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Formononetin attenuates hydrogen peroxide (H_2O_2)-induced apoptosis and NF-κB activation in RGC-5 cells

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Abstract. – OBJECTIVES: Diabetic retinopathy is a common diabetic eye disease caused by changes in retinal ganglion cells (RGCs). Several studies suggest that the oxidative stress plays a role in the pathogenesis of diabetic retinopathy in adults. Formononetin is a flavone with powerful antioxidant properties that exists naturally in various plants and Chinese medicine. In the present study, an attempt has been made to investigate the antioxidative effects of formononetin on H_2O_2-induced apoptosis of RGC-5 cells.

MATERIALS AND METHODS: Exposure of retinal ganglion cells (RGCs) to the indicated concentrations of formononetin and H_2O_2 for 24 h, analyzed by MTT assay. Cells were stained with Annexin V-FITC and PI, analyzed by flow cytometry. And the level of superoxide anions, malondialdehyde (MDA, a marker of lipid peroxidation), 8-hydroxy-2-deoxyguanosine (8-OHdG, indicator of oxidative DNA damage) and Mn-SOD (manganese superoxide dismutase) activity were measured by kits.

RESULTS: Formononetin reduced hydrogen peroxide (H_2O_2)-induced apoptosis and improved the levels or activity of indicators of oxidative stress. Formononetin also inhibited the activation of nuclear factor-kappaB (NF-κB), which is a significant transcription factor for RGC-5 apoptosis.

CONCLUSIONS: Formononetin may be developed as a antioxidant drug to treat diabetic retinopathy.

Keywords:
RGC-5; NF-κB; Oxidative stress; H_2O_2; Formononetin; Apoptosis

INTRODUCTION

Diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years. The National Eye Institute data is very alarming; it suggests that about half of the people with diabetes in the United States have at least some form of retinopathy, and about 700,000 have some serious retinal disease. Diabetic retinopathy is affecting approximately 65,000 people in the United States alone causing 12,000 to 24,000 new cases of blindness each year [1].

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Oxidative stress plays an important role in diabetic complications and reactive oxygen species (ROS) are considered a causal link between elevated glucose and metabolic abnormalities important in the development of diabetic complications. Retina and capillary cells experience increased oxidative damage in the diabetic milieu, and the antioxidant defense mechanism is impaired [2]. Many studies have shown that oxidative stress played the key role on diabetic retinopathy, which directly caused cell dysfunction or apoptosis in retinal ganglion cells (RGCs) [3]. H_2O_2 could induce oxidative stress and apoptosis in RGC-5 cell line [4]. However, diabetic retinopathy is also well-known as the result of microvascular retinal changes. Oxidative stress activated vascular endothelial growth factor (VEGF) survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration[5]. Hyperglycemia-induced intramural pericyte death and thickening of the basement membrane lead to incompetence of the vascular walls. These damages change the formation of the blood-retinal barrier and also make the retinal blood vessels become more permeable[6].

Nuclear Factor-kappaB (NF-κB) is actually a family of structurally-related proteins that are involved in the control of a large number of normal cellular and body functions, such as immune and inflammatory responses, developmental processes, cellular growth and apoptosis [7, 8]. The general term NF-κB traditionally refers to the p50/p65 (p50/RelA) heterodimer, which is an apoptotic

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gene regulator. NF-κB-p65, a subunit of NF-kappaB transcription complex, provides the gene regulatory function and plays a crucial role in the development of diseases [9]. And downregulation of NF-kappaB signaling by mutant huntingtin proteins induces oxidative stress and cell death [10].

H_2O_2 induced apoptosis and NF-kappaB expressions in vascular endothelial cells and retinal ganglion cells (RGCs)[8,11]. However, NF-κB, as important transcription factors, mediates various biological functions *in vivo*, but not as final effector molecules. Previous studies reveal that NF-κB acts in synergy with other transcription factors such as Ap-1 (activating protein-1) or Sp1 (specific protein-1) in order to mediate an effective transcriptional activation. This suggests that a distinct combination of binding sites for different transcription factors within individual gene promoters contributes to the selective regulation of gene expression [12].

Formononetin (biochanin B) is an *O*-methylated isoflavone phytoestrogen from the root of *Astragalus membranaceus*. Several mechanisms have been proposed to explain the *in vitro* anti-inflammatory actions of formononetin, such as antioxidant activity, inhibition of eicosanoid-generating enzymes or the modulation of the production of pro-inflammatory molecules [13]. And flavones significantly suppress inflammation-associated gene expression by blocking NF-kappaB and AP-1 activation pathway in animal models [14-16].

Based on the results described above, the aim of the present study was to investigate the effects of formononetin on hydrogen peroxide (H_2O_2)-induced apoptosis using the transformed rat retinal ganglion cell line RGC-5.

Materials and Methods

Reagents

Formononetin (HLPC content 98%) was purchased from Shanghai Winherb Medical S & T Development Co. Ltd (Shanghai, China) Dulbecco's modified Eagle media (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). ATP assay kit was obtained from Beyotime (Jiangsu, China). The Annexin-V-FITC Apoptosis Detection Kit was purchased from BD Biosciences, San Jose, CA, USA. Competitive ELISA kit for MDA, 8-OHdG and MnSOD was purchased from Cayman Chemical Co., Ann Arbor, MI, USA.

Cell Culture

RGC-5 cells (American Type Culture Collection: ATCC, Manassas, VA, USA) were routinely maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 g/mL streptomycin (Gibco). Cells were grown in a humidified incubator of 95% air and 5% CO2 at 37 °C. Cells were passaged when 80% confluent.

MTT Assay

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded in 96-well dishes at 1 × 104 to 2 × 104 cells per well, and pretreated with or without formononetin for 2h. Each well was then supplemented with 10 μL MTT (Sigma, Aldrich, St Louis, MO, USA) and incubated for 4 h at 37 °C. The medium was then removed, and 150 μL dimethyl sulfoxide (Sigma) were added to solubilize the MTT formazan. The optical density (OD) was read at 490 nm.

Flow Cytometry

To estimate the number of apoptotic cells, cells were fluorescently labeled by addition of 20 μL of binding buffer, 5 μL of Annexin V-FITC and 5 μL of propidium iodide. After the incubation at room temperature in dark for 15 min, cells were applied to flow cytometry analysis. A minimum of 10,000 cells in the gated region was analyzed by BD FACS Calibur Flow Cytometer, Franklin Lakes, NJ, USA. Results were interpreted by the percentage of total cells appearing in each quadrant.

ATP assay

The level of intracellular ATP was determined using the ATP Bioluminescence Assay Kit (Roche Applied Science, Indianapolis, IN, USA). Cultured cells were lysed with a lysis buffer, followed by centrifugation at 12,000 × g for 1 min at 4°C. Finally, the level of ATP was determined by mixing 50 μl of the supernatant with 50 μl of luciferase reagent, which catalyzed the light production from ATP and substrate. The emitted light was linearly related to the ATP concentration and measured using a microplate luminometer.

Oxygen consumption

To evaluate the ability of cellular oxygen consumption (VO_2) during drug treatment, the Micro Respirometry System (Strathkelvin, Mitocell S200, North Lanarkshire, MLI 5RX, Scotland) was used to measure oxygen content of culture media. Be-

fore the assay, primary hepatocytes just isolated from mice were pre-treated in low oxygen conditions for 2 h in suspension culture with drugs.

Detection of the level of superoxide anion ($O_2^{\cdot-}$), MDA, 8-OHdG and MnSOD

We used the probe dihydroethidium (DHE; Molecular Probes,Eugene, OR, USA) to detect intracellular $O_2^{\cdot-}$. $O_2^{\cdot-}$ oxidizes DHE to ethidium, which generates a red fluorescent signal. Fluorescence spectrometry of tissue $O_2^{\cdot-}$ production was performed according to previous methods [17].

Cell samples were prepared as a 10% homogenate in 0.9% saline using a homogenizer on ice according to their respective weight. Then the homogenate was centrifuged, and the supernatant was collected and diluted. The assay of the MDA levels was performed according to the manufacturer's instructions for OxiSelect™ MDA Adduct ELISA Kit (STA-332, Carlsbad, CA, USA).

Competitive ELISA for 8-OHdG was performed according to the manufacture's protocol of OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation, STA-320, Franklin Lakes, NJ, USA). Sample DNA assays were performed in triplicate. Standard 8-OHdG was assayed over a concentration range of 0.125-20 ng/ml in duplicates for each experiment. The average concentration of 8-OHdG per microgramof DNA for each group was calculated for each sample. Controls without added DNA and appropriate blanks were also incorporated into experiments.

The enzyme activity of MnSOD was measured in 5-10 μg of cell protein using Thermo MnSOD Induction Kit (8407001, Rockford, IL, USA). The method utilizes tetrazolium salt to quantifying $O_2^{\cdot-}$ generated by xanthine oxidase and hypoxanthine. The standard curve was generated using a quality-controlled SOD standard. MnSOD activity was determined by performing the assay in the presence of potassium cyanide to inhibit Cu-ZnSOD, thus measuring the residual MnSOD activity.

Transient Transfection and Luciferase Reporter Assay

The luciferase reporter construct 3 × NF-κB-LUC (NF-κB responsive elements) was transiently transfected into RGC-5 cells grown in 24 well plates respectively using the lipofectamine 2000 reagent according to the manufacturer's instructions. The luciferase reporters construct driven by three copies of the NF-κB response elements from B. M. Forman (Department of Gene Regulation and Drug Discovery, Beckman Research Institute of City of Hope National MedicalCenter, Duarte, CA, USA). A plasmid expressing the gene encoding β-galactosidase driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was simultaneously cotransfected as an internal control. The medium was replaced 4 h after transfection. Twenty-four hours after transfection, the cells were treated with the indicated concentrations of H_2O_2 and formononetin for an additional 24 h and harvested for luciferase reporter assays as described previously [18].

Statistical analysis

Differences between groups were analyzed using the two-sided *t* test and ANOVA with $p < 0.05$ considered statistically significant.

RESULTS

Formononetin (Form) Protects RGC-5 cells from H_2O_2-Induced Apoptosis

More and more research pinpoints oxidative stress as a root cause of diabetic retinopathy[19]. Chronic exposure to H_2O_2 promoting NF-kappaB activation led to apoptosis in RGC-5 cells[20]. To investigate the effect of formononetin on the apoptosis of retinal ganglion cells (RGCs), cell viability was determined in RGC-5 cells using MTT assays. Formononetin significantly inhibited the decrease of RGC-5 cell viability induced by H_2O_2 in a dose-dependent manner (Figure 1A). Quantitative evaluation of apoptosis through annexin V-FITC/PI staining was analyzed by Flow Cytometry. As shown in Figure 1B, the rate of apoptotic cells were risen to 38.94% with the treatment of H_2O_2 (0.1 mmom/l) for 24 h. Furthermore, pretreatment with formononetin prevented H_2O_2-induced apoptosis in a dose-dependent manner. As formononetin accelerated the amount of RGC-5 cells, the production of ATP (Figure 1C) and the total oxygen uptake (Figure 1D) were also promoted significantly, compared with the group treated with H_2O_2 (0.1 mmom/l). Taken together, it suggested that formononetin had a strong anti-apoptotic effect in RGC-5 cell.

Formononetin (Form) reduces oxidative stress induced by H_2O_2 in RGC-5 cells

Oxidative stress has been implicated in the pathogenesis of diabetic retinopathy[21, 22]. To estimate the anti-oxidative effect of formononetin, superoxide anion (DHE), MDA and 8-OHdG levels and MnSOD activity in the retina tissues were

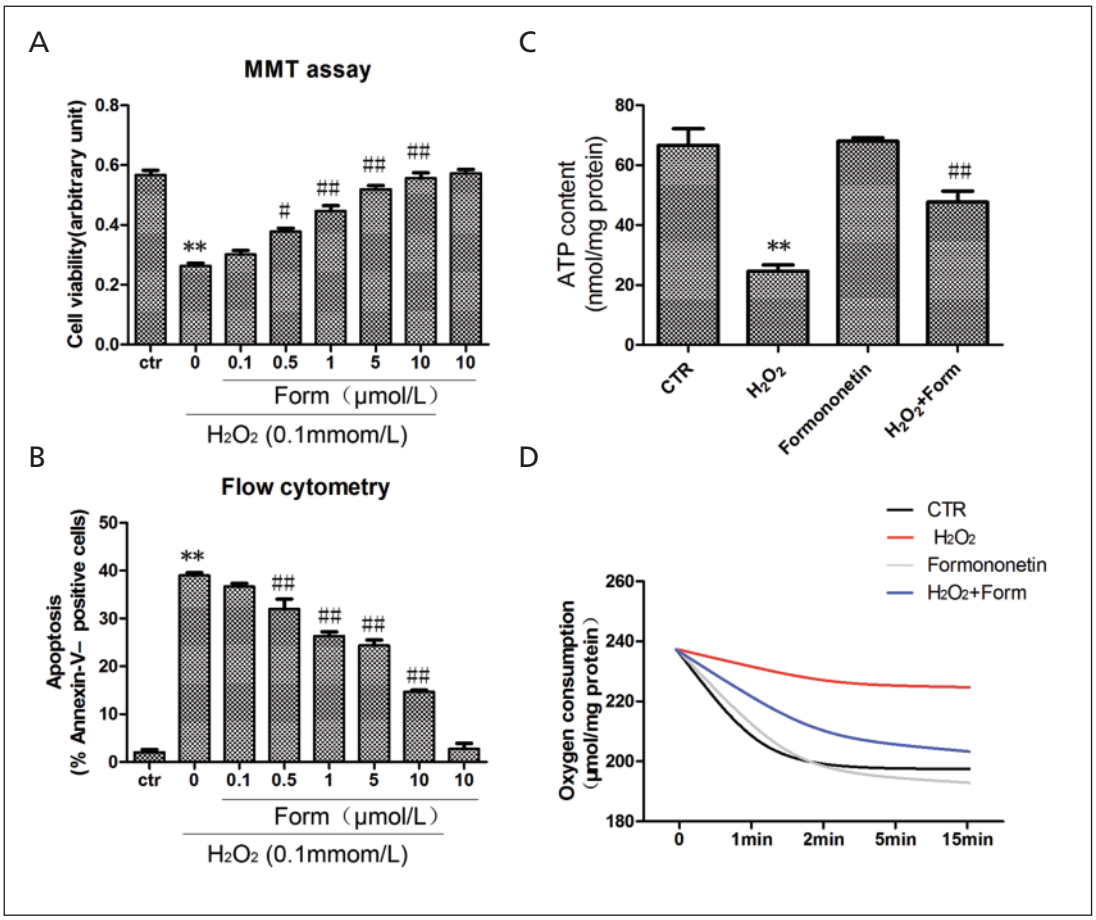

Figure 1. Formononetin inhibited H_2O_2-induced apoptosis in RGC-5 cells. (A)Cells were treated with the indicated concentrations of formononetin and H_2O_2 for 24 h, analyzed by MTT assay. (B) Cells were stained with Annexin V-FITC and PI, analyzed by flow cytometry. Data are expressed as % of Annexin V-FITC- positive and PI-negative cells (early stage of apoptosis). (C) The intracellular ATP concentrations were evaluated after exposure to formononetin and H_2O_2 for 24 h. Before the oxygen consumption test (E), RGC-5 cells were pre-treated in low oxygen conditions for 2 h in suspension culture with formononetin and H2O2. Values are the means ± SD (n = 3) of three independent experiments. * $p < 0.05$, ** $p < 0.01$ *vs*. control (DMSO: dimethyl sulfoxide), $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* group treated with H_2O_2 (0.1μmol/L).

measured. As an initial indicator of ROS, we used DHE staining, which is a probe for $O_2^{\cdot-}$ and produces red fluorescence as a result of the complex between ethidium and DNA as described by previous study[23]. To quantitate changes in $O_2^{\cdot-}$ levels, we measured total DHE fluorescence in RGC-5 cell. $O_2^{\cdot-}$ levels was induced in H_2O_2 control, and formononetin reversed such effects (Figure 2A). MnSOD is a pivotal enzyme scavenging ROS *in vivo*. MnSOD activity in RGC-5 cell was significantly increased after treatment with formononetin (Figure 2B). Oxidative stress, as determined by the concentrations of MDA (as a marker of lipid peroxidation) and 8-OHdG (indicator of oxidative DNA damage), remained elevated in H_2O_2 control. Administration of formononetin inhibited the increase of MDA and 8-OHdG levels (Figure 2C and D) in RGC-5 cell. Therefore, formononetin show an anti-oxidative effect in RGC-5 cell line.

Formononetin (Form) Suppressed H_2O_2 Induced Activation of NF-κB in RGC-5 Cells

Nuclear factor kappa B (NF-κB), as a transcription factor, is thought to play an important role in onset of RGCs apoptosis mediated by H_2O_2[24,25]. To determine the effects of formononetin on the inhibition of NF-κB during apoptosis using the luciferase reporter assay, the plasmid (pGL3-3 × NF-κB-Luc) was transiently transfected into RGC-5 cells. The results showed that formononetin dose-dependently decreased the level of NF-κB activation induced by H_2O_2 (Figure 3A). In our studies, H_2O_2 had no signifi-

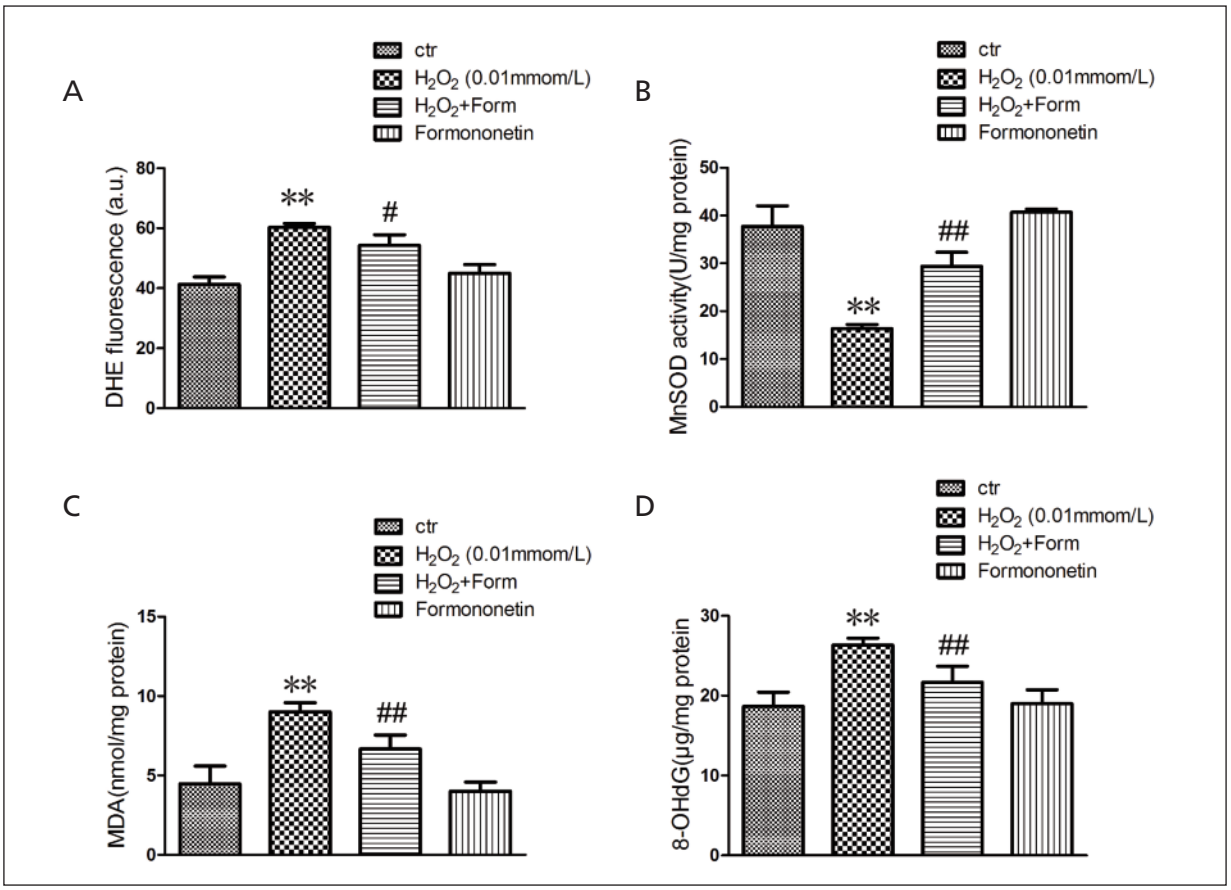

Figure 2. Effects of superoxide(DHE), MDA and 8-OHdG levels and MnSOD activity in RGC-5 cells. (A)superoxide level (DHE), (B) MnSOD activity, (C)MDA production and (D)8-OHdG level were measured according to the manufacturer's instructions for each assay. Values are the means ± SD (n = 3) of three independent experiments. * $p < 0.05$, ** $p < 0.01$ *vs*. control (DMSO: dimethylsulfoxide), $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs*. group treated with H_2O_2 (0.01μmol/L).

cant effect on activation of AP-1 (Figure 3B). These data indicated that NF-κB might be a mediator for H_2O_2-initiated toxicity in RGC-5 cells and H_2O_2-induced increases of NF-κB activation were significantly reversed by formononetin.

DISCUSSION

As nature's most powerful antioxidant, flavones have been documented to show a wide range of benefits in human clinical studies on several serious health concerns[26,27]. Bio-flavonoids comprise a group of phenolic secondary plant metabolites that are widespread in nature [28]. A good number of studies have already demonstrated many biological activities of flavonoids using different experimental models and treatments. Formononetin (biochanin B), is a major phytoestrogen found in alfa and clover sprouts, that has been reported to have beneficial effects for Alzheimer's disease (AD) and anti-oxidant properties[13,19,20]. However, the role of formononetin on eye disease from oxidative stress is not clear. Our results provided a new insight into potential beneficial effects of antioxidants in the treatment of retinopathy in patients. Formononetin attenuated H_2O_2-induced apoptosis and oxidative stress in the transformed rat retinal ganglion cell line RGC-5. Therefore, formononetin may be used in diabetic retinopathy.

It is well-known that the development of diabetic retinopathy is caused by chronic multi-factors such as gluco-lipotoxicity, inflammatory mediators and oxidative stress[1,31,32]. Many diabetes-induced metabolic abnormalities are implicated in its development, and appear to be influenced by elevated oxidative stress; the exact mechanism of its development remains elusive. In our studies, formononetin could decrease the level of oxidative stress that caused dysfunction, apoptosis and ATP depletion (Figure 1). Moreover, oxidative stress played the key role on diabetic retinopathy, which

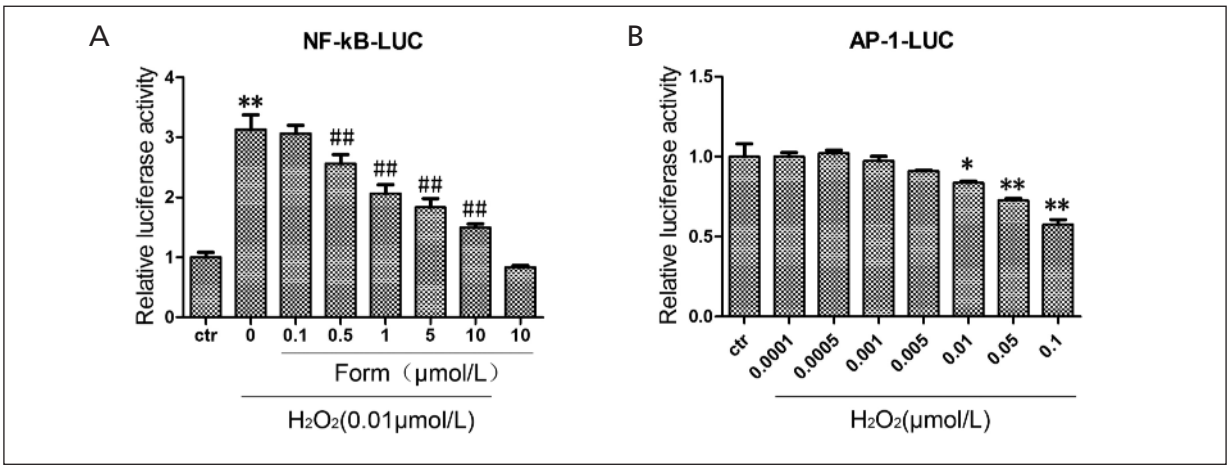

Figure 3. The effect of formononetin on H_2O_2-induced activation of NF-κB in RGC-5 cells. Cells were treated with the indicated concentrations of formononetin and H_2O_2 for 24 h. (A) Formononetin dose-dependently reduced NF-κB activation in luciferase reporter assay. (B) After cells were treated with the indicated concentrations of H_2O_2 for 24 h, AP-1 activation was determined by luciferase reporter assay. Values are the means ± SD (n = 3) of three individual experiments. * $p < 0.05$, ** $p < 0.01$ *vs*. ctr (DMSO: dimethylsulfoxide); # $p < 0.05$, ## $p < 0.01$ *vs*. group treated with H2O2 excluding formononetin.

directly caused cell dysfunction or apoptosis in retinal ganglion cells (RGCs)[19], and activated VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration [33]. Thus, oxidative stress and antioxidant defenses may be as a target for the treatment of diabetic eye disease.

Nuclear factor kappa B (NF-κB) is a transcription factor thought to play an important role in onset of cell apoptosis[34]. To determine the effects of NF-κB inhibition during apoptosis, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was given (data not shown). And NF-κB has become a candidate target for new anti-inflammatory and anti-apoptosis treatment[35]. The classical pathway of nuclear factor-kappa B (NF-kappaB) activation by several inducers mainly involves the phosphorylation of IkappaBalpha by a signalsome complex composed of IkappaBalpha kinases (IKKalpha and IKKbeta). However, in some cell types hydrogen peroxide (H_2O_2) has been shown to activate an alternative pathway that does not involve the classical signalsome activation process [36]. These results suggest that NF-kappaB activation is involved in H_2O_2-induced apoptosis in many cells.

CONCLUSIONS

In this study, we investigate the effects of formononetin on H_2O_2-induced apoptosis and oxidative stress in the transformed rat retinal ganglion cell line RGC-5. Therefore, formononetin may be developed as a new drug to treat diabetic eye disease from oxidative stress.

Conflict of interest

The Authors declare that they have no conflict of interests.

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