Quercetin protect cigarette smoke extracts induced inflammation and apoptosis in RPE cells

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ABSTRACT

Background: Age-related macular degeneration (AMD) is the leading cause of blindness in elderly population in the developed world. Dysfunction of retinal pigment epithelium (RPE) likely triggers early AMD stages. The effect of Quercetin on the early AMD in-vitro model remained unclear.

Methods: The effect of Quercetin in the cell viability was detected with CCK8 methods in control, CSE treated, and CSE with Quercetin treatment group. The apoptotic status in each group was detected with tunnel assay. The oxidative and inflammation biomarkers were detected by ELISA. The expression levels of Keap1/Nrf2/ARE in RPE cells were measured by western blot after pretreatment of Quercetin followed by CSE treatment.

Results: It was found that Quercetin could improve the cell viability and decrease cellular apoptotic rate in the CSE treated RPE group. The expressions of inflammatory and apoptotic biomarkers were significantly decreased in Quercetin treatment group. Furthermore, Quercetin exerts protective effects via activation Keap1/Nrf2/ARE pathway in CSE treated RPE cells.

Conclusions: Quercetin demonstrated significant protective effects in an in-vitro model of early AMD and it might be a new therapeutic strategy for the management of early AMD.

Introduction

Age-related macular degeneration (AMD), which is a chronic degenerative disease that can lead to visual loss the elderly, was now a leading cause of blindness [1,2]. There are two subtypes of AMD, dry AMD, characterized by the development of yellowish deposits called drusen and the dysfunction of the retina and, wet AMD (neovascular AMD), characterized by a development of new choroidal vessels that invades the retina [3]. The neovascular AMD would lead to leakage of fluids, and result in the destruction of the macular. Anti-VEGF could be effective for wet AMD, while it could lead to potential risk of endophthalmitis, retinal detachment, vitreous hemorrhage and increased incidence of geographic atrophy [4,5]. To prevent the additional risk of visual loss, inhibiting the progression of dry AMD to wet AMD would provide significant effect.

There were both genetic and environmental risk factors for the incidence of AMD. It is believed that the AMD is associated with advancing age and tobacco smoking [6]. Previous study has previously implicated the environmental cigarette smoke extract (CSE) expose, which is found in cigarette smoke, pollutants, and plastics, as a potential cause of dry AMD. Previous study had shown that aged mice fed a diet with low-dose CSE develop AMD-like sub-retinal pigment epithelium (RPE) deposits. Additionally, exposure of cultured RPE cells to CSE triggers numerous nonlethal injury responses (cytoskeletal disruption, cell blebbing, increased collagen synthesis, etc.) via activation of specific cytoplasmic signaling cascades (such as ASK1, p38 MAPK, pHSP25, others) [7]. Based on some preliminary studies, it was proposed the conceptual hypothesis that CSE and other AMD-relevant triggers promote subRPE deposit formation via induction of mitochondrial dysfunction [8]. Linking in vitro observations of RPE cell culture to in vivo RPE biology has proven quite challenging because existing mouse models of subRPE deposits require aging, genetic manipulations, high-fat diet, or other injury (i.e. blue light, complement, etc.).

Quercetin, which is a common flavonol found in vegetables and fruits, was reported to be a major flavonoid in human diet [9]. Quercetin is abundantly found in different food, including red wine, apples, onions, and green tea, berries [10]. It has been found to possess strong antioxidant, anti-inflammatory, antiangiogenic, neuroprotective, and apoptosis properties and would demonstrate significant protective effect [11,12]. There were also some studies focusing on the protective effects of Quercetin on the retina. A study about dry AMD showed that Quercetin could inhibit apoptosis and inflammation via inhibition of NF-KB p65 translocation, C3 activation, and PARP cleavage [13].

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Another study was conducted to demonstrate the inflammatory protein expression regulated by VEGF using mouse photoreceptor-derived cells and the protective effect of Quercetin against VEGF-induced inflammatory response. All these events were reversed by quercetin. Zona occludins-1 and β-catenin decreased by VEGF were recovered by quercetin [14]. In a previous study based on the H₂O₂ induced RPE damage model, it was found that quercetin phospholipid complex significantly protects against oxidative injury in ARPE-19 cells associated with activation of Nrf2 pathway [15].

The purpose of this study is to support exploratory studies of a better understanding of the effect of Quercetin on the early AMD. This study was based on the effect of Quercetin on the CSE treated RPE cells. Also, we conducted advanced study to characterize relevant biochemical mechanisms of the protective effects of Quercetin in the in vitro model. The result of this study will support our conceptual hypothesis that Quercetin will demonstrate regulation effect as a novel therapeutic method for dry AMD.

Materials and methods

Cell culture and reagents

ARPE-19, a human RPE cell line, was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) (both from HyClone; GE Healthcare Life Sciences, Logan, UT) and antibiotics under a humidified atmosphere with 5% CO₂. Confluent cells were switched to a serum-free medium for 24 h before treatment. CSE was obtained from Murty Pharmaceuticals, Inc., Lexington, KY, and is 40 mg/mL condensate, and 6% nicotine. According to the manufacturer, CSE was prepared by smoking University of Kentucky’s 3R4F Standard Research Cigarettes on an FTC Smoke Machine. The smoke on the filter is calculated by the weight gain of the filter after smoking. The amount of DMSO is calculated that will dissolve a 4% (40 mg/mL) solution.

Cell viability measurement

Cell viability was evaluated with the LIVE/DEAD® Assay (Invitrogen) according to the manufacturer’s protocol. Cells were grown to visual confluence in 96-well plates, serum starvation for 24 h, and treated with 0–500 µg/mL CSE in DMSO for 24 h. The number of live and dead cells was counted using the Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific, Waltham, MA). Hoechst staining was used to identify the total number of the cells.

Fluorescence microscopic analysis of cell death

Cells were treated with different group: control, CSE and CSE + Quercetin treatment for 24 h. The typical morphological features of apoptotic degeneration were analyzed by the use of confocal microscopy with the nuclear dye Hoechst 33342. Cells were fixed with a solution of methanol/acetic acid (3:1 v/v) for 30 min, washed three times in PBS and incubated for 15 min at 37 °C with 0.4 mg/mL Hoechst dye. After being rinsed in water, cells were visualized for determination of nuclear chromatin morphology with the use of confocal laser scanning microscopy (CLSM; Zeiss LSM700). Each scanning was individually digitalized by a high sensitivity PMT. All acquisitions were performed with ZEN-2010 software.

Apopotic cells were recognized on the basis of nuclear condensation and/or fragmented chromatin. Each condition was reproduced in three dishes per experiment. Both apoptotic and normal cells were counted from three fields per dish in a fixed pattern.

Antioxidant and inflammatory parameters

Inflammatory factors, including IL-1β, IL-6, IL-8, and MCP-1 levels in cell supernatant of all the groups were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quidel, Inc., San Diego, CA), respectively as per the manufacturer’s instructions.

Estimations of antioxidant parameters such as MDA, SOD, and GSH were performed using commercially available kits from Cayman Chemicals Ltd (Ann Arbor), as per manufacturer’s instructions. Enzyme activities were measured in cell supernatant for MDA, SOD, and GSH.

Western blotting

Cultured cells in all the groups were washed with PBS and lysed in buffer containing 10 mM Tris, pH 7.5 (Sigma-Aldrich Corp.), 1 mM EDTA, and 0.1% Triton X-100 (Sigma-Aldrich Corp.) to yield total protein. Lysates were centrifuged at 13,000 g for 20 min at 4°C. Extracted proteins (25 µg) obtained from cellular samples of all the groups were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked in a 5% fat-free dried-milk solution and incubated overnight with partially purified human Keap1, Nrf2 and ARE primary antibody. GAPDH was used as an internal control to confirm equivalent total protein loading. Signal intensities in the control lanes were arbitrarily. Western blots were repeated three times.

Statistical analysis

All data were expressed as means ± standard deviation. One-way analysis of variance was performed to compare the statistical differences among multiple groups with Graphpad Prism 5.0 package (San Diego, CA). p values less than .05 was considered as statistically significant.

Results

Effect of quercetin on cell viability

Quercetin is a common flavonol extracted from vegetables and fruits and could produce significant protective effect (Figure 1(A)). To detect the effect of Quercetin for the CSE...
treated RPE cells, a series of studies were conducted to demonstrate the effect of Quercetin on ARPE-19 cell viability in both the control and CSE-treated groups. Cell viability was measured by MTT assay. Quercetin in 1 nM and 10 nM demonstrated no significant protective effect when compared with the CSE-treated group. In this in vitro experiment, Quercetin could improve the cell viability when the Quercetin concentration was over 50 nM. As showed in Figure 1(B), the protective effect of Quercetin on CSE treated RPE cells in a dose-dependent manner. So, we would like to use 500 nM of Quercetin as a treatment for the following studies.

**Effect of quercetin on CSE induced apoptosis in RPE cells**

The protective role performed by Quercetin was related to their anti-apoptotic effect which preserves monolayer alteration during hypoxic process. The presence of morphological signs of nuclear damage and chromatin fragmentation has been analyzed by using the Hoechst staining technique. As reported in Figure 2, it was reported that CSE could induce cellular die by apoptosis. Also, it could be reported that Quercetin would protect the apoptosis in the CSE-treated group.

**Quercetin protected CSE induced inflammation**

Also, there was a significant anti-inflammation effect of Quercetin in different disorders. In this study, it was found that exposure of CSE would induce the inflammation in ARPE-19 cells comparing with the control group. It was found that the expression of several inframammary factors, including IL-1β, IL-6, and IL-8, were significantly increased \( (p < .001) \). The effect of Quercetin could be applied on the CSE induced inflammation and the expression of IL-1β, IL-6, and IL-8 were significantly reduced after the treatment of Quercetin comparing with the CSE group. However, when the expression of MCP-1 in control and CSE treated ARPE-19 cells was considered, the expression of MCP-1 are consistent in control and CSE-treated group. When the treatment of the Quercetin was detected, it was found that Quercetin did not influence the expression level of MCP.

**Effect of quercetin on oxidative biomarkers in CSE treated RPE cells**

Given that oxidative stress is quite important in the effect of RPE cells, we conducted experiments to measure the levels of three oxidative stress biomarkers, MDA, SOD, and GSH in cultured ARPE-19 cells and detected the effect of Quercetin on the oxidative stress in the CSE treated RPE cells. Expression of MDA was higher in the CSE group, while SOD and GSH expression was significantly lower \( (p < .001) \). In addition, levels of the anti-apoptotic proteins, SOD and GSH,
were significantly higher in the CSE treated ARPE-19 cells treated with Quercetin than in the only CSE group. However, the MDA level was significantly lower after incubation with Quercetin in the CSE group \( (p < .001) \). The MDA level was downregulated with the treatment of Quercetin used in this study, and SOD and GSH were upregulated accordingly. The results for these oxidative stress biomarkers in aging RPE cells with and without Quercetin treatment are presented in Figures 3 and 4.

**Quercetin activates Keap1/Nrf2/ARE pathway in CSE treated RPE cells**

To assess the effect of Quercetin on the activation of Keap1/Nrf2/ARE pathway, the expression of Keap1/Nrf2/ARE in control, CSE and Quercetin treated CSE groups by western blot. As shown in Figure 5, the levels of all the three proteins of Keap1/Nrf2/ARE pathway were significantly decreased after CSE stimulation. To further investigate the role of Quercetin on the effect of the Keap1/Nrf2/ARE signaling pathway in the CSE treated RPE cells, we detect the expression of the related expressions of the signaling protein after Quercetin treatment in the CSE group. The expression levels of Keap1/Nrf2/ARE were significantly up-regulated after Quercetin treatment.

**Discussion**

Quercetin is a flavonoid widely distributed in nature and it is derived from quercetum (oak forest). It is a naturally occurring polar auxin transport inhibitor. To date, the anti-inflammatory activity of this compound has been described [16,17].
In this experimental study, to assess the anti-inflammatory and anti-apoptotic activity of Quercetin, we analyzed the expression of inflammatory and apoptotic biomarkers in the treated group. Furthermore, our results revealed that Quercetin exerts protective effects via activation Keap1/Nrf2/ARE pathway in CSE treated RPE cells.

Accumulating evidence has documented the protective effect of Quercetin on different disorders and could produce significant anti-inflammation effects [18]. A recent was conducted to investigate the effects of a natural phenolic compound quercetin on surgical-induced osteoarthritis (OA) in rabbits. Quercetin can up-regulate SOD and TIMP-1, down-regulate MMP-13, and improve the degeneration of OA through weakening the oxidative stress responses and inhibiting the degradation of cartilage extracellular matrix [19]. Quercetin was chosen to detect the anti-inflammatory effect in its wound healing potential in an animal model induced by bacterial lipopolysaccharide and the results demonstrated significant protective effects, which is the acceleration of the healing of the wound via a diminished inflammation [20].

Also, there were studies focusing the effect of Quercetin in the early AMD and it was found that Quercetin could demonstrate important protective effects. Dry AMD is characterized by the accumulation of lipofuscin in RPE cells. In a study based on early AMD model, it was reported that Quercetin could inhibit C3 complement activation and poly (ADP-ribose) polymerase (PARP) cleavage and advanced pathway analyses showed that Quercetin could demonstrate an inhibitory effect on AP1 and NF-kB activity as estimated in a reporter gene assay. In addition, Quercetin activated the gene expression of aryl hydrocarbon receptor target genes (CYP1A1, CYP1B1) in TCDD-treated RPE cells [13]. We also conduct a series of in vitro studies to detect the protective effects of Quercetin in early AMD. Our current findings showed that inflammation and apoptosis was significant upregulated under CSE stimulation in RPE cells. It was found that Quercetin could improve cellular viability significantly.

To investigate the effect of Quercetin in the treatment of early AMD, we measured the protein expression levels of genes involved in inflammation and apoptosis in both RPE cells after Quercetin treatment under CSE conditions. Therefore, Quercetin treatment could demonstrate significant protective effect through down-regulation of inflammation and apoptosis induced by CSE.

We also try to conduct experiments to detect the signaling pathway related to the function of Quercetin. Keap1/Nrf2/ARE pathway is a well-known cytoprotective transcription factor and it plays an irreplaceable role in the cellular defense against various oxidative stress-induced tissue injuries including different diabetes related disorders. Oxidative stress aroused by advanced glycation-end products (AGEs) is a culprit in the pathological progression of diabetic nephropathy. In a study based on in vivo and in vitro study, it was found that Keap1/Nrf2/ARE antioxidative pathway exert crucial inhibitory effects on the development of diabetic nephropathy [21]. There was also a recent study focus the effect of Keap1/Nrf2/ARE pathway in retinal disorders and the underlying mechanism found that concomitant with deacetylation and reducing the ubiquitination levels of Nrf2, Sirt1 significantly enhanced the activity of Keap1/Nrf2/ARE pathway including decreasing Keap1 expression, promoting the nuclear content, ARE-binding ability, and transcriptional activity of Nrf2, augmenting the protein levels of heme oxygenase 1, a target gene of Nrf2, which eventually quenched ROS overproduction and alleviating FN and TGF-β1 accumulation in AGES-treated glomerular mesangial cells [21]. In this study, we try to demonstrate the potential influence pathways involving the effect of Quercetin application. Once stimulated by CSE in the RPE cells, it could induce significant oxidant and inflammation reactions and the treatment of Quercetin could demonstrate significant antioxidant response element and regulate the expression antioxidant and anti-inflammation effects. Thus, the effect of Quercetin is significant and could be used as a potential treatment target for early AMD.

While it is a study based on in vitro study, more experiments based on primary cultured RPE cells and in-vivo model would be conducted in the following studies.

Conclusions

The present in vitro study has revealed a protective effect of Quercetin on early AMD. Also, the results were also reported to reveal the underlying mechanism, and Quercetin decreases CSE induced apoptosis through activating Keap1/Nrf2/ARE pathway, indicating that Quercetin might be a new therapeutic strategy for the management of early AMD.

Disclosure statement

The authors do not have any financial or personal relationships with other people or organizations that could influence the work described in this manuscript.

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