Coumestrol inhibits inflammation, apoptosis and oxidative stress in retinal cells of diabetic retinopathy rats by SIRT1 Running title: CMS & SIRT1 on DR rats

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May 5, 2020

Abstract

Diabetes-induced oxidative stress is the key factor that initiates neuronal damage in the diabetic retina leading to diabetic retinopathy. This study was to investigate the possible effects of coumestrol (CMS) on inflammation, apoptosis and oxidative stress of retinal cells in streptozotocin-nicotinamide (STZ)-induced DR. Initially, a rat model of STZ was established by intraperitoneal injection of streptozotocin, and the expression of SIRT1 in retina tissues was determined by RT-qPCR. Next, the regulatory roles of CMS in oxidative stress, inflammation, and apoptosis of retinal cells were analyzed through detecting the expression of ROS, MDA, SOD, NO, iNOS, IL-6, TNF-α, and CRP, apoptotic factors (Cyt-C and Caspase-3) after ARPE-19 cells were treated with CMS. Moreover, the relationship between CMS and SIRT1 was analyzed. SIRT1 was lowly expressed in retina tissues. CMS treatment suppressed expression of ROS, MDA, iNOS, NO, IL-6, TNF-α, and CRP, as well as expression of Cyt-C and Caspase-3, but induced SOD expression in retina cells. CMS activated the expression of SIRT1, thereby inhibited oxidative stress, inflammation and apoptosis of retinal cells induced by DR. Taken together, the present study defines that CMS ameliorated DR by inhibiting inflammation, apoptosis and oxidative stress of retinal cells through the activation of SIRT1.

Background

As one of the major microvascular complication of diabetes mellitus (DM), diabetic retinopathy (DR) is considered as the leading cause of visual impairment and blindness worldwide (Stitt, Curtis, 2016). It is estimated that 2.4 million cases of blindness globally are resulted from DR (Dow, Mancini, 2018). Accompanied by the annual incidence of 2·2% to 12·7%, the progression of DR ranged from 3·4% to 12·3% (Sabanayagam, Banu, 2019). As a sight-threatening disorder, DR is characterized by neuronal and vascular dysfunction in the retina at early stages, and subsequently further impairment of visual acuity through neovascularization (Wang, Ling, 2017). The pathogenesis of DR is closely related to angiogenesis, oxidative stress, as well as chronic inflammation (Maghbooli, Emamgholipour, 2018). Multiple risk factors including hyperglycaemia, hypertension, advanced age, insulin treatment, induced fasting blood glucose level, longer DM duration, higher haemoglobin A1c concentration, and diet have close links with the occurrence and progression of DR (Dow, Mancini, 2018, Nawaz, Rezzola, 2019, Song, Yu, 2018). Although progress has been made in improving the vascular alterations, DR remains a major challenge in clinical therapy (Rubsam, Parikh, 2018). Thus, new and more accurate predictors to help provide better therapeutic strategies of DR are necessary.

Phytoestrogens, as natural chemicals, possess important modulatory activities on estrogen receptors, therefore affecting glucose homeostasis, and preventing DM among women (Glisic, Kastrati, 2018, Ye, Yang, 2019). For example, daidzein has been reported to be a potential candidate for the treatment of DM (Das, Sarkar, 2018). Coumestrol (CMS), as a coumestan isoflavone, exerts paramount roles in the treatment of estrogen-linked pathologies, such as DM (Li, Zhang, 2019). Besides, it was reported that 10-hydroxy-coumestrol

showed strong activity working as antihyperlipidemic agent in streptozotocin (STZ) induced diabetes (Seida, El-Hefnawy, 2015). Furthermore, CMS was found to promote mitochondrial biogenesis through the activation of Sirtuin 1 (SIRT1) in the skeletal muscle cells (Seo, Jeong, 2014). More importantly, activated SIRT1 has been used for treating DR with good outcomes. For example, a recent study has pointed out that down-regulating microRNA-377 inhibited high glucose and hypoxia-induced angiogenic functions and suppressed pro-inflammatory cytokines release via enhancing SIRT1, thereby attenuating DR (Maghbooli, Emamgholipour, 2018). In addition, a previous study demonstrated that formononetin treatment together with induced SIRT1 in kidney tissues of diabetic rats contributed to reduction of oxidative stress burden (Oza and Kulkarni, 2018). From aforementioned reports, we can make the speculation that a potential regulatory relationship among CMS, SIRT1, and DR might exist. Thus, the aim of the present study was to investigate the effects of CMS on oxidative stress, inflammatory response, and cell apoptosis of DR in relation to SIRT1.

Materials and methods

Ethics Statement

The study was strictly in accordance with the statement on the ethical use of animals in eye research of association for research in vision and ophthalmology (ARVO) and conducted with the principle of completing experiments with a minimum number of animals and minimizing the suffering of experimental animals.

Streptozotocin (STZ)-induced Diabetic Retinopathy (DR) Model Establishment

A total of 45 specific pathogen-free (SPF) grade male Sprague-Dawley (SD) rats aged 8 weeks old with an average weight of 250 - 300 g were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). The STZ induced DR model was established according to the following methods: STZ was dissolved with 0.1 mol/L citrate buffer (PH = 4.5) and 60 mg/kg of the mixture was injected intraperitoneally into rats after 12 h of fasting. After 72 h, blood samples were taken from the tail vein and blood glucose level was measured. The model was considered as successfully established when blood glucose level exceeded 16.7 mmol/L. Rats were then grouped into the sham, STZ, and CMS + STZ groups (with 15 rats in each group). Rats in the sham group were intraperitoneally injected with an equal dose of normal saline. Rats in the CMS + STZ group were treated with CMS as follows: from the day of establishment of STZ rat model, STZ induced DR rats were injected subcutaneously with 10 mg/kg, 50 mg/kg and 100 mg/kg CMS for 8 weeks, respectively, while rats in the sham and STZ groups were injected with equal doses of normal saline. 60 d after modeling, the rats were euthanatized and bilateral eyeballs were removed to detaching retinas. Retinas were homogenized using appropriate amount of phosphate buffer solution (PBS) immediately and centrifuged at 4°C for 20 min (12,000 rpm). Then the supernatant was transferred into another 1.5 mL centrifugal tube and stored at -80°C.

Cell Culture and Treatment

ARPE-19 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in dulbecco's modification of eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 UL/mL penicillin, and streptomycin in a 37°C, 5% CO2 incubator. The cells were sub-cultured every 2 d and treated with 0.25% trypsin. Because diabetes was characterized by hyperglycemia and hyperlipidemia, high glucose (HG) was used to induce DR cell model in ARPE-19 cells. Cells were grouped into four groups namely control, shRNA against SIRT1 (sh-SIRT1), CMS, and CMS + sh-SIRT1. Cells were inoculated to a 6 cm medium at a density of 2 × 105 and put into an incubator of 5% CO2 at 37°C until cells grew to 70% - 80%. On the day before transfection, cells were trypsinized and then dissociated into single cell suspension with culture medium. Then single cell suspension was inoculated to a 6-well plate at a density of 2 × 105 cells per well. After that, 10 μL of shRNA was mixed with Opti-MEM to a total volume of 250 μL for 5 min at room temperature, which then incubated with an equal volume of liposome for 20 min at room temperature to form a shRNA-liposome complex. Next, the complex was added into the 6-well plate at a density of 0.5 nL per well and mixed gently. Then each well was added with 1.5 mL serum-free DMEM medium and cultured at 37°C in 5% CO2 for 6 h. After that, medium was renewed with a normal serum-containing DMEM medium and continued to culture with 5% CO2 at 37°C for a proper period of time .

Detection of Reactive Oxygen Species (ROS) Level

The ROS level in the cells was measured using a ROS detection kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, cells were trypsinized, harvested, and then stained with dichlorofluorescein diacetate (DCFH-DA) according to the instructions of the manufacture. The signal was detected using a spectroscopic cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (Luo, Zheng, 2015). The positive control were cells treated only by the ROSup which was provided by the ROS detection kit.

Nitrite Test

Since nitric oxide (NO) was unstable and easy to form nitrates and nitrites in cells, the nitrite in the supernatant was measured as an indicator of NO production using an oxidized nitrite kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, according to the instructions, the supernatant was sequentially mixed with an equal volume of Gris reagent I and Gris reagent II, and then the concentration of nitrites was calculated according to the optical density (OD) value at 540 nm measured using a smart-spec plus spectrophotometer (Bio Rad laboratories, Hercules, CA, USA) (Stepuro, Chaikovskaya, 1997).

Enzyme Linked Immunosorbent Assay (ELISA)

The levels of IL-1β and TNF-α in the cell supernatant were detected by a ELISA kit (R & D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions with a smart spec-plus spectrophotometer (Bio Rad laboratories, Hercules, CA, USA) (Saferding, Puchner, 2017).

Hematoxylin-eosin (HE) Staining

Paraffin sections were dewaxed with xylene and rinsed successively with 99.9%, 97%, 75%, 50% ethanol and distilled water. Then paraffin sections were stained by hematoxylin for 1 - 3 min, rinsed with tap water for 1 min after each step, differentiated with 1% hydrochloric acid alcohol for 20 s, backed to blue with PBS for 30 s. Next, the sections were stained with eosin for 1 min, dehydrated with gradient ethanol, cleaned by xylene, and then sealed with Histomount. The retinas of rats in each group were observed and photographed.

Detection of Leakage Rate of Lactate Dehydrogenase (LDH)

LDH was determined by a CytoTox 96® Non-Radioactive Cytotoxicity assay kit (Promega Corporation, Madison, WI, USA). Cells were inoculated into a 96-well plate at a density of 105 - 106 and incubated with 5% CO2 at 37°C. After cells were treated with high glucose and free fatty acids (HG-FFA) for 1 h, 15 μL of lysis buffer [9% (v/v) Triton X-100 dissolved in water] was added to each well, and cells were incubated at 37°C for 45 min. Then 50 μL supernatant was transferred to another 96-well plate, which was then reacted with a pre-formulated substrate mixture (50 μL) at room temperature for 30 min in dark. Finally, 50 μL of stop solution was added to each well to terminate the enzyme reaction. And the OD value was measured at 490 nm by enzyme labeling instrument.

Transmission Electron Microscopy (TEM)

The anterior segment was removed immediately after eyeball extraction, and immersed in 2.5% glutaraldehyde solution for retina separation. Then retinas were immersed in 2.5% glutaraldehyde for 1 h, and fixed in 1% osmic acid for 1 h. Next, retinas were dehydrated with ethanol, embedded with Epon epoxy resin, followed by treated with electron staining, uranium acetate, lead citrate (each for 15 min). Retinas were then observed and photographed under TEM (HT7700; Exalens, Hitachi Plant Technologies Ltd, Tokyo, Japan).

TdT-mediated dUTP-biotin Nick end-labeling (TUNEL) Staining

The TUNEL assay was performed using DeadEnd Fluorometric TUNEL System kit (Promega, Madison, WI, USA). In brief, cells were fixed with 4% paraformaldehyde at 4°C for 25 min, penetrated with 0.2% Triton X-100 for 5 min, and incubated with 100 μL of equilibration solution per well at room temperature for 5 - 10 min. After aspirating the equilibration solution, 50 μL of the terminal deoxyribonuclease reaction solution

containing 45 μL of equilibration buffer, 5 μL of nucleotide mixture, and 1 μL of terminal deoxynucleotidyl transferase was added to each well, and the reaction was carried out at 37°C for 1 h. Then each well was mixed with 50 μL of 2 × soluble solids content (SSC) at room temperature for 15 min in the dark to terminate the reaction. After washing three times in PBS, cells were stained with 4',6-diamidino-2-phenyl indole (DAPI) (1 μL/mL) at room temperature for 15 min, followed by rinsed with PBS for three times. Next, cells were subjected with anti-fade solution (Molecular Probe, Cat#57461) and photographed under fluorescence microscope (IX71-F22FL/DIC, Olympus America Inc., Bethlehem, USA). Five visual fields of each group were randomly selected for observation, and the number of cells was counted.

Flow Cytometry

Cells in logarithmic phase (1 × 106) were collected for cell cycle detection, cells were fixed with 70% cold ethanol, and stained with 1 mL propidium iodide (PI) dye (50 μg/mL; Becton Dickinson Biosciences, Mountain View, CA, USA) in dark for 30 min. Cell cycle was detected by fluorescein isothiocyanate (FITC) Calibur flow cytometry (Becton Dickinson Biosciences, Mountain View, CA, USA).

Then same amount of cells in logarithmic phase were collected for cells apoptosis detection, cells were suspended with 1 × Annexin buffer and stained with 5 μL Annexin-V-FITC (Becton Dickinson Biosciences, Mountain View, CA, USA) at room temperature for 10 min. Then cells were suspended with 300 μL of 1 × Annexin. The apoptosis rate was detected by flow cytometry.

Immunofluorescence Staining

The frozen sections of rat retina were washed, penetrated with Triton and blocked with goat serum blocking solution for 30 min. Sections were incubated with rabbit anti-rat SIRT1 polyclonal antibody (ab189494, 1 : 100; Abcam, Cambridge, UK) overnight, respectively. Next, sections were incubated with fluorescent second antibody for 1 h, stained by DAPI and sealed with glycerinum. The sections were finally observed under a confocal microscope to obtain the image of staining and images were analyzed by a confocal software.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were extracted from cells using a Trizol kit (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary DNA (cDNA) according to the instructions of TaqMan MicroRNA Assays Reverse Transcription Primer (4427975; Applied Bio-systems, Foster City, CA, USA). The conditions of reverse transcription reaction were 37°C for 30 min and 85°C for 5 s. Then 5 μL of cDNA obtained above was taken as template for quantitive polymerase chain reaction (PCR) amplification using a QuantiTect SYBR Green RT-PCR kit. The reaction conditions were comprised of pre-denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s. The expression of each gene was analyzed. Real-time quantitative PCR results were analyzed using the 2-Dct theory. U6 was served as an internal reference for miRNA, and β-actin was served as an internal reference for the other genes. The fold changes were calculated by means of relative quantification (2-ΔΔCt method) (Livak and Schmittgen, 2001). The primers are depicted in Table 1.

Western Blot Analysis

Total proteins were extracted from cells using radio-immune precipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and quantified by Bradford assay. Next, 40 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBST) buffer at room temperature for 1 h. The membrane was probed with primary antibodies: rabbit anti-CD11b antibody (Cat. No. PA5-29633; 1 : 3000), rabbit anti-Iba-1 antibody (Cat. No. PA5-21274; 1 : 3000), rabbit anti-iNOS antibody (Cat. No. PA3-030A; 1 : 2000), rabbit anti-β-actin antibody (No. PA1-183; 1 : 2000) at 4 °C overnight. All the antibodies above were purchased from Invitrogen (Carlsbad, CA, USA). Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA; 1 : 4000) was added and incubated with the membranes at room temperature for 1 h. β-actin was served as an internal reference. Enhanced chemiluminescence detection system (ECL; Amersham

Pharmacia Biotech, Piscataway, NJ) was used to develop the protein band and Image Pro Plus 7.0 software (Media Cybernetics, Inc., Rockville, Maryland, USA) was used for analysis.

Statistical Analysis

All data were processed by SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA). Data was presented as mean ± standard deviation. The comparison between multiple groups should be analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical significance was assumed when $p < 0.05$.

RESULTS

SIRT1 was Lowly Expressed in Rat Model of STZ

In order to study the effects of CMS on DR, the STZ rat model was established by intraperitoneal injection of STZ and the rats were divided into sham, STZ and CMS + STZ groups. Retinal tissues were collected and observed after HE staining, which presented that the vascular intima of retinal tissues in sham-operated rats consisted of a layer of intact and continuous endothelial cells without hyperplasia. Compared with the sham-operated rats, the rats injected with STZ exhibited obvious edema, capillary wall thickening, endothelial cell proliferation and fibrous tissue proliferation, which were partially rescued by CMS in a dose-dependent manner (Figure 1A). After that, the ultrastructure of retinas was observed under TEM, which showed that compared with sham-operated rats, the retinal capillary endothelial cells in rats treated with STZ were markedly swollen, the cell body became round and protruded into the lumen with swelled and vacuolated mitochondria as well as thickened basement membrane thickened, which were partially blocked by treatment of CMS in a dose-dependent manner (Figure 1B). In addition, the results of RT-qPCR and western blot analysis showed that compared with sham-operated rats, the expression of SIRT1 was notably decreased in rats treated with STZ, which was rescued by CMS in a dose-dependent manner (Figure 1C - D). Therefore the expression of SIRT1 was significantly inhibited in STZ rats, and CMS was able to enhance the expression of SIRT1 in STZ rats.

CMS Alleviated Oxidative Stress, Inflammation and Apoptosis in the Retina of DR Rats

Further, we examined oxidative stress and inflammation in STZ rats. It was shown that compared with the sham-operated rats, the reactive oxygen species (ROS) and 3,4-methylenedioxyamphetamine (MDA) levels were significantly increased, while superoxide dismutase (SOD) level was decreased in the retinal tissue of rats injected with STZ, which could be blocked by co-treatment of CMS in a dose-dependent manner (Table 2). In addition, the results of RT-qPCR and western blot analysis suggested that compared with sham-operated rats, the expression of iNOS was notably increased in rats injected with STZ, which was inhibited by CMS in a dose-dependent manner (Figure 2A - B). Nitrite test showed that compared with sham-operated rats, the expression of NO was notably increased in rats injected with STZ, which was blocked by CMS in a dose-dependent manner (Figure 2C). According to the result of ELISA, compared with sham-operated rats, rats injected with STZ presented notably increased expression of interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and C-reactive protein (CRP). And compared with rats injected with STZ, rats injected with CMS + STZ presented a significantly decreased expression of IL-6, TNF-α, and CRP, and the effect of CMS was dose-dependent (Figure 2D - F). Then cell apoptosis was detected by TUNEL, which found that apoptosis in rats injected with STZ notably increased when compared with sham-operated rats. And compared with rats injected with STZ, apoptosis in rats injected with CMS + STZ obviously reduced, and the effect of CMS was dose-dependent (Figure 2G). Besides, western blot analysis showed that the expression of apoptotic factors (Cyt-C and Caspase-3) in rats injected with STZ was higher than that in sham-operated rats. And compared with rats injected with STZ, rats injected with CMS + STZ showed a reduced expression of apoptotic factors (Cyt-C and Caspase-3), and the effect of CMS was dose-dependent (Figure 2H). Taken together, it was concluded that CMS was beneficial to alleviate the increased oxidative stress, inflammation and apoptosis in retina in the STZ rat model induced by DR.

CMS Activated SIRT1 Expression in a STZ Cell Model

Next, the relationship between CMS and SIRT1 was investigated. The results of RT-qPCR and western blot analysis demonstrated that expression of SIRT1 in ARPE-19 cells transfected with sh-SIRT1-1, sh-SIRT1-2, or sh-SIRT1-3 all declined dramatically with sh-SIRT1-2 exhibiting the highest effect (Figure 3A - B), therefore sh-SIRT1-2 was selected for the following study. Immunofluorescence staining revealed that stimulation with HG-FFA notably decreased activity and nuclear accumulation of SIRT1; also, the activity and nuclear accumulation of SIRT1 were significantly higher in cells treated with CMS than in cells stimulated with HG-FFA, the effect of CMS on SIRT1 was dose-dependent, and the increase in cells treated with 2-CMS was the highest (Figure 3C - D). According to the result of RT-qPCR, compared with the controls, the expression of SIRT1 was decreased in ARPE-19 cells stimulated with HG-FFA, while the expression of SIRT1 was notably higher in cells stimulated with CMS than in cells stimulated with HG-FFA, the effect of CMS on SIRT1 was dose-dependent, and the increase in cells treated with 2-CMS was the highest (Figure 3E). Collectively, CMS could activate the expression of SIRT1 in a STZ cell model, and sh-SIRT1-2 and 2-CMS were selected for the following study.

CMS Inhibited Oxidative Stress and Inflammation of Retinal Cells through Activating SIRT1

In order to evaluate whether CMS regulated oxidative stress and inflammation in retinal cells with DR through SIRT1, we silenced SIRT1 expression in ARPE-19 cells. ELISA was performed to detect the expression of oxidative stress-related factors, which observed that CMS treatment obviously reduced the expression of ROS and MDA, but increased SOD expression in ARPE-19 cells stimulated with HG-FFA, which could be blocked by sh-SIRT1 (Table 3). In addition, the results of RT-qPCR and western blot analysis demonstrated that iNOS expression in ARPE-19 cells stimulated with HG-FFA was declined by CMS treatment, which could be reversed by sh-SIRT1 (Figure 4A - B). Nitrite test revealed that CMS treatment notably decreased the expression of NO in HG-FFA stimulated ARPE-19 cells, which could be rescued by sh-SIRT1 (Figure 4C). According to the result of ELISA, in ARPE-19 cells stimulated with HG-FFA, notably decreased expression of IL-6, TNF-α, and CRP was observed after CMS treatment, which could be reversed by sh-SIRT1 (Figure 4D - F). Therefore, CMS was beneficial to alleviate the oxidative stress and inflammation in retinal cells of DR through activating SIRT1.

CMS Inhibits Apoptosis of Retinal Cells of DR

At last, to measure the effect of CMS on apoptosis, we analyzed cell apoptosis and examined the expression of apoptosis related factors in ARPE-19 cells stimulated with HG-FAA. Flow cytometry suggested that the apoptosis rate of ARPE-19 cells were notably decreased after CMS treatment, which could be reversed by sh-SIRT1 (Figure 5A - B). Besides, western blot analysis showed that ARPE-19 cells treated with CMS presented lower expression of Cyt-C and Caspase-3, which could be enhanced by sh-SIRT1 (Figure 5C). Taken together, all these data suggested that CMS could inhibit apoptosis of retinal cells of DR through activating the expression of SIRT1.

DISCUSSION

As a progressive ophthalmopathy, DR was resulted from pathological alterations mediated by long-term accumulations of hyperglycemia in the retina of DM patients (Nawaz, Rezzola, 2019). Several treatment approaches, such as physiotherapy and drug therapy, have been demonstrated to be able to alleviate DR (Pardue and Allen, 2018). A previous finding has suggested that phytoestrogens such as biochanin A could play a therapeutic role in DR treatment (Mehrabadi, Salemi, 2018). By establishing a STZ model in rats, the current study explored the potential roles of CMS and SIRT1 in oxidative stress, inflammatory response, and cell apoptosis of retinal cells, and got the conclusion that CMS induced the activation of SIRT1 and effectively inhibited oxidative stress, inflammatory response, as well as cell apoptosis in rats with DR.

Initially, based on our findings, SIRT1 was expressed at a low level in rats with DR. SIRT1, as a nuclear protein, was found to regulate inflammation and apoptosis as well as other metabolic pathways through deacetylation of histones, non-histones and transcription factors (Kowluru, Santos, 2014a). Consistent with our study, Li *et al.*also demonstrated that SIRT1 was down-regulated in the endothelial cells of STZ-induced diabetic mice and enhancing SIRT1 could increase resistance to oxidative stress and inhibit diabetic vascular

endothelial dysfunction (Li, Kim, 2016). In addition, activation of retinal SIRT1 was reported to protect the retina from damage in the diabetic milieu (Maghbooli, Emamgholipour, 2018).

Next, the relationship between CMS and SIRT1 in mice with DR was studied. We proved that SIRT1 was activated by CMS, indicating that the functions of CMS on DR might be associated with SIRT1. Furthermore, our study found that CMS inhibited oxidative stress and inflammatory response in rats in with DR through the activation of SIRT1, indicated by decreased levels of ROS, MDA, iNOS, NO, increased level of SOD, and reduced levels of inflammatory factors. Induction of ROS production was proved to be one of the major reasons for retinal cell death in DR by mitochondrial dysfunction (Pearsall, Cheng, 2019). It was reported that increased levels of MDA, NO and decreased iNOS, SOD levels were found in the DM-model rats, blocking of which would contribute to enhanced anti-oxidation (Long, Yu, 2012). DR was related to elevated release of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α (Gao, Li, 2017, Sharma, Purohit, 2015). A previous study demonstrated that CMS inhibited production of hydrogen peroxide-induced ROS, lipid peroxidation, and prevented reduction of intracellular glutathione levels and SOD activity (Jeon, Seo, 2012). Besides, CMS has been observed to prevent IL-1β-induced catabolic effects by inhibiting inflammation and inflammatory cytokines in chondrocytes (You, Cho, 2017). Furthermore, enhanced SIRT1 was associated with reduced oxidative stress and ameliorated DR (Kowluru, Santos, 2014b). In addition, activated SIRT1 was proved to down-regulate pro-inflammatory cytokines, inhibit oxidative stress and cell inflammation, thereby improve DR (Karbasforooshan and Karimi, 2018).

Furthermore, we proved that CMS suppressed apoptosis of retinal cells, thereby ameliorated DR through the activation of SIRT1, as supported by reduced levels of Cyt-C, Caspase-3. Phytoestrogens, including CMS, genistein, and daidzein, were reported to suppress apoptosis by working with 17β-estradiol (Schmidt, Michna, 2005). Genistein, for example, inhibited chemical hypoxia-induced cell apoptosis by suppressing the mitochondrial apoptotic pathway and level of Caspase-3 (Shi, Zhang, 2019). What's more, activated SIRT1 together with silenced protein arginine methyltransferases was involved in suppressing the oxidative stress-induced apoptosis of retinal pigment epithelial cells in DR, as supported by reduced level of Caspase-3 (Kim, Park, 2015). Moreover, another study also proved that activation of SIRT1 was related to inhibited HG-induced apoptosis and inflammation cytokines production in DR (Tong, Peng, 2019). Collectively, our study suggested that CMS might be an important reagent for DR treatment, which regulated the pathogenesis of DR by activating SIRT1.

In summary, we have proved that CMS could inhibit oxidative stress, inflammatory response, and cell apoptosis in rats with DR through the activation of SIRT1, which may have potentially important therapeutic implications in the treatment of oxidative stress and inflammatory response in patients suffering from DR.

ACKNOWLEDGEMENTS

We acknowledge and appreciate our colleagues for their valuable efforts and comments on this paper.

CONFLICTS OF INTEREST

None.

FUNDING

None.

AUTHOR CONTRIBUTIONS

Weiying Sun designed the study. Chunzhi Li , Hongcheng Gao and Juan Miao collated the data, carried out data analyses and produced the initial draft of the manuscript. Weiying Sun contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

ADDITIONAL STATEMENT

We declare that this is an original unpublished work and is not beingsubmitted for publication elsewhere at the same time.

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FIGURE LEGENDS

FIGURE 1 SIRT1 was down-regulated in STZ induced DR rats. A, Retinal tissues observed by HE staining ($\times$ 400). B, Ultrastructure of retinas of rats observed by TEM ($\times$ 10000). C, The expression of SIRT1 measured by RT-qPCR. D, The expression of SIRT1 measured by Western blot analysis. * $p < 0.05$ compared to the sham-operated rats, and $^{\#}$ $p < 0.05$ compared to the rats injected with STZ. The results

were measurement data and expressed as mean ± standard deviation. Comparisons between multiple groups should be analyzed by one-way ANOVA with Tukey's post hoc test. (n = 15). DR, diabetic retinopathy; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SIRT1, Sirtuin 1; TEM, transmission electron microscopy; ANOVA, analysis of variance; n, number.

FIGURE 2 CMS reduced oxidative stress, inflammation and apoptosis in the retina of DR rats. Rats were treated with sham, STZ, CMS + STZ, respectively. A, The expression of iNOS in rats determined by RT-qPCR. B, The protein level of iNOS in rats measured by Western blot analysis. C, The expression of NO in rats measured by nitrite test. D - F, The expression of IL-6, TNF-α, and CPR in rats measured by ELISA. G, Cell apoptosis in rats detected by TUNEL (× 400). H, The expression of apoptotic factors (Cyt-C and Caspase-3) in rats determined by Western blot analysis. * $p < 0.05$ compared to the sham-operated rats, and $^{\#}$ $p < 0.05$ compared to the rats injected with STZ. The results were measurement data, which were expressed as mean ± standard deviation. Comparisons between multiple groups should be analyzed by one-way ANOVA with Tukey's post hoc test (n = 15). iNOS, inducible nitric oxide synthase; CMS, coumestrol, NO, nitric oxide.

FIGURE 3 CMS activated the expression of SIRT1. ARPE-19 cells were transfected with sh-SIRT1-1, sh-SIRT1-2, and sh-SIRT1-3, respectively and treated with HG-FFA, 0.5-CMS, 1-CMS, and 2-CMS, respectively. A, The expression of SIRT1 in ARPE-19 cells detected by RT-qPCR. B, Protein levels of SIRT1 in ARPE-19 cells detected by Western blot analysis. C - D, The SIRT1 activity and nuclear accumulation in ARPE-19 cells detected by immunofluorescence staining (× 400). E, The expression of SIRT1 in ARPE-19 cells detected by RT-qPCR.* $p < 0.05$ compared to the controls, and $^{\#}$ $p < 0.05$ compared to cells stimulated with NC. The results were measurement data and expressed as mean ± standard deviation. Comparisons between multiple groups should be analyzed by one-way ANOVA with Tukey's post hoc test. (n = 15). NC, negative control.

FIGURE 4 CMS suppressed oxidative stress and inflammation of retinal cells induced by DR through activating SIRT1. A, The expression of iNOS in ARPE-19 cells determined by RT-qPCR. B, Protein level of iNOS in ARPE-19 cells determined by Western blot analysis. C, The expression of NO in ARPE-19 cells measured by nitrite test. D - F, The expression of IL-6, TNF-α, and CPR in ARPE-19 cells measured by ELISA.* $p < 0.05$ compared to the controls, and $^{\#}$ $p < 0.05$ compared to cells stimulated with sh-SIRT1. The results were measurement data and expressed as mean ± standard deviation. Comparisons between multiple groups should be analyzed by one-way ANOVA with Tukey's post hoc test. (n = 15)

FIGURE 5 CMS suppressed apoptosis of retinal cells of DR. A - B, Cell cycle and apoptosis of retinal cells detected by flow cytometry analysis. C, Protein levels of Cyt-C and Caspase-3 in ARPE-19 cells detected by Western blot analysis. * $p< 0.05$ compared to the controls, and $^{\#}p < 0.05$ compared to cells stimulated with sh-SIRT1. The results were measurement data and expressed as mean ± standard deviation. Comparisons between multiple groups should be analyzed by one-way ANOVA with Tukey's post hoc test. (n = 15)