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Paeonol attenuates oxidative stress and inflammation in experimental diabetic retinopathy by regulating the Nrf2/NF-κB signaling

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Abstract. This study aimed to investigate the protective mechanism of paeonol against diabetic retinopathy (DR) *via* modulation of the Nrf2/NF-κB signaling pathway. Primary mouse retinal Müller cells exposed to high glucose (HG) and streptozotocin-induced diabetic mice were used as *in vitro* and *in vivo* models, respectively. Key techniques included RT-qPCR, Western blot, DCFH-DA-based ROS assays, and ELISA. Paeonol (40–80 μg/ml) significantly reduced HG-induced ROS accumulation ($p < 0.01$) and restored Nrf2 nuclear translocation, upregulating antioxidant genes HO-1 and NQO1 ($p < 0.01$). It suppressed NF-κB activation (p-IκBα and p-p65 reduction; $p < 0.001$) and proinflammatory cytokines (TNF-α, IL-1β, IL-6; $p < 0.01$) in Müller cells and diabetic retinas. Blood-retinal barrier (BRB) integrity was preserved *via* upregulation of tight junction proteins (claudin-5, occludin, ZO-1; $p < 0.01$). In conclusion, paeonol attenuates DR progression by activating Nrf2-mediated antioxidant responses and inhibiting NF-κB-driven inflammation, highlighting its potential as a novel therapeutic agent for DR.

Key words: Paeonol — Diabetic retinopathy — Oxidative stress — Nrf2 — Blood-retinal barrier — Inflammation

Introduction

Diabetic retinopathy (DR), a leading cause of blindness in working-age adults, is driven by oxidative stress and chronic inflammation that disrupt retinal homeostasis (Kowluru 2023). Hyperglycemia-induced overproduction of ROS disrupts retinal homeostasis, triggering mitochondrial dysfunction, DNA damage, and activation of proinflammatory cascades that collectively drive vascular leakage (Hussain et al. 2023). Despite therapeutic advances such as anti-VEGF agents and laser photocoagulation, these approaches predominantly target late-stage neovascularization and edema, failing to address the root molecular mechanisms – oxidative injury and inflammatory glial activation – that initiate early DR progression (Moulin 2019; Bonfiglio 2020). Moreover, frequent intravitreal injections of anti-VEGF drugs are burdened by tachyphylaxis, ocular complications, and high costs (Hartman 2021), underscoring the urgent need for safer, mechanism-based therapies.

Müller cells, the principal glial cells of the retina, play a pivotal role in DR pathogenesis (Coughlin 2017). Under hyperglycemic stress, Müller cells undergo reactive gliosis, characterized by upregulated glial fibrillary acidic protein (GFAP) and aberrant secretion of inflammatory mediators (e.g., TNF-α, IL-1β), which perpetuate retinal inflammation and capillary degeneration (Zhu 2022; Yang 2024). Activated Müller cells further exacerbate oxidative damage by suppressing antioxidant defenses (e.g., Nrf2/HO-1 signaling) (Albert-Garay 2022) while activating NF-κB (Park 2023), a master regulator of proinflammatory gene transcription. The vicious cycle of oxidative-inflammatory injury accelerates blood-retinal barrier (BRB) breakdown and photoreceptor loss (Carpi-Santos 2022). Targeting Müller cell-mediated oxidative-inflammatory crosstalk thus represents a promising therapeutic avenue.

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Paeonol, a phenolic compound derived from *Paeonia suffruticosa*, exhibits well-documented antioxidant and anti-inflammatory properties in metabolic and neurodegenerative disease (Tayanloo-Beik 2022; Sun 2023). Preclinical studies demonstrate its potent antioxidant capacity to scavenge free radicals, upregulate Nrf2-dependent cytoprotective genes (HO-1, SOD1), and suppress NF-κB-driven inflammation in diabetic neuropathy and nephropathy (Zhang 2018; Adki and Kulkani 2021). While a prior study reported paeonol's antioxidant effects in DR rat models (Adki and Kulkani 2022), the mechanistic interplay between Nrf2 and NF-κB in this context – and its relevance to Müller cell dysfunction – has not been elucidated.

Therefore, this study aimed to investigate whether paeonol attenuates DR progression by rescuing Nrf2 antioxidant signaling and suppressing NF-κB-mediated inflammation in hyperglycemia-stressed Müller cells and diabetic murine models.

Materials and Methods

Cells

Primary mouse retinal Müller cells were isolated from 3-day-old C57BL/6 mice and authenticated using established protocols as previously described (Du 2020). Cells were incubated in DMEM containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) with 5% CO_2 at 37°C. To mimic diabetic conditions, Müller cells were exposed to 33.3 mM glucose (HG) treatment for 24 h, with 5 mM glucose-treated cells serving as the normal glucose (NG) control group. For pharmacological intervention, cells were co-treated with paeonol (MedChemExpress, Shanghai, China; purity ≥98%) at specified concentrations (20–100 μg/ml) for 24 h to assess cytotoxicity and dose-dependent therapeutic effects.

Transfection

To investigate the functional role of Nrf2, Müller cells were transfected with small interfering RNA (siRNA) targeting Nrf2 (si-Nrf2) or a scrambled negative control siRNA (si-NC; GenePharma, Shanghai) using Lipofectamine™-based siRNA Transfection Reagent (MedChemExpress) according to the manufacturer's protocol. After 48 h, transfection efficiency was validated by RT-qPCR and Western blot. Cells were subsequently subjected to HG and/or paeonol treatment as described in the experimental design.

CCK-8 assay

Müller cells were seeded in 96-well plates (5×10^3 cells/well) and allowed to adhere for 24 h. Cells were then exposed to paeonol (0–100 μg/ml) and/or HG for 24 h. Then, 10 μl CCK-8 (MedChemExpress) was added into each well and incubated at 37°C for 3 h in the dark. Absorbance was measured at 450 nm using a CLARIOstar microplate reader (BMG Labtech, Germany). All experiments were performed in triplicate.

Animal models

All experimental procedures were approved by the Laboratory Animal Welfare & Ethics Committee of Bestcell Model Biological Center (Approval Number. BSMS2023-11-14A; Date, 2023.11.10). Eight-week-old male C57BL/6 mice were acclimatized for 7 days under controlled conditions (23 ± 1°C, 55–60% humidity, 12-h light/dark cycle) with free access to food and water. Mice were randomly divided into 4 groups (n = 10/group): normal (healthy mice treated with citrate buffer), DR (untreated DR model), DR+PBS (diabetic mice treated with PBS as solvent control), and DR+paeonol (diabetic mice treated with paeonol). The DR model was induced *via intraperitoneal* (*i.p.*) injection of streptozotocin (STZ; Sigma-Aldrich) at 50 mg/kg/day for 5 consecutive days after overnight fasting. Blood glucose levels were monitored weekly using an Accu-Chek glucometer (Roche), and mice with fasting blood glucose ≥16.7 mmol/l in two consecutive measurements were included in the diabetic groups. Four weeks post-STZ induction, the DR+paeonol group received 150 mg/kg/day paeonol *via* oral gavage for 8 weeks (Xu 2019), while the DR+PBS group was administered equivalent volumes of PBS. At the endpoint, mice were euthanized *via* cervical dislocation method under anesthesia. Retinas were immediately dissected, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent analyses. Retinas from all 10 mice *per* group were collected. For each experimental assay, retinas from distinct subsets of mice were allocated as independent biological replicates: ROS detection (n = 8 retinas/group), RT-qPCR (n = 7 retinas/group), Western blot (n = 6 retinas/group), and ELISA (n = 8 retinas/group). Where technically feasible, a retina contributed to more than one assay, but no retina was used for all assays.

ROS detection

ROS production in Müller cells and retinas was evaluated using the fluorescent probe DCFH-DA (MedChemExpress). For *in vitro* assays, Müller cells seeded in 24-well plates (1×10^4 cells/well) were treated with paeonol (40 or 80 μg/ml) and/or HG for 24 h. After treatment, cells were washed twice with PBS and incubated with 10 μM DCFH-DA in serum-free DMEM at 37°C for 20 min in the dark. For retinal tissue analysis, frozen retinal sections (10 μm thickness) from experimental mice were incubated with 10 μM DCFH-DA in PBS under identical conditions. Fluorescence intensity was

quantified using a Nikon Ti2-U fluorescence microscope, and images were analyzed using ImageJ 1.53t (NIH) and expressed as relative fold change compared to control groups.

RT-qPCR

Total RNAs were isolated from murine retinas using TRIzol (Invitrogen) and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed using the SYBR Premix Ex Taq^{TM} (Takara) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following conditions: initial denaturation at 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the endogenous control. The used primer sequences are shown in Table 1.

Western blot

Protein lysates from retinal tissues or Müller cells were prepared using RIPA buffer (Beyotime, Shanghai) containing protease and phosphatase inhibitors (MedChemExpress). Equal amounts of protein (30 μg *per* lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBST (TBS + 0.1% Tween-20) for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies: Nrf2 (AF0639, 1:1000; Affinity Biosciences), HO-1 (ab52947, 1:2000; Abcam), NQO1 (ab80588, 1:10,000; Abcam), IκBα (AF5002, 1:2000; Affinity Biosciences), phospho-IκBα (AF2002, 1:2000; Affinity Biosciences), p65 (AF5006, 1:2000; Affinity Biosciences), phospho-p65 (AF2006, 1:2000; Affinity Biosciences), GFAP (DF6040, 1:1000; Affinity Biosciences), Histone H3 (BF9211, 1:2000; Affinity Biosciences), β-actin (4970, 1:1000; Cell Signaling Technology), and GAPDH (AF7021, 1:3000; Affinity Biosciences). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:5000; Cell Signaling Technology) for 1 h at room temperature. Protein bands were visualized using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged on a ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis was performed using ImageJ 1.53t (NIH).

ELISA

For *in vitro* experiments, culture supernatants of Müller cells treated with HG and/or paeonol were collected. For *in vivo* experiments, retinal tissues from DR mice were homogenized in PBS, centrifuged at 12,000×*g* for 15 min, and supernatants were harvested. Levels of IL-6, IL-1β, and TNF-α in Müller cell supernatants and retinal tissue lysates were measured using commercial ELISA kits (Beyotime) according to the manufacturer's protocols.

Table 1. Primer sequences for RT-qPCR

Gene	Sequence (5'→3')	
VE-cadherin	F:	TGGAAGGTCTGCACCTGCTA
	R:	TTTGGCCCACGGGATTG
Claudin-5	F:	TCTGCTGGTTCGCCAACAT
	R:	CGGCACCGTCGGATCA
Occludin	F:	TGTGGGATAAGGAACACATTTATGA
	R:	CAGACACATTTTTAACCCACTCTTCA
ZO-1	F:	TGAACGCTCTCATAAGCTTCGTAA
	R:	ACCGTACCAACCATCATTCATTG
GAPDH	F:	TGTTCGTCATGGGTGTGAAC
	R:	ATGGCATGGACTGTGGTCAT

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (IBM) with data expressed as mean ± standard deviation (SD). For *in vivo* experiments, 6–8 independent biological replicates per group (one retina *per* mouse) were performed. *In vitro* studies included three independent biological replicates (cells from separate culture batches). One-way ANOVA with Tukey's *post hoc* test was used for multi-group comparisons. Statistical significance was defined as * $p < 0.05$.

Results

Paeonol activates Nrf2 antioxidant signaling and suppresses ROS accumulation in HG-exposed Müller cells

CCK-8 assays (Fig. 1A) revealed that paeonol at 0–80 μg/ml under NG conditions caused minimal cytotoxicity, while 100 μg/ml significantly suppressed cell viability ($p < 0.05$). Similarly, in HG-treated Müller cells (Fig. 1B), paeonol at 20–80 μg/ml showed no cytotoxicity, whereas 100 μg/ml induced marked cell death ($p < 0.05$). Based on these findings, concentrations 40 and 80 μg/ml were selected for subsequent experiments as they represent the highest non-toxic concentrations under both NG and HG conditions. Subcellular fractionation analysis revealed impaired Nrf2 nuclear translocation under HG stress, evidenced by decreased nuclear Nrf2 accumulation and concomitant cytoplasmic retention ($p < 0.001$; Fig. 1C). Paeonol treatment dose-dependently restored nuclear Nrf2 localization while reducing cytoplasmic levels ($p < 0.01$ or $p < 0.001$). This nuclear translocation correlated with upregulated expression of Nrf2-target antioxidant genes: HO-1 and NQO1

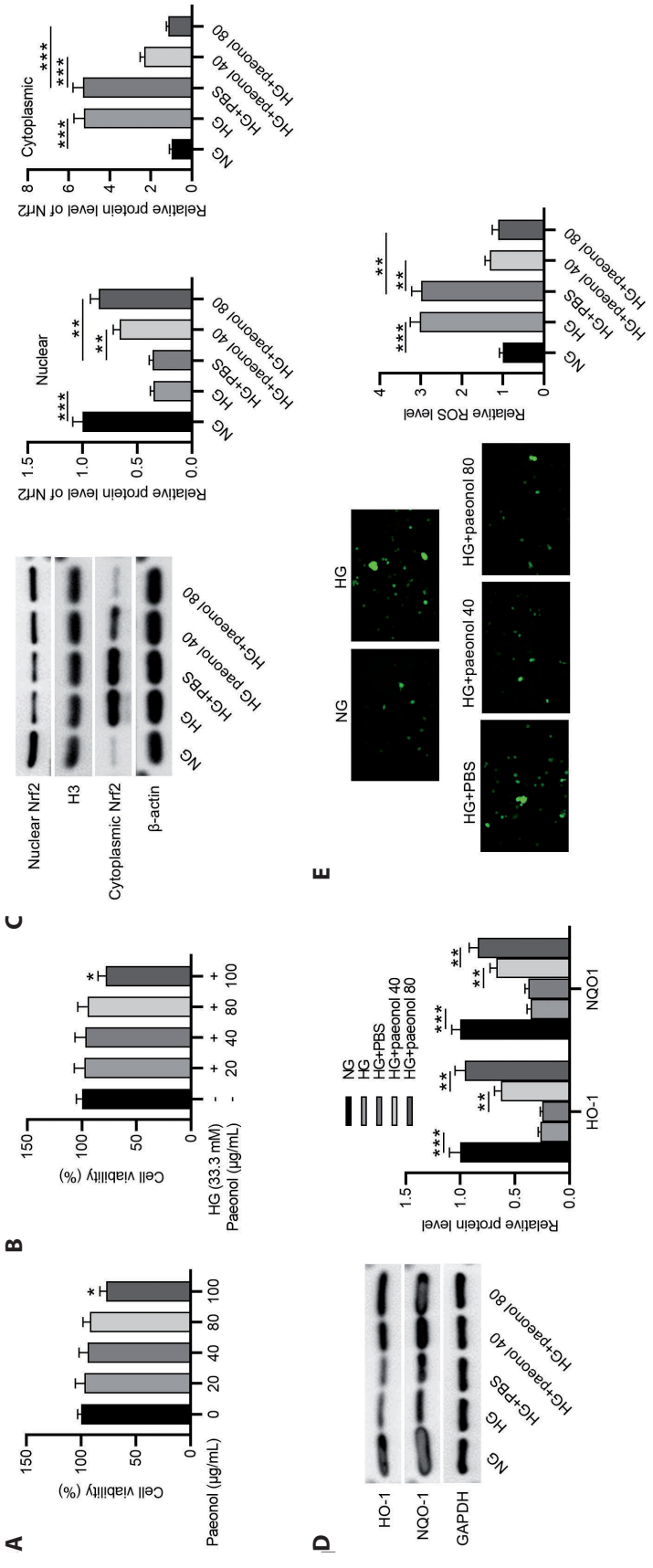

Figure 1. Paeonol mitigates high glucose-induced cytotoxicity and oxidative stress in Müller cells. **A.** Dose-dependent effects of paeonol (0–100 μg/ml) on Müller cell viability under normoglycemic conditions (NG, 5 mM glucose) assessed by CCK-8 assay. **B.** Viability of Müller cells co-treated with HG (33.3 mM glucose) and paeonol (20–100 μg/ml) for 24 h. **C.** Representative immunoblots and quantification of Nrf2 protein levels in nuclear and cytoplasmic fractions. **D.** Expression of antioxidant proteins HO-1 and NQO1 under indicated treatments. **E.** Intracellular ROS levels measured by DCFH-DA fluorescence. $n = 3$ biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in the paeonol group compared to HG controls ($p < 0.01$; Fig. 1D). Consistent with enhanced antioxidant defenses, paeonol attenuated HG-induced ROS overproduction ($p < 0.01$; Fig. 1E).

Paeonol mitigates NF-κB-driven inflammation and glial activation in hyperglycemic Müller cells

HG stimulation triggered robust proinflammatory responses, elevating extracellular TNF-α, IL-1β, and IL-6 levels compared to NG controls ($p < 0.001$). These cytokine surges were significantly attenuated by paeonol ($p < 0.01$; Fig. 2A). Western blot analysis of NF-κB pathway components revealed HG-induced phosphorylation of IκBα and p65, indicating pathway activation ($p < 0.001$). Paeonol treatment suppressed these phosphorylation events compared to HG-exposed cells ($p < 0.001$; Fig. 2B). Concurrently, HG-induced glial activation (GFAP upregulation) was reduced by paeonol ($p < 0.001$; Fig. 2B), demonstrating its dual capacity to inhibit both inflammatory signaling and reactive gliosis.

Inhibition of Nrf2 reverses the protective effects of paeonol

In subsequent mechanistic studies (Fig. 3), the 80 μg/ml dose of paeonol was selected based on its superior efficacy in activating Nrf2 signaling and suppressing oxidative-inflammatory responses compared to 40 μg/ml, while maintaining no cytotoxicity (Fig. 1,2). Paeonol treatment significantly upregulated HO-1 and NQO1 expression in HG-stimulated Müller cells, an effect that was abolished by Nrf2 siRNA transfection ($p < 0.001$; Fig. 3A). DCFH-DA staining demonstrated that silencing Nrf2 abolished paeonol's inhibitory effects on ROS accumulation in Müller cells ($p < 0.01$; Fig. 3B). Similarly, Nrf2 knockdown reversed paeonol-mediated suppression of NF-κB pathway activation and GFAP overexpression in HG conditions ($p < 0.001$; Fig. 3C). Concordantly, the anti-inflammatory effects of paeonol – evidenced by reduced TNF-α, IL-1β, and IL-6 levels – were negated upon Nrf2 inhibition ($p < 0.01$; Fig. 3D).

Paeonol attenuates NF-κB-driven inflammation and gliosis in diabetic retinas

For *in vivo* experiments (Figs. 4,5), diabetic mice received 150 mg/kg/day paeonol – a dosage previously shown to improve metabolic parameters in diabetic models (Xu 2019). Retinal Nrf2 protein expression was significantly downregulated in diabetic mice compared to normal controls, an alteration rescued by paeonol treatment ($p <$

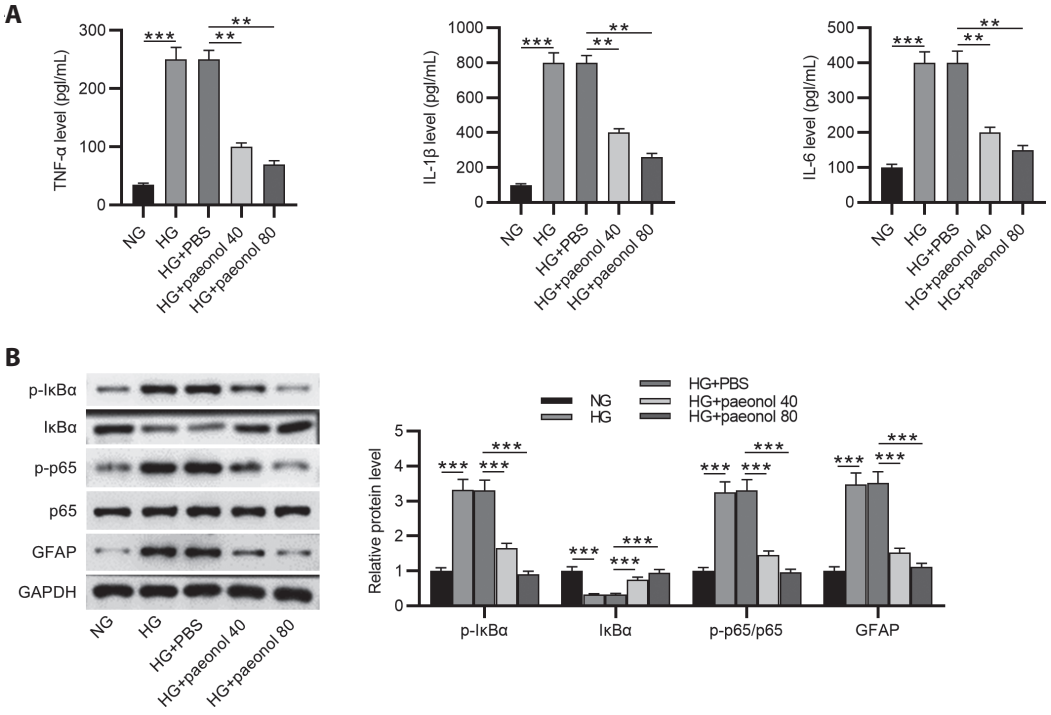

Figure 2. Paeonol suppresses HG-induced glial activation and NF-κB-mediated inflammation in Müller cells. **A.** ELISA quantification of TNF-α, IL-1β, and IL-6 in cell supernatants. **B.** Immunoblot analysis of NF-κB pathway components (p-IκBα, IκBα, p-p65, p65) and glial activation marker GFAP. $n = 3$ biological replicates. ** $p < 0.01$, *** $p < 0.001$.

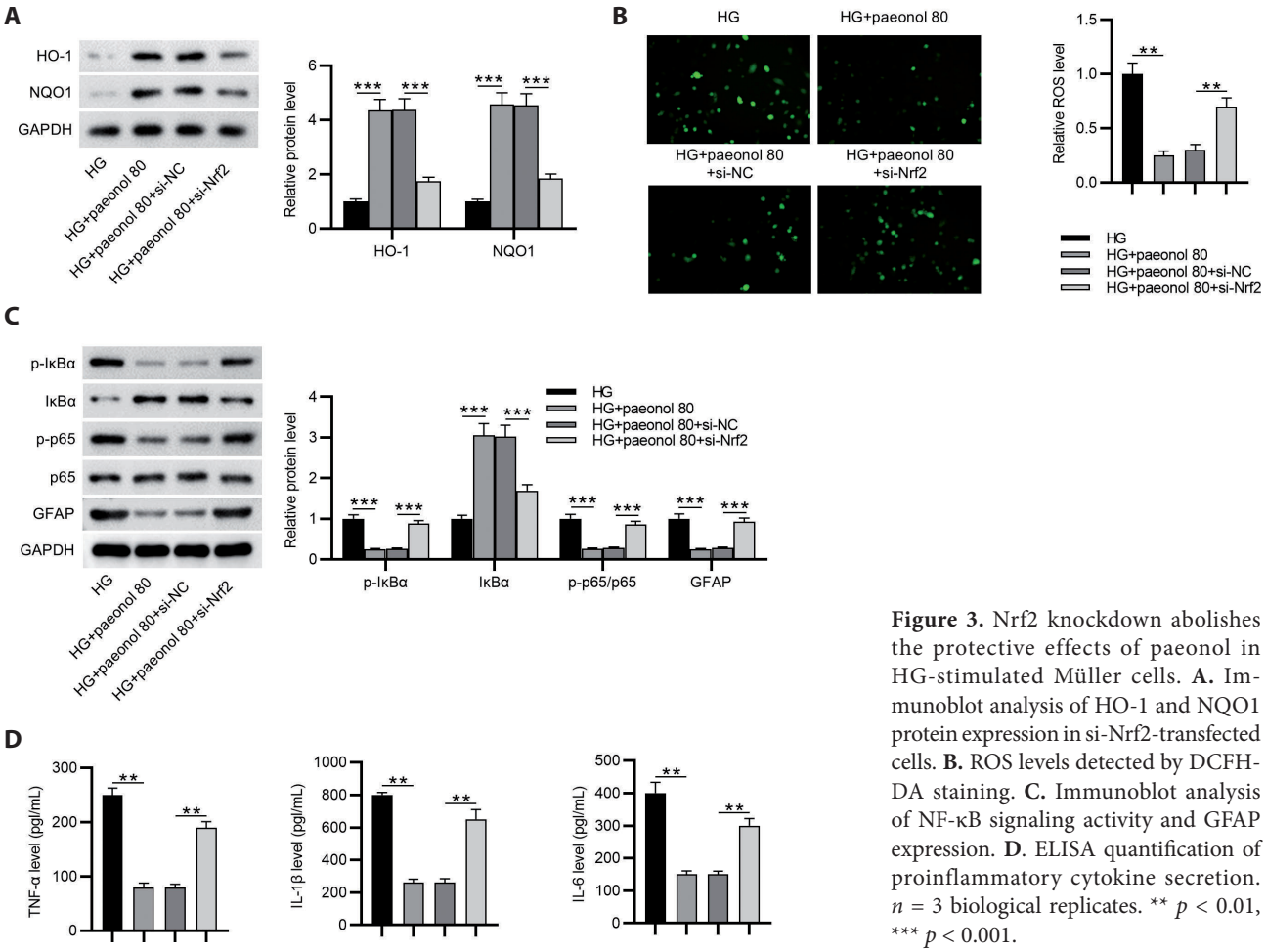

Figure 3. Nrf2 knockdown abolishes the protective effects of paeonol in HG-stimulated Müller cells. **A.** Immunoblot analysis of HO-1 and NQO1 protein expression in si-Nrf2-transfected cells. **B.** ROS levels detected by DCFH-DA staining. **C.** Immunoblot analysis of NF-κB signaling activity and GFAP expression. **D**. ELISA quantification of proinflammatory cytokine secretion. $n = 3$ biological replicates. ** $p < 0.01$, *** $p < 0.001$.

0.01; Fig. 4A). Paeonol administration restored diabetes-induced reductions in HO-1 and NQO1 protein levels ($p < 0.001$; Fig. 4B) and mitigated retinal ROS overproduction ($p < 0.01$; Fig. 4C). Western blot analysis further revealed that paeonol suppressed diabetes-associated NF-κB activation (evidenced by reduced p-IκBα and p-p65 and increased p-IκBα) and attenuated reactive gliosis (decreased GFAP) in retinal tissues ($p < 0.001$; Fig. 4D). ELISA confirmed paeonol's anti-inflammatory efficacy, with significant reductions in retinal TNF-α, IL-1β, and IL-6 levels compared to untreated diabetic mice ($p < 0.01$; Fig. 4E).

Paeonol preserves BRB integrity in DR mice

Quantitative RT-qPCR analysis demonstrated that diabetic retinas exhibited marked downregulation of junctional proteins critical for BRB integrity – VE-cadherin, claudin-5, occludin, and ZO-1 – all of which were significantly restored by paeonol treatment ($p < 0.01$; Fig. 5).

Discussion

Oxidative-inflammatory crosstalk in DR pathogenesis

Recent advances underscore that hyperglycemia initiates a self-perpetuating cycle of oxidative stress and inflammation, driving retinal neurodegeneration and vascular leakage in DR (Kowluru 2023; Rani 2023). Our findings align with this paradigm, demonstrating that HG suppresses Nrf2 nuclear translocation in Müller cells, a critical regulator of antioxidant defenses, thereby exacerbating ROS accumulation. This oxidative milieu directly activates NF-κB signaling, amplifying the production of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and Müller cell gliosis (GFAP upregulation). These events mirror clinical observations where retinal oxidative damage precedes microvascular complications (Callan 2024), and NF-κB-driven inflammation accelerates BRB breakdown (Lim 2024). Our data extend prior work by delineating the sequential pathway linking HG-induced Nrf2 suppression to NF-κB activation and BRB disruption (Fig. 6).

Nrf2/NF-κB axis as a therapeutic target

The interplay between Nrf2 and NF-κB in DR has gained prominence as a dual therapeutic target. While Nrf2 activators like dh404 (Deliyanti 2018) and chlorogenic acid (Ouyang 2022) mitigate oxidative stress in diabetic retinas, their effects on NF-κB suppression remain underexplored in glial cells. This study provides the first demonstration of a natural phenolic compound (paeonol) uniquely bridging antioxidant and anti-inflammatory responses through coordinated Nrf2 activation and NF-κB inhibition – a therapeutic strategy not previously reported for phenolic compounds in diabetic retinal glial cells. This dual modulation distinguishes paeonol from single-pathway agents (e.g., anti-VEGF therapies) and aligns with emerging strategies targeting oxidative-inflammatory crosstalk (Chalke 2021; Tu 2021; Xu 2024). The selection of paeonol concentrations (40–80 μg/ml) for mechanistic studies was guided by rigorous cytotoxicity profiling. These doses represent the maximal non-cytotoxic range under both normoglycemic and hyperglycemic conditions, balancing therapeutic efficacy with cellular safety. This precaution ensures observed effects reflect genuine pharmacodynamic actions rather than confounding cytotoxicity.

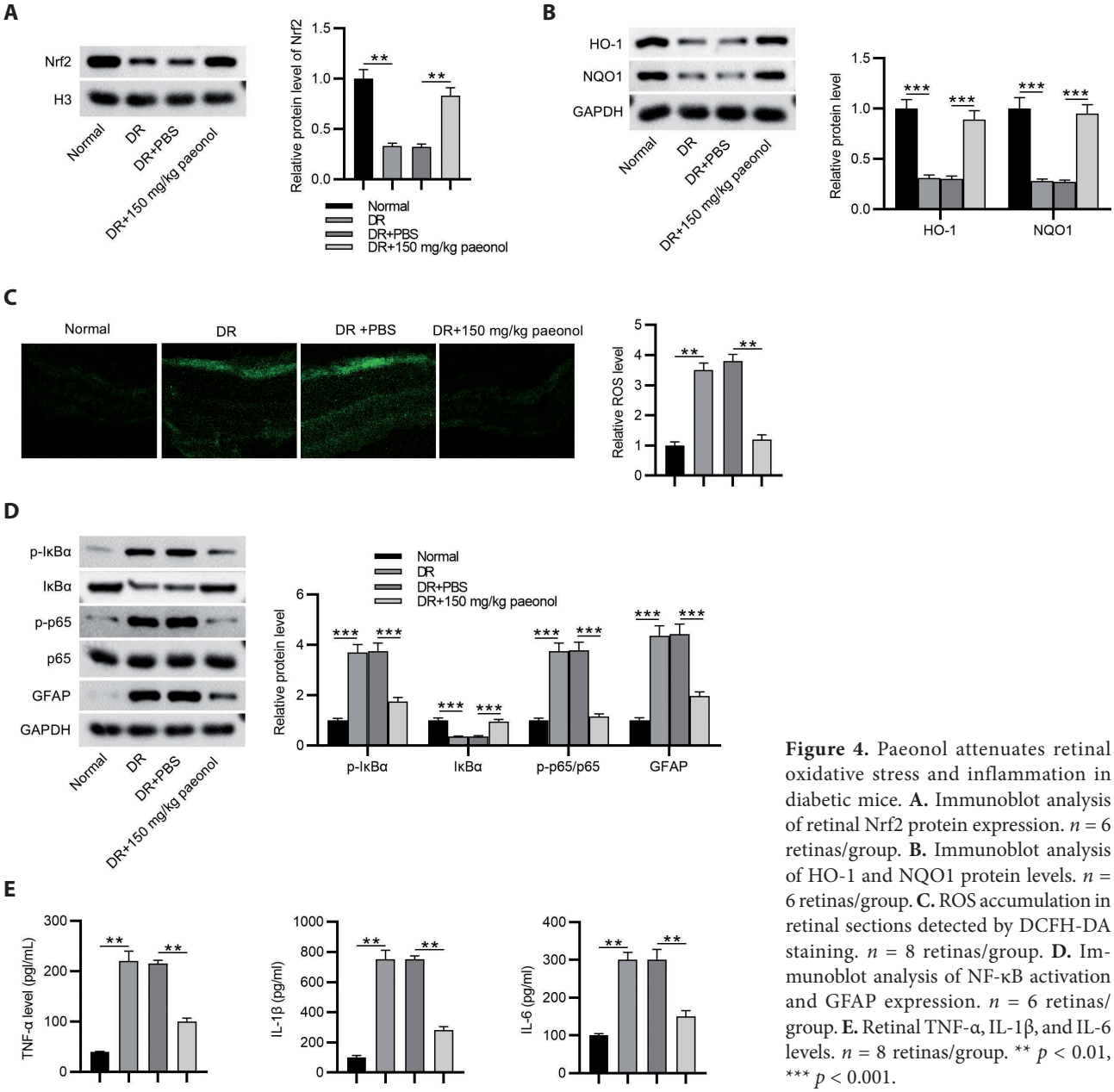

Figure 4. Paeonol attenuates retinal oxidative stress and inflammation in diabetic mice. **A.** Immunoblot analysis of retinal Nrf2 protein expression. $n = 6$ retinas/group. **B.** Immunoblot analysis of HO-1 and NQO1 protein levels. $n = 6$ retinas/group. **C.** ROS accumulation in retinal sections detected by DCFH-DA staining. $n = 8$ retinas/group. **D.** Immunoblot analysis of NF-κB activation and GFAP expression. $n = 6$ retinas/group. **E.** Retinal TNF-α, IL-1β, and IL-6 levels. $n = 8$ retinas/group. ** $p < 0.01$, *** $p < 0.001$.

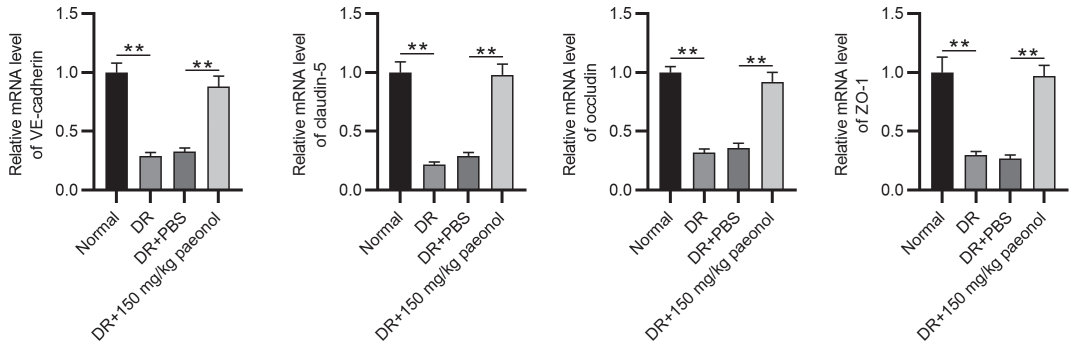

Figure 5. Paeonol preserves BRB integrity in diabetic mice. RT-qPCR analysis of mRNA expression of tight junction proteins (VE-cadherin, claudin-5, occludin, ZO-1) in retinas. $n = 7$ retinas/group. ** $p < 0.01$.

Paeonol's novel role in glial-vascular protection

Müller cells are central to DR progression, yet few studies have explored natural compounds targeting their dysfunction. Prior work by Adki and Kulkarni (2022) demonstrated paeonol's antioxidant effects in DR rats but did not link these to Nrf2/NF-κB crosstalk or BRB repair. Our study bridges this gap, showing paeonol reverses HG-induced GFAP overexpression and restores BRB integrity by upregulating junctional proteins – effects dependent on Nrf2 activation. This glial-centric mechanism complements recent findings that cyanidin-3-O-glucoside and verbascoside (Anfuso 2022) and morin (Jiang 2020) protect BRB *via* antioxidant pathways but emphasizes paeonol's unique dual action on Müller cells and vasculature. Importantly, our data align with clinical evidence that BRB stabilization slows DR progression (Li 2021).

Limitations and future directions

While our study establishes paeonol's efficacy in preclinical models, translational limitations must be acknowledged. First, the STZ-induced DR model does not fully replicate human DR's chronic progression. Second, systemic paeonol administration may have off-target effects; future studies should assess intravitreal delivery to enhance retinal bioavailability (Adu-Agyeiwaah 2023). Lastly, the crosstalk between Nrf2 and other pathways (e.g., NLRP3 inflammasome) (Zhang 2020) in paeonol's mechanism warrants exploration. Nevertheless, our findings provide a foundational rationale

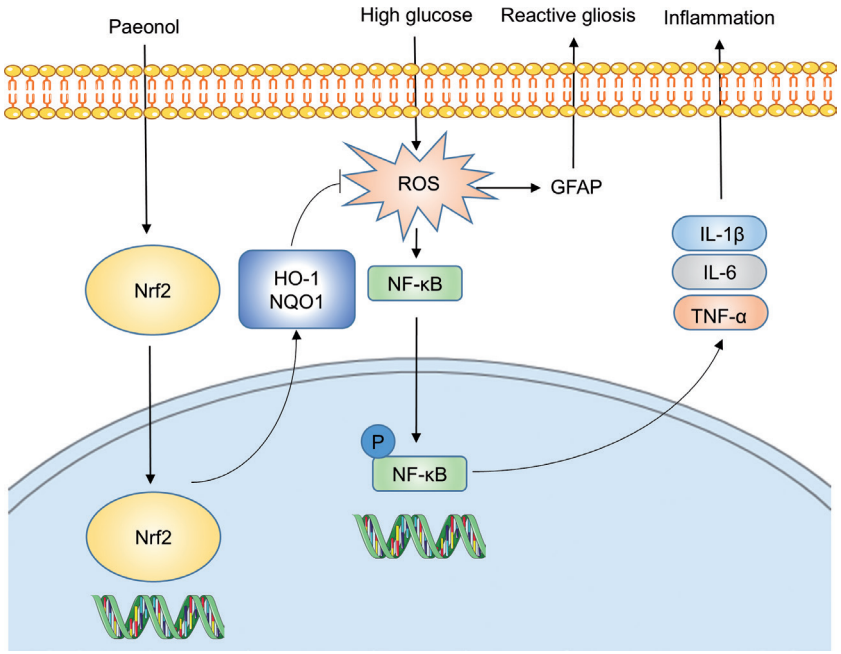

Figure 6. Proposed mechanism of paeonol in DR. Schematic illustrating paeonol-mediated dual regulation of Nrf2 antioxidant signaling and NF-κB inflammatory pathway in Müller cells and diabetic retinas.

for evaluating paeonol in combinatorial therapies with existing anti-VEGF or anti-inflammatory agents.

Conclusion

In summary, this study demonstrates that paeonol attenuates DR by activating Nrf2 to suppress oxidative stress, inhibit NF-κB-driven inflammation, and restore BRB integrity in hyperglycemic Müller cells and murine models. These findings provide the first evidence of paeonol's dual modulation of the Nrf2/NF-κB axis in retinal glial cells, distinguishing it from single-target therapies. Our work supports paeonol's potential as a supplementary therapy for early-stage DR. Future research should prioritize clinical translation, including pharmacokinetic profiling and long-term safety assessments, to evaluate its viability for DR management.

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Conflict of interests. The authors declare that they have no competing interests.

Ethic approval. All experimental procedures were approved by the Laboratory Animal Welfare & Ethics Committee of Bestcell Model Biological Center (Approval Number. BSMS2023-11-14A; Date, 2023.11.10).

Authors' Contributions. XL conceived and designed the experiments. XL and KW carried out the experiments. XL and KW analyzed the data. XL and KW drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Data Availability Statement. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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