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Naringenin prevents diabetic retinopathy via inhibition of apoptosis, oxidative stress and inflammation through heme oxygenase-1 upregulation

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Despite the high prevalence of diabetic retinopathy, early interventions remain limited due to a lack of therapies targeting oxidative stress and inflammation simultaneously. This study investigates naringenin, a flavonoid with potential antioxidant and anti-inflammatory properties, as a novel therapeutic candidate targeting HO-1 upregulation to mitigate retinal damage. In this regard, a rat model of streptozocin-induced diabetic retinopathy was established and naringenin was administrated (40 mg/Kg/day and 80 mg/Kg/day). Two other untreated groups of diabetic and non-diabetic rats were used to compare with naringenin-treated groups. The findings revealed that naringenin decreased the overexpression of the pro-inflammatory factors IL-1β, IL-6, and TNF-α in the retinal tissue. Moreover, naringenin inhibited the overexpression of TLR4, NF-κB, and CASP-3, caused heme oxygenase-1 (HO-1) overexpression, upregulated BCL-2, reduced the levels of malondialdehyde, and elevated the levels of superoxide dismutase, catalase, and glutathione peroxidase (P-value<0.05). Intraperitoneal injection of the HO-1 inhibitor zinc protoporphyrin (ZnPP) blocked the protective effect of naringenin. These findings suggest that naringenin exerts therapeutic effects in diabetic retinopathy possibly by inducing HO-1 expression.

Keywords: Flavonoid, Retinal damage, TLR4, NF-κB, Streptozotocin, Signaling pathway

Diabetic retinopathy, a leading cause of blindness, involves oxidative stress and inflammation[1]. Given the critical importance of early intervention for effective treatment, there is an urgent need for innovative preventive measures and early-stage therapeutic agents that demonstrate high efficacy[1]. Hyperglycemia exacerbates these pathways via TLR4/NF-κB activation, highlighting the need for therapies targeting these mechanisms[2,3]. Toll-like receptors (TLRs), a prominent group of pattern recognition receptors (PRRs), play a pivotal role in triggering inflammatory and immune responses. Specifically, it is suggested that there is an upregulation of TLR4 expression in the diabetic retina associated with hyperglycemia, which activates multiple pathways that contribute to the development of diabetic retinopathy[4]. Evidence suggests that TLR4 is essential in streptozotocin (STZ)-induced diabetic retinopathy, particularly concerning the induction of inflammatory cytokines such as interleukins (ILs) and tumor necrosis factor-alpha (TNF-α)[5]. Oxidative stress is linked, either directly or indirectly, to diabetic retinopathy and its associated complications[6]. In fact, hyperglycemia could disrupt the balance between the production and elimination of reactive oxygen species (ROS), thereby impairing the antioxidant defense mechanisms[7,8]. Oxidative stress resulting from elevated glucose levels can contribute to heightened apoptosis within the retinal tissue through modifications in signaling pathways such as nuclear factor-kappa B (NF-κB), thereby facilitating the advancement of diabetic retinopathy[9].

Naringenin, a widely distributed flavonoid known for its diverse biological activities, is prevalent in various plant species[10]. Naringenin is a dihydroflavonoid isolated from grapefruit peel with bitter taste and is naturally present in the peel and pulp of grapefruit, tangerine, and orange, which are plants of the Rutaceae family. It is also one of the main active ingredients of traditional Chinese medicines such as *Drynaria fortunei* and *Citrus aurantium*. The content of naringenin in various plants varies greatly depending on the origin and usually is higher in immature fruits[11,12]. The beneficial effects of naringenin similar to other phytochemicals may be partially linked to its ability to induce heme oxygenase-1 (HO-1)[13]. Nevertheless, the specific role of naringenin in diabetic retinopathy and the mechanisms involved remain poorly understood. This

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research aimed to explore the therapeutic effects of naringenin and elucidate its mechanisms of action in the context of diabetic retinopathy.

Materials and Methods

Reagents

The chemicals used in this study included naringenin (CAS No. 480-41-1; molecular weight: 272.25 g/mol) and Zinc (II) Protoporphyrin IX (ZnPP, CAS No. 15442-64-5; molecular weight: 626.03 g/mol), both obtained from Sigma Chemical Company (St. Louis, MO). STZ, sourced from Sigma-Aldrich (St. Louis, Missouri, USA), was utilized in the experimental setup. RIPA Lysis Buffer and the BCA Protein Assay Kit were acquired from Beyotime Biotechnology (Shanghai, China), and enzyme-linked immunosorbent assay (ELISA) Kits were purchased from different companies mentioned where applicable. Anesthetic solutions containing xylazine and ketamine were supplied by Alfasan (Woerden, Netherlands).

Experimental animals

All animal research protocols and experiments were subject to comprehensive review and all procedures adhered to the guidelines outlined in the 1996 National Institutes of Health Guide for the Care and Use of Laboratory Animals and according to ARRIVE guidelines. The study involved 40 adult male Wistar rats, aged eight weeks, with body weights ranging from 200 to 250 grams. The animals had *ad libitum* access to food and water and were housed in a controlled environment with temperatures maintained at room temperature (22 ± 3°C) and relative humidity levels between 50 ± 5%. A 12-hour light-dark cycle was implemented for all animals. The study included five distinct experimental groups, each consisting of eight rats. Control group (Con): This group consisted of normoglycemic rats that were not subjected to diabetes induction and did not receive any experimental treatments; STZ group (STZ): Diabetes was induced in rats using a standardized STZ protocol; Naringenin 40 group (Nar40): STZ-induced diabetic rats were supplemented with naringenin at a dose of 40 mg/Kg via oral gavage; Naringenin 80 group (Nar80): STZ-induced diabetic rats were supplemented with naringenin at a dose of 80 mg/Kg via oral gavage; Naringenin+ZnPP group (Nar80+ZnPP): In this group, STZ-induced diabetic rats received a combination of high-dose naringenin (80 mg/Kg) and ZnPP (30 mg/Kg).

Diabetes was induced in the experimental group using established protocols[14]. Following a 12-hour fasting period, each rat received a single intraperitoneal injection of STZ at a dose of 60 mg/Kg body weight, dissolved in a 0.1 M citrate buffer (pH 4.5). Control animals were administered an equivalent volume of citrate buffer without STZ. Blood glucose levels were carefully measured 72 h post-STZ injection. Rats with blood glucose concentrations exceeding 200 mg/dL in three consecutive readings were classified as diabetic and selected for subsequent analysis. Rats with fasting blood glucose levels between 80-100 mg/dL and not exposed to STZ were considered normoglycemic and non-diabetic Con group.

Diabetic rats confirmed 72 h after STZ injection, received naringenin via oral gavage at 8:00 a.m. once daily. All animals including Control and STZ groups were treated with an equal volume of saline (1 mL) via oral gavage. ZnPP (30 mg/Kg; Sigma-Aldrich) was administered intraperitoneally once every two weeks, starting 72 hours after STZ injection. Rats in the other four groups received an equivalent volume of saline (1 mL) intraperitoneally once every two weeks, matching the frequency of ZnPP administration in the Nar80+ZnPP group. After 16 weeks of diabetes induction and treatment, the animals were euthanized under general anesthesia, induced by a combination of xylazine (2%) and ketamine (10%) solutions. The eyes were carefully excised, and rinsed with sterile PBS, and the retinas were dissected from each eyeball. The retinal tissue was preserved at -80°C for long-term storage. For protein analysis, the tissue was homogenized, and the supernatant was stored at -4°C for short-term use.

Measurement of protein levels

Upon completion of the treatment protocol, the experimental animals were euthanized in accordance with ethical guidelines, and tissue samples were subsequently collected. One retina from each animal, weighing approximately 10 milligrams, was processed by homogenization in RIPA buffer containing protease inhibitors. The homogenates were then subjected to centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant fractions were carefully aliquoted and stored at -4°C for future analysis. Quantification of various biomolecules and inflammatory cytokines was performed using ELISA kits. The specific targets included Toll-like receptor 4 (TLR4, #E-EL-R0990, Elabscience, USA), nuclear

factor kappa-light-chain-enhancer of activated B cells (NF-κBp65, #E-EL-R0674, Elabscience, USA), heme oxygenase-1 (HO-1, #ab279414, Abcam, USA), interleukins (IL-1β [#ab100768], IL-6 [#ab234570], IL-18 [#ab213909], Abcam, USA), and tumor necrosis factor-alpha (TNF-α, #ab236712, Abcam, USA). ELISA assays were conducted in strict accordance with the manufacturer′s instructions to ensure accurate and reproducible results.

RT-PCR protocol

Retinal tissue samples were initially homogenized to facilitate RNA extraction. Total RNA was isolated using the PureLink RNA Mini Kit (#12183018A, Thermo Fisher Scientific, USA), following the manufacturer's recommended protocol. RNA quality and concentration were assessed using the Biotek Nanodrop spectrophotometer. Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (#4368814, Thermo Fisher Scientific, USA). The resulting cDNA was subsequently analyzed by quantitative polymerase chain reaction (qPCR) using the StepOne Real-Time PCR System (Applied Biosystems, USA) in conjunction with the Maxima SYBR Green qPCR Master Mix (#K0253, Thermo Fisher Scientific, USA). Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for comparative analysis. Expression data were calculated as fold changes relative to control samples using the $2^{-\Delta\Delta CT}$ method. The specific primers used in this study are provided in Table 1. A three-step thermal cycling protocol was employed, which included an initial 5 min denaturation step at 95°C, followed by 35 amplification cycles consisting of 20 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 72°C for primer annealing and extension.

Oxidative stress indicators

The level of oxidative stress in homogenized rat retinal tissues was assessed through a detailed quantitative analysis of several critical enzymes. The concentrations of catalase (CAT, #ab83464, Abcam, USA), superoxide dismutase (SOD, #ab119520, Abcam, USA), and glutathione peroxidase 4 (GPX4, #ab210574, Abcam, USA) were measured using enzyme-linked immunosorbent assays (ELISAs). The MyBioSource ELISA assay kit was utilized carefully, following the manufacturer's guidelines and protocols throughout the experimental process.

Malondialdehyde (MDA), a key indicator of lipid peroxidation, was quantified through a colourimetric assay. In this procedure, 400 µL of the homogenate supernatant was combined with 1600 µL of a ThioBarbituric Acid (TBA) reagent solution, which contained 0.375% TBA, 15% trichloroacetic acid, and 0.25 mol/L hydrochloric acid. The mixture was then heated at 95°C for 60 min in a water bath. After cooling rapidly, the sample was centrifuged at 8,000 × g for 15 min at 4°C. The absorbance of the resulting pink-colored supernatant was measured at 532 nm. MDA concentrations were calculated using tetraethoxypropane as the calibration standard and were expressed as micromoles of MDA per milligram of protein[15].

Statistical analysis

Data are presented as means ± standard deviations. To assess statistical significance, the one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test for pairwise comparisons. All statistical analyses were performed using SPSS software (version 24.0, IBM, Chicago, IL, USA), while graphical figures were created using GraphPad Prism (version 8, San Diego, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Naringenin downregulated NF-κB and TLR4 levels through HO-1 upregulation in the retina of STZ-induced diabetic rats

The findings of this investigation revealed that STZ-induced diabetes increased HO-1 gene expression (*P*-value=0.002) and protein levels (*P*-value=0.001) in the retina (Fig. 1). Interestingly, administration of 40 mg/Kg of naringenin did not reveal any significant difference with the STZ group in terms of HO-1 gene expression (*P*-value=0.162)

Table 1 — Primer sequences

Primer	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
GAPDH	GGTGGACCTCATGGCCTACAT	GCCTCTCTCTTGCTCTCAGTATCCT
TLR4	TTGAAGACAAGGCATGGCATGC	TCTCCCAAGATCAACCGATG
NF-κB	TGCAGAAAGAAGACATTGA	AGGCTAGGGTCAGCGTATGG
HO-1	CAGGTGTCCAGAGAAGGCTTT	TCTTCCAGGGCCGTGTAGAT
BCL-2	CCTGTGGATGACTGAGTACC	GAGACAGCCAGGAGAAATCA
CASP-3	GTGGAACTGACGATGATATGGC	CGCAAAGTGACTGGATGAACC-3

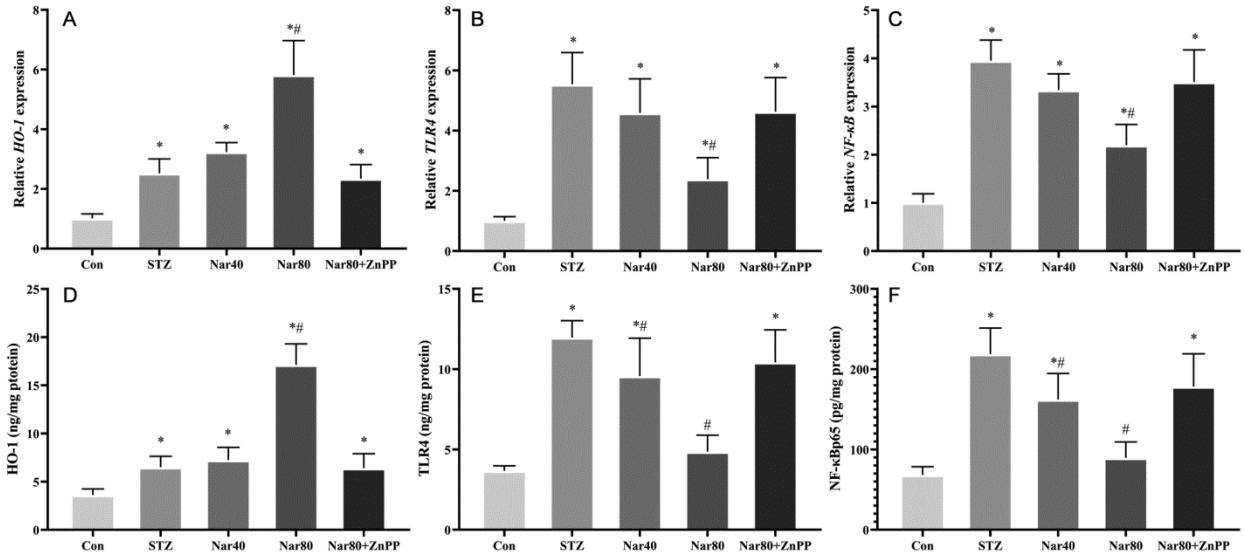

Fig. 1 — Naringenin modulated the expression of HO-1, TLR4, and NF-κB. The gene expression of HO-1 (A), TLR4 (B), and NF-κB (C) along with protein levels (D, E, and F, respectively) were measured. [*: significant difference with Con; #: significant difference with STZ; *#: significant difference with both Con and STZ groups; *P*-value<0.05 was considered significant].

and protein levels (*P*-value=0.812), although Nar40 administration caused a significant increase compared to Con in terms of HO-1 gene expression (3.19-times increase, *P*-value<0001) and protein levels (2.01-times increase, *P*-value<0.001). Whereas, in the group treated with 80 mg/Kg of naringenin, a 5.74-fold and 2.31-fold increase in *HO-1* gene expression, a 4.73-fold and 2.63-fold increase in HO-1 protein level was found compared to Con and STZ animals, respectively (*P*-value<0.001). Administration of ZnPP caused no significant difference in *HO-1* gene expression and protein level compared to the STZ group (*P*-value>0.05).

Prior studies have suggested that HO-1 upregulation may improve diabetic retinopathy by inhibiting TLR4 and NF-κB pathways[14]. Our results align with this hypothesis, as naringenin-induced HO-1 overexpression correlated with downregulation of TLR4 and NF-κB. In this regard, the findings indicated an overexpression of *TLR4* and *NF-κB* genes in the retina of STZ rats compared to Con, which was followed by a significant increase in TLR4 and NF-κB protein levels (*P*-value<0.001). Although administration of 40 mg/Kg of naringenin did not show a significant change compared to the STZ group (*P*-value>0.05), the expression of *TLR4* and *NF-κB* genes was remarkably suppressed by 56.87% and 44.46%, respectively, after treatment with 80 mg/Kg of naringenin in comparison with STZ animals. Align with that, a significant downregulation of TLR4 (59.43%) and NF-κB (59.07%) proteins was found after treatment with 80 mg/Kg of naringenin compared to the STZ-treated group. The data obtained did not reveal a significant difference in TLR4 and NF-κB gene expression and protein levels between Nar80 and Con groups (*P*-value>0.05). Importantly, in the retina of Nar80+ZnPP animals, gene expression and corresponding levels of TLR4 and NF-κB proteins did not show a significant difference compared to the STZ group (*P*-value>0.05). While TLR4 and NF-κB gene expression showed no significant difference between Nar80 and Con groups (*P*-value>0.05), protein levels were significantly reduced in the Nar80 group compared to STZ (*P*-value<0.05), suggesting potential post-transcriptional or post-translational modulation by naringenin.

Naringenin prevented apoptosis via HO-1 upregulation in the retina of diabetic rats

The expression status of genes encoding *BCL-2*, a major inhibitor of apoptosis, and *CASP-3*, a critical mediator of apoptotic cell death, was investigated along with protein level measurements. The expression of the *BCL-2* gene in STZ, Nar40, Nar80, and Nar80+ZnPP groups showed a significant decrease of 77.95%, 71.46%, 26.85%, and 60.09%%, respectively, compared to Con animals (*P*-value <0.05). Nevertheless, in the Nar80 (3.32-fold) and Nar80+ZnPP (1.81-fold) groups, the expression of the *BCL-2* gene was significantly overexpressed compared to the STZ group (Fig. 2). Moreover,

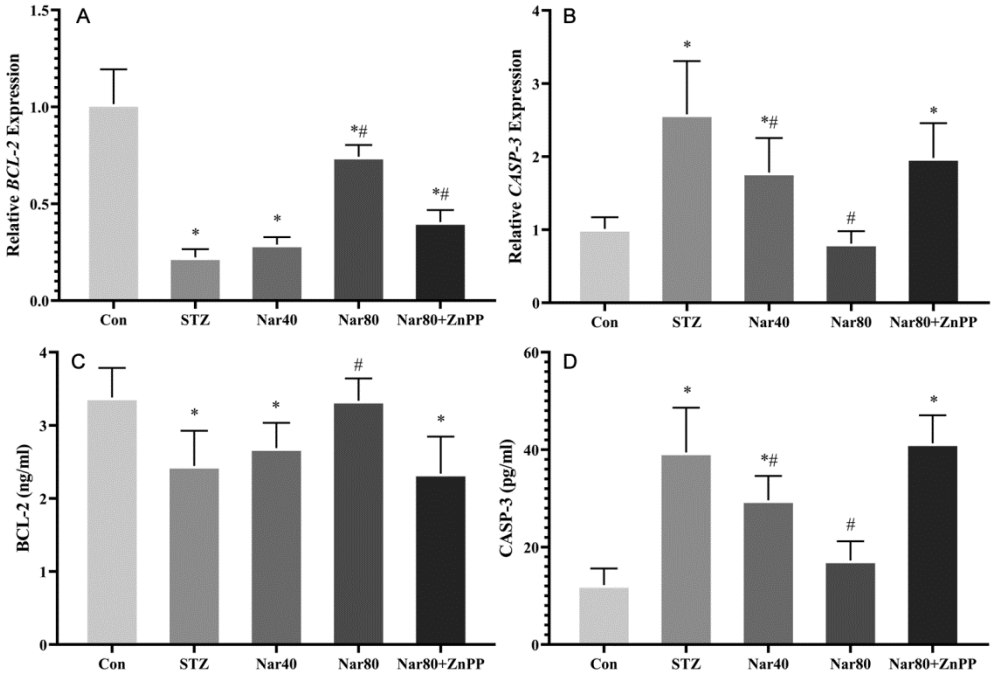

Fig. 2 — Naringenin suppressed apoptosis in the retina of diabetic rats via induction of HO-1. The gene expression of *BCL-2* (A) and *CASP-3* (B) as well as the protein levels (C & D, respectively) are illustrated. [*: significant difference with Con; #: significant difference with STZ; *#: significant difference with both Con and STZ groups; *P*-value<0.05 was considered significant].

significant downregulation of BCL-2 levels was found in STZ (27.71%, *P*-value=0.001), Nar40 (20.50%, *P*-value=0.0175), and Nar80+ZnPP (30.82%, *P*-value=0.002) groups compared to Con. Administration of 80 mg/Kg of naringenin significantly upregulated BCL-2 levels by 1.24 times compared to the STZ group (*P*-value=0.002). Administration of HO-1 inhibitor resulted in no significant difference in BCL-2 levels between STZ and Nar80+STZ groups (*P*-value>0.05).

In contrast, the gene encoding *CASP-3* was significantly overexpressed in STZ (2.55-fold, *P*-value<0.001), Nar40 (1.76-fold, *P*-value=0.013), and Nar80+ZnPP (1,96-fold, *P*-value=0.001) groups compared to Con animals, while no significant difference was found between Nar80 rats and Con group (*P*-value>0.05). Accordingly, STZ-induced diabetes caused a significant 3.23 times upregulation of CASP-3 in the retina compared to Con animals. Notably, administration of 40 (*P*-value=0.015) and 80 (*P*-value<0.001) mg/Kg naringenin significantly reduced CASP-3 levels compared to the STZ group, although a significant difference was found between Nar40 and Con groups (*P*-value<0.001). Administration of HO-1 inhibitor abolished the changes in CASP-3 levels caused by naringenin administration in the retina of diabetic rats.

Naringenin suppressed inflammatory mediators in the retina of diabetic animals

An ELISA approach was used to determine the inflammatory status in the retina by measuring the levels of three different inflammatory mediators (Fig. 3). In the STZ group, ELISA revealed significant increases in IL-6 (5.40-fold), IL-1β (3.18-fold), and TNF-α (3.44-fold) compared to Con (*P*-value<0.001). Naringenin treatment attenuated these effects as administration of 40 and 80 mg/Kg doses of naringenin resulted in a significant decrease in the levels of inflammatory mediators IL-6 (22.93% and 60.65%, respectively) and TNF-α (14.11% and 57.61%, respectively) compared to STZ group (*P*-value<0.05), while IL-1β levels showed a significant 38.03% decrease only after administration of 80 mg/Kg of naringenin compared to STZ group. Importantly, the Nar80+ZnPP group did not show a significant difference from the STZ-induced diabetic group in terms of the levels of inflammatory cytokines measured (*P*-value>0.05).

Oxidative stress was ameliorated by naringenin in the retina of diabetic animals

In STZ-induced diabetic animals, a significant decrease in CAT (41.10%), SOD (47.40%), and GPx (46.70%) levels was found compared to Con rats (*P*-value<0.001, Fig. 4), whereas comparing the STZ

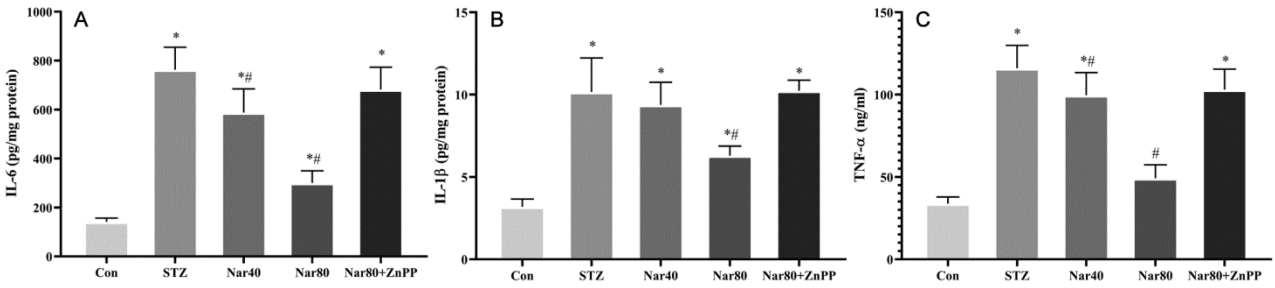

Fig. 3 — Inflammation in the retina was suppressed after naringenin treatment by HO-1 overexpression. The administration of naringenin caused a significant reduction in the levels of IL-6 (A), IL-1β (B), and TNF-α (C). [*: significant difference with Con; #: significant difference with STZ; *#: significant difference with both Con and STZ groups; *P*-value<0.05 was considered significant].

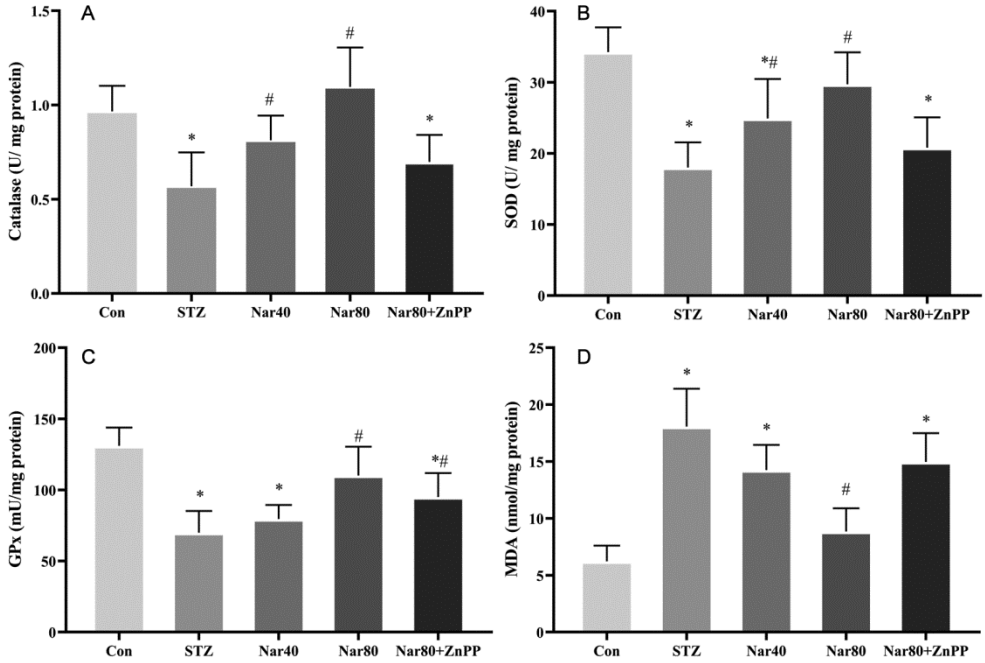

Fig. 4 — Oxidative stress in the retina was ameliorated by naringenin dependent on HO-1 overexpression. Naringenin increased catalase (A), SOD (B), and GPx (C) levels, whereas reduced MDA (D) levels. [*: significant difference with Con; #: significant difference with STZ; *#: significant difference with both Con and STZ groups; *P*-value<0.05 was considered significant].

group with Con demonstrated a 2.90-fold increase in MDA levels. Administration of 40 mg/Kg of naringenin caused a significant increase in CAT (1.43-fol, *P*-value=0.036) and SOD (1.38-fold, *P*-value=0.024) levels compared to STZ group, although Nar40 animals showed a 21.20% decrease in MDA levels compared to STZ group. Nevertheless, no significant difference was found between the Nar40 and STZ in terms of GPx levels (*P*-value>0.05). Notably, in the retina tissue of Nar80 animals, the levels of all three antioxidant enzymes including CAT(1.97-fold), SOD (1.65-fold), and GPx (1.58-fold) increased compared to STZ animals, while administration of 80 mg/Kg of naringenin caused a significant decrease in MDA levels compared to STZ group. Importantly, groups STZ and Nar80+ZnPP showed a significant difference only in terms of GPx levels (*P*-value=0.019).

Discussion

Diabetic retinopathy is a prevalent microvascular complication associated with diabetes mellitus. The underlying mechanisms of diabetic retinopathy encompass inflammation, stress, and the irreversible degeneration of retinal neurons[16]. At present, existing targeted therapeutic strategies are insufficient[17]. In this study, we explored the protective effects of naringenin on diabetic retinopathy. Our results

demonstrated that naringenin mitigated the pathological alterations induced by hyperglycemia in the retinas of rats with STZ-induced diabetes. Additionally, we discovered that the retinal protective effects of naringenin may be linked to its ability to inhibit the TLR4/NF-κB/inflammation pathway, reduce apoptosis, and alleviate oxidative stress through the induction of HO-1. These findings underscore the therapeutic promise of naringenin in the context of diabetic retinopathy, highlighting its cell-protective, anti-inflammatory, and antioxidant properties.

Naringenin, a prominent member of the natural flavonoid family, has been shown to offer benefits in conditions such as age-related ophthalmopathy and retinal ischemia-reperfusion injury[18,19]. Previous research has primarily concentrated on its antioxidant capabilities, attributing naringenin's protective effects on retinopathy largely to its antioxidant and anti-inflammatory actions within retinal cells[20]. However, recent studies have begun to investigate naringenin's anti-apoptotic and neuroprotective properties[21]. Nevertheless, the specific mechanisms through which naringenin exerts its neuroprotective effects remain inadequately understood.

Recent investigations have demonstrated that the modulation of the Nrf2/HO-1 signaling pathway causes protection against inflammation and oxidative stress in diabetic retinopathy[22]. The Nrf2/HO-1 axis is recognized as a crucial element of the cellular defense mechanism against oxidative damage[22]. An earlier research indicated that HO-1 is vital for the protective effects of flavonoids against acute alcoholic liver injury[23]. Nevertheless, the relationship between naringenin and HO-1 in the context of diabetic retinopathy has not been fully elucidated. In the current study, we observed that the downregulation of HO-1 markedly diminished the neuroprotective effects of naringenin in diabetic retinopathy, as evidenced by the damage to ganglion cells in the Nar80+Znpp group. Given that HO-1 is a well-established antioxidant enzyme, its role in mitigating oxidative stress in diabetic retinopathy is not unexpected. Consequently, we explored additional pathways to better understand the mechanisms underlying naringenin's neuroprotective effects in diabetic retinopathy. HO-1 has been postulated as the regulator of the upstream effector of inflammation and oxidative stress, respectively TLR4 and NF-κB[14]. Inflammation and oxidative stress are two intracellular events that induce damage and ultimately apoptotic cell death[24]. Indeed, damage leading to apoptosis is considered a key factor in the pathophysiology of diabetic retinopathy[7]. We observed that naringenin administration suppressed apoptosis in the retina through upregulation of BCL-2, an inhibitor of apoptosis, and downregulation of CASP-3, a serine protease that promotes cell death. Accordingly, the anti-apoptotic activity of naringenin has been elucidated in hypoxic-ischemic brain damage[25], lung injury[26], ischaemic stroke damage[27], etc. Importantly, the suppression of apoptosis by naringenin was dependent on HO-1 induction, as administration of the HO-1 inhibitor restored the changes observed in BCL-2 and CASP-3 levels.

Our results demonstrating naringenin′s HO-1-dependent suppression of TLR4/NF-κB align with emerging studies on flavonoid-mediated retinal protection. It has been reported that HO-1 induction via Nerolidol ameliorated oxidative stress and inflammation in diabetic retinopathy by inhibiting Nrf2/NF-κB crosstalk[28], mirroring our observed NF-κB downregulation. Notably, epigenetic modifications in patients with diabetic retinopathy have been linked to oxidative stress[29], supporting our mechanistic focus on naringenin′s antioxidant effects. Unlike prior work, our study uniquely links naringenin′s anti-apoptotic effects (via BCL-2 upregulation and CASP-3 suppression) to HO-1/TLR4 axis modulation, a pathway not yet explored in recent diabetic retinopathy literature. These findings position naringenin as a multi-target therapy, bridging gaps in current antioxidant and anti-inflammatory approaches.

In recent years, the chronic inflammatory response in tissues has been recognized as a significant contributor to diabetic retinopathy[30]. The data from the present study showed that naringenin administration was accompanied by downregulation of the upstream regulator of inflammation, TLR4, and subsequently a significant reduction in inflammatory cytokines including IL-6, IL-1β, and TNF-α. Notably, the anti-inflammatory effects of naringenin were due to the induction of HO-1 expression, as administration of ZnPP as an HO-1 inhibitor restored the desired therapeutic changes. Consistent with our findings, it has previously been shown that suppression of TLR4 by naringenin leads to a reduction in inflammatory injury in pathological conditions such as colitis[31], endotoxemia[32], osteoarthritis[33], and neurological diseases[21,34]. However, the connection between these

factors in diabetic retinopathy remains ambiguous. Inflammatory responses appear to be linked to the activation of NF-κB, leading to the disruption of the blood-retinal barrier[35], and the induction of oxidative stress[36]. In the context of hyperglycemia-induced damage, Hu *et al*. reported concurrent elevations in TLR4 and inflammatory cytokines (e.g. IL-1β, and IL-18) in ganglion cells exposed to varying glucose concentrations[37]. These observations align with our findings in the STZ group as we observed analogous alterations in inflammatory cytokines, including IL-6, IL-1β, and TNF-α. Additional research is warranted to explore the relationship between these factors in diabetic retinopathy.

Dysregulation of oxidative stress is a critical factor that predisposes individuals to or exacerbates the progression of various pathological conditions and diseases. Recent research has provided significant insights into the essential role of ROS in various fundamental cellular processes, including cell proliferation, inflammation, apoptosis, and gene expression[3]. The generation of free radicals within the ROS framework has a profound impact on a diverse array of biomolecules, including proteins, lipids, and DNA, resulting in cellular damage and disruption of essential cellular functions[3]. Notably, the pathogenesis of several ocular diseases, such as age-related macular degeneration, glaucoma, and diabetic retinopathy, is closely linked to oxidative stress[3,38]. It is posited that oxidative stress and the release of inflammatory mediators serve as early indicators of diabetic retinopathy, occurring prior to any observable histological alterations in the retina. Furthermore, increased oxidative stress within the retina activates the NF-κB signaling pathway, which has been identified as a key regulator in the initiation and progression of diabetic retinopathy[39]. The role of inflammation and oxidative stress in the damage to the retina caused by diabetes has prompted extensive research into polyphenols, which possess antioxidant and anti-inflammatory properties, as a potential approach for managing diabetic retinopathy[40]. In this study, we found that naringenin administration downregulated NF-κB, the negative upstream regulator of oxidant response mechanisms, which was accompanied by a significant increase in the level of antioxidant defensive enzymes including catalase, SOD, and GPx, as well as a decrease in the level of lipid peroxidation. The restoration of the effects of naringenin after administration of HO-1 inhibitor may indicate that the antioxidant activity of this flavone is dependent on the induction of HO-1. Consistent with the present findings, it has been previously shown that modulation of the HO-1/NF-κB pathway can counteract diabetes-related oxidative stress-induced damage[14,41]. Nevertheless, further research is required to clarify the correlation between HO-1 and NF-κB and the response to oxidants under the impact of naringenin administration in diabetic retinopathy models.

Conclusion

Diabetic retinopathy was associated with overexpression of HO-1, TLR4, and NF-κB, which ultimately led to the induction of apoptosis, inflammation, and oxidative stress in the retina. Administration of naringenin at a dose of 80 mg/Kg resulted in the induction of expression, which resulted in the downregulation of TLR4 and NF-κB. Administration of naringenin also suppressed apoptosis, inflammation, and oxidative stress in the retina of diabetic animals. Importantly, inhibition of HO-1 induction by naringenin resulted in the abrogation of its therapeutic effects on TLR4 and NF-κB downregulation as well as suppression of oxidative stress, inflammation, and apoptosis. Therefore, one may conclude that naringenin inhibits oxidative stress, inflammation, and apoptosis induced by diabetes in the retina through the induction of HO-1 expression.

Conflict of interest

The authors declare that they have no competing interests.

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