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Combination of Lutein and DHA Alleviate H_2O_2 Induced Cytotoxicity in PC12 Cells by Regulating the MAPK Pathway

Yan HU, Xu ZHANG, Fuzhi LIAN, Jun YANG* and Xianrong XU**

Department of Nutrition and Toxicology, School of Public Health, Hangzhou Normal University, 2318 Yuhangtang Road, Hangzhou, 311121, PR China

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Summary Docosahexaenoic acid (DHA) and lutein are important nutrients for brain health. Whether there were synergistic effects of DHA and lutein on the protection against neuronal cell damage induced by oxidative stress remained unclear. The present study was designed to investigate the synergistic effects of DHA and lutein against hydrogen peroxide (H_2O_2)-induced oxidative challenge in PC12 cells. PC12 cells were divided into different groups and received H_2O_2 (80 μM), lutein (20 μM)+H_2O_2 (80 μM), DHA (25 μM)+H_2O_2 (80 μM), and lutein (20 μM)+DHA (25 μM)+H_2O_2 (80 μM), respectively. The results indicated that pre-treatment of cells with lutein, DHA and DHA+lutein could significantly antagonize the H_2O_2-mediated growth inhibition and morphological changes in PC12 cells ($p<0.05$). Molecular-level studies indicated that the DHA+lutein combination can significantly inhibit the mRNA expression of AMAD10 and BAX. Furthermore, Western blot analysis demonstrated that DHA+lutein synergistically inhibits the phosphorylation of JNK1/2. The results of the present study suggest that DHA and lutein in combination may be utilized as potent anti-oxidative compounds, with potential preventative or palliative effects on age-related neurodegenerative diseases.

Key Words lutein, DHA, oxidative stress, MAPK signal pathway, PC12 cell

With the extension of life expectancy and the rising percentage of older individuals in the general population, aging has become a serious challenge and a heavy burden on the modern society (*1*). It is now well established that ageing is one of the most common risk factors for most human diseases (*2*), including neuronal degenerative diseases (*3*, *4*). Though the precise causes of aging are complicated and mostly remain unknown, there is increasing evidence that oxidative stress, which represents an imbalance between oxidant and antioxidant mechanisms, is a major contributor to several alterations observed in age-related conditions (*5*). Due to the high oxygen consumption, demand of polyunsaturated fatty acid and low antioxidant capacity, brain is particularly sensitive to oxidative stress. Finding nutritional or pharmacological resources that mitigate or prevent oxidative stress in aging brain continues to be a great challenge and requires additional effort from researchers.

There is evidence that omega-3 fatty acids, especially docosahexaenoic acid (DHA), play essential roles in preventing age-related brain deterioration (*6*). An adequate amount of DHA in the brain may attenuate stress responses (*7*) and influence the neuronal and astroglial functions that govern and protect synaptic transmission (*8*). The brain DHA status also influences neurogenesis (*9*, *10*), nested in the hippocampus, which helps maintain cognitive function throughout life. However, the results from human studies suggested that DHA supplements has no significant effect on age-related cognitive decline (*11*, *12*). This discrepancy may probably be due to the fact that DHA was highly peroxidable with the presence of multiple double bonds in their carbon chains, and the elevated oxidative stress in aging brain would influence the biological effects of DHA (*13*). This hypothesis was supported by the findings obtained in colon cancer cells cultured in vitro, in which it was demonstrated that the combined use of DHA and resveratrol, a naturally occurring polyphenolic antioxidant (*14*), resulted in more efficient anti-irritant and anti-inflammatory effects than free DHA in the cells (*15*, *16*). Therefore, combined use of antioxidants with DHA may be a feasible stagey to improve the beneficial effects of DNA in preventing brain aging.

Lutein, a non-provitamin A dietary carotenoid found in spinach, kale, eggs, and corn (*17*), is known to be an effective antioxidant that preferentially accumulate in the macula of the eye and form macular pigments (MP) (*18*). Lutein also preferentially accumulates in the human brain and is the predominant carotenoid in human brain tissue (*19*). There was evidence that content of lutein in human brain was consistently and inversely associated with age-related cognitive decline in elderly (*20*) and lutein supplementation could significantly improve the cognitive performance in healthy older women (*21*). Besides, lutein could inhibit the oxidation of DHA in brain tissue in adult rhesus macaques (*22*), and lutein and DHA co-supplementation signifi-

*, **To whom correspondence should be addressed.
*E-mail: gastate@zju.edu.cn
**E-mail: xuxianrong@hznu.edu.cn

cantly improved memory scores and rates of learning in healthy older women (*21*). Taken together, these observations suggest that lutein may be an ideal antioxidant to preserve the biological effects of DHA in aging brain.

However, the effects of lutein and DHA co-administration on the oxidative stress in neuronal cells and its underlying mechanisms have not been fully studied. In this study, an in vitro experiment was performed to investigate the potential protective effects of lutein and DHA co-administration against oxidative stress and the underlying mechanisms. PC12 cell, the rat pheochromocytoma cells that are useful neuronal models for studying neuronal degeneration disorders and are also extensively used to investigate reactive oxygen species (ROS) biochemical pathways involved in cell death and neuroprotection (*23*, *24*), is used in our study. The oxidative stress in PC12 cell was induced by H_2O_2, an oxidizing agent commonly used to cause irreversible oxidative damage in various cell models. As lutein is unstable and has low water solubility, poor absorption, and low bioavailability in cell culture, we synthesized lutein-loaded nanoparticles, which were composed of lutein, phosphatidylcholines, (*1*) alpha-tocopherol acetate, and surfactant with the method described by Zhang et al. (*25*). We focused on the possible synergistic effects of lutein and DHA on oxidative stress, trying to gain further understanding of the roles of lutein and DHA on oxidative injury in neuronal cells.

MATERIALS AND METHODS

Materials. H_2O_2 solution (29–32%, v/v) was purchased from Alfa Aesar (Fisher Scientific, NY, USA). NaOH solution (1 mM) was purchased from Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan). Lutein (purity >96%, Sigma), soy L-α-lecithin (purity >96%, Sigma), DL-α-tocopherol acetate (purity >98%, Sigma), kolliphor® HS15, bovine serum albumin (fatty acid free, purity >96%, Sigma) and DHA (purity >98%, Sigma) were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM high glucose cell culture medium was purchased from GIBCO Invitrogen. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA). Primary antibodies were obtained from Cell Signaling Technology (CST, Boston, MA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sangon Biotech (Shanghai, China).

DHA solution preparation. DHA-supplemented media were prepared following the protocol described previously with slight modification (*26*). Briefly, 32.85 mg DHA was dissolved in 5 mL ethanol, dried under nitrogen and then saponified with 10 mL 0.1 M NaOH for 5 min at 55˚C. Two point two grams fatty acid free BSA was added into the liquid and saponified for another 5 min at 55˚C. The pH was adjusted to 7.0±0.1 using HCl and NaOH. The concentration of the stocking DHA solution was 10 mM. Then, DHA stock solutions with appropriate volumes were mixed DMEM culture medium to achieve the final volume and used immediately.

Lutein nanoparticle preparation. Lutein nanoparticle was prepared following the method reported in previous studies (*27*). Briefly, lutein was dissolved in tetrahydrofuran to obtain lutein stock solution. Then, lutein stock solution with appropriate volumes was mixed with DL-α-tocopherol acetate solution (100 mg/mL in ethanol), soy L-α-lecithin solution (200 mg/mL in ethanol) and kolliphor® HS15 (200 mg/mL in ethanol). The weight ratios of DL-α-tocopherol acetate, L-α-lecithin and kolliphor® HS15 in the nanoparticle was 7 : 11 : 11, respectively. The lipid mixture in ethanol was dried under nitrogen to form a lipid film. Lutein nanoparticle was produced by reconstituting the dried lipid film in 10 mL hot PBS followed by homogenization (PowerGen 125, Fisher Scientific, Pittsburgh, PA) for 2 min and sonication for additional 2 min using a Branson Sonifier S-450. The particle size and polydispersity index were measured using a BI-MAS particle size analyzer, and zeta potential was measured using a Zeta PALS analyzer (Brookhaven Corporation, Holtsville, NY, USA). The final concentration of lutein used in the DMEM culture medium for cell treatment was 20 μM.

Cell culture and treatments. The PC12 cells were supplied from Shanghai Institutes for Biological Sciences (CAS), maintained routinely in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in humidified atmosphere of 5% CO_2 at 37˚C. All cells were cultured in poly-L-lysine-coated culture dishes at an appropriate density (1×10^6 cells per mL). PC12 cells after five to seven passages were used for further experiments.

The CCK-8 method was applied to determine the final dosage of H_2O_2, DHA, lutein and DHA+lutein used for cell treatment. We found that 80 μM H_2O_2 treatments for 2 h could significantly inhibit cell growth. The pretreatment of cells with 25 μM DHA and 20 μM lutein for 2 h have no significant cytotoxicity and could significantly antagonize the growth inhibition caused by H_2O_2. Therefore, in the current study, 80 μM H_2O_2, 25 μM DHA, 20 μM lutein, and 25 μM DHA+20 μM lutein concentrations were applied for the experiments.

Cells were divided into control, H_2O_2, H_2O_2+lutein, H_2O_2+DHA and H_2O_2+lutein+DHA groups. In the control group, cells received a normal culture medium and blank nanoparticle. In the H_2O_2+lutein, H_2O_2+DHA and H_2O_2+lutein+DHA groups, cells were pretreated with 20 μM lutein, 25 μM DHA, and 20 μM lutein+25 μM DHA, respectively, for 2 h; after this, 80 μM H_2O_2 was added to the medium for another 2 h incubation. In the H_2O_2 group, the cells were treated with 80 μM H_2O_2 for 2 h. At the end of incubation, cell morphology was visualized using a microscope (Olympus CKX31, Tokyo, Japan) and imaged (Nickon D810, Tokyo, Japan).

Measurement of cell viability. Cell viability was determined using the CCK-8 assay (Boster, Wuhan, China) according to the manufacturer's protocol. Briefly, approximately 2×10^3 cells were plated into 96-well plates. After different treatments, 10 μL CCK-8 solution was added to each well and incubated at 37˚C for 1 h.

The absorbance was read at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated by (experimental group absorbance value/control group absorbance value)×100%.

qRT-PCR. Total RNA was isolated from PC12 cells using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription was performed by the Multiscribe RT kit (Applied Biosystems, Foster, CA, USA). A SYBR Green PCR kit (Takara Shuzo Co., Ltd., Dalian, Shandong, China) was used to quantify the messenger RNA (mRNA) levels of target gene with appropriate primers (Supplemental Online Material, Table S1), including ADAM metallopeptidase domain 10 (ADAM10), presenilin-1 (PSEN1), B-cell CLL/lymphoma 2 (BCL-2), BCL2 associated X (BAX), heme oxygenase 1 (HO-1), thioredoxin reductase 1 (TXNRD1), and Peroxiredoxin 1 (PRDX1). β-Actin was amplified as control. The relative expression levels of were calculated using the $2^{-\Delta\Delta CT}$ method (*28*).

Western blot analysis. The cells were collected and lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Samples of the lysates were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with the indicated primary and secondary antibodies. The primary antibodies used in this study included anti-ERK1/2 (CST, 1 : 1,000), anti-p-ERK1/2 (CST, 1 : 2,000), anti-JNK (CST, 1 : 1,000), anti-p-JNK (CST, 1 : 1,000), anti-p38 MAPK (CST, 1 : 1,000), anti-p-p38MAPK (CST, 1 : 1,000) and anti-GAPDH (1 : 5,000, Sangon Biotech, Shanghai, China). Then, the membranes were incubated with an HRP-conjugated anti-rabbit secondary antibody. Finally, the bands were detected by an imaging analyzer (Tanon-5200, Tanon Science and Technology Co., Ltd., Shanghai, China) using the ECL substrate (Sangon Biotech).

Statistical analysis. Data analysis was conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (SPAA, Inc., Chicago, USA). One-way analysis of variance followed by Tukey HSD or Dunnett's C post hoc test was performed to compare multiple groups. Differences were considered statistically significant at $p<0.05$. Data in figures were expressed as means±standard deviation (S.D.).

RESULTS

Characteristics of lutein-loading nanoparticle

Lutein-loaded nanoparticle was successfully synthesized. As shown in Fig. 1, lutein-loading nanoparticle significantly increased the solubility and stability of lutein. The average size, zeta potential, and polydispersity index of nanoparticle was 102±2.4 nm, −24.23±7.32 mV, and 0.25 ±0.03, respectively.

Cell viability and morphological changes of the PC12 cells

As shown in Fig. 2, incubation of cells with 80 μM H_2O_2 for 2 h led to a dramatic inhibition of cell viability, while the pre-treatment of cells with lutein, DHA and DHA+lutein could antagonize the H_2O_2-mediated growth inhibition. Morphological changes of the cells were also observed. The H_2O_2-treated cells were characterized by the shrinkage of cell membrane, smaller size and loss of connection between cells (Fig. 2A). The pre-treatment with lutein and/or DHA could alleviate the growth inhibiting effect of H_2O_2 and maintain the normal cell morphological characteristics (Fig. 2B).

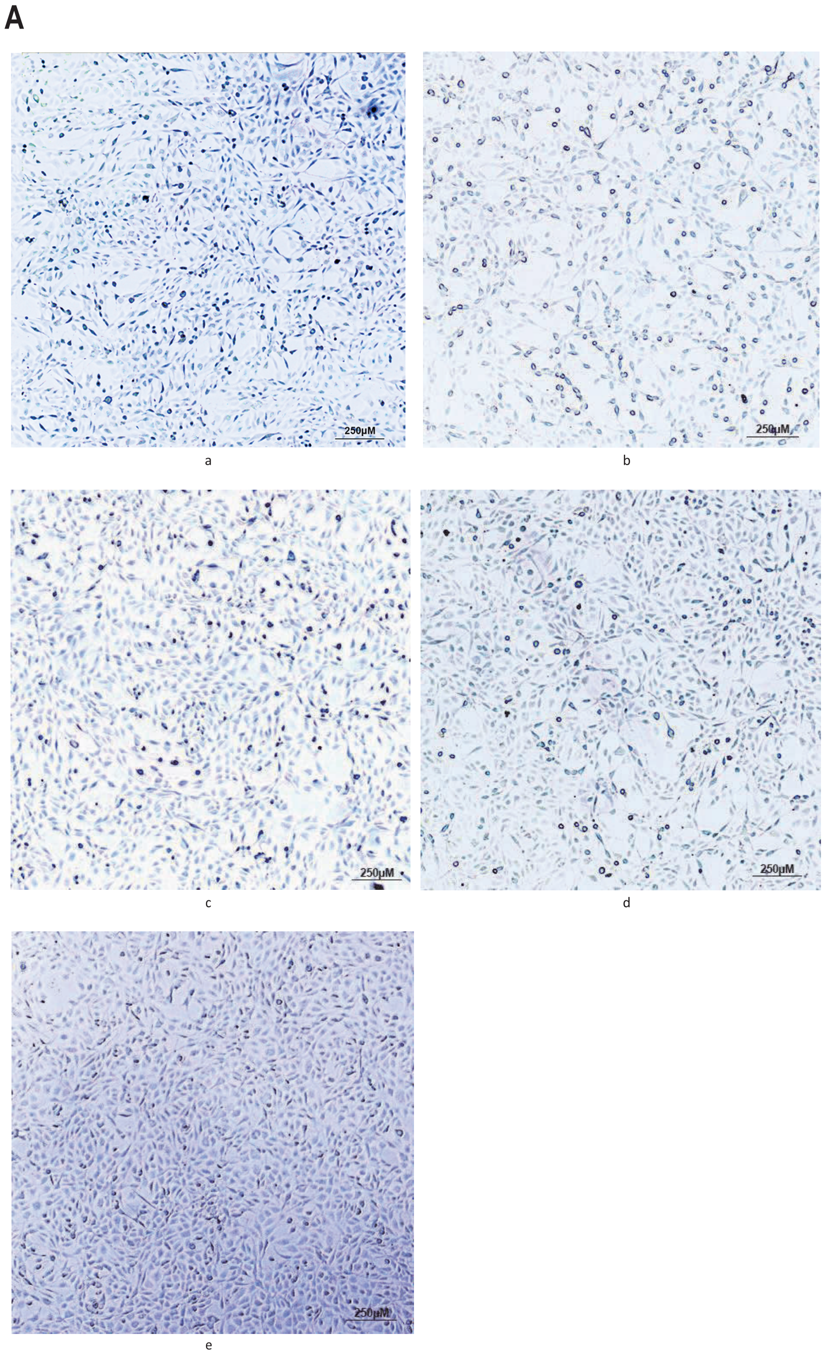
Fig. 1. Characteristics of lutein-loading nanoparticle. The lutein-loading nanoparticle significantly increased the solubility and stability of lutein (right).

Expression of oxidative stress associated gene mRNA (HO-1, TXNRD1 and PRDX1) in PC12 cells

The expression of the mRNA of the genes associated with oxidative stress, including HO-1, TXNRD1 and PRDX1, were detected using RT-PCR following H_2O_2, H_2O_2+lutein, H_2O_2+DHA and H_2O_2+lutein+DHA treatment in PC12 cells. The results indicated that though H_2O_2 treatment had no significant effect on the expression of HO-1 mRNA (compared with control group, $p>0.05$), DHA, lutein and DHA+lutein pre-treatment significantly inhibited the expression of HO-1 mRNA, and the strongest down-regulating effect was observed in the lutein-treated group ($p<0.01$). In the contrast, lutein, DHA, and lutein+DHA pre-treatment have no significant effects on H_2O_2-mediated expression of TXNRD1 mRNA ($p>0.05$). DHA and DHA+lutein pre-treatment significantly enhanced the expression of PRDX1 mRNA ($p<0.01$), with the strongest up-regulating effect was observed in the DHA-treated group ($p<0.01$) (Fig. 3).

The expression of ADAM10, PSEN1, BCL-2 and BAX mRNA in PC12 cells

As shown in Fig. 4, H_2O_2 treatment inhibited the mRNA expression of PSEN1 ($p<0.05$), but had no effects on the expression of ADAM10, BCL-2 and BAX mRNA (compared with control group, $p>0.05$). Pre-treatment with DHA and DHA+lutein slightly antagonized the inhibition BCL-2 an BAX mRNA expression ($p<0.05$). Pre-treatment with lutein, DHA, and DHA+lutein significantly inhibited the ADAM10 mRNA expression ($p<0.05$). On the contrast, pre-treatment with lutein, DHA, and DHA+lutein had no effects



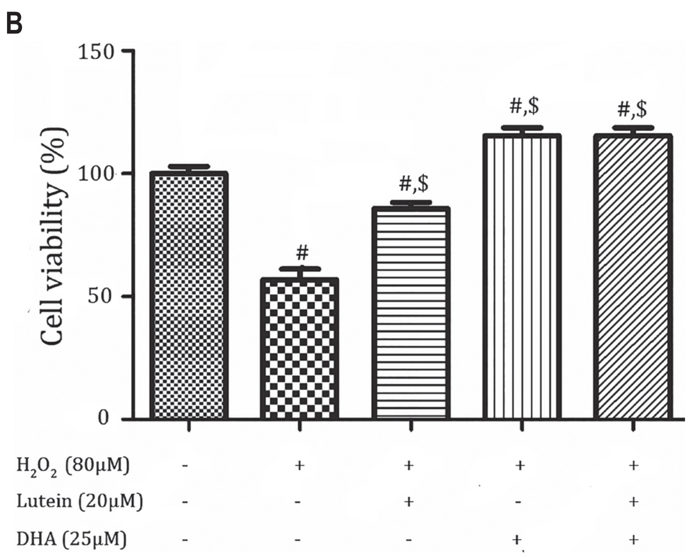


Fig. 2. The morphology (A) and cell viability (B) of PC12 cells after different treatment. (A) a, blank control; b, model control (80 μM H_2O_2); c, L group (80 μM H_2O_2+20 μM lutein); d, D group (80 μM H_2O_2+25 μM DHA); e, L+D group (80 μM H_2O_2+20 μM lutein+25 μM DHA); (B) # significantly different from blank control group; $ significantly different from model control group.

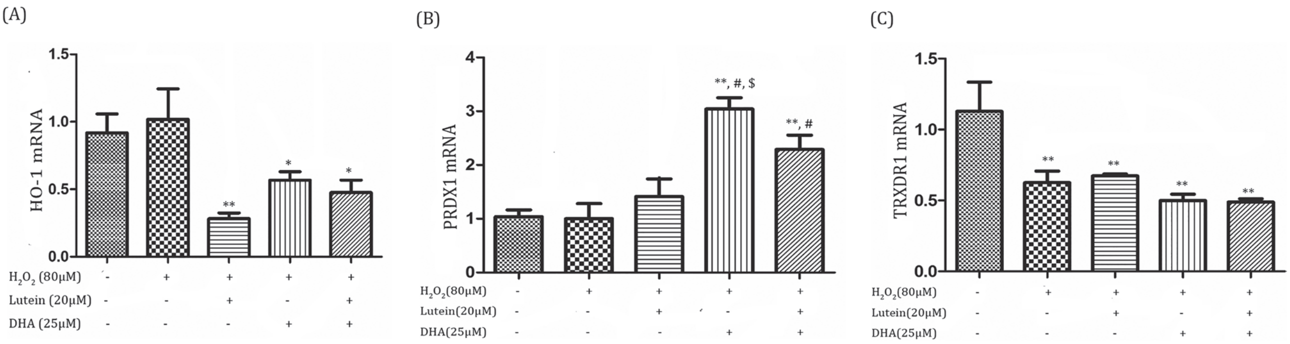


Fig. 3. The expression of oxidative stress associated genes in PC12 cells after different treatment. HO-1, heme oxygenase 1; TXNRD1, thioredoxin reductase 1; and PRDX1, peroxiredoxin 1. ** Significantly different from blank control group ($p<0.01$); # significantly different from model control group ($p<0.05$); $ significantly different from L group ($p<0.05$).

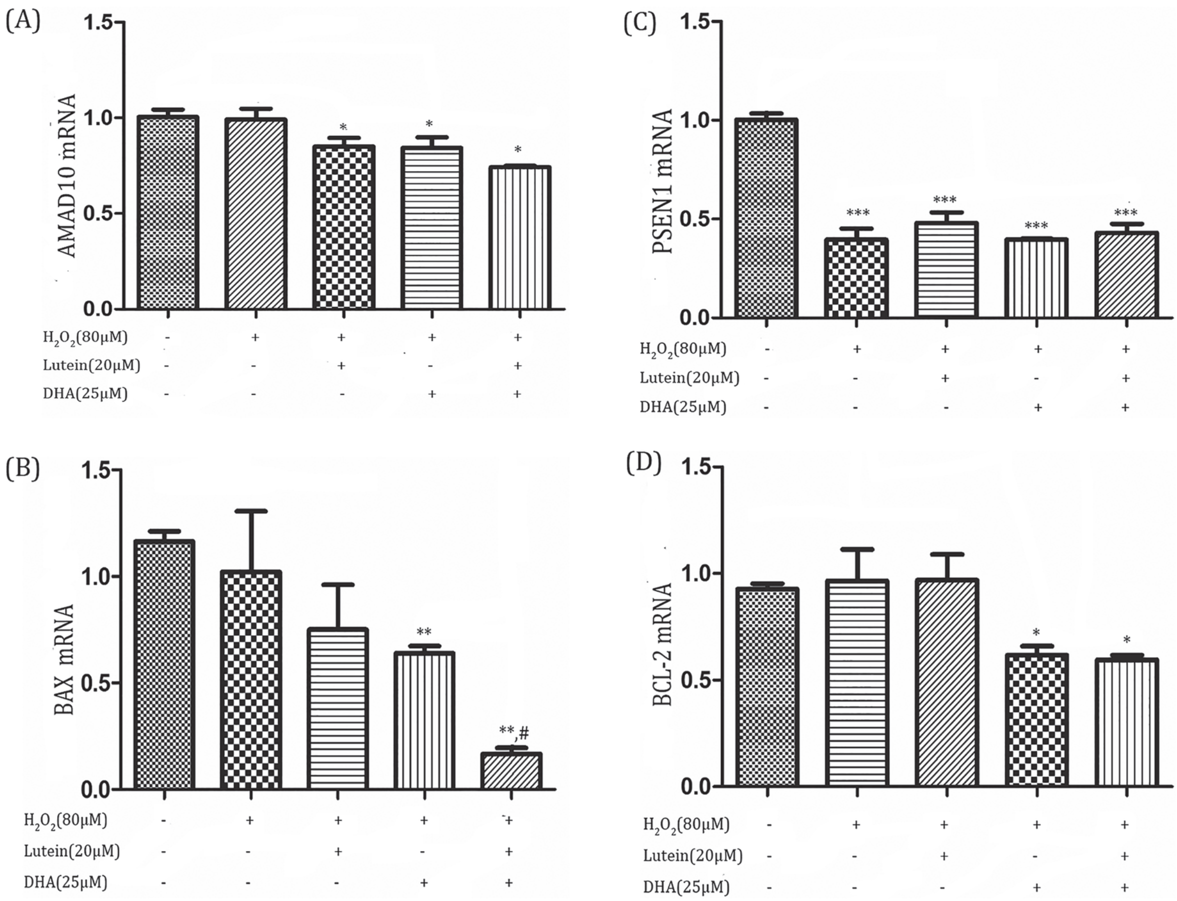


Fig. 4. The expression of aging associated genes in PC12 cells after different treatment. ADAM10, ADAM metallopeptidase domain 10; PSEN1, presenilin-1; BCL-2, B-cell CLL/lymphoma 2; BAX, BCL2 associated X. * Significantly different from blank control group ($p<0.05$); ** significantly different from blank control group ($p<0.01$); *** significantly different from blank control group ($p<0.001$); # significantly different from D group ($p<0.05$).

on the H_2O_2-induced inhibition of PSEN1 mRNA expression.

The expression of MAPK signaling pathway proteins in PC12 cells

The expression of the proteins ERK, p-ERK, JNK, p-JNK, p38 MAPK, and p-p38 MAPK associated with the MAPK signaling pathway was measured using Western blot analysis following H_2O_2, H_2O_2+lutein, H_2O_2+DHA, and H_2O_2 lutein+DHA treatment in PC12 cells. The results demonstrated that lutein, DHA, and lutein+DHA could significantly inhibit the activation of ERK1/2 (Fig. 5), JNK (Fig. 6) pathway in PC12 cells. Compared with those in H_2O_2, H_2O_2+lutein group, the activations of p38 MAPK were also inhibited in H_2O_2+DHA and H_2O_2+lutein+DHA groups, though there was no significant difference (Fig. 7). Compared with lutein and DHA single treatment group, the inhibition effects were stronger in H_2O_2+lutein+DHA group,

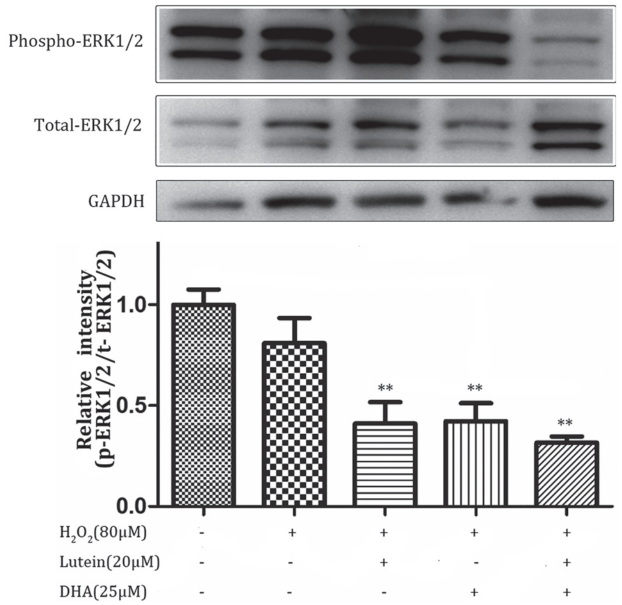


Fig. 5. The phosphorylation of ERK1/2 in different groups. ** Significantly different from blank control group ($p<0.01$).

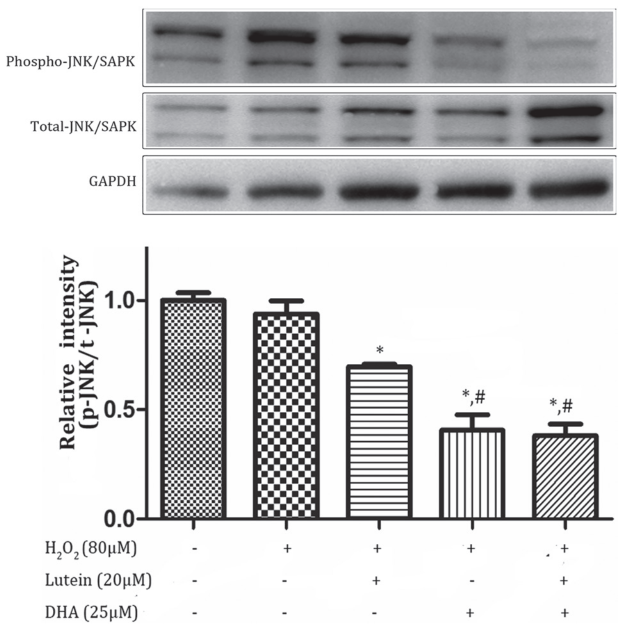


Fig. 6. The phosphorylation of JNK in different groups. * Significantly different from blank control ($p<0.05$); # significantly different from L group.

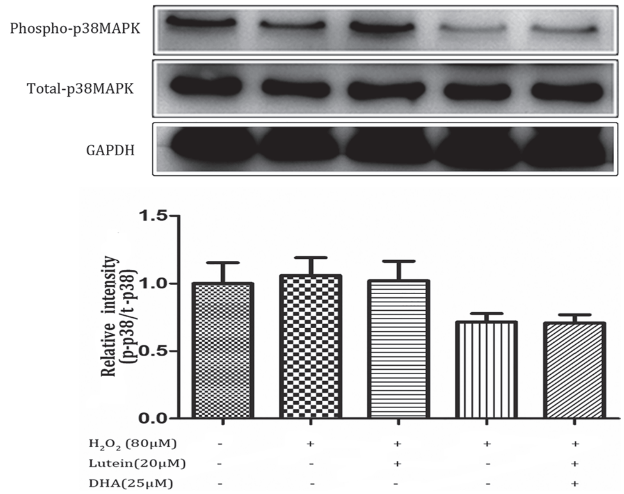


Fig. 7. The phosphorylation of p38 MAPK in different groups.

though there were also no significant difference.

DISCUSSION

The neuroprotective effects of DHA and lutein have been extensively demonstrated by in vivo and in vitro experiments. However, the mechanism by which DHA and lutein exert their neuroprotective effects has not been clearly elucidated. Besides, whether there was synergistic neuroprotective effect of combined DHA and lutein against oxidative stress in neuronal cells was still unclear. In the current study, we carried out an in vitro experiment to explore the roles of DHA or/and lutein in antagonizing the H_2O_2 mediated neurotoxicity in PC12 cells. Our data indicated that H_2O_2 significantly decreased cell viability, and the neurotoxicity caused by H_2O_2 could be partially inhibited by the pre-treatment of the cells with lutein, DHA, or DHA+lutein. These results indicate that DHA and lutein could protect PC12 cells from H_2O_2-induced cytotoxicity and damage.

Oxidative damage has been suggested to contribute to the pathological progress of Alzheimer's disease (AD) (*29*). The results from our study suggested that lutein and DHA might act against H_2O_2-induced cytotoxicity via the regulation of HO-1 and PRDX1. HO-1 is an inducible enzyme and its up-regulation is recognized as a pivotal mechanism of cell adaptation to stress (*30*). Furthermore, there was also evidence highlighted that HO-1 expression was associated with neuronal damage and neurodegeneration, especially in Alzheimer's and Parkinson's diseases (*31*). These results indicated that lutein and DHA might attenuate H_2O_2-induced oxidative stress and prevent the abnormal up-regulation of HO-1 under certain conditions in neuronal cells. PRDX1 is a critical peroxidase enzyme that plays dominant roles in regulating ROS levels and downstream signaling pathways within cells (*32*). PRDX1 is involved in the pathology of various age-related diseases and cancers, including AD and Parkinson's disease (*33*). In the present study, it was shown that DHA but not lutein could up-regulate the expression of PRDX1 mRNA in PC12 cell. These results indicated that lutein and DHA might exert anti-AD effects via different mechanisms.

ADAM10 (*34*), PSEN1 (*35*), BCL-2 and BAX (*36*) were important genes involved in brain aging and AD. In the present study, lutein, DHA, and lutein+DHA pre-treatment could significantly inhibit the expression of ADAM10 in PC12 cells. DHA and DHA+lutein down-regulated the expression of BCL-2 and BAX mRNA expression. Besides, lutein and DHA seem to have a synergistic effect on the expression of BAX. Whereas, these treatments had no significant effects on H_2O_2-induced down-regulation of PSEN1 mRNA. According to these data, we can infer that lutein and DHA might exert neuroprotective effects on PC12 through influencing the metabolism of amyloid precursor protein (APP) and inhibiting cell apoptosis. These results seem to be in consistent with other research in which DHA could prevent $A\beta_{25\text{-}35}$-induced SH-SY5Y cells apoptosis by inhibiting the expression of BCL-2 and BAX (*37*). Che et al. also found that in Chinese hamster ovary cells stably transfected with APP and PSEN1 (CHO-APP/PS1 cells) and senescence accelerated mice P8 (SAMP8 mice) fed with high fat diet, DHA could suppress oxidative stress and inhibit the expression of APP (*38*). More studies were warranted to confirm the findings in our study.

There is evidence that MAP kinase signal transduction pathway plays a central role in the production of neuroinflammatory mediators and neurodegeneration (*39*). Neuroinflammatory mediators, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) (*40*), were all inducers of MAP kinase, and MAP kinases activation leads to the release of more inflammatory mediators and thus increasing neuroinflammation, which may form a positive feed-back loop and vicious cycle that prolongs inflammation and finally leads to neuronal damage and death (*41*, *42*). Moreover, ERK1/2 and JNK were found to promote the phosphorylation of APP and the induction of ERK resulted in uncontrolled phosphorylation of both APP and tau (*43*). Upregulation of MAP kinase phosphatase 1 (MKP-1), a negative modulator of MAP kinases, decreased amyloid β-protein (Aβ) production and neuritic plaque formation, prevented synaptic deficits and ameliorated the cognitive impairments in AD transgenic mice (*44*). In this study, DHA and lutein were found to inhibit the activation of MAP kinases signaling pathway, including the activation of ERK1/2 and JNK. Besides, the combination administration of DHA and lutein seem to synergistically inhibit the phosphorylation of JNK. These results were consistent with those of Si et al., who found that DHA could inhibit the activation of ERK1/2 and JNK and the combination use of DHA and quercetin (QE), a common flavonol with strong antioxidant activity, synergistically inhibit the phosphorylation of ERK1/2 and JNK (*45*). The synergistic effects of DHA and lutein on the reduction of ERK and JNK phosphorylation may complement their synergistic effects on the suppression of BAX level. These results suggested the potential benefits of DHA and lutein on the oxidative stress induced neurotoxicity and Aβ production.

In conclusion, combining DHA (25 μM) and lutein (20 μM) treatment enhances their anti-neurotoxicity effects in H_2O_2-induced PC12 by regulating the levels of oxidative stress (HO-1 and PRDX1), metabolism of APP and ADAM10, and inhibiting cell apoptosis (BAX). This combination also exerts an enhanced effect on the expression and phosphorylation of MAPK signaling pathway proteins compared with their effects individually. Future studies should investigate the anti-inflammatory effects of different lutein and DHA doses in combination. However, in the current study only the protective effects of pre-treatment were assessed. Since pathological progression of neurodegenerative diseases can also be induced by ROS in early phases, the effects of post-treatment on oxidative damage in PC12 cells should be examined as well, and such studies are currently underway in our laboratory. Nonetheless, the present study provides insight into the benefits of foods containing these molecules. The findings of the current study should be investigated further in future in in vivo studies by examining lutein and DHA as nutritional supplements to exert preventative or palliative effects on brain aging and neurodegenerative diseases.

Authorship

YH, XZ and XX performed experiments and analyzed the data. XX and FL wrote the draft. YH and JY revised the manuscript. All authors read and approved the manuscript.

Disclosure of state of COI

No conflicts of interest to be declared.

Acknowledgments

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Supporting information

Supplemental online material is available on J-STAGE.

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