

Naringin attenuates diabetic retinopathy by inhibiting inflammation, oxidative stress and NF- κ B activation *in vivo* and *in vitro*

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ABSTRACT

Objective(s): Naringin, an essential flavonoid, inhibits inflammatory response and oxidative stress in diabetes. However, whether naringin has beneficial effects on diabetic retinopathy (DR) remains unknown.

Materials and Methods: Streptozotocin (STZ, 65 mg/kg) was intraperitoneally injected into male rats (8 weeks old weighting 200-250 g) to establish diabetic model, then naringin (20, 40 or 80 mg/kg/day) was intraperitoneally injected into the diabetic rats for twelve weeks. Glial fibrillary acidic protein (GFAP) level, thickness of ganglion cell layer (GCL) and ganglion cell counts were assessed in diabetic retina *in vivo*. Naringin (50 μ M) that significantly inhibited high glucose (HG, 25 mM)-induced cell proliferation was used to treat rat Muller cell line (rMC1) *in vitro*. Inflammatory response, oxidative stress and activation of nuclear factor kappa B (NF- κ B) p65 were evaluated in retina *in vivo* and in rMC1 cells *in vitro*.

Results: Naringin alleviated DR symptoms as evidenced by the increased retinal ganglion cells and decreased GFAP level in rat retina. Naringin exhibited anti-inflammatory and antioxidative effects as confirmed by the down-regulated pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), and the up-regulated antioxidants, glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in DR rats. Moreover, we found that naringin inhibited HG-induced proliferation, abnormal inflammatory response and oxidative stress in rMC1 cells. In addition, the enhanced nuclear translocation of NF- κ B p65 in diabetic rat retina and HG-induced rMC1 cells was suppressed by naringin.

Conclusion: Naringin attenuates inflammatory response, oxidative stress and NF- κ B activation in experimental models of DR.

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Introduction

Diabetes is an epidemic disease affecting a large proportion of the population worldwide (1). Neuropathy, microangiopathy (retina, glomerulus) and macroangiopathy (heart, lower limbs) are the main pathological features among diabetic complications (2, 3). Diabetic retinopathy (DR) is the most common diabetic complication, which is caused by retinal vascular leakage, inflammatory response, and neovascularization (4). DR remains the leading cause of blindness among working age people worldwide (5). The lines of therapy for treatment of DR are laser photocoagulation treatment (6, 7), hyperbaric oxygen therapy (8) and drug treatment (9). Unfortunately, current approaches exhibit adverse side effects (10).

Hence, more efficient therapeutic strategies are needed to be investigated for patients with DR. Recent studies revealed that inflammation and oxidative stress played important roles in the pathogenesis of DR (11-13). Therefore, we investigated whether natural extract, which has no adverse side effects, can suppress the inflammation and oxidative stress in experimental models of DR.

Citrus flavonoids have been demonstrated to provide protection against many diseases, including diabetes and diabetic complications by attenuating oxidative stress and inflammatory reaction (14, 15). Flavanones, the major and typical flavonoid class, may be regarded as the precursors of all other flavonoid classes (16, 17). Among flavanones, naringin

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(4',5,7-trihydroxy flavanone-7-rhamnoglucoside), was the most abundant flavonoid in grapefruit, sour orange and *Citrus* seed extract (18, 19), while investigations of its therapeutic effects were limited. Naringin is present in Chinese herbal medicines and citrus fruits (20, 21), which can prevent oxidative injury or disease by up-regulating the activities of antioxidative enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and paraoxonase (PON) (22). Naringin also possesses numerous other biological and pharmacological activities, including anti-inflammation (23), anti-ulcer (24), anti-cancer activities (25, 26). Naringin has been reported to improve the healing of diabetic foot ulcer by inhibiting hyperglycemia, oxidative stress, inflammation and growth factor expressions (27). Naringin also modulated the endogenous biomarker to inhibit neuropathic pain in streptozotocin (STZ)-induced diabetic neuropathy in rats (28) and alleviated diabetic kidney disease by inhibiting nuclear factor- κ B (NF- κ B) signaling pathway (29). However, the therapeutic effects of naringin on DR are still unknown and the underlying mechanisms need further investigations. We hypothesize that naringin can suppress oxidative stress, inflammation and regulate the activation of NF- κ B signaling pathway in experimental models of DR.

Therefore, we investigated whether naringin inhibited oxidative stress and inflammation in STZ-induced diabetic rats and high glucose (HG)-induced rat Muller cell line (rMC1). Also, this study assessed the effects of naringin on proliferation ability in HG-induced rMC1, and expression level of NF- κ B p65 was assessed in retinal tissues of STZ-induced rats *in vivo* and in HG-induced rMC1 cells *in vitro*. This study first provides insights into the role of naringin in resistance to DR.

Materials and Methods

Animal model and tissue samples

The healthy rats were obtained from Vital River Laboratory Animal Technology Co Ltd (Beijing, China). Male rats (8 weeks old) weighting 200-250 g were used as experimental animals. The rats were intraperitoneally injected with STZ (65 mg/kg; Solarbio, Beijing, China), and serum glucose levels were detected five days later. The rats with glucose levels > 16.7 mmol/l were considered as diabetes and selected for further experiments. The experiments were allocated into six groups: group 1 served as control rats, group 2 served as naringin control rats (80 mg/kg), group 3 served as diabetic control rats, group 4 served as diabetic rats with naringin (20 mg/kg), group 5 served as diabetic rats with naringin (40 mg/kg), group 6 served as diabetic rats with naringin (80 mg/kg). In groups 2, 4, 5 and 6, the rats were intraperitoneally injected with the corresponding dose of naringin each day for 12 weeks. In groups 1 and 3, the rats were intraperitoneally injected with the same volume of

physiological saline. Twelve weeks after the injection, the rats were weighted and the serum glucose levels were determined. After an overnight fast, the rats were anaesthetized and blood was drawn from inferior vena cava. The rats were killed and retinal tissues were obtained and kept in -80 °C. Partly retinal tissues were stored with 10 % formaldehyde solution. The animals used in this study were maintained following the guidelines Institutional Animal Care and Use Committee.

Histological analysis

To investigate the effects of naringin on thickness and cell numbers of ganglion cell layer (GCL), hematoxylin and eosin (H&E) staining was performed. The fixed retinal tissues were dehydrated with a graded series of ethanol, embedded in paraffin and cut into 5 μ m thick sections. The sections were deparaffinized with xylene and rehydrated with a graded series of ethanol. Thereafter, the sections were stained with hematoxylin (Solarbio) and eosin (Sinopharm, Beijing, China), and then GCL was observed with a microscope at a magnification of 400 \times (Olympus, Tokyo, Japan).

Immunohistochemistry (IHC)

IHC assay was performed to detect the effects of naringin on expression of NF- κ B p65 in rat retinal tissues. Sample sections (5 μ m) were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed with sodium citrate buffer in a microwave for 10 min. The sections were added with 3 % H₂O₂ for 15 min at room temperature (RT). Thereafter, the sections were blocked with goat serum (Solarbio) for 15 min at RT. After serum deprivation, the sections were incubated with NF- κ B p65 primary antibody (1:50; Santa cruz, Santa Cruz, CA, USA) at 4 °C overnight and then with a Biotin-labeled Goat Anti-Rabbit IgG(H+L) secondary antibody (1:200; Beyotime, Beijing, China) at 37 °C for 30 min. Then the sections were incubated with HRP-labeled streptavidin (Beyotime) at 37 °C for 30 min and color was developed using DAB (Solarbio). The sections were counterstained with hematoxylin (Solarbio). The positive staining was visualized using a microscope (Olympus).

Cell culture

rMC1 cells were purchased from CHI Scientific, Inc. (Jiangyin, Jiangsu, China). rMC1 cells were cultured with Dulbecco's modified Eagle medium (DMEM; Gibco Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37 °C in 5 % CO₂ humidified environment.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

For analysis of the effects of naringin on rMC1 cell proliferation at different time periods after HG

addition, MTT assay was carried out. Briefly, cells at 90 % confluence were transferred to a 96-well plate (3×10^3 cells/well) in quintuplicate. The cells were cultured with 0.2 % serum medium and treated with different concentrations of naringin (1, 5, 10, 25, 50, or 100 μ M) for 2 hr. After incubation, cells were treated with 25 mM HG for 24 hr, 48 hr and 72 hr, respectively. Then the treated cells were added with 5 mg/ml MTT. After incubation for 4 hr, the purple formazan crystals were dissolved with 200 μ l of DMSO (Sigma, St. Louis, MO, USA). The absorbance values at 490 nm were measured using a microplate reader (BioTek, Winooski, VT, USA).

Based on cell proliferation analysis, the naringin with 50 μ M which significantly reduced the HG-induced cell proliferation was selected for the later experiments. Thereafter, the rMC1 cells were treated with naringin (50 μ M) for 2 hr, and then treated with 25 mM HG for 48 hr.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to detect the effects of naringin on inflammatory reaction. The levels of inflammatory cytokines (tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6)) were determined in retinal tissues and in rMC1 cells by corresponding ELISA kits according to the manufacturer's instructions (USCN, Wuhan, China). In the rat retinal tissues, tissue homogenate was centrifuged and the protein samples were harvested. Protein concentrations were determined by Enhanced BCA Protein Assay Kit (Beyotime) according to the manufacturer's instructions. The results were read using a microplate reader (BioTek) and the concentrations of inflammatory cytokines were calculated by generating a standard curve.

Determination of oxidative stress markers

The levels or activities of oxidative stress markers were detected to analyze the effects of naringin on oxidative stress. The level of glutathione (GSH), and activities of superoxide dismutase (SOD) and catalase (CAT) were determined in retinal tissues and in rMC1 cells using specific assay kits according to the manufacturer's instructions (Nanjing Jiancheng Co., Ltd, Nanjing, China).

Western blot

Western blot was performed to analyze the effects of naringin on NF- κ B activation. The protein was harvested with RIPA (Beyotime) and quantified with Enhanced BCA Protein Assay Kit (Beyotime) according to the manufacturer's instructions. Equal amounts of proteins were separated on a SDS-PAGE gel and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Thereafter, the membrane was blocked with 5% non-fat dried milk for 1 hr. The membrane was incubated with NF- κ B

p65 primary antibody (1:400; Boster, Wuhan, China) at 4 °C overnight. After washing with TTBS, the membrane was incubated with HRP-labeled Goat Anti-Rabbit IgG(H+L) secondary antibody (1:5000; Beyotime) at 37 °C for 45 min. The membrane was then treated with ECL reagent (7 sea biotech, Shanghai, China) following the manufacturer's instruction. The protein expression levels were detected using a gel imaging system (Liuyi, Beijing, China). The expression of NF- κ B p65 in the cytoplasm was normalized to β -actin and in the nucleus to histone H3.

Immunofluorescence assay (IF)

IF was carried out to analyze glial fibrillary acidic protein (GFAP) expression in rat retinal tissues and the distribution of NF- κ B p65 in rMC1 cells. The retinal sections embedded in paraffin were deparaffinized before antigen retrieval. The rMC1 cells seeded on glass cover slips were treated with 4% paraformaldehyde for 15 min and then permeabilized with 0.1% tritonX-100 for 30 min. The tissue sections or cell slides were blocked with goat serum (Solarbio) and incubated with GFAP primary antibody (1:100; Santa cruz) or NF- κ B p65 primary antibody (1:200; Santa cruz) at 4 °C overnight. The secondary antibody Cy3-labeled Goat Anti-Mouse IgG(H+L) (1:500 for tissue sections or 1:50 for cell slides; Beyotime) was applied for 60 min at RT. The nucleus was stained with DAPI (Biosharp, Beijing, China). The images were captured by a fluorescence microscope (Olympus).

Statistical analysis

Data analysis was performed with SPSS20.0 (IBM SPSS Inc., Chicago, IL, USA). Comparisons were determined using one-way ANOVA followed by Duncan test, with $P < 0.05$ considered significant. All data were presented with a standard deviation (SD).

Results

Naringin regulates DR in rat model

The weight and serum glucose values in rat model were determined and analyzed. Body weight of diabetic rats treated with naringin was significantly higher than those of diabetic rats, naringin improved body weight in a dose-dependent manner (20 mg/kg naringin, $P < 0.05$; 40 or 80 mg/kg naringin, $P < 0.01$) (Figure 1A). Serum glucose values in diabetic rats treated with naringin were significantly decreased by almost a quarter ($P < 0.01$) than corresponding values in diabetic rats (Figure 1B). Meanwhile, GCL thickness ($P < 0.01$) and ganglion cell number ($P < 0.05$) were significantly increased after treatment with naringin (80 mg/kg) (Figure 1C-E). Results also showed that GFAP level was reduced after naringin treatment in diabetic rats (Figure 1F).

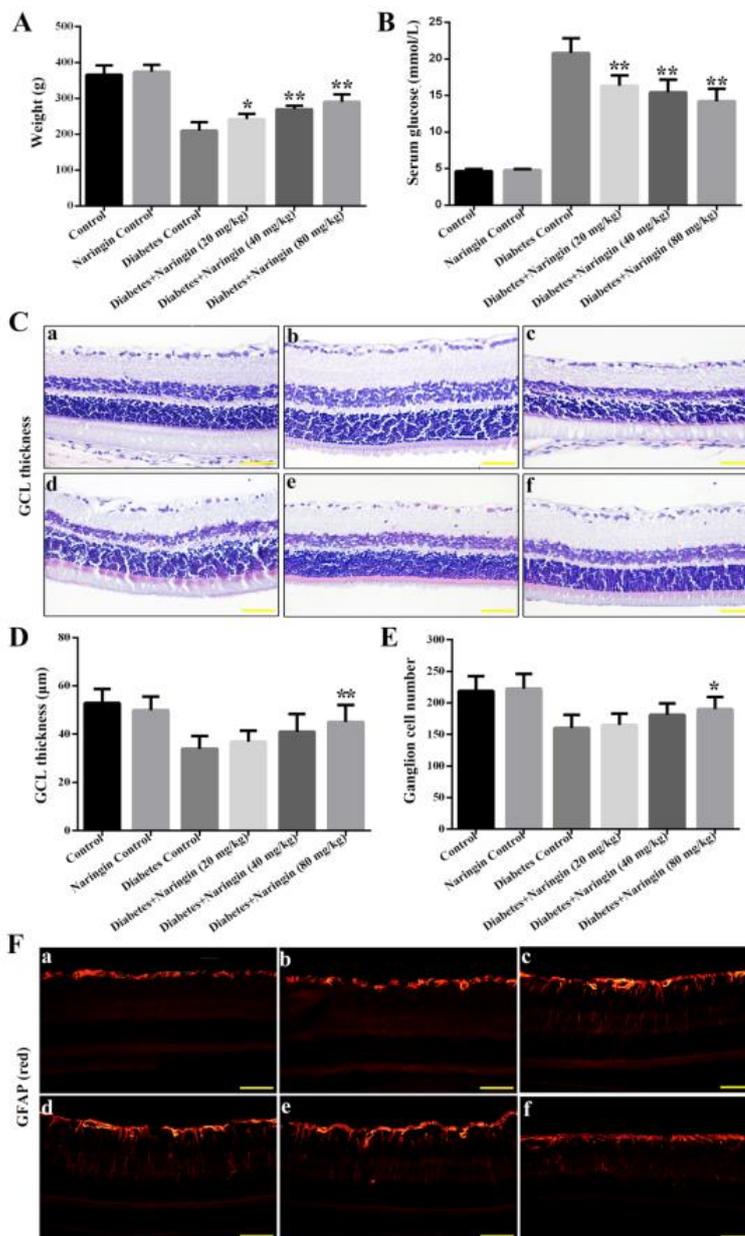


Figure 1. Naringin alleviated DR in STZ-induced diabetic rats. The body weights (A) and serum glucose concentrations (B) of diabetic model after 12 weeks of treatment with different doses of naringin. (C) GCL in retinal tissues staining with hematoxylin and eosin. The quantification of GCL thickness (D) and ganglion cells number (E). (F) The expression levels of GFAP in retinal tissues. For (C) and (F), (a) control group, (b) 80 mg/kg naringin group, (c) diabetes control group, (d) diabetes administered with 20 mg/kg naringin, (e) diabetes administered with 40 mg/kg naringin, (f) diabetes administered with 80 mg/kg naringin. Bars represented standard deviations. * $P < 0.05$, ** $P < 0.01$ versus diabetes control group. Scale bar, 50 μm

Naringin inhibits inflammatory response, oxidative stress and NF- κ B p65 activation in diabetic model

All three inflammatory cytokines TNF- α , IL-1 β and IL-6 were significantly down-regulated by about half ($P < 0.01$) by naringin (80 mg/kg) treatment (Figure 2A-2C). Meanwhile, the level of GSH ($P < 0.01$) and activities of SOD ($P < 0.01$) and CAT ($P < 0.05$) were significantly up-regulated by 50% to 100% in diabetes rats which were administered with naringin (40 or 80 mg/kg) (Figure 2D-2F). The expression

level of NF- κ B p65 in retinal tissues obtained from diabetic model was determined by IHC assay. The NF- κ B level was significantly increased under diabetic condition, while the level was decreased with the treatment with naringin (Figure 2G). From the above results, we found that naringin significantly attenuated inflammatory response and oxidative stress, and suppressed NF- κ B signaling pathway activation in STZ-induced rat model of DR.

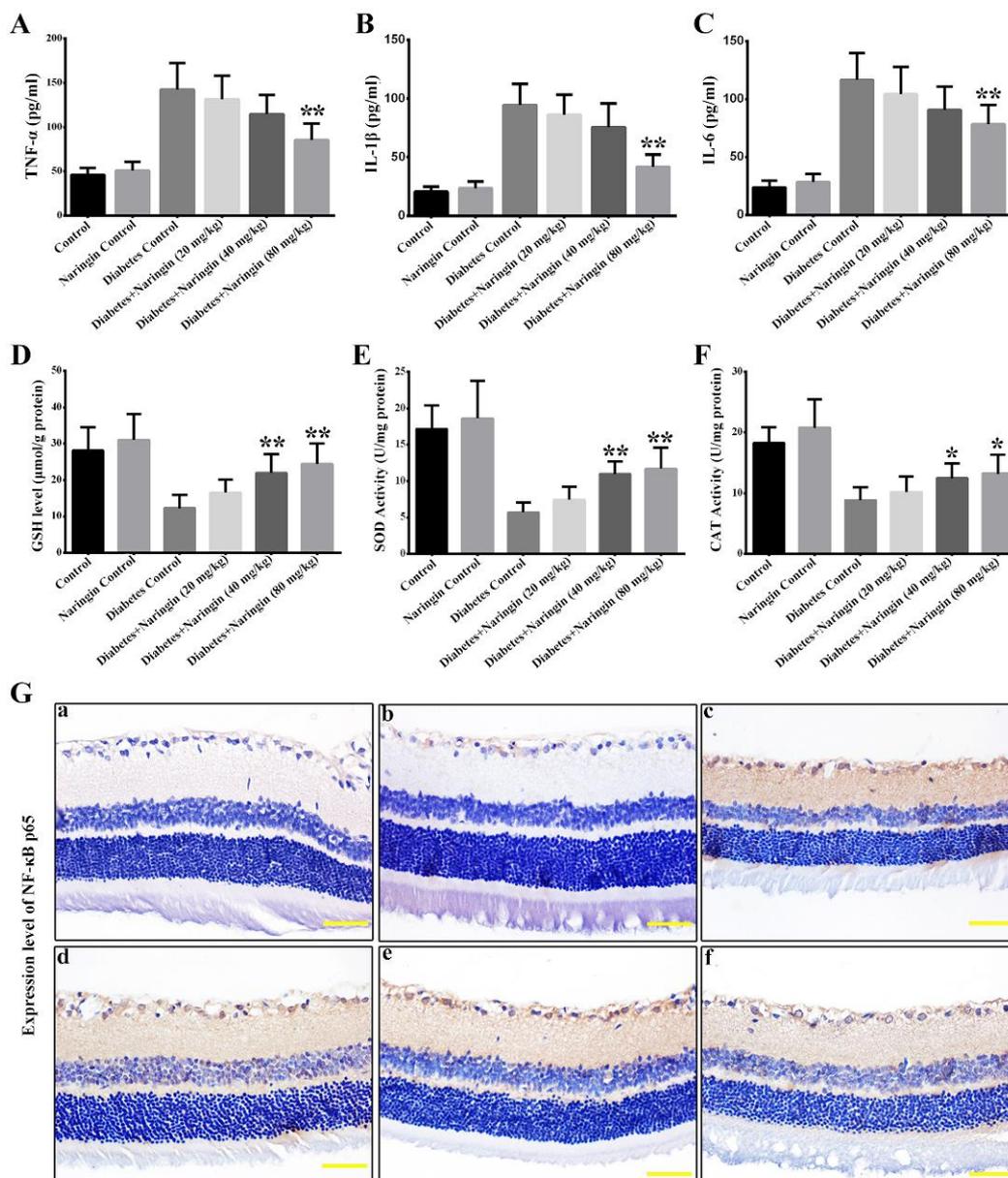


Figure 2. Effects of naringin on the levels of inflammatory cytokines, antioxidants and NF-κB p65 in STZ-induced rat model of DR. Inflammatory cytokines TNF-α (A), IL-1β (B), IL-6 (C) levels were decreased by naringin treatment in a dose-dependent manner. (D-E) The level of GSH (D), activities of SOD (E) and CAT (F) were increased by naringin treatment in a dose-dependent manner. (G) The NF-κB p65 levels among groups were determined by IHC. Bars represented standard deviations. * $P < 0.05$, ** $P < 0.01$ versus diabetes control group

Naringin restrains HG-induced cell proliferation, inflammatory response and oxidative stress in rMC1 cells

Cell proliferation of rMC1 cells was detected by MTT assay. Naringin treatment significantly inhibited HG-induced proliferation in rMC1 cells at 48 hr (50 and 100 μM, $P < 0.01$) and 72 hr (1, 5, 10 μM, $P < 0.05$, 25, 50, and 100 μM, $P < 0.01$) (Figure 3A). Results also showed that inflammatory cytokines (TNF-α, IL-1β, and IL-6) were significantly decreased by almost half ($P < 0.05$), and level of GSH, activities of SOD and CAT had nearly doubled ($P < 0.05$) by naringin treatment compared with HG group (Figure 3B-3G).

Naringin suppresses NF-κB p65 activation in rMC1 cells

The level of NF-κB p65 in the cytoplasm and nucleus of rMC1 cells was determined by western blot and IF assay. Results from western blot and IF showed that NF-κB p65 was mainly located in the cytoplasm in control cells, while HG treatment induced NF-κB p65 translocation into the nucleus. Naringin treatment efficiently attenuated NF-κB p65 translocation into the nucleus induced by HG (Figure 4).

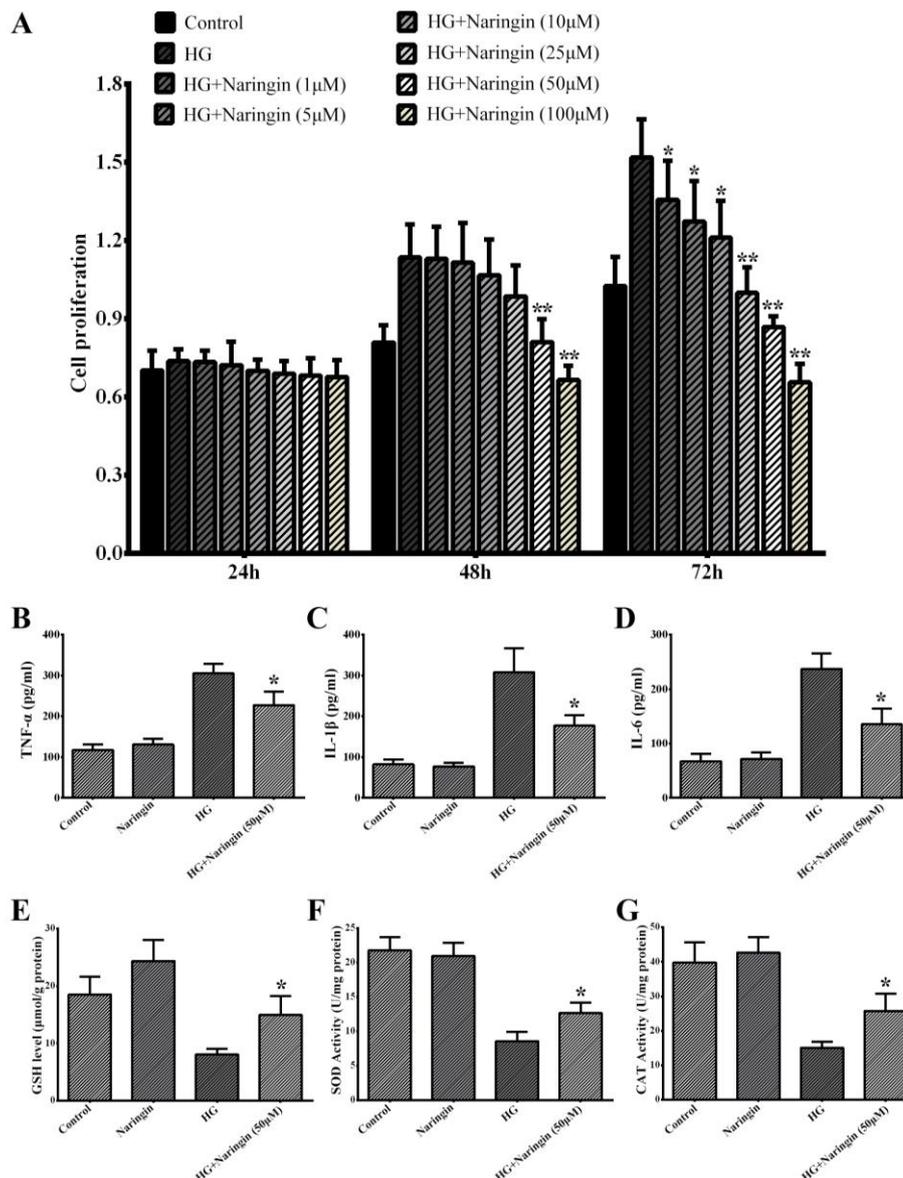


Figure 3. Effects of naringin on cell proliferation, inflammatory response and oxidative stress in HG-induced rMC1 cells. (A) HG-induced cell proliferation was inhibited by naringin treatment. The inflammatory cytokines TNF- α (B), IL-1 β (C), IL-6 (D) levels were decreased by naringin treatment. The levels or activities of antioxidants GSH (E), SOD (F) and CAT (G) were increased by naringin treatment. Each experiment was run in triplicate and bars represented standard deviations. * $P < 0.05$, ** $P < 0.01$ versus HG group

Discussion

Naringin, the main active ingredient of Chinese herbal medicines and citrus fruits, has recently been demonstrated to have pharmacological effects on diabetes and diabetic complications through inhibiting oxidative stress and inflammatory reaction (14, 29). In the present study, we investigated the regulation and function of naringin in HG-treated rMC1 cells *in vitro* and in retinal tissues from STZ-induced rats *in vivo*. Naringin increased body weight, GCL thickness as well as ganglion cell number; decreased serum glucose levels and GFAP expression. Naringin down-regulated inflammatory cytokines (TNF- α , IL-1 β , IL-6) levels and up-regulated antioxidants (GSH, SOD, CAT) both *in vivo* and *in vitro*.

Exogenous naringin reduced proliferation ability of rMC1 cells, and suppressed NF- κ B p65 translocation into the nucleus in rMC1 cells *in vitro* and in retinal tissues *in vivo*.

DR, a microvascular complication of diabetes, has also been considered as a neurodegenerative dimension as well (30). Activation of GFAP (a marker for glia activation), loss of ganglion cells and thinning of GCL represent the neurodegeneration in DR (31, 32). According to our results, naringin increased body weight, GCL thickness as well as ganglion cell number; decreased serum glucose level and GFAP protein level, which suggested that naringin prevented against neurodegeneration in DR.

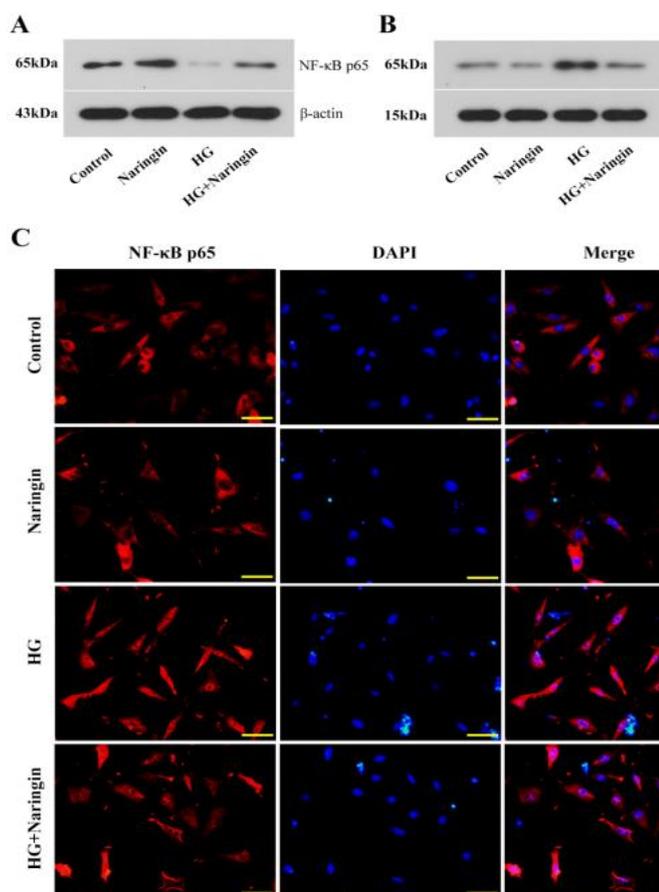


Figure 4. Effects of naringin on the translocation of NF- κ B p65 in HG-induced rMC1 cells. The NF- κ B p65 levels in the cytoplasm (A) and in the nucleus (B) were detected by western blot. (C) The expression level of NF- κ B p65 in rMC1 cells was detected by IF. The red fluorescence indicated NF- κ B p65 and blue fluorescence for nuclei. Scale bar, 50 μ m

Elevated inflammatory reaction and oxidative stress regulated by inflammatory cytokines and antioxidants, respectively, play important roles in the pathogenesis of DR (11, 33). Several natural extracts in flavanone subclass such as hesperetin and eriodictyol have been reported to provide therapeutic benefits on DR by attenuating retinal oxidative stress and inflammation in diabetic rats (34, 35). However, whether naringin can regulate oxidative stress and inflammation in DR has not been investigated. A previous study showed that naringin exhibited antidiabetic effects in type 2 diabetic rats by down-regulating malondialdehyde (MDA), nitric oxide (NO), TNF- α and IL-6 levels, and up-regulating GSH, vitamin C, and vitamin E levels (14). Naringin also has been reported to alleviate diabetic foot ulcer by promoting angiogenesis and inhibiting endothelial apoptosis through down-regulating inflammatory mediators, TNF- α , IL-1 β and IL-6, and up-regulating the expressions of growth factors, insulin-like growth factor-1 (IGF-1), transforming growth factor- β (TGF- β) and vascular endothelial growth factor c (VEGF-c)

(27). Naringin attenuated diabetic kidney disease by down-regulating oxidative stress markers MDA, reactive oxygen species (ROS) production, and inflammatory factors TNF- α , MCP-1, ICAM-1 and VCAM-1 levels, and up-regulating antioxidants SOD, GSH-Px levels (29). These data illustrated that naringin attenuated diabetes and several diabetic complications through inhibiting oxidative stress and inflammation. In this study, we investigated the therapeutic effects of naringin on oxidative stress and inflammation in HG-induced rMC1 cells and STZ-induced rat model of DR. Our results indicated that naringin significantly decreased TNF- α , IL-1 β and IL-6 levels, and significantly increased GSH, SOD and CAT both in rMC1 cells and in retinal tissues of diabetic rats. Hence, we concluded that naringin attenuated retinal oxidative stress and inflammation *in vivo* and *in vitro*. It was worth mentioning that naringin markedly up-regulated SOD level in diabetic retina, more efficient than hesperetin (34). We speculated that naringin might have more efficient effects on inhibiting retinal oxidative stress in diabetic rats as compared to hesperetin, but further evaluation should be done in the future.

NF- κ B is a pro-inflammatory transcription factor that is responsible for retinal inflammatory reaction in diabetes (36). Previous data have shown that oxidative stress is implicated in the pathogenesis of diabetes and its complications (37), which is partly related with NF- κ B activation (38). Hence, we speculated that NF- κ B signal pathway played critical roles in the pathogenesis of DR. In previous studies, naringin has been reported to attenuate several diseases by inhibiting the activation of NF- κ B signaling pathway, including acute lung injury in LPS-treated mice (39) and cisplatin-induced striatum injury in Wistar aged rats (40). One study showed that naringin attenuated oxidative damage and pathological alterations partly through decreasing NF- κ B expression in high-fat diet (HFD)-STZ-induced type 2 diabetic rats (41). In addition, a recent study confirmed that naringin inhibited oxidative stress by activating Nrf2 signaling pathway and attenuated inflammatory reaction by inhibiting NF- κ B signaling pathway in diabetic kidney disease (29). However, this study didn't investigate whether naringin regulated oxidative stress in diabetic kidney disease through NF- κ B signaling pathway. Our results illustrated that naringin treatment efficiently inhibited the activation of NF- κ B both in STZ-induced diabetic rat model and HG-induced rMC1 cells. We demonstrated that naringin may inhibit retinal inflammatory reaction and oxidative stress in diabetic rats, at least in part by inhibiting the activation of NF- κ B signaling pathway, but the underlying mechanisms should be elucidated deeply in the future.

Conclusion

Naringin exerted protected effects on retinal tissues of STZ-induced rat model *in vivo* and HG-induced rMC1 cells *in vitro*. Naringin attenuated DR by inhibiting inflammatory reactions and oxidative stress *in vivo* and *in vitro*. Furthermore, naringin attenuated DR at least by inhibiting NF- κ B signaling transduction pathway. Although further studies on the underlying mechanisms will be required, this study provides evidence that naringin may be a novel and efficient therapy for DR.

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