induced RPE cell death by an apoptotic pathway whereas H_2O_2 at higher concentrations (>600 µM) mediated cell necrosis rather than apoptosis.[18] However, another study by Hanus and colleagues demonstrated that necrotic but not apoptotic was the major feature in RPE cell death induced by H_2O_2 (300 and 500 µM).[19] Oxidative stress induced necrotic pathway in RPE cells might be mediated by calcium overload.[20] In the present study, H_2O_2 (400 µM) mediated RPE cell damage predominately through the loss of membrane integrity which could be the result of necrosis and/or apoptosis. Small increase in caspase-3 activity suggested that apoptosis had minimal role in H_2O_2 induced ARPE-19 cell damage. Although significant increase in LDH release was expected to be the result of necrotic pathway in this study, other characteristics such as ATP depletion and/or mitochondrial swelling might be

useful to clarify this type of cell death. Taken all together, both apoptosis and necrosis could be found in oxidative stress-mediated RPE damage.

Lutein acting as an antioxidant has been previously shown to protect ARPE-19 cells from blue and UV light, and various oxidative insults.[9-11,21-23] Similar to previous reports using H_2O_2 to induce RPE cytotoxicity,[10,24] the present study demonstrated that lutein protect oxidative damage to RPE cells by inhibiting the intracellular ROS formation and especially lipid peroxidation. The latter can be ascribed to the high fat solubility of lutein thus protecting cell membranes while DCFH is water soluble and largely confined to the cytosol where the lutein concentrations would be lower.

The three luteins clearly had different potencies which contrasts with our previous, study which compared silk lutein extract and std. lutein but using UVB-induced cell damage.[9] However, here lutein predominantly absorbs UV and primarily quenches singlet oxygen while its antioxidant actions are manifest against H_2O_2 damage. This accords with the idea that scavenging efficiency varies with the nature of the oxidizing agent and antioxidant.[25]

Since the luteins had the same molecular identity, the superiority of the silk extract must lie in other constituents in the product. Fatty acids and "waxes" present in silk lutein extract could be responsible for this observation. According to fatty acids analysis, silk lutein extract contains approximately 10% free fatty acids which are 44% saturated, 31% monounsaturated, and 25% polyunsaturated (data not shown). Although the present study showed no direct evident of the role of polyunsaturated fatty acids (PUFA) in ARPE-19 cells, the influences of PUFA on cell functions was documented as to maintain membrane fluidity and facilitate membrane processes such as molecular movement and membrane-bound protein interactions.[26] Based on their chemical structures, PUFA are susceptible for being attacked by free radicals.[26] The level of lipid peroxidation products in silk lutein extract were very small compared to the oxidation products generated by membrane lipids and thus they rarely interfere the measurement of lipid peroxidation in RPE cells.

In addition, silk lutein extract contained substantial amounts of waxes (80% total weight). These compounds might be unintentionally useful for silk lutein preparation either protecting lutein against oxidation by atmospheric oxygen or preventing lutein degradation, or may improve cell penetration. Indeed, association with fats enhances intestinal epithelial transport,[8] but whether there is also increased penetration across the blood retinal barrier requires *in vivo* testing.

In agreement with a previous report,[10] this study showed that lutein preserved the antioxidant functions of SOD, catalase, and especially GPx in H_2O_2-treated RPE cells possibly by directly protection the enzyme molecules. These antioxidant enzymes are vulnerable to oxidative loss of function.[27] Furthermore, luteins may also spare the endogenous small molecule antioxidant, glutathione (GSH) as shown for xanthophylls with cultured RPE cells in H_2O_2.[10] Also, lutein in combination with chlorophyll b prevented the reduction of GSH levels in the in vivo oxidative stress induced by cisplatin that directly interacted with GSH.[28] In the present study, we proposed that the effect of lutein on GSH might in turn facilitate cellular anti-oxidation processes particularly GPx as its activity was dominantly enhanced.

Conclusion

H_2O_2 has lethal effects on 19-ARPE cells due to the increase in intracellular ROS and especially lipid peroxidation, and decreased activities of the crucial antioxidant enzymes SOD, GPx and catalase. Such oxidative cell damage could be partially prevented by all three tested lutein samples; silk lutein extract exhibited the most effective protective effect; reflecting that other constituents in lutein preparations may provide additional benefits which go beyond direct lipid peroxide scavenging.

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