Baicalin Alleviates Age-Related Macular Degeneration via *miR-223*/*NLRP3*-Regulated Pyroptosis

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Keywords

miR-223 · NOD-like receptor family pyrin domain-containing 3 · Pyroptosis · Age-related macular degeneration · Baicalin

Abstract

Background: Age-related macular degeneration (AMD), a major eye degenerative disease, ultimately causes irreversible vision loss. Baicalin was identified to attenuate laser-induced choroidal neovascularization, indicating a therapeutic role in AMD. However, the exact mechanisms for baicalin in AMD remain unknown. **Methods:** MTT assay was performed to access the suitable concentration of baicalin or A β for treating ARPE-19 cells. CCK-8, morphology, and flow cytometry analysis were performed to evaluate cell viability and pyroptosis of baicalin in A β -evoked ARPE-19 cells. Quantitative real-time polymerase chain reaction and western blot analysis were subjected to measure the correlation between miR-223 and NLRP3. Luciferase reporter assay was performed to determine their direct relationship. Western blot analysis was subjected to determine pyroptosis-related proteins. **Results:** Baicalin inhibited A β -evoked pyroptosis in ARPE-19 cells. Mechanistically, baicalin significantly induced upregulation of miR-223 and downregulation of NLRP3, thus suppressing pyroptosis triggered by NLRP3 inflammasome signaling, yet such beneficial effects were reversed by miR-223 knockdown. Additionally, MCC950, a

NLRP3 inhibitor, restored anti-pyroptosis activity of baicalin under miR-223 silencing. **Conclusion:** Baicalin alleviates intracellular pyroptosis and viability damage resulted from A β inducement in human retinal pigment epithelium cells via negative crosstalk of miR-223/NLRP3 inflammasome signaling, indicating that baicalin may be considered as a potential candidate for AMD therapy.

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Introduction

Age-related macular degeneration (AMD) is a major eye degenerative disease in aged individuals in the developed countries [1]. Increased drusen deposition, retinal pigment epithelium (RPE), cell death, and secondary photoreceptor degeneration have been the main hallmarks of AMD [2]. As it progresses, AMD ultimately causes irreversible vision loss. Clinically, AMD is divided into 2 main phenotypes, geographic atrophy and neovascular/exudative AMD. Unfortunately, there is no cure to both AMD forms, AMD has been regarded as the global eye health problem causing substantial economic and social burden [3]. Thus, developing a potential candidate agent for treating AMD is urgent. Although AMD pathogenesis is complex and obscure, chronic oxidative stress and inflammation play key role in the progress of AMD [4–6]. Persistent inflammation activation has been re-

ported to accelerate the formation of drusen. Various inflammatory molecules are also proposed as crucial biomarkers of AMD [7]. Inflammation has been closely linked with the development of AMD. Therefore, pharmacological targeting inflammation inhibition might be an attractive strategy for AMD treatment.

Inflammasomes are a class of cytoplasmic protein complexes sensing endogenous or exogenous pathogen-associated molecular patterns or danger-associated molecular patterns that induce autoactivation of caspase-1 and the subsequent cleavage of proinflammatory cytokines, including pro-interleukin (IL)-1 β and pro-IL-18 into their mature and secreted forms, which stimulate intracellular inflammation response [8]. NOD-like receptor family pyrin domain-containing 3 (NLRP3), a widely studied inflammasome, was shown to be involved in various immune and inflammatory diseases including AMD [9]. Previous studies have indicated that activated NLRP3 aggravates RPE death and potential visual loss. Meanwhile, the NLRP3 activation in the immune cells accumulating in the retinal area also contributes to the pathogenesis of AMD [9, 10]. Reversely, blocking the NLRP3-mediated inflammasome signaling in the RPE slows down the AMD progress [11]. It indicates that targeting NLRP3 is an important pharmacological mechanism for AMD therapy.

Pyroptosis is a form of programmed cell death triggered by perturbations of extracellular or intracellular homeostasis related to innate immunity [12]. Due to leading to ruptures in the plasma membrane and subsequent releases of abundant inflammatory factors (e.g., IL-1 β and IL-18) in a Caspase1-dependent manner, pyroptosis is identified as a novel proinflammatory regulated cell death [12]. In general, pyroptosis acts as a defence mechanism protecting cells from infection by inducing pathological inflammation [13]. However, persistent inflammation will result in multiple diseases take place, such as metabolic disorder, autoinflammatory diseases, AMD, and so on [4]. Previously, it is reported that pyroptosis could increase RPE cells susceptibility to photooxidative damage-mediated cell death and contribute to RPE degeneration [4]. Moreover, activation of pyroptosis mediated via NLRP3/Caspase1 signaling is a main mechanism of cellular death in AMD [8, 10, 14]. It suggests that inhibiting pyroptosis is an important insight of AMD intervention.

Scutellaria baicalensis Georgi, a common plant, is distributed in China, Korea, Mongolia, and Russia. In China, *Scutellaria baicalensis* Georgi has been regarded as the most widely used traditional Chinese medicine for treating pulmonary diseases, jaundice, hypertension, and so on [15]. Baicalin, a main effective flavonoid compound,

isolated from the raw root of *Scutellaria baicalensis* Georgi [16], similarly possesses various biological properties, including anti-inflammatory, antimicrobial, and antioxidant activities and targeting NLRP3 inflammasome and nuclear factor-kappa B (NF- κ B) signaling is proposed as one of the main regulatory mechanisms [17–20]. Interestingly, it is demonstrated that baicalin also exerts active therapeutic in ocular diseases, such as glaucoma, retinopathy and AMD via antiangiogenesis, anti-apoptosis, anti-inflammatory, and antioxidant oxygen species [16, 21–23]. However, the underlying molecular mechanism of baicalin in AMD is mainly unknown.

Here, we found that baicalin could alleviate A β -induced pyroptosis via increasing miR-223 expression while downregulating NLRP3 expression. Moreover, miR-223 overexpression suppressed NLRP3-triggered pyroptosis by targeting its 3'-untranslated region (3'-UTR). Therefore, we demonstrated that baicalin increased the expression of miR-223 and promoted miR-223/NLRP3/Caspase-1 feedback signaling, which mediated its anti-inflammation and anti-pyroptosis activity in A β -induced ARPE-19 cells. Our data clarified the protective role of baicalin in AMD in vitro and shed the possibility on baicalin treatment improving AMD in vivo.

Materials and Methods

Cell Culture and Treatment

The human RPE cell line ARPE-19 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were seeded at a density of 20,000 cells/cm² and cultured in high glucose Dulbecco's Modified Eagle Medium containing with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies) at 37 °C (95% air, 5% CO₂). Cells were subjected to A β treatment, and then MTT, CCK-8, fluorescence activated cell sorter (FACS), gene expression, or reporter analysis were performed for further exploration in the absence or presence of baicalin (YuanYe Biotech, Shanghai, China) or MCC950 (Sigma-Aldrich, St. Louis, MO, USA) treatment (baicalin and MCC950 were dissolved in dimethylsulfoxide, Sigma-Aldrich) at indicated concentrates and time points.

MTT Assay

ARPE-19 cells were seeded at a density of 20,000 cells/cm² in 96-well plates and incubated with A β _{1–42} (Sigma-Aldrich) or baicalin at indicated concentrates. Then, MTT (10 μ L of 5 mg/mL, Sigma-Aldrich) was added into cell. After another 3-h incubation, dimethylsulfoxide (200 μ L) was added, and absorbance was measured on a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm and background absorbance measured at 630 nm. Background absorbance was subtracted from signal absorbance to obtain normalized absorbance values. The colorimetric signal obtained was proportional to the cell number.

CCK-8 Determination

ARPE-19 cells were seeded at a density of 20,000 cells/cm² in 96-well plates and incubated with A β ₁₋₄₂ (10 μ M) or baicalin (50 μ g/mL) for 24, 48, and 72 h. Subsequently, cell was determined colorimetrically at 450 nm using the CCK-8 Kit (Dojindo, Tokyo, Japan).

Flow Cytometry and Pyroptosis Evaluation

A cell population with positive propidium iodide, PI (Abcam, Cambridge, UK) and caspase1 (Abcam) activities were enriched and detected by flow cytometry. The flow cytometry data were assessed using BD FACSDiva Software version 7.0 (Becton-Dickinson, USA).

Transfection

For cell transfection, different expression plasmids (anti-miR, anti-miR-223, miR-NC, miR-223, wild or mutant 3'-UTR reporter of NLRP3) were transiently transfected into ARPE-19 cells or HEK293T cells (American Type Culture Collection) with FuGENE-HD (Roche) according to the ratio: 1 μ g plasmid/5 μ L FuGENE. After 48-h transfection, cells were used to following experiments. The anti-miR, anti-miR-223, miR-NC, and miR-223 were purchased from RiboBio Co., Ltd. (Guangzhou, China).

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA from ARPE-19 cells was isolated using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Then the RNA was reverse-transcribed to cDNA using reverse transcriptase (Takara Dalian, China). First-strand cDNA was synthesized with a cDNA synthesis kit (Fermentas, Madison, WI, USA). Quantitative real-time polymerase chain reaction (PCR) was carried out using SYBR Green PCR Kit (ABI, USA). Individual transcripts in each sample were repeated at least 3 times and values were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Total Protein Extraction and Western Blot

The whole-tissue protein from ARPE-19 cells was extracted using RIPA buffer and separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies of NLRP3, Caspase 1, apoptosis-associated speck-like protein (ACS; 1:1,000; Cell Signaling Technology, USA), and GAPDH were used by 1:5,000 dilution in 5% bovine albumin at 4°C overnight followed by HRP-linked goat antirabbit antibody at room temperature for 2 h. Immunoreactive proteins were visualized using LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), according to the manufacturer's in-

structions. The relative protein levels were normalized to GAPDH. All of the primary antibodies were purchased from Cell Signaling Technology.

Dual-Luciferase Reporter Assay

The predicted miR-223 binding site of the NLRP3 3'-UTR sequence or mutant sequence was cloned into the pMIR-REPORT vector (Ambion®; Thermo Fisher Scientific, Inc.) to construct the NLRP3-3'-UTR-WT plasmid or the NLRP3-3'-UTR-Mut plasmid. For NLRP3 3'-UTR reporter assay, the expression plasmids for miR-NC, miR-223, wild or mutant 3'-UTR reporter of NLRP3, and pREP7 (Renilla luciferase) were cotransfected into HEK293T cells with FuGENE-HD reporter, and Renilla was used to normalize transfection efficiencies. After 48 h, cells were collected for luciferase activity determination by using the dual luciferase reporter assay system (Promega Corp., Madison, WI, USA).

Statistical Analysis

All values were expressed as means $\pm$ standard deviation (SD) and analyzed using the statistical package for the social science (SPSS, version 18.0). Paired or unpaired two-tailed t-tests were used to detect a difference in the mean values of treatment group and control and analysis of variance for the difference among more than 2 groups. Differences with *p* values <0.05 were considered to be statistically significant and denoted as (*).

Results

Baicalin Alleviates A β -Induced Pyroptosis in RPE Cell

A β , a major constituent of drusen, has been proved to induce the AMD phenotype [24]. Therefore, A β -induced ARPE-19 (human RPE cell line) was chosen as a model to explore the pharmacology effects of baicalin on regulating AMD progress. First, we optimized the concentration of A β in ARPE-19 cells for further experiments. According to MTT assay, A β (10 μ mol/L) might be the most suitable dose, which could successfully cause approximately 50% cells viability loss in ARPE-19 cells (Fig. 1a). Additionally, the result of CCK-8 assay showed that cell viability was significantly suppressed by A β treatment (Fig. 1b). Morphologically, after stimulating

Fig. 1. Baicalin exerts anti-pyroptosis activity in A β -induced ARPE-19 cells. **a** ARPE-19 cells were incubated with various A β concentrations (0, 0.1, 1, 10, and 30 μ mol/L) for 24 h and then were collected for MTT assay. * *p* < 0.05 and ** *p* < 0.01 vs. Control group (0 μ mol/L). **b** CCK-8 assay in ARPE-19 cells with or without A β (10 μ mol/L) stimulation at different indicated time (24, 48, and 72 h). * *p* < 0.05 vs. Control. **c** Optical microscopy morphology pictures of ARPE-19 cells with or without A β (10 μ mol/L) stimulation (Scale bar: 400 μ m). **d** Pyroptosis in A β -induced ARPE-19 cells was detected by flow cytometry. ** *p* < 0.01 vs. Control. **e** MTT assay was subjected in ARPE-19 cells incubated with baicalin (0,

12.5, 25, 50, and 100 μ g/mL) for 48 h. ** *p* < 0.01 vs. Control. **f** Baicalin increased ARPE-19 cells viability by CCK-8 assay. ** *p* < 0.01 vs. Control and ** *p* < 0.01 vs. A β . **g** Optical microscopy morphology pictures of A β -induced ARPE-19 cells with or without baicalin (50 μ g/mL, 48h) treatment and untreated control ARPE-19 cells (Scale bar: 400 μ m). **h** The flow cytometry assay was performed to measure the effects of baicalin on pyroptosis in A β -induced ARPE-19 cells. ** *p* < 0.01 vs. Control and * *p* < 0.05 vs. A β . The results represent 3 independent experiments, and data are analyzed and presented as means $\pm$ SD (*n* = 3).

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A β (10 μ mol/L), more number of dead cells were observed in ARPE-19 cells as compared with untreated control group (Fig. 1c). Recently, the pyroptosis triggered by A β could cause RPE dysfunction in the pathogenesis of AMD [25]. Similarly, FACS analysis revealed that A β significantly increased positive cell proportion with PI/Caspase1 dual staining in ARPE-19 cells (Fig. 1d). Interestingly, such dysfunction in ARPE-19 cells induced by A β was reversed by 50 μ g/mL baicalin (the suitable dose was determined by MTT assay in Fig. 1e) treatment. As shown in Figure 1f–h, baicalin protected cell damage and pyroptosis from A β insult. Thus, these data indicated that baicalin could alleviate A β -induced pyroptosis in ARPE-19 cells in vitro.

Baicalin Inhibits Pyroptosis via Upregulating miR-223 Expression in ARPE-19 Cells

miRNA-223, a myeloid-enriched microRNA, has been regarded as a negative regulator of inflammation in various conditions involving in many inflammatory disorders, infections, and cancers [26–28]. Meanwhile, previous studies have reported that NLRP3 transcription and activity are negatively regulated by miR-223 in numerous cells line, which contributes to anti-pyroptosis [29]. However, whether miRNA-223/NLRP3 interplay mediates baicalin attenuating pyroptosis remains unclear. Excitingly, in A β -challenged ARPE-19 cells, baicalin markedly increased miRNA-223 expression, while it reduced pyroptosis markers (NLRP3, Caspase1 and ACS) and proinflammation cytokines (IL-1 β and IL-18) expression (Fig. 2a, b). To further clarify the correlation between miRNA-223 expression and pyroptosis in ARPE-19 cell, the transfection of miR-223 inhibitor (anti-miR-223) was applied. Results showed that miRNA-223 silencing largely decreased miRNA-223 expression (Fig. 2c), but induced NLRP3, IL-1 β , and IL-18 mRNA levels in condition of A β incubation (Fig. 2d). FACS and western blot analysis also indicated that downregulation of miRNA-223 promot-

ed NLRP3, Caspase1, and ACS pyroptosis-related protein levels and block cell pyroptosis in A β -insulted ARPE-19 cells (Fig. 2e, f). Our data suggested baicalin might exert anti-pyroptosis activity via upregulating miR-223.

Identifying NLRP3 as a miR-223 Target Gene

It is predicted that miR-223 could interact with the 3'-UTR of NLRP3 according to mRNA TargetScanHuman (<http://www.targetscan.org>; Fig. 3a). To further confirm the direct crosstalk between the 2 molecules, a dual-luciferase reporter assay was performed. As shown in Figure 3b, cotransfection with miR-223 markedly reduced the luciferase activity of the plasmid expressing the wild type of the respective fragment of NLRP3 3'-UTR. However, the luciferase activity of mutant NLRP3 3'-UTR reporter was not affected by cotransfection with miR-223 mimics or negative control. Gene expression analysis similarly presented that miR-223 significantly decreased the expression of NLRP3, while miR-223 silencing showed the opposite result (Fig. 3c, d). These results indicated that miR-223 could directly bind to the 3'-UTR of NLRP3 and negatively regulate NLRP3 expression.

Baicalin Alleviates A β -Induced Pyroptosis through Regulating miR-223/NLRP3 Axis

To further understand whether miR-223/NLRP3 regulatory network plays an essential role in baicalin-mediated pyroptosis in RPE cell, flow cytometry, quantitative real-time-PCR, and western blot analysis were subjected to evaluate the anti-pyroptosis and anti-inflammation activity of baicalin in the presence or absence of anti-miR-223 in A β -challenged ARPE-19 cells. As illustrated, baicalin protected the cells from A β -induced pyroptosis (Fig. 4a) and upregulated miR-223 expression (Fig. 4b). Meanwhile, baicalin reduced pyroptosis markers (NLRP3, Caspase1, and ACS) and proinflammation cytokines (IL-1 β and IL-18) expression (Fig. 4b, c). Expectedly, such beneficial effects were

Fig. 2. Baicalin promotes miR-223 expression and alleviates pyroptosis in ARPE-19 cells. The relative mRNA expression levels (a) and protein levels (b) were determined by qRT-PCR and western blot analysis in baicalin-treated (50 μ g/mL) ARPE-19 cells in the presence of A β (10 μ mol/L) stimulation. * p < 0.05 and ** p < 0.01 vs. A β . c Transfection of anti-miR-223 decreased miR-223 mRNA level in ARPE-19 cells. * p < 0.05 vs. anti-miR. d qRT-PCR analysis in A β -induced ARPE-19 cells transfected with anti-miR or anti-miR-223. * p < 0.05 and ** p < 0.01 vs. A β +anti-miR. e Transfection of anti-miR-223 enhanced A β -induced pyroptosis in ARPE-

19 cells according to flow cytometry assay. ** p < 0.01 vs. A β + anti-miR. f Western blot analysis was subjected to measure the expression of proteins (NLRP3, Caspase1, ACS). * p < 0.05 and ** p < 0.01 vs. A β + anti-miR. For all genes expression analysis, GAPDH was used as an internal control for normalizing the mRNA levels and protein levels. The results represent 3 independent experiments, and data are analyzed and presented as means $\pm$ SD (n = 3). NLRP3, NOD-like receptor family pyrin domain containing 3; ACS, apoptosis-associated speck-like protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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reversed with miR-223 knockdown by anti-miR-223 transfection (Fig. 4a–c), indicating that miR-223 might be a crucial participant in baicalin-mediated pyroptosis attenuation. Finally, to clarify whether NLRP3 is a key downstream mediator of miR-223, MCC950, a NLRP3 antagonist, was coincubated with A β , anti-miR-223, and baicalin in ARPE-19 cells. As presented in FACS analysis (Fig. 4d), MCC950 treatment obviously re-

duced pyroptosis caused by miR-223 knockdown in condition that A β and baicalin coincubation. Consistently, in the same condition, MCC950 exactly reduced miR-223-mediated pyroptosis markers (NLRP3, Caspase1, and ACS) expression in protein levels (Fig. 4e). Thus, these findings identified that miR-223/NLRP3 axis is involved in baicalin-mediated pyroptosis suppression in A β -induced ARPE-19 cells.

Fig. 3. NLRP3 is a target of miR-223. **a** Scheme of predicted potential binding sites between miR-223 and NLRP3. **b** A dual-luciferase reporter assay was performed. Luciferase activities of plasmids with WT or Mutant NLRP3-3'-UTR reporters were assessed in HEK293T cells cotransfected with miR-223 mimics or nonhomologous NC (negative control), * $p < 0.05$. Gene expression of NLRP3 in ARPE-19 cells transfected with anti-miR, anti-miR-223, miR-NC, and miR-223 was evaluated by qRT-PCR (**c**) and western blot

(**d**). ** $p < 0.01$ vs. Anti-miR. * $p < 0.05$ and ** $p < 0.01$ vs. miR-NC. GAPDH was used as an internal control for normalizing the mRNA and protein levels. The results represent 3 independent experiments, and data are analyzed and presented as means $\pm$ SD ($n = 3$). NLRP3, NOD-like receptor family pyrin domain containing 3; 3'-UTR, 3'-untranslated region; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 4. Baicalin alleviates pyroptosis in ARPE-19 cells via regulating miR-223/NLRP3 axis. **a** Downregulation of miR-223 suppressed the anti-pyroptosis effect of baicalin in A β -induced ARPE-19 cells in flow cytometry assay. * $p < 0.05$ vs. A β and ** $p < 0.01$ vs. A β + Baicalin + anti-miR. qRT-PCR (**b**) and western blot (**c**) analysis in A β control (dimethylsulfoxide), baicalin, baicalin + anti-miR, and baicalin + anti-miR-223 group ARPE-19 cells in condition that A β challenge. * $p < 0.05$ and ** $p < 0.01$ vs. A β , * $p < 0.05$ and ** $p < 0.01$ vs. A β + baicalin + anti-miR. **d** MCC950 restored the anti-pyroptosis activity of baicalin in A β -induced ARPE-19 cells, transfected with anti-miR-223, according to flow cytometry

assay. * $p < 0.05$ vs. A β + baicalin + anti-miR-223. **e** Western blot analysis in (A β + baicalin + anti-miR-223) ARPE-19 cells with or without MCC950 treating. * $p < 0.05$ and ** $p < 0.01$ vs. A β + baicalin + anti-miR-223. For all genes expression analysis, GAPDH was used as an internal control for normalizing the mRNA levels and protein levels. The results represent 3 independent experiments, and data are analyzed and presented as means $\pm$ SD ($n = 3$). NLRP3, NOD-like receptor family pyrin domain containing 3; ACS, apoptosis-associated speck-like protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Discussion

Previous researches have demonstrated that baicalin plays a therapeutic role in metabolic diseases, cancer, fibrosis diseases, especially in inflammatory and degenerative diseases, via its antioxidant, anti-inflammatory, anti-apoptotic, and antitoxicity properties [19, 20, 30, 31]. Meanwhile, many flavonoids have been reported to reduce the risk of ocular diseases such as cataract, glaucoma, and AMD [22]. However, the protective effects and pharmacological mechanisms of baicalin in AMD are still limited. Our current study provides the evidence that baicalin exerts protective roles in improving AMD via its anti-pyroptosis and anti-inflammatory functions mediated by modulating miR-223/NLRP3 axis in A β -induced ARPE-19 cells. It suggested baicalin might be considered for the treatment of AMD in vivo.

Although the pathogenesis in AMD development is not fully understood, pyroptosis, a highly inflammatory form of programmed cell death in Caspase-1-dependent, is still regarded as a main risk factor in the pathogenesis of AMD. A β , the main component of drusen in AMD patients, can trigger inflammation and pyroptosis in RPE cells via Caspase-1 activation [12, 14]. To evaluate the activity of baicalin in anti-AMD, we subjected ARPE-19 cells to baicalin treatment with A β stimulation. As observed in our studies, baicalin treatment inhibited A β -induced damage and caspase-1 activation in ARPE-19 cell. Such results suggested us baicalin could alleviate AMD development via blocking pyroptosis in ARPE-19 cell.

NLRP3 inflammasome is indicated be involved in promoting inflammation and pyroptosis in AMD progress [9, 14]. Increased mRNA level of NLRP3 in AMD lesions of the RPE cells has been observed [32]. Oxidative stress could induce pyroptosis in AMD via the NLRP3 inflammasome activation [5]. Inversely, NLRP3 inflammasome blockade inhibits VEGF-A-induced AMD, and small molecule NLRP3 inhibitors have been shown to be effective on treating AMD in vivo [11]. Due to the important role of NLRP3 inflammasome pathway in the pyroptosis and pathogenesis of AMD, targeting NLRP3 inflammasome signaling is regarded as a novel and promising therapy for AMD treatment. Previously, the anti-inflammatory property of baicalin via NF- κ B signaling inhibition has been observed [19]. Additionally, it is reported that baicalin suppress LPS-stimulated NLRP3 inflammasome signaling [31]. Thus, we speculated baicalin would reduce pyroptosis via hindering NLRP3-mediated inflammatory response in RPE cell. Expectedly, in gene

expression analysis, we found baicalin markedly decreased NLRP3 inflammasome signaling genes, NLRP3, Caspase1, and ACS expression levels in ARPE-19 cell, it reflected baicalin inhibited NLRP3 inflammasome signaling. IL-1 β and IL-18 are major inflammatory effector cytokines of inflammasome activation [25, 33]. These chronic proinflammatory cytokines would induce persistent stress to RPE cells and result in pyroptosis in RPE cells [33]. It is established NLRP3 silencing in human RPE greatly decrease the expression of inflammatory IL-1 β and IL-18 and subsequent pyroptosis in RPE cells [9]. Similarly, baicalin indeed decreased the IL-1 β and IL-18 expression. Our results proved that baicalin blocked NLRP3 inflammasome signaling and thereby protected the cells from A β -evoked pyroptosis and inflammation in RPE cells.

miRNAs are small noncoding RNA molecules that typically suppress the translation and the stability of transcripts through binding to complementary sequences in the 3'-UTR of their target mRNAs. miRNAs are implicated in playing essential roles in almost all biological functions [34, 35]. miR-223, a myeloid-enriched microRNA, has been connected with a large number of inflammatory conditions [26, 36]. miR-223 has been shown to directly suppress the canonical NF- κ B Pathway in basal keratinocytes [28]. miR-223 loss results in continuous neutrophil recruitment and the excessive neutrophilic inflammation [37]. miR-223 responds to stimuli to control the production of IL-6 and IL-1 β in immune cells [38]. Moreover, NLRP3 inflammasome activation is found to be largely miR-223 dependent [29]. Bioinformatics analysis and previous studies have highly suggested the negative regulation of NLRP3 expression by miR-223 that exists in many cell lines. However, a directly regulatory between miR-223/NLRP3 cross talk and their role in inflammasome activation in AMD are not well established in RPE cells. To do so, we firstly used NLRP3 3'-UTR reporter and NLRP3 gene expression analysis to confirm the causal link between the 2 molecules. Consistently, miR-223 decreased the NLRP3 3'-UTR reporter activity and inhibited NLRP3 expression, while such effects were abolished by miR-223 knockdown. Next, we analyzed the miR-223 functional role in regulating NLRP3 inflammasome signaling and pyroptosis in A β -evoked ARPE-19 cells. Interestingly, miR-223 knockdown promoted NLRP3 inflammasome-related pyroptosis. Our data provided us that miR-223 exerted a negative regulation for NLRP3 inflammasome and pyroptosis in RPE cells. Meanwhile, we found miR-223 expression levels were increased by

baicalin treatment in ARPE-19 cells. It hinted us that miR-223 may be involved in pharmacological mechanisms of baicalin. To investigate it, miR-223 was repressed in ARPE-19 cells, we observed miR-223 knock-down block the beneficial effects of baicalin on protecting ARPE-19 cells from A β -induced activation of NLRP3 inflammasome and pyroptosis, but such adverse manifestations were rescued by NLRP3 inhibitor, MCC950 cotreatment. It provided us the proof that the miR-223/NLRP3 axis could mediate the protection of on A β -induced damage in RPE cells.

In summary, we explored the activities and mechanisms of anti-apoptosis and anti-inflammation of baicalin in A β -induced ARPE-19 cells and demonstrated that baicalin exerted protective effects on improving inflammation and pyroptosis through miR-223/NLRP3 axis. Although considering the poor bioavailability of baicalin, it still puts forward a possibility of improving AMD by using baicalin or baicalin-contained traditional medicines in clinic.

Acknowledgment

This work was supported by Zhejiang Provincial Science and Technology program of Traditional Chinese Medicine (No. 2019ZA072).

Statement of Ethics

No humans were involved in this research.

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Author Contributions

H.-J.S.: conceptualization; H.-J.S.: design; X.-M.J.: clinical study; J.X.: intellectual content; X.-M.J.: literature research; Q.X.: data analysis; Q.X.: statistical analysis; H.-J.S.: writing review and editing.

References

- Mehta S. Age-Related Macular Degeneration. *Prim Care*. 2015 Sep;42(3):377–91.
- Wang H, Hartnett ME. Regulation of signaling events involved in the pathophysiology of neovascular AMD. *Mol Vis*. 2016 Feb;22:189–202.
- Klein R, Chou CF, Klein BE, Zhang X, Meuer SM, Saaddine JB. Prevalence of age-related macular degeneration in the US population. *Arch Ophthalmol*. 2011 Jan;129(1):75–80.
- Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. *Cell Mol Life Sci*. 2016 May;73(9):1765–86.
- Shaw PX, Stiles T, Douglas C, Ho D, Fan W, Du H, et al. Oxidative stress, innate immunity, and age-related macular degeneration. *AIMS Mol Sci*. 2016;3(2):196–221.
- Nita M, Grzybowski A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. *Oxid Med Cell Longev*. 2016;2016:3164734.
- Dick AD. Doyné lecture 2016: intraocular health and the many faces of inflammation. *Eye (Lond)*. 2017 Jan;31(1):87–96.
- Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. The inflammasome: a caspase-1 activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol*. 2009 Mar;10(3):241–7.
- Ildefonso CJ, Biswal MR, Ahmed CM, Lewin AS. The NLRP3 Inflammasome and its Role in Age-Related Macular Degeneration. *Adv Exp Med Biol*. 2016;854:59–65.
- Aachoui Y, Sagulenko V, Miao EA, Stacey KJ. Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. *Curr Opin Microbiol*. 2013 Jun;16(3):319–26.
- Shao BZ, Xu ZQ, Han BZ, Su DF, Liu C. NLRP3 inflammasome and its inhibitors: a review. *Front Pharmacol*. 2015 Nov;6:262.
- Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol*. 2009 Feb;7(2):99–109.
- Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev*. 2015 May;265(1):130–42.
- Gao J, Cui JZ, To E, Cao S, Matsubara JA. Evidence for the activation of pyroptotic and apoptotic pathways in RPE cells associated with NLRP3 inflammasome in the rodent eye. *J Neuroinflammation*. 2018 Jan;15(1):15.
- Jia Y, Xu R, Hu Y, Zhu T, Ma T, Wu H, et al. Anti-NDV activity of baicalin from a traditional Chinese medicine in vitro. *J Vet Med Sci*. 2016 Jun;78(5):819–24.
- Jung SH, Kang KD, Ji D, Fawcett RJ, Safa R, Kamalden TA, et al. The flavonoid baicalin counteracts ischemic and oxidative insults to retinal cells and lipid peroxidation to brain membranes. *Neurochem Int*. 2008 Dec;53(6-8):325–37.
- Nagaki Y, Hayasaka S, Kadoi C, Nakamura N, Hayasaka Y. Effects of scutellariae radix extract and its components (baicalein, baicalin, and wogonin) on the experimental elevation of aqueous flare in pigmented rabbits. *Jpn J Ophthalmol*. 2001 May-Jun;45(3):216–20.
- Chen H, Xu Y, Wang J, Zhao W, Ruan H. Baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat. *Int J Clin Exp Pathol*. 2015 Sep;8(9):10139–47.
- Fu S, Liu H, Xu L, Qiu Y, Liu Y, Wu Z, et al. Baicalin modulates NF- κ B and NLRP3 inflammasome signaling in porcine aortic vascular endothelial cells infected by Haemophilus parasuis causing Glässer's disease. *Sci Rep*. 2018 Jan;8(1):807.
- Srinivas NR. Baicalin, an emerging multi-therapeutic agent: pharmacodynamics, pharmacokinetics, and considerations from drug development perspectives. *Xenobiotica*. 2010 May;40(5):357–67.
- Yang SJ, Jo H, Kim JG, Jung SH. Baicalin attenuates laser-induced choroidal neovascularization. *Curr Eye Res*. 2014 Jul;39(7):745–51.
- Nakamura N, Hayasaka S, Zhang XY, Nagaki Y, Matsumoto M, Hayasaka Y, et al. Effects of baicalin, baicalein, and wogonin on interleukin-6 and interleukin-8 expression, and nuclear factor-kappaB binding activities induced by interleukin-1 β in human retinal pigment epithelial cell line. *Exp Eye Res*. 2003 Aug;77(2):195–202.
- Xiao JR, Do CW, To CH. Potential therapeutic effects of baicalein, baicalin, and wogonin in ocular disorders. *J Ocul Pharmacol Ther*. 2014 Oct;30(8):605–14.

- 24 Ding JD, Johnson LV, Herrmann R, Farsiu S, Smith SG, Groelle M, et al. Anti-amyloid therapy protects against retinal pigmented epithelium damage and vision loss in a model of age-related macular degeneration. *Proc Natl Acad Sci USA*. 2011 Jul;108(28):E279–87.
- 25 Liu RT, Gao J, Cao S, Sandhu N, Cui JZ, Chou CL, et al. Inflammatory mediators induced by amyloid-beta in the retina and RPE in vivo: implications for inflammasome activation in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2013 Mar;54(3):2225–37.
- 26 Haneklaus M, Gerlic M, O'Neill LA, Masters SL. miR-223: infection, inflammation and cancer. *J Intern Med*. 2013 Sep;274(3):215–26.
- 27 Wang H, Hao P, Zhang H, Xu C, Zhao J. MicroRNA-223 inhibits lipopolysaccharide-induced inflammatory response by directly targeting Irak1 in the nucleus pulposus cells of intervertebral disc. *IUBMB Life*. 2018 Jun; 70(6):479–90.
- 28 Zhou W, Pal AS, Hsu AY, Gurol T, Zhu X, Wirbisky-Hershberger SE, et al. MicroRNA-223 Suppresses the Canonical NF- κ B Pathway in Basal Keratinocytes to Dampen Neutrophilic Inflammation. *Cell Rep*. 2018 Feb;22(7):1810–23.
- 29 Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V. NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol*. 2012 Oct; 189(8):4175–81.
- 30 Yao J, Cao X, Zhang R, Li YX, Xu ZL, Zhang DG, et al. Protective Effect of Baicalin Against Experimental Colitis via Suppression of Oxidant Stress and Apoptosis. *Pharmacogn Mag*. 2016 Jul-Sep;12(47):225–34.
- 31 Ye C, Li S, Yao W, Xu L, Qiu Y, Liu Y, et al. The anti-inflammatory effects of baicalin through suppression of NLRP3 inflammasome pathway in LPS-challenged piglet mononuclear phagocytes. *Innate Immun*. 2016 Apr;22(3):196–204.
- 32 Wang Y, Hanus JW, Abu-Asab MS, Shen D, Ogilvy A, Ou J, et al. NLRP3 Upregulation in Retinal Pigment Epithelium in Age-Related Macular Degeneration. *Int J Mol Sci*. 2016 Jan;17(1):17.
- 33 Cookson BT, Brennan MA. Pro-inflammatory programmed cell death. *Trends Microbiol*. 2001 Mar;9(3):113–4.
- 34 Gottesman S. Small RNAs shed some light. *Cell*. 2004 Jul;118(1):1–2.
- 35 Soifer HS, Rossi JJ, Sætrom P. MicroRNAs in disease and potential therapeutic applications. *Mol Ther*. 2007 Dec;15(12): 2070–9.
- 36 Fukao T, Fukuda Y, Kiga K, Sharif J, Hino K, Enomoto Y, et al. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell*. 2007 May;129(3):617–31.
- 37 Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, et al. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1 β production in macrophages by targeting STAT3. *PLoS One*. 2012;7(8):e42971.
- 38 Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, McInnes IB, et al. Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1 β production. *J Immunol*. 2012 Oct;189(8): 3795–9.