Cordyceps Militaris Carotenoids Protect ARPE-19 Cells From Oxidative Stress Damage Induced By Hydrogen Peroxide

Lin Lan
Xihua University
YuZhi Ren
Xihua University
XiangYu Zhou
Xihua University
ShengYu Wang
Xihua University
JinSen Zhang
Xihua University
Ling Xie
Xihua University
YeQian Qian
Xihua University
YuHang Li
Xihua University
YuFeng Li (yufeng_li19651123@163.com)
Xihua University
Liang Zhang
Xihua University

Research Article

Keywords: Age-related macular degeneration (AMD), Cordyceps militaris carotenoids, Retinal pigment epithelial cell, Oxidative stress

Posted Date: April 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-384293/v1

Abstract

A retinal disorder known as age-related macular degeneration (AMD) can be very damaging, which may result in vision loss. Cordyceps militaris has antioxidant, anti-inflammatory, anticancer activity and immunomodulatory functions. carotenoids were considered potential therapeutic agents for the treatment of age-related macular degeneration. In this study, we evaluated for the first time the protective effect of purified carotenoids (CMCT) extracted from Cordyceps militaris on hydrogen peroxide (H_2O_2)-induced damage to human retinal pigment epithelial cells (ARPE-19). The pretreatment of ARPE-19 cells with CMCT (1, 2.5μg/ml) for 12h after exposure to H_2O_2 (400μM) greatly improved cell viability and injury tolerance. and reduced reactive oxygen species production (ROS) and decreased malondialdehyde (MDA) formation. Furthermore, Bax and Caspase-3 showed increased protein expression and less protein expression of Bcl-2 in cellular oxidative stress by flow cytometry analysis but showed the opposite expression by adding CMCT. Besides, cells were treated with H_2O_2 and then CMCT restored catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) enzyme activities to normal levels. Our results suggest that CMCT can protect RPE cells from oxidative stress damage by regulating oxidative activity and anti-apoptotic function. It indicates that CMCT has a potential therapeutic role in oxidative stress-directed protection and prevention of AMD.

Introduction

Age-related macular degeneration (MDA) has become a common blinding disease with increasing age [1] , and was driven by other lifestyle and environmental factors [2, 3]. By 2020, it is estimated that 196 million people worldwide will be affected by age-related macular degeneration, with the number increasing to 288 million by 2040 [4]. As a public illness its pathogenesis is complex and no targeted countermeasures have been developed, while it can be classified into early, intermediate and late stages according to macular characteristics [5-7]. In AMD's pathophysiology, oxidative stress in the retinal pigment epithelium was considered a crucial role, more importantly, according to reports in the retinal pigment epithelium (RPE) where oxidative damage and mechanism malfunctions appear in the early stages of AMD. Protecting the damage to the retinal pigment by oxidative stress thus plays a significant role in stopping or slowing the macular degeneration pathological phase [8, 9].

This RPE is situated at the interface between the neural and choriocapillaris retinas. It was composed of a single layer of pigment epithelial cells, which are arranged very regularly and form part of the retina's external protection [10]. Drusen will store lipid materials amassing under the retinal pigment epithelium (RPE). What's more, pale-yellow stains on the retina may be shown. It plays an important role as the most active cell in the cellular metabolism of ocular tissues, promotes the regeneration and repair of photoreceptor cells, and prevents toxic molecules and blood from entering the retina to promote cellular metabolism [11-13]. Because of the particular structure and function of RPE under prolonged exposure to photo-oxidative effects, RPE has a higher sensitivity to stress and is more susceptible to damage to generate oxidative substances ROS, which leads to retinal damage over, peroxidation including continuous degradation of lipid-rich photoreceptor outer mesenchyme discs, the intracellular formation of

lipofuscin and deposition of toxic waste such as extracellular vitreous membrane coelomic [14, 15]. An increasing number of reports have demonstrated that oxidative stress causes in vivo ROS production in cells showing retinal cell damage and apoptosis ultimately leading to AMD [16, 17].With increasing age, RPE cell density decreases and the number of lipofuscin granules increases, weakening the protective mechanisms that cause oxidative cell damage. Therefore, it may be possible to slow down AMD progression by reducing the protection of RPE cells from stress damage.

A large amount of research data proves that the beneficial effects of natural antioxidants extracted from plants are related to bioactive photochemical, by protecting the retinal epithelium from oxidative stress and thus for treating AMD [18]. Such substances extracted can attenuate oxidative stress by protecting the RPE from damage and regulate the imbalance between free radicals thus protecting cells. For example, flavonols, cyanidins, curcumin, polyphenols, resveratrol, vitamin C, vitamin E and cryptochrome are widely distributed in (plants, fruits, vegetables, legumes and medical herbs) and have the ability to mitigate age-related changes in functional retinal damage, microcirculation of the retinal tissue and defense from oxidative stress [19-21]. Daily natural vegetative antioxidant supplementation in moderation has become a topic of research interest for alleviating or slowing down AMD development. Carotenoids, lutein and zeaxanthin are potential mechanisms for the antioxidant and free radical scavenging properties of these natural extracts in preventing AMD pathology [22]. At the same time, Cordyceps militaris has multiple pharmacologically active functional components, many studies have reported anti-inflammatory, antioxidant anti-aging, anti-tumor, hypotensive and vasodilator activities, free radical scavenging, antibacterial, anti-fibrotic, improved insulin secretion and anti-diabetic properties of Cordyceps militaris [23]. In particular, Cordyceps militaris was currently identified as the strain with the highest carotenoid content among the large-use herbal fungi reported. In addition to acting as antioxidants, carotenoids were known to function as prooxidants in high oxygen stress, high concentrations of carotenoids and unbalanced intracellular redox conditions [24]. However, the protective effect of CMCT against H_2O_2-induced stress in retinal epithelial cells remains unclear. Therefore, in the current research on the protective effect of CMCT on H_2O_2-stimulated ARPE-19 cells, we performed in vitro experiments on ARPE-19 cells and explored the possible protection mechanisms of oxidized cells under the action of CMCT.

Materials And Methods

Reagents and test kits

Cordyceps militaris were obtained commercially from Nanjiang Hongxing Biological Company, Bazhong City, Sichuan Province of China. Dulbecco's Modified Eagle's Medium (DMEM). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acetonitrile and dimethyl sulfoxide (DMSO)were purchased from Sigma–Aldrich, Inc (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit was obtained from ALPCO (Salem, NH, USA). Fetal bovine serum (SV3087), penicillin, streptomycin (SV3010), and trypsin (0.25 % concentration) were recruited from Biotechnology Hyclone Inc (Los Angeles, U.S.A). and HP Macroporous Adsorption Resin was purchased from Mitsubishi Chemical Holdings (Japan). Superoxide

dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehyde (MDA) and total antioxidant capacity test kit were obtained from Jiancheng Institute of Bioengineering. (Nanjing, China). The four principal antibodies were present Bax, Bcl-2, caspase-3 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Isolation and purification of Carotenoids from Cordyceps militaris

The fresh fruit bodies of Cordyceps Sinensis was air-dried at 60°C at a constant weight, and then the dried fruit bodies were ground into powder by passing through a 60mesh sieve. Weigh (2g) with acetone-ethanol (2:1,20ml), then add complex enzyme (0.5%) to adjust the pH of the extraction system to 4. The enzymatic digestion time was 45 min at 50°C, and then the mixture was sonicated for 1.5h. The supernatant was taken by centrifugation for 10 min at 4500 rpm and 100 times diluted. The resulting solution was the crude extract of Cordyceps carotenoids (CMC).

The crude extract CMC pigment supernatant was loaded onto an HP-20 macroporous resin adsorption column for further purification by complete adsorption (particle size 0.3-1.2 mm) ≥ 90%, and the eluate was desorbed with 60% ethanol as the carotenoid purification (CMCT) pigment purification solution. The eluent was placed on a rotary evaporator and concentrated under reduced pressure to a paste (60 rpm for min at 50℃), collected the vacuum freeze-dried CMCT purify, which were used for further studies. Bintong Total Antioxidant Capability Kit (T-AOC) to detected the total antioxidant activitied of pigments.

Antioxidant analysis and UV spectrum analysis

The DPPH was removed by colorimetric method using pyrogallol auto-oxidation to remove O_2 in combination with sodium salicylate complexation to remove OH. The total antioxidant activity of the extracted pigments was determined by colorimetric method (T-AOC) test kit and using a microplate reader, the absorbance was determined. (Bio-Tek, Winooski, VT, USA) In the wavelength range of 380-600 nm, in the wavelength range for UV-Vis spectral scanning.

Determination of pigment composition

The composition of carotenoids of Cordyceps Sinensis was determined by ultra-performance liquid chromatography (UPLC). The carotenoids of Cordyceps Sinensis were dissolved in pure methanol through a 0.22μm filter protected from light. A Waters C18 reversed-phase column with reversed-phase specifications (2.1×50mm, 1.7μm) was used, the column temperature was held constant at 30°C, and the flow rate was (0.25Ml/min), the injection volume (2μl), the elution gradient temperature (32-40min), and the detection wavelength was 445nm. The drying temperature for HRMS detection was (450℃). The DAD wavelength was (380nm-600nm) The drying gas flow rate was 40L/min. For HRMS detection. The pigment was mixed with potassium bromide at 1:100 to a powder to a wavelength of 500-4000 cm^{-1}.

ARPE-19 Cell Culture

Human retinal epithelial cells ARPE-19 cells were purchased from the American (ATCC) model culture collection and Dulbecco's Modified Eagle Medium (DMEM)/F12 preserved the ARPE-19 cells. Taken 10% heat-inactivated fetal bovine serum (Los Angeles, U.S.A.) 100 U/ml of penicillin (Los Angeles, U.S.A.) and 100 μg/ml of 10% streptomycin (SV3010 Los Angeles, U.S.A.) Cells were kept in an incubator and monitored in a humidified environment at 37 ℃ and 5% CO_2 and RPE cells were selected during the first 5-7 passages when they reached 90% confluence passed with 0.25% Trypsin (Biotechnology Hyclone Inc Los Angeles, U.S.A), every 3-4 days, and placed into appropriate culture plates for each experiment.

Induction of oxidative stress using H_2O_2.

Oxidative stress tests in serum-free or serum-containing media were conducted. After fresh serum-free base cell processing, ARPE-19 cells were plated on 96-well plates at a concentration of 1-10^5 cells/mL and enabled cell replication to cling to the bottom of the board and fusion overnight. Add DMEM/F12 medium with a final concentration of H_2O_2 (0-500μM) and incubate for 12h. Untreated cells served as controls to obtain final concentrations depending on the experiments, followed by a 12h exposure of different concentrations of CMCT.

MTT assays

The MTT test was used as the cell viability test measure. Briefly, the viability of ARPE-19 was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (St. Louis, MO, USA). The spread of cells ARPE-19 cells was plated at 1×10^5 cell/mL at 96-well plates. ARPE-19 cells cultivated in DMEM/F12 were pretreated for 12 hours at various CMCT concentrations. The medium was discarded, washed twice with PBS, and continuously exposed to H_2O_2 for 1h. After various treatments, MTT solutions were prepared in advance, stored in keeping in darkness, and then added to 96-well plates with 20μm of 5mg/mL MTT solution per well to reflect the mitochondrial activity of the cells through the transformation and dissolution of eszopiclone MTT methyl crystals to form particles, and combed at 37°C for 4h. After incubation the medium solution in each well was discarded 150μl of dimethyl sulfoxide (DMSO) was added, and the reaction was stopped and placed on an electric shaker for 10 min. The wells' crystals were then fully dissolved. The absorption of each well with a microplate reader at 492 nm was calculated (Model Devices SpectraMaxi3X; American Valley Molecular Instruments, Inc.), and all experiments were performed in triplicate. The average of the results was read for calculation. The absorbance values represent cell viability normalized to the untreated control and represent 100% cell viability the cells' relative viability as a percentage of the control.

Detection of cell death by ARPE-19

Apoptosis assays were evaluated by Annexin V-FITX/PI kit according to the manufacturer's instructions. ARPE-19 cells were incubated on 1 × 10^5-well plates with or without CMCT treatment for 12h after exposure to H_2O_2 for 12h. Afterward, cells were collected by washing twice in ice-cold PBS liquid by centrifugation at 4°C for 5 min and resuspended in fresh medium and 5μl Annexin V-FITX was added for

staining in the dark for 15 min. Immediately after staining, Flow cytometry was used to analyze the cells, and the number of apoptotic cells was measured using Cell Quest analysis tools. Results were expressed as Annexin-V negative-PI negative for normal cells, Annexin-V positive-PI negative for cells in early apoptosis, and Annexin-V positive-PI positive for cells in late apoptosis or necrotic cells and all assay data were performed in triplicate.

Intracellular ROS levels are measured

A fluorescent probe DCFH-DA staining determined the number of reactive oxygen species in cells. Briefly, ARPE-19 cells in 94-well plates were re-stained with H_2O_2 (400μM) for 12h, extended and excess liquid discarded then ARPE-19 cells were pretreated with CMCT for 12h. Cells were incubated with 10μM DCFH-DA reagent from the kit for 30min at 37°C in the dark, and serum-free cell culture medium was washed twice to remove excess DCFH-DA and resuspended in PBS. The cells' absorbance wavelength was recorded immediately with a microplate reader. Flow cytometry was used to record the intracellular fluorescence strength during therapy at 488 nm excitation and 525 nm emission wavelengths.

Detection of SOD activity, CAT and GSH, MDA levels

Upon various treatments, the effects of the CMCT on oxidative stress in different ARPE-19 cells were measured with four specific oxidative biomarkers including SOD, MDA, CAT and GSH. Briefly, dismutase (SOD), malondialdehyde (MDA) and catalase (CAT) activity were observed using the test kit obtained, refer to instructions of the manufacturer.

SOD activity assay, the cells were pretreated at 37°C and attached to the plate, two cells were collected and washed with PBS and centrifuged for 5 minutes to separate the sediment the resulting cell pellets were incubated in the buffer (25 mM NaCl, 0.5% Triton X-100, 60 mM Tris-HCl (pH7.4), 1mM Na3VO4, 20 mM NaF, 10 mM Na4P2O7. For 60 min, the mixture was incubated with ice water, with Centrifuge at 4°C for 30 minutes. The supernatant was collected, and the package operation for superoxide dismutase has measured the maker's directions.

CAT activity assay, the method was based on detecting the reaction decomposition of CAT in the sample under the adaptive H_2O_2 concentration. In this procedure, prepare pelleted cells and cell supernatants according to the previous activity detection analysis, di- dilute the volume to 50μl with phosphate buffer solution (pH 7.0), and added with 20ul H_2O_2 matrix solution and carry out the enzyme-catalyzed reaction at 25°C for 15 min, then detect the sample at 528 nm before. A standard bovine liver CAT curve was used for determining the function of the enzyme.

GSH activity assay, the same operation was used to prepare 20μl of cell-free supernatant in the sample for SOD activity determination. The detection kit was used to analyze the total protein, and the supernatant was centrifuged for glutathione determination. (4℃ 15000*9 10min). Read the absorbance at a wavelength of 405nm according to the manufacturer's instructions. The quantification of intracellular glutathione was based on its use solution as a standard.

The impact of CMCT on the identification of oxidation biomarkers in cells was calculated using the MDA activity assay. Briefly, cells were treated with H_2O_2 (100μM) for 1h and CMCT overnight. After completion of treatment, the cells were then washed with PBS, and the oxidation biomarkers were obtained by centrifugation and identified using the test set as directed by the manufacturer. For both antioxidant enzymes and MDA amounts, nmol per mg of protein has been expressed.

Western blot analysis

Cells were pretreated. Briefly, two washes of ice-cold PBS were performed on the cells. After treatment, the cells were collected that the cells were laced with RIPA buffer for nuclear tissue. At 4°C for 15min the lysate was centrifuged at 12,000 × g and the supernatant were extracted. The protein content in the samples was detected in the test kit and according to the directions of the manufacturer. 20μg protein samples were electrophoresed on polyacrylamide (PAGE) 10% sodium dodecyl sulfate (SDS), moved to the membrane of polyvinyl fluoride (PVDF), and sealed with 5% skim milk at a temperature of 4°C. Incubate the antibody characterizing overnight, wash with PBS and then incubate the eggs at room temperature for 1h. with the following secondary antibody. The ECL Western blotting assay kit is used to detect protein bands.

Statistical Analysis

All experiments were repeated at least three times, and data are shown as mean ± standard deviation or percentages. origin9.0 was used for plot analysis, and variance analysis between groups was compared using one-way ANOVA or ordinary two-way ANOVA, and variability with *P<0.05, **P<0.01 and #P<0.05, ##<0.01. was considered statistically significant.

Results

Preparation and characterization of CMCT

The crude cordyceps militaris carotenoid was prepared according to the reported method with certain variations. The Cordyceps militaris carotenoids were intracellular pigments, destroy the cell wall and then extract CMCT by Cellulase and Pectinase. (the extraction ratio is 2:1, absorbance 3.322), Determine the acetone and 60% ethanol solution to dissolve the crude pigment extract and measure the absorbance at 445nm (Figure 1.A). Configure standard curve as follows: y=0.1093x+0.0801, R2 =0.9992(Y was Absorbance and X was Pigment concentration). For subsequent cell experiments using higher purity carotenoids, CMCT was further fractionated by macroporous adsorbent resin as shown in the figure (Figure 1.B) giving a purified carotenoids CMCT. Absorbance OD_{455nm}, the sample is colorless, determined by the color reaction, concentrated sulfuric acid is blue-green and the chloroform solution of antimony trichloride is green. The color characteristics were in line with the general features of olefin carotenoids. The five prominent peaks were prepared by the UPLC method (Figure 1.C), the exact prime numbers were measured in the positive ion [M+H] + mode, Dry under vacuum at 60℃, UV spectrophotometers tested carotenoids dissolved in methanol the absorption maximums of the five

carotenoids had the same absorption spectrum and a same conjugate system of bond, According to its UV-MS and Infrared spectrograms, the filtered carotenoids CMCT fulfill the specific characteristics when contrasting the retention period and peak region of the standard under the same conditions.

Effects of H_2O_2 and CMCT in ARPE-19 Cells

The impact of CMCT on cell viability of ARPE-19 was first investigated using the MTT system, and the cell survival rate is expressed as the control group's survival rate group (untreated cells). As shown in (Figure 2.A) there was no significant difference in cell viability ($P<0.05$) after treating ARPE-19 cells with 1μg/ml to 7.5μg/ml of CMCT after 12h treatment with different concentrations of CMCT. But beyond this concentration, we will find that 10μg/ml of CMCT reduces cell viability, reaching 71% of the control group's survival rate ($p>0.05$). The cell viability of ARPE-19 cells induced by high concentrations of H_2O_2 was also tested to monitor for CMCT defense against harm caused by H_2O_2. Under the same conditions, the attention of H_2O_2 was treated from 0-500μM for 12h (Figure 2.B). Cell survival rate showed a dose-dependent decrease from 37 ± 0.79% at 500μM, 51 ± 1.09% at 400μM, 63 ± 3.68% at 250μM, 66 ± 3.06% at 250μM, 78 ± 3.41% at 100μM, 90 ± 1.94% at 50μM, respectively, with the statistical difference between the cell survival rate and the control group. The cell survival rate of H_2O_2 (500μM) treatment concentrations is too low and does not meet the modeling conditions. As the picture shows (Figure 2.C), we selected H_2O_2 (400μM) as the working concentration in the following experiments.

Next, the protective role of CMCT on H_2O_2-induced cell death from ARPE-19 was examined. ARPE-19 cells were subjected for 12 h to H_2O_2(400μM) and then incubated for 12h in a cultivated medium comprising CMCT (1, 2,5, 5, 7.5μg/ml) (Figure 2.D). The survival rate of treated cells of H_2O_2 (400μM) decreased by 35% relative to the control group. however, an elevated ability to reverse cell activity protection occurred in the presence of added CMCT. Cell survival increased 75.6±4.7%,69.4±6.5%,60.8±7.4%,61.7±8.2% at different CMCT concentrations of（1, 2.5, 5, 7.5μg/ml）. Respectively, the findings indicate that CMCT causes fewer dose-dependent death of retinal epithelial cells owing to H_2O_2. Therefore, we used (1, 2.5μg/ml) as the next experiment.

Protection of CMTC against H_2O_2-induced apoptosis in ARPE-19 cells

Death of the retinal pigment is normally caused by oxidative stress. We investigated whether CMCT has a protective effect against H_2O_2-induced ARPE-19 cell death. Therefore, ARPE-19 cells were incubated with H_2O_2(400μM) for 12h to induce cell death and then co-incubated with different concentrations of CMCT (1, 2.5μg/ml) for 12 h by the morphological fruit of the cells shown (Figure 3 .A). The percentage of apoptotic cells was detected by V-FITC/PI staining on flow cytometry as shown in (Figure 3.B). The total percentage of early and late apoptosis of cells (63.87±1.78、5.16±0.34%) was significantly elevated after exposure of cells to H_2O_2（400μM） for 12h compared to the real apoptosis rate of untreated controls (Figure 3.C). However, the addition of CMCT (1, 2.5μg/ml) to cells treated at H_2O_2(400μM) after 12h

exposure significantly attenuated the apoptosis of cells stressed,(57.87±1.78%、38.71±1.58%) respectively decreasing compared to H_2O_2-induced apoptosis.（p<0.05）

CMCT affects the expression of associated proteins in apoptotic ARPE-19 cells.

To further explore the defensive role of CMCT in ARPE-19 cells on H_2O_2-induced apoptosis, Western blot was used to analyze modifications in the proteins of Bax, Bcl-2 and Caspase3 under various conditions and to test the expression of associated proteins that cause apoptosis, as shown in (Figure 4). The Bcl-2 down-regulation and substantial up-regulation of Bax and Cleaved caspase-3 relative to the control cells were seen in the ARPE-19 cells subjected to H_2O_2 (400μM). After incubating the cells in H_2O_2（400μM）and then treating ARPE-19 cells with different doses of CMCT (1, 2.5μg/ml) for 12h, Bax expression was down-regulated, Cleaved caspase-3 was differentially down-regulated, and protein expression of Bcl-2 was slightly up-regulated according to the dose-dependence.

CMCT affects the expression of intracellular ROS activity induced by H_2O_2 in ARPE-19 cells.

Many experiments have shown that the oxidative stress of the human retinal pigment epithelium (RPE) was due to imbalances between the excessive in vivo formation of free, reactive radicals and the premature reaction of oxidants to the stimulation of the RPE. Therefore, we further investigated the mechanism of cellular oxidative stress caused by H_2O_2 and whether CMCT treatment of ARPE-19 influences the activity of reactive species of oxygen as shown in (Figure 5). The ARPE-19 cells were exposed to H_2O_2(400μM) and treated with CMCT (1,2.5μg/ml) in the present sample. And the cellular ROS levels were determined with the DCFH-DA package according to the directions of the manufacturer. In comparison to control cells, we observed that treatment with H_2O_2 significantly increased the development of receptive oxygen types in ARPE-19 cells. However, CMCT treatment blocked the upregulation of reactive oxygen activity in cells subjected to H_2O_2.

CMCT promotes H_2O_2-induced intracellular antioxidant activity down modulation in ARPE-19 cells.

Oxidative stress was necessary for normal cellular function; we investigated the expression of three antioxidant biomarkers (SOD, CAT, and GSH) activity in ARPE-19 cells treated during specific treatment periods and assessed the levels of the lipid oxidation indicator (MDA). As showed in (Figure 6.A). We observed a substantial increase in MDA levels in ARPE-19 cells after H_2O_2 induction and decreased MDA levels after CMCT treatment relative to the untreated cell group (p<0.05). Besides, CMCT treatment of cells alone did not affect SOD, CAT, and GSH. In contrast, the three oxidative parameters were substantially reduced in ARPE-19 cells after H_2O_2 treatment and standardized after reduced SOD stimulation, CAT, and GSH levels up-regulated by CMCT treatment in culture. (Figure 6. B, C and D). Differences were observed compared to the control group. (p<0.05 or p<0.01)

Discussion

An increasing amount of studies have shown that oxidative stress was usually caused by excessive ROS induction, cell mitochondrial dysfunction, and damage to the antioxidant system bring about the degeneration of human retinal epithelial cells. However, In the pathogenesis of AMD, oxidative stress plays a significant function [25, 26] . The disparity between the generation of active free radicals and the capacity of biological scavenging was the critical cause of oxidative stress. As the environment changes and age increases, fat accumulation, cleavage mutations of nucleic acid molecules, protein degeneration and inactivation, the repair and regeneration of photosensitive cells decrease [27]. Therefore, slowing the oxidative stress state of cells was essential to promote the development of potential vision loss treatment options.

It can be seen that, according to clinical and experimental research data, the intake of vitamin C, vitamin E, lutein, zeaxanthin, anthocyanins, it's possible that a regular dose of and other antioxidants is needed to keep the retina in good shape. And a potential strategy for the function of photoreceptor cells [22, 28-31]. There are indications that carotene may defend retinal epithelial cells against oxidative stress harm [32]. Cordyceps militaris has various natural biologically active ingredients, different pharmacological functions such as immunomodulation, antioxidant, anti-aging, anti-tumor and anti-bacterial [33]. For medicinal purposes and tonic health food, it has been used extensively in China and other Asian countries. Meanwhile, Cordyceps militaris various medicinal properties come from the unique chemical composition, mainly including polysaccharides, phenols, ascorbic acid, proteins, cordycepin, adenosine, ergosterol, carotenoids, etc. Especially, carotenoids have the natural properties of immunomodulation, antioxidant, antioxidant, aging, tumor and anti-bacterial [34, 35]. In particular, carotenoids have natural antioxidant properties, and Cordyceps militaris has a high carotenoid content and is also a highly water-soluble carotenoid that the body can easily absorb. It can scavenge free radicals through single electron transfer, protect the retina from light and aging-induced oxidative stress by filtering phototoxic short-wavelength visible light [36] . Through removing single electron transmission, they may block and slow down AMD by filtering free radicals, filtering short-wavelength visible light with phototoxicity and function as antioxidants to defend the retina against light and aging oxidative stress, DPPH, FRAP and inhibitions of lipid peroxidation [37, 38]. However, the biochemical and oxidative synthesis of eye carotenoids has not been completely known. Therefore, this research was intended to detect the possible protection impact on the death of ARPE-19 caused by H_2O_2 of Cordyceps militaris carotenoids. RPE cells remain behind photoreceptor cells.H_2O_2 was a mature oxidative stress model in epithelial retinal pigment cells and thus vulnerable to oxidative stress injury. Therefore, in the present study, stress damage to retinal pigment epithelial cells stimulated by cell exposure to H_2O_2 resulted in increased cellular ROS levels, upregulation of lipid antioxidant capacity, apoptosis, and cell necrosis mimicking the pathogenesis of AMD in oxidative stress [39]. The effect of CMCT on damage to human epithelial retinal pigments through oxidative stress was also hypothesized. Compared to normal cells, by experiment, we observed reduced survival of H_2O_2-stimulated ARPE-19 cells and a higher rate of apoptosis at adverse oxidative levels, and the results of the experimental data are consistent with previously reported results. However, ARPE-19 H_2O_2 exposure cells showed a significant increase in cell survival with CMCT treatment for 12h,

as well as a decrease in apoptosis rate and improvement in oxidative stress levels. These findings show a beneficial impact on retinal pigment epithelial cells of CMCT against oxidative stress.

Many experiments have shown the inextricable linkage of apoptosis caused by oxidative stress in retinal pigment epithelial cells to the production of certain reactive oxygen species, and antioxidant enzymes, as an antioxidant, can help the body to scavenge free radicals and peroxides to maintain relative stability of the organism [40]. So, Studies also confirmed antioxidant enzyme inhibition of reactive oxygen species and a greater degree of lower apoptosis [41]. In ARPE-19 cells, we studied ROS levels and the activity of SOD, CAT, GSH/GPX, and MDA expression. CMCT protects retinal pigment epithelial cells by regulating intracellular ROS levels, SOD, CAT, GSH, MDA, etc, while CMCT pretreatment reduces intracellular MDA production and increases GSH to attenuate the H_2O_2-induced stress response [42-44]. CMCT is interested in the retinal pigment epithelial protective antioxidant by regulating intracellular ROS, SOD, CAT, GSH, MDA, etc. It increases the expression level of reduced GSH and reduces the accumulation of endophytic products.

Various studies have shown that apoptotic pathways activation caused by ROS has an important role in AMD pathogenesis [45]. It is reported that the apoptosis About RPE cells prompted oxidative stress is profoundly connected with mitochondria-related apoptotic signaling, which usually involves decreased anti-apoptotic (Bcl-2) protein expression and improved pro-apoptotic (Bax and Cleaved caspase-3) protein expression [46, 47]. The Western blotch findings show that ARPE-19 cells stimulated with H_2O_2 showed strong growth in protein expression in Bax and Cleaved caspase-3; however, a decrease in protein expression in the Bcl-2 community compared with control groups. On the contrary, Pretreatment of CMCT cells for 12h before H_2O_2 exposure effectively reversed the shift, as shown by the decreased Bax and Cleaved Caspase-3 protein expression and increased Bcl-2 protein. By all tests of oxidative stress biomarkers and apoptosis-related proteins, this result indicated the intracellular synthesis of ROS was closely associated with Apoptosis caused by H_2O_2 in cells of ARPE-19.

In conclusion, Cordyceps militaris carotenoids was our first application to defend the human retinal epithelial cells from harm caused by H_2O_2. This study provides evidence that CMCT treatment protects H_2O_2 oxidative disruption ARPE-19 cells through anti-apoptotic and anti-oxidant properties. These findings help prevent or alleviate ocular diseases such as AMD, and further studies will follow to investigate the detailed mechanism of action for pharmacological applications.

Declarations

Acknowledgements

We are thankful to Professor YuFeng Li and Professor Liang Zhang (Xihua University) for kindly providing suggestions and advice for this research.

Author Information

Affiliations

Molecular Biology Laboratory, college of Food and Biological Engineering, Xihua University, Chengdu, 610039, Sichuan, China

Lin Lan, YuZhi Ren, XiangYu Zhou, ShengYu Wang, JinSen Zhang, Ling Xie, YeQian Qian, YuHang Li, YuFeng Li & Liang Zhang

Author Contributions

Conception and Research Design: L.Lan, YF.Li and L.Zhang; Methodology and experiments: L.Lan, XY.zhou; Data analysis and introduction: L.Lan, YY.Ren, XY.Zhou, SY.Wang, JS.Zhang, L.Xie, YH.Li, and YQ.Qian; Writing and reviewing manuscripts: L.Lan, XY.zhou, L.Zhang; Study supervision: YF.Li and L.Zhang.

Corresponding author

Correspondence to YF.Li and L.Zhang

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Figures

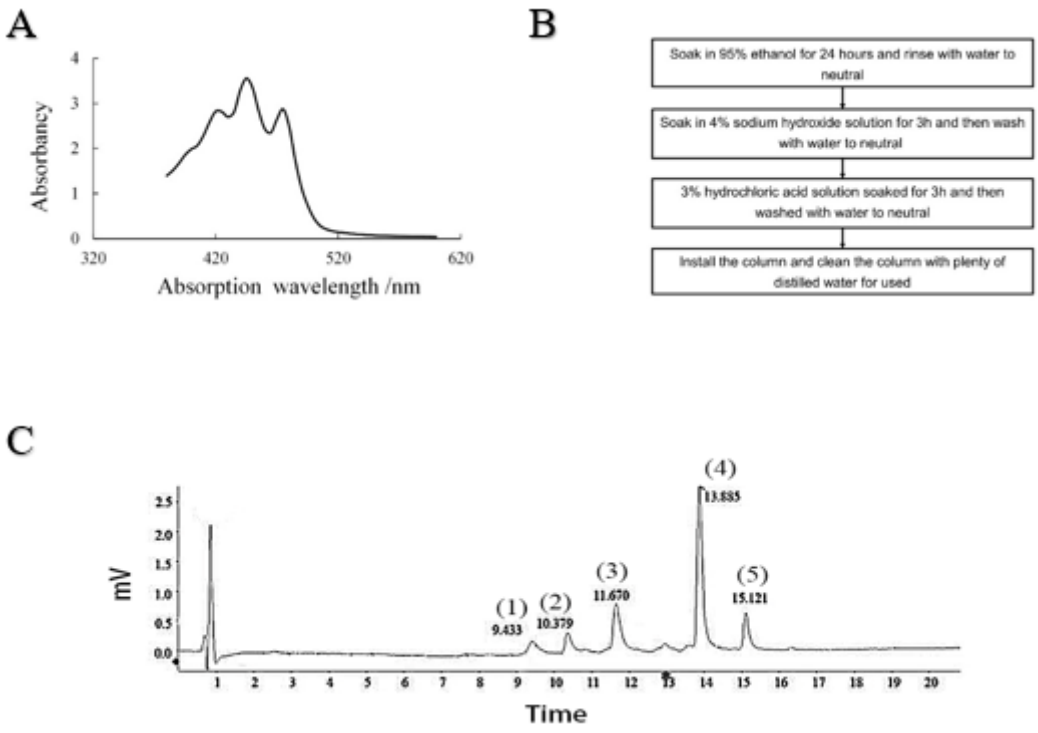

Figure 1

Isolation and purification of Cordyceps militaris (A) Spectrum Picture of Carotenoids' extraction solution in Cordyceps militaris, the three characteristic absorption peaks appear at 420nm, 445nm, and 475nm. The carotenoid extract of Cordyceps militaris used in this experiment has the most prominent peak at 445nm. (B) The activation process of the macroporous resin. (C) UPLC Chromatogram of Cordyceps militaris carotenoids.

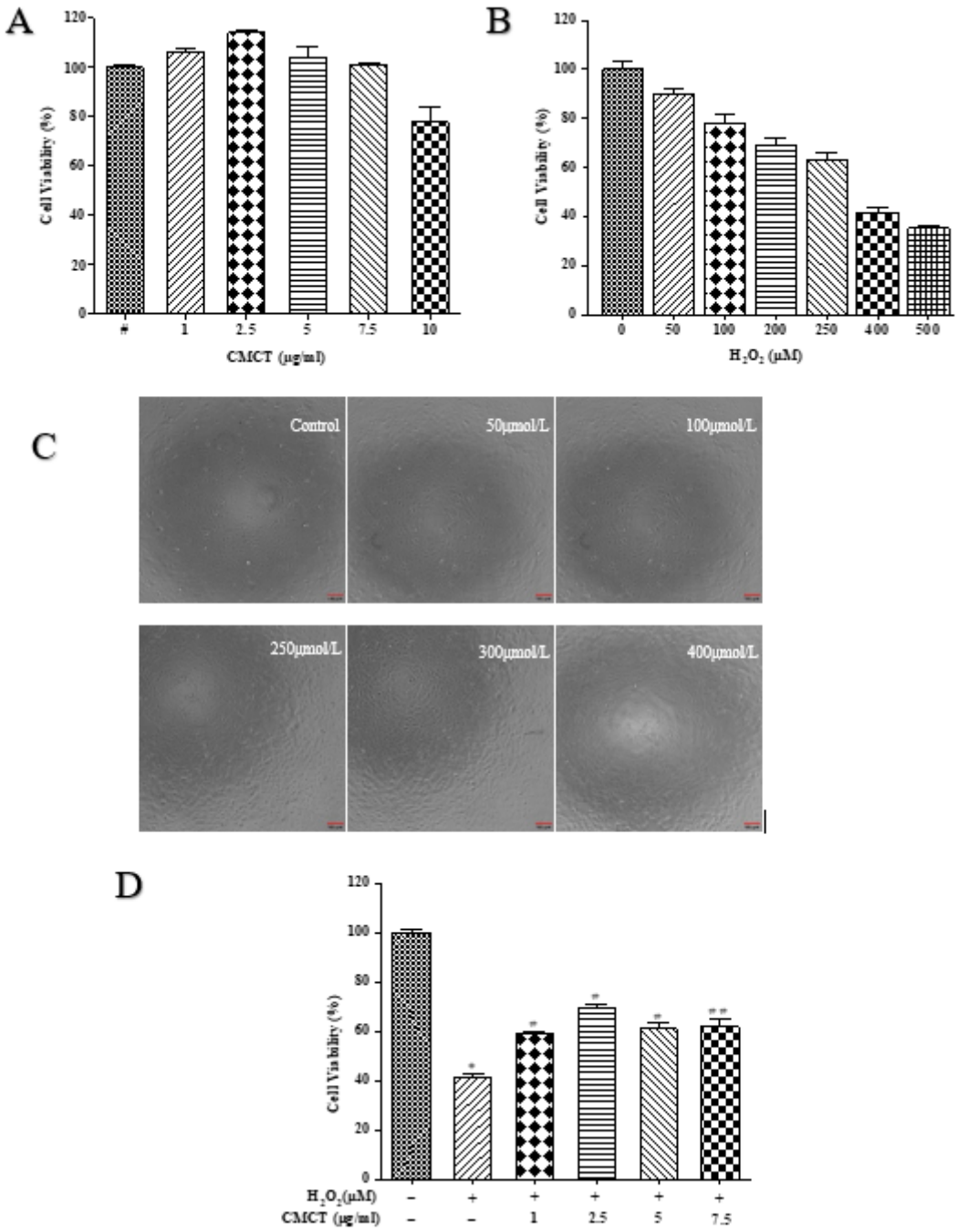

Figure 2

Effect of H2O2 and Cordyceps militaris carotenoids CMCT on cell viability. (A) Effect of CMCT on cell viability of ARPE-19. ARPE-19 cells have been treated for 12 h with differing CMCT levels (0-10μg/ml). (B) Effect of H2O2 on cell viability of ARPE-19. ARPE-19 cells received various concentrations of H2O2 (0-500μM) for 12h. (C) Morphological images of ARPE-19 cells after treatment with various H2O2 (0-400μM) concentrations. (D) Effect of CMCT on H2O2-induced of ARPE-19 cytotoxicity. ARPE-19 cells were pretreated for 12h with various CMCT (0-7.5μg/ml) concentrations and then treated for 12h with H2O2 (400μM). Compare to untreated control cells and the data are presented as the means ± S.D of an

average of three experiments. *P <0.05, **P <0.01 P value compared with the control group. #P<0.05, ##P<0.01 value compared with the H2O2 control group.

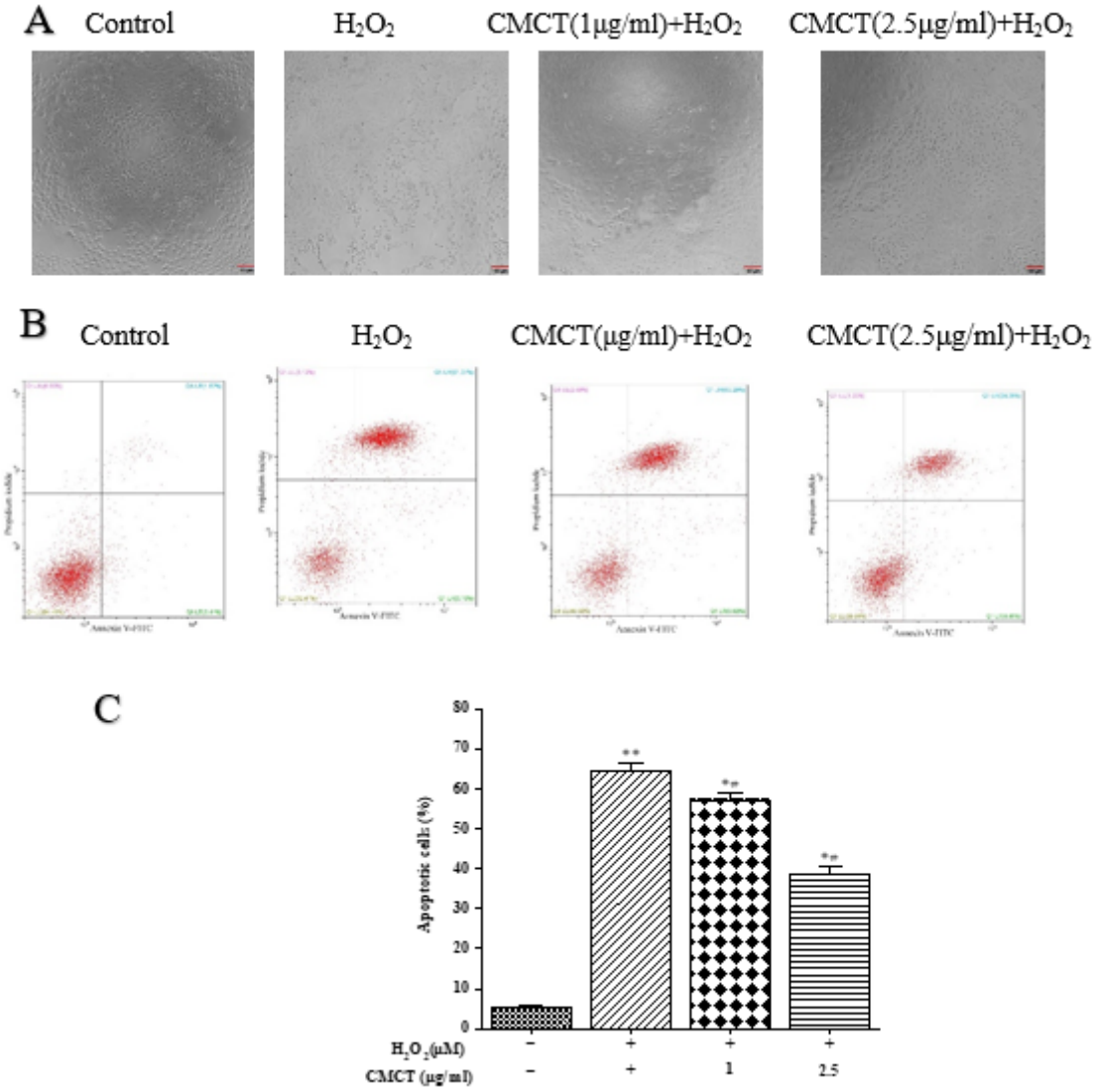

Figure 3

Protection of CMTC against H2O2-induced apoptosis in ARPE-19 cells. (A)Morphological images of cells ARPE-19 following treatment with CMCT. (B) CMCT effect in ARPE-19 cells on H2O2-induced apoptosis. ARPE-19 cells were pre-treated for 12 h at various concentrations of CMCT (1.2.5 μg/ml) and then treated for 12 h by H2O2 (400μM). (C) Analysis of apoptosis rate by flow cytometry. All data are expressed as the mean ± standard deviation of three experiments, including three replicates. *P<0.05, **P<0.01 vs. normal controls. #P<0.05, versus H2O2 control.

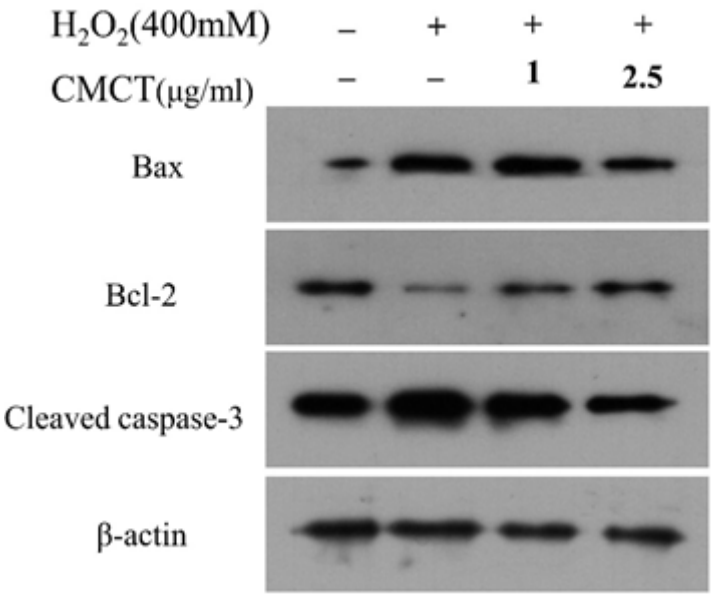

Figure 4

CMCT affects the expression of associated proteins in apoptotic ARPE-19 cells. Evaluation of the effect of CMCT (1, 2.5μg/ml) on protein expression of Bax, Bcl-2, Cleaved caspase-3 in post-protein ARPE-19 H2O2 blotting cells. The 12h pretreatment of ARPE-19 cells with different CMCT (1.2.5μg/ml), accompanied by 12h treatment with H2O2 (400μM). All data are expressed as the mean ± standard deviation of three experiments, including three replicates. *P<0.05, **P<0.01 vs. normal controls. #P<0.05, ##P<0.01 versus H2O2 control.

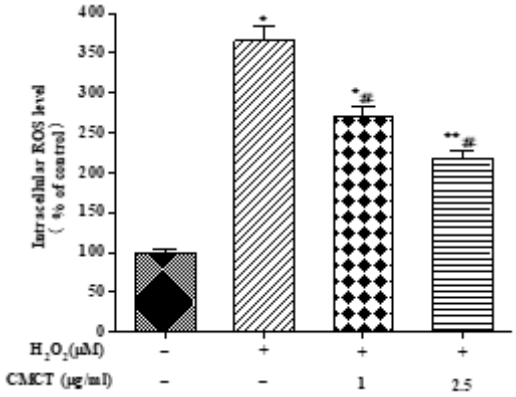

Figure 5

CMCT affects the expression of intracellular ROS activity induced by H2O2 in ARPE-19 cells. Effect of CMCT processing on human ARPE-19 cells generated by intracellular reactive oxygen species (ROS) induced by H2O2. All results were represented as Mean ± SD for three tests. *P<0.05, **P<0.01, compared with the standard control category. #P<0.05, according to the category H2O2control.

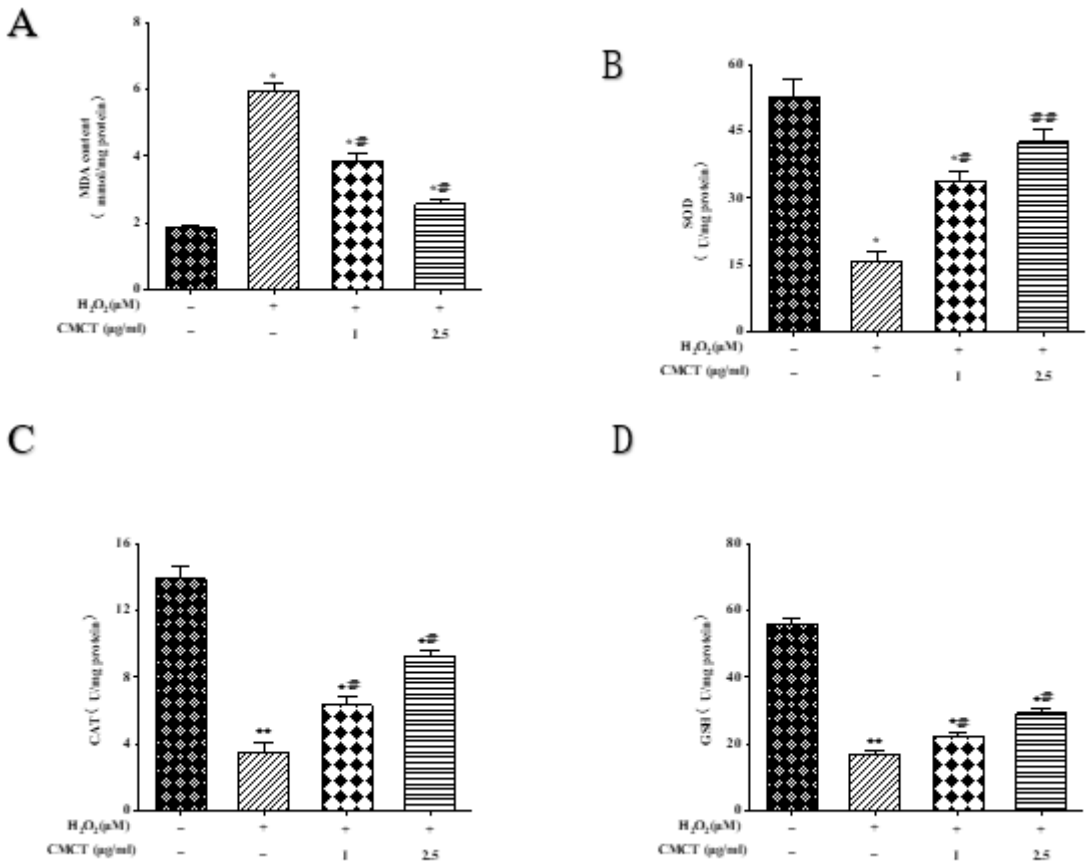

Figure 6

CMCT promotes H2O2-induced intracellular antioxidant activity down modulation in ARPE-19 cells. CMCT promotes H2O2-induced intracellular antioxidant activity down-modulation in ARPE-19 cells. (A) Evaluation of the influence of CMCT (1,2.5μg/ml) on lipid peroxide degraded malondialdehyde in ARPE-19 post-treated H2O2 cells. (B)Evaluation of the impact of CMCT (1,2.5μg/ml) on ARPE-19 post-treated H2O2 SOD behaviors. (C) Evaluation of the effect of CMCT (1, 2.5μg/ml) on CAT activities in ARPE-19 cells post-treated with H2O2 (D) Evaluation of the effect of CMCT (1,2.5μg/ml) on GSH activities in ARPE-19 cells post-treated with H2O2. All data were represented as three experiments' Mean ± SD, and each experiment contained triple repeats. *P<0.05, **P<0.01, vs regular power. #P<0.05, #P<0.01 vs H2O2 control.