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Ganoderic acid protects against diabetic retinal injury *via* modulating inflammatory, apoptotic, and antioxidant signaling pathways

Shu−Jing Zhao, He−Chen Li, Qian Li, Lei Wang✉

Department of Ophthalmology, the Second People's Hospital of Jinan City, Jinan, 250001, Shandong, China

ABSTRACT

Objective: To investigate whether ganoderic acid can alleviate streptozotocin-induced diabetic retinopathy and explore its underlying mechanisms.

Methods: Streptozotocin (55 mg/kg) combined with nicotinamide (100 mg/kg body weight) was used to establish a diabetes mellitus rat model. Rats were then orally administered ganoderic acid at doses of 12.5, 25, and 50 mg/kg. Body weight, blood glucose, organ weights, and serum insulin levels were determined. Hepatic and renal biochemical indices, as well as markers related to oxidative stress, angiogenesis, inflammation, and apoptosis in retinal tissues, were also assessed.

Results: Ganoderic acid treatment significantly (P<0.001) lowered blood glucose and improved insulin levels and body and organ weights. The treatment remarkably attenuated blood-retinal barrier disruption, reduced acellular capillaries, and ameliorated hepatic and renal pathological changes, accompanied by decreased levels of IL-1β, TNF-α, IL-6, IL-17, COX-2, and PGE_2. In addition, ganoderic acid upregulated Nrf2 and HO-1 and modulated the mRNA expression of *Bax* and *Bcl−2*.

Conclusions: Ganoderic acid ameliorates streptozotocin-induced diabetic retinopathy by inhibiting inflammation and apoptosis and enhancing antioxidant activities.

KEYWORDS: Diabetic retinopathy; Ganoderic acid; Glucose; Nrf2/HO-1; Insulin

1. Introduction

Diabetes mellitus (DM) is a hormonal disorder characterized by impaired insulin action. It is a progressive disease that affects over 230 million people worldwide. DM damages both microvascular and macrovascular structures across the body[1]. This metabolic dysregulation arises from either peripheral tissue insulin resistance (non-insulin-dependent diabetes mellitus, or Type 2 diabetes) or autoimmune destruction of pancreatic β cells (insulin-dependent diabetes mellitus, or Type 1 diabetes)[2]. Chronic elevation of blood glucose levels can lead to complications and damage organs such as the heart, kidneys, eyes, and nerves. Maintaining glycemic control is crucial for preventing long-term complications and preserving the health of these vital organs[3]. Chronic hyperglycemia predominantly damages the ocular microvasculature, giving rise to conditions such as diabetic retinopathy, lenticular opacities, keratopathy, and neovascular glaucoma. These complications can directly impact vision and pose serious threats to ocular health, with advanced disease carrying a significant risk of blindness[1,4]. Hyperglycemia increases aldose reductase activity and sorbitol production *via* the neurotoxic polyol pathway[5]. This hyperglycemic metabolic perturbation also promotes advanced glycation end product

Summary

Question: Can ganoderic acid alleviate streptozotocin-induced diabetic retinopathy in rats?

Findings: Compared with untreated diabetic rats, ganoderic acid significantly lowered blood glucose, improved insulin levels, and mitigated diabetic retinal injury by suppressing inflammatory cytokine and apoptosis while boosting antioxidant defenses.

Meaning: Ganoderic acid mitigates diabetic retinopathy in rats by inhibiting inflammation and apoptosis and enhancing antioxidant activities.

✉To whom correspondence may be addressed. E-mail: fesan@163.com

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(AGE) formation, protein kinase C activation, and the hexosamine biosynthetic pathway, which together contribute to diabetic microvascular complications[6].

Diabetic retinopathy (DR) is a major complication of DM and a leading cause of diminished visual acuity and acquired blindness in working-age adults, affecting both developed and developing nations[7]. Early pathological manifestations of DR observed in diabetic rodents and human patients include neurodegeneration, vascular dysfunction, and glial dysfunction[8,9]. Although laser techniques exhibit therapeutic potential for DR, the existing treatments for DR are considered unsatisfactory. Given the escalating global incidence of DR, there is a pressing need for more targeted and effective treatment options[10].

Numerous reports on individuals with chronic diabetes demonstrate that elevated blood glucose correlates with lens opacification and ocular edema[11]. Uncontrolled hyperglycemia promotes sorbitol accumulation within the lens, potentially increasing the risk of cataract development. Retinal vascular endothelial dysfunction and impairment of retinal proteins further contribute to microvascular injury and pathological neovascularization in DR[12]. Furthermore, lysine-residue glycation of lens and macular proteins together with dysregulated antioxidant enzyme activity are implicated in the pathogenesis of diabetic ocular injury, including cataract and retinopathy[11].

DR is characterized by inflammatory responses and vascular oxidant dysfunction. Locally generated inflammatory cytokines, chemokines, and reactive oxygen species (ROS) play a crucial role in mediating the pathogenesis of DR and act as primary triggers for multiple downstream pathological events[13]. Accordingly, both inflammation and hyperglycemia are pivotal drivers of DR progression, and their relative contributions vary depending on diabetes subtype. In diabetic patients, both inflammation and hyperglycemia constitute core pathogenic targets in DR[14]. Increased systemic inflammatory cytokines independently induce retinal injury through blood-retinal barrier (BRB) disruption, enhanced ROS production, elevated vascular permeability and macrophage recruitment, and microglial activation; activated microglia subsequently induce neuronal apoptosis and pericyte loss within the retina[13,15].

Ganoderic acid was selected as the research candidate in this study owing to its well-documented pharmacological activities, particularly antioxidant, anti-inflammatory, and anti-apoptotic properties[16,17]. Excessive oxidative stress, persistent inflammation, and activation of apoptotic pathways are closely involved in neuronal and vascular damage during diabetic retinal injury[18]. Previous studies have demonstrated that ganoderic acid modulates cellular antioxidant defense systems and inflammatory signaling. Nevertheless, its protective role and molecular mechanisms in diabetic retinal injury are poorly understood. Accordingly, the current study aimed to explore the protective effects of ganoderic acid against retinal injury in a streptozotocin-induced diabetic rat model, focusing on its regulation of oxidative stress, inflammation, apoptosis, and related signaling pathways.

2. Methods

2.1. Animals and ethical approval

Male Wistar rats weighing (200±20) g (12-14 weeks old) were used in this study. All experimental animals were sourced from the institutional animal housing facility and housed in standard polyethylene cages, with six rats per cage. The animals were maintained under controlled laboratory conditions [a 12 h dark/light cycle, relative humidity of (60 ± 5)%, and ambient temperature of (22 ± 5) ℃], with free access to food and drinking water. All experimental procedures involving animals were approved by the Ethical Committee of the Second People's Hospital of Jinan City (Approval No. 20231022d; approved on 22 October 2023) and were performed in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and applicable ethical requirements for animal experimentation.

2.2. Induction of diabetes

For diabetic model establishment, all rats underwent an 18-hour overnight fast with *ad libitum* access to drinking water before treatment. Streptozotocin (STZ) was dissolved in citrate buffer to obtain a homogeneous solution and intraperitoneally injected into rats at a dose of 55 mg/kg body weight (b.w.)[11]. Nicotinamide (100 mg/kg b.w.) was administered intraperitoneally to rats prior to STZ administration[11]. The blood glucose level was measured 72 h after the combined injection of STZ and nicotinamide. Rats with blood glucose levels higher than 300 mg/dL were considered diabetic and included in subsequent experiments.

Fasting blood glucose level measurement was performed using a commercial Johnson & Johnson OneTouch® glucometer, with matched supporting test strips. Rats were fasted overnight with free access to water before measurement. After gentle tail warming to facilitate peripheral blood circulation, a small drop of fresh tail vein blood was collected and immediately loaded onto the test area of the glucose strip. The glucometer automatically detected and displayed the blood glucose concentration, with all results recorded in mmol/L.

2.3. Experimental protocol

After induction of DM, the rats were divided into the following groups (n=6): Group A: normal (received saline only); Group B: STZ control (received saline only); Group C: STZ + Ganoderic acid (12.5 mg/kg); Group D: STZ + Ganoderic acid (25 mg/kg); Group E: STZ + Ganoderic acid (50 mg/kg); and Group F: STZ + Gliclazide (1 mg/kg), respectively.

The dose of ganoderic acid was selected on the basis of previously reported doses[19]. The rats received the above-mentioned treatment for 56 d, and food and water intake were monitored weekly throughout the treatment period. The body weight and fasting blood glucose levels were measured on a weekly basis[20].

At the end of the study, all rats were fasted overnight and anesthetized *via* intraperitoneal injection of a mixed anesthetic solution (1 mL/kg), consisting of 80 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis) and 12 mg/kg xylazine hydrochloride (Rompun)[13]. Major organs including the retina, pancreas, liver, and kidney were dissected and weighed. Complete anesthesia was confirmed by the absence of response to noxious stimuli, with an effective anesthetic duration of 45-60 min. Cardiac blood samples were collected into 1.5 mL plain tubes, followed by centrifugation at 5000 ×*g* for 10 min to separate serum. The isolated serum was then frozen and stored at −20 ℃ until use. After blood collection, rats were euthanized *via* cervical dislocation. Ocular tissues were rapidly enucleated, and redundant surrounding muscle and connective tissues were removed in ice-cold phosphate-buffered saline. For retinal sample preparation, isolated retinal tissues were homogenized in ice-cold phosphate-buffered saline (pH 7.4) at a weight-to-volume ratio of 1∶9. The homogenates were centrifuged at 10000 ×*g* for 10 min, and the resulting supernatants were collected and stored at −80 ℃ until biochemical detection.

2.4. Estimation of plasma insulin

Briefly, standard solutions and serum samples were dispensed into the designated wells of an insulin antibody-precoated microplate. After incubation, the wells were thoroughly washed to remove unbound residues, followed by the addition of enzyme-linked detection reagents. Following a further incubation and washing step, chromogenic substrate was added to each well, and color development proportional to insulin concentration was observed. The reaction was terminated by stop solution and the absorbance was measured at 450 nm using a microplate reader. A standard curve was established based on the detected optical densities of insulin standards, and the serum insulin concentrations of samples were calculated accordingly and expressed as μIU/mL.

2.5. Estimation of BRB breakdown

Retinal vascular permeability was assessed by using the previously described Evans blue dye extravasation technique with slight modifications[21].

2.6. Quantification of acellular capillaries and retinal area

Retinal flat mounts were prepared, and stained as appropriate for visualization of retinal microvasculature. Representative images were captured with a fluorescence microscope using identical acquisition settings. Acellular capillaries were defined as hollow basement membrane tubes lacking endothelial cells and pericytes. Acellular capillaries were quantified in non-adjacent fields of retinal parenchyma, and expressed as the number of acellular capillaries per unit area retina. The acquired images were analyzed using ImageJ by selecting the retinal tissue/area of interest and determining the respective area. All measurements were done in a blinded manner, and statistical analysis was conducted based on the average values derived from multiple microscopic fields per retinal sample.

2.7. Detection of hydrogen sulfide (H_2S) level

H_2S levels in rat retinal tissues and plasma were determined using a previously reported method with minor modifications[22].

2.8. Detection of retinal peroxynitrite ($ONOO^-$) and superoxide radical (O_2^-) production

Retinal $ONOO^-$ and O_2^- production were detected using the method described by Elks *et al.*, with minor modifications[23].

2.9. Hepatic and renal function and serum electrolyte analysis

Serum hepatic function parameters (alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase), renal function parameters (gamma-glutamyl transferase, total protein, globulin, albumin, creatinine, and urea), as well as serum electrolytes (potassium, sodium, and chloride) were measured using commercial ELISA kits in strict accordance with the manufacturer's instructions.

2.10. AGEs determination

Serum AGE concentrations were determined using a commercial rat AGE ELISA kit following the manufacturer's protocols and a previously established assay procedure. Briefly, AGE standards and serum samples were added in duplicate to antibody-precoated microplate wells. The plate was subsequently washed to remove non-specifically bound substances. Subsequently, 50 μL of enzyme-conjugated detection reagent was added to each well for further incubation. After additional incubation and washing steps, the chromogenic substrate was added and the reaction was terminated using stop solution. The absorbance value of each well was measured at 450 nm with a microplate reader, and AGE levels were calculated according to the standard curve provided by the manufacturer.

2.11. Detection of protein carbonyl (PCO) content

Commercial protein carbonyl assay kit (Bioxytech Protein Carbonyl Kit, Oxis International, Portland, Oregon) was used for measuring free carbonyl in tissue homogenates following the manufacturer's recommendations. Briefly, protein samples were incubated with

2,4-dinitrophenylhydrazine, which reacts with oxidized amino acid residues to form protein hydrazones. The resulting derivatives were then subjected to the incubation and washing steps specified in the corresponding assay protocol and measured spectrophotometrically at the appropriate wavelengths. PCO content was calculated using the standard curve and formula provided and expressed as nmol/mg protein.

2.12. Detection of antioxidant and inflammatory parameters

A series of oxidative stress and inflammatory biomarkers were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, CA, USA) in accordance with the manufacturer's protocols. The detected oxidative stress indicators included reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), heme oxygenase-1 (HO-1) and Nrf2. The inflammatory and angiogenic mediators comprised interleukin-1β (IL-1β), interleukin-17 (IL-17), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and prostaglandin E_2 (PGE_2).

2.13. Quantitative real–time polymerase chain reaction (qRT–PCR) analysis of mRNA expression

Total RNA was extracted from rat retinal tissues using the Total RNA kit (Life Technologies Inc., Gaithersburg, MD, USA) as per the manufacturer's instructions. Complementary DNA (cDNA) was subsequently synthesized from purified RNA following the corresponding kit protocol. qRT-PCR was performed on a Bio-Rad CFX96 system using Ssofast Evergreen Supermix to detect the mRNA expression levels of target genes. The 10 μL reaction system consisted of 5 μL of supermix master mix, 1 μL of template cDNA (500 ng), 0.2 μL of forward primer (200 nM), 0.2 μL of reverse primer (200 nM), and 3.6 μL of nuclease-free water. The amplification was performed as follows: (1) initial pre-denaturation at 95℃ for 30 s; (2) 34 cycles of denaturation at 95℃ for 30 s; (3) annealing at 60℃ for 30 s; and (4) a final melting step at 95℃ for 1 s to verify the specificity of amplification products. The relative mRNA expression levels of target genes were normalized to the internal reference gene *β–actin* and calculated using the $2^{-\Delta\Delta Ct}$ formula. The primer sequences used in this study are listed in Table 1.

2.14. Statistical analysis

All the experimental data were expressed as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was applied to evaluate the differences between experimental groups, followed by Tukey's multiple comparisons test. A P-value < 0.05 was considered significantly different.

3. Results

3.1. Effect of ganoderic acid on body weight, organ weight, as well as blood glucose and insulin levels

Rats in the DR group showed reduced body weight compared with the normal control group (P<0.001). Ganoderic acid treatment significantly (P<0.05) increased body weight in a dose-dependent manner (Figure 1A). In addition, rats in the DR group exhibited

Table 1. List of primers used in RT-PCR analysis.

Genes	Forward	Reverse
Bcl–2	TGGGATGCCTTTGTGGAACT	TCTTCAGAGACTGCCAGGAGAAA
Bax	ATGGAGCTGCAGAGGATGATT	TGAAGTTGCCATCAGCAAACA
β–actin	TACCCAGGCATTGCTGACAG	AGCCACCAATCCACACAGAG

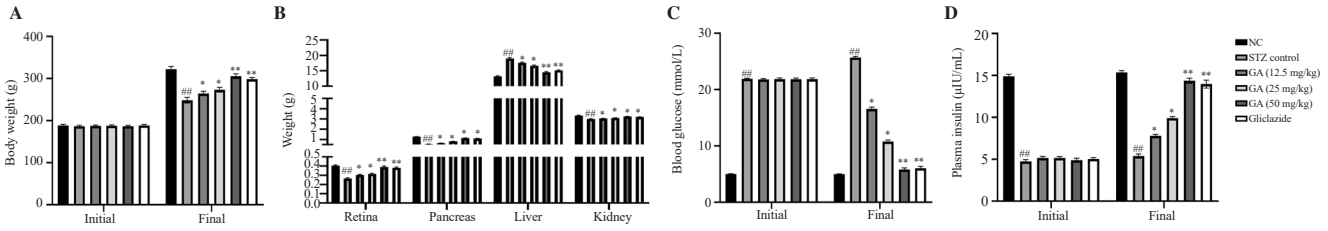


Figure 1. Effect of ganoderic acid on (A) body weight, (B) organ weights, as well as (C) blood glucose and (D) insulin levels in rats with streptozotocin (STZ)-induced diabetic retinopathy. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the normal control (NC) group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group. GA: ganoderic acid.

altered weights of organs including the retina, pancreas, liver, and kidney (P<0.001), whereas ganoderic acid dose-dependently restored organ weights to near-normal levels (P<0.05) (Figure 1B).

The DR group displayed elevated blood glucose (Figure 1C), and reduced insulin levels were observed in the DR group (P<0.001) (Figure 1D). In contrast, ganoderic acid-treated DR rats showed significantly decreased blood glucose and increased insulin levels (P<0.05).

3.2. Effect of ganoderic acid on BRB breakdown, acellular capillaries and retinal area

Figure 2 exhibits increased BRB breakdown (Figure 2A), greater numbers of acellular capillary segments (Figure 2B), and a reduced retinal area (P<0.001) (Figure 2C) in DR rats. Ganoderic acid treatment significantly (P<0.05) attenuated BRB breakdown, decreased the number of acellular capillaries, and alleviated the reduction in retinal area.

3.3. Effect of ganoderic acid on H_2S level

Serum and retinal H_2S levels were markedly decreased in DR rats (P<0.001) (Supplementary Figure 1). In contrast, ganoderic acid treatment significantly (P<0.05) elevated these H_2S levels.

3.4. Effect of ganoderic acid on hepatic and renal parameters

The DR group exhibited altered serum levels of gamma-glutamyl transferase, total protein, globulin and albumin, together with changed activities of alkaline phosphatase, aspartate

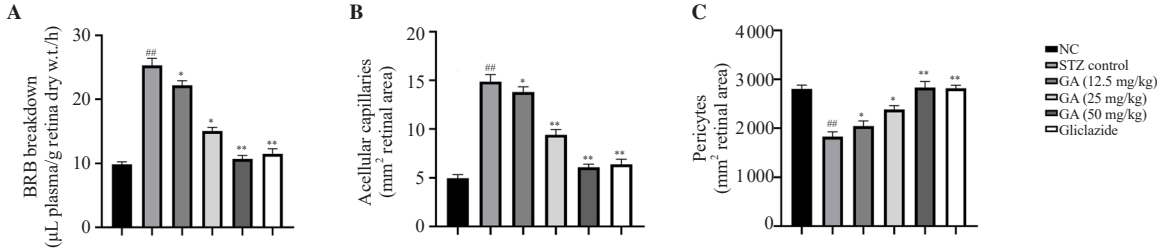


Figure 2. Effect of ganoderic acid on (A) BRB breakdown, (B) acellular capillaries and (C) retinal area in rats. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group. BRB: blood-retinal barrier.

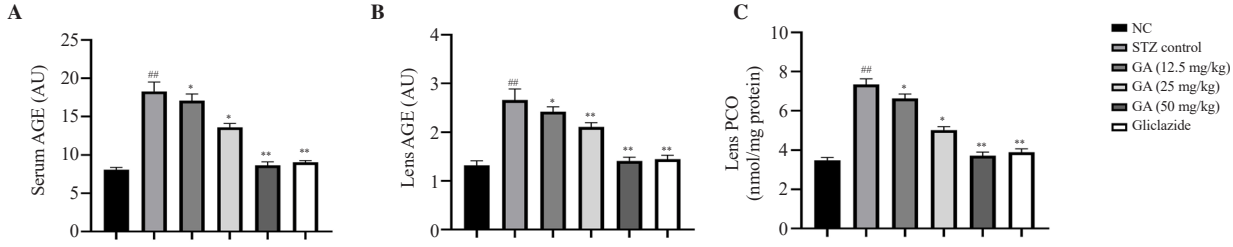


Figure 3. Effect of ganoderic acid on (A-B) AGE levels in serum and lens, and (C) lens PCO levels in rats. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group. AGE: advanced glycation end product; PCO: protein carbonyl.

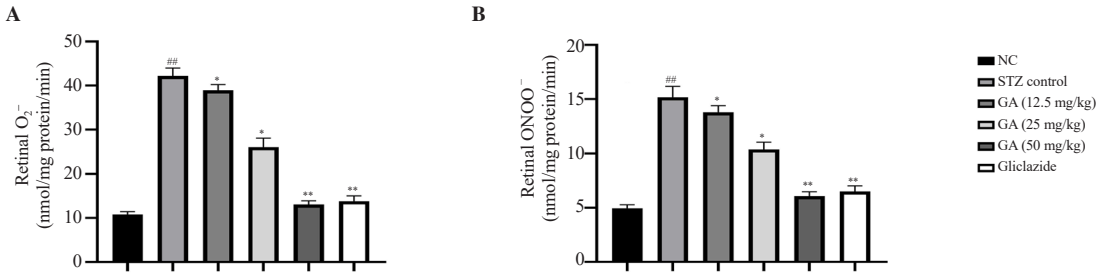


Figure 4. Effect of ganoderic acid on retinal O_2^- and $ONOO^-$ levels in rats. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group.

aminotransferase, and alanine aminotransferase (Supplementary Figure 2). However, ganoderic acid treatment remarkably reversed these biochemical alterations (P<0.05).

Moreover, altered serum levels of potassium, creatinine, sodium, urea, and chloride were found in DR rats (P<0.001) (Supplementary Figure 3), which were improved by ganoderic acid treatment.

3.5. Effect of ganoderic acid on AGE and PCO levels

DR rats exhibited notably elevated AGE levels in serum (Figure 3A) and lens tissue (Figure 3B) (P<0.001), whereas ganoderic acid treatment markedly reduced AGE concentrations in both serum and lens (P<0.05). Figure 3C shows increased lens PCO in the DR group (P<0.001); ganoderic acid treatment significantly attenuated lens PCO (P<0.05).

3.6. Effect of ganoderic acid on retinal O_2^- and $ONOO^-$ levels

Superoxide (O_2^-) and peroxynitrite ($ONOO^-$) levels were markedly increased in the retina of DR rats (P<0.001). Ganoderic acid treatment significantly suppressed O_2^- and $ONOO^-$ levels (P<0.05) (Figure 4).

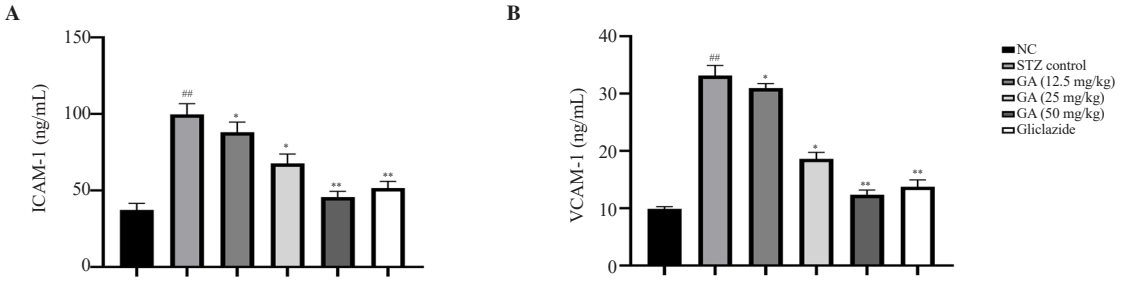


Figure 5. Effect of ganoderic acid on ICAM-1 and VCAM-1 level in rats. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group. ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1.

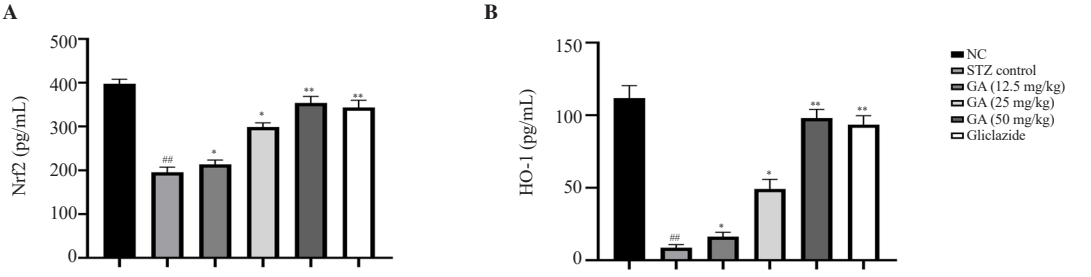


Figure 6. Effect of ganoderic acid on Nrf2 and HO-1 levels in rats. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group. HO-1: heme oxygenase-1.

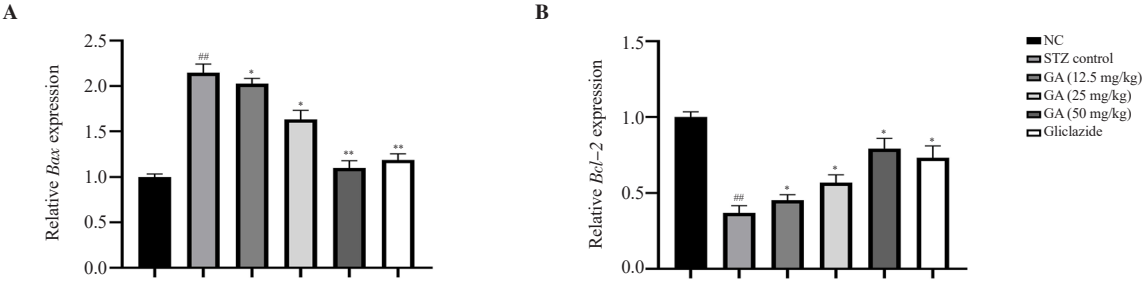


Figure 7. Effect of ganoderic acid on the mRNA expression of *Bax* and *Bcl–2*. *Bax* and *Bcl–2* expression was normalized against *β–actin*. Data are presented as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. $^{\#\#}P$<0.001 compared with the NC group. $^{*}P$<0.05, $^{**}P$<0.001 compared with the STZ control group.

3.7. Effect of ganoderic acid on antioxidant parameters

DR rats showed markedly reduced levels of CAT, SOD, and GSH and increased MDA content (P<0.001) (Supplementary Figure 4). However, ganoderic acid treatment significantly restored antioxidant levels (P<0.05).

3.8. Effect of ganoderic acid on inflammatory cytokines and angiogenic parameters

Rats in the DR group exhibited elevated levels of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-17 (P<0.001) (Supplementary Figure 5), whereas ganoderic acid significantly reduced these cytokine levels (P<0.05).

Likewise, DR rats displayed upregulated expression of COX-2, PGE_2, and VEGF (P<0.001) (Supplementary Figure 6). Ganoderic acid treatment significantly lowered these inflammatory and angiogenic mediators (P<0.05).

3.9. Effect of ganoderic acid on ICAM-1 and VCAM-1

The DR group exhibited markedly elevated expression levels of adhesion molecules ICAM-1 and VCAM-1 (P<0.001) (Figure 5). In contrast, ganoderic acid treatment significantly downregulated the expression of ICAM-1 and VCAM-1 in DR rats (P<0.05).

3.10. Effect of ganoderic acid on Nrf2 and HO-1

The expression of antioxidant-related proteins Nrf2 (Figure 6A) and HO-1 (Figure 6B) was significantly reduced in DR rats (P<0.001). Ganoderic acid administration effectively rescued the downregulation of Nrf2 and HO-1 and remarkably upregulated their expression levels in a dose-dependent manner (P<0.05).

3.11. Effect of ganoderic acid on apoptosis

Compared with the normal control group, DR rats showed significantly upregulated Bax and downregulated Bcl-2 expression (P<0.001) (Figure 7). Administration of ganoderic acid significantly ameliorated these alterations in DR rats (P<0.05).

4. Discussion

DM is a chronic metabolic disorder characterized by abnormal glucose homeostasis, which primarily arises from impaired insulin secretion, reduced insulin sensitivity, or a combination of both[20,24]. Despite substantial advances in glycemic regulation *via* nutritional therapy, herbal medicine, and pharmacological interventions, the clinical management of diabetic complications, including visual impairments, remains challenging[25]. Due to the limitations of conventional drug treatments for diabetes and its complications, especially DR, alternative and complementary therapeutic strategies have attracted increasing research attention.

BRB impairment, predominantly in the retinal ganglion cell layer, is a key driver of retinal neuronal loss, and retinal layer thinning is recognized as a hallmark neurodegenerative alteration during DR pathogenesis. Multiple pathogenic mechanisms are implicated in DR development, including AGE deposition, hyperglycemia, inflammation, oxidative stress, and protein kinase C activation. Chronic hyperglycemia triggers low-grade inflammation and excessive ROS accumulation[26], which play a crucial role in inducing retinal pigment epithelial cell apoptosis and accelerating DR progression. Long-term uncontrolled hyperglycemia results in AGE accumulation beneath the endothelial layer. Specifically, persistent hyperglycemia promotes AGE formation *via* the formation of Amadori products between glucose and cellular proteins. Excessive AGE accumulation alters vascular structure, increases vascular stiffness, and activates intracellular signaling pathways, ultimately resulting in elevated oxidative stress and inflammation[26,27]. In the late stage of the Maillard reaction, browning, cross-linking, biological recognition, and fluorescence occur, along with interactions with specific receptors on macrophages. These processes may contribute to diabetic complications such as retinopathy and cataracts. Elevated intraocular glucose levels can lead to superoxide radicals and AGE formation in the aqueous humor. Additionally, glucose also interacts with cell-surface receptors on lens epithelial cells, further stimulating the generation of hydroxyl and superoxide radicals. Accumulated AGEs further drive retinopathy progression by upregulating the expression of intracellular adhesion molecules[28,29]. Consistent with these pathogenic mechanisms, the present study found that ganoderic acid treatment significantly reduced serum and lens AGE levels in diabetic rats, indicating its protective potential against DR lesions.

Persistent hyperglycemia leads to protein glycation and glucose autooxidation-mediated cellular disruption, which in turn induces excessive oxidative stress[11]. Several reports suggest that sustained hyperglycemia triggers excessive ROS generation, which results in severe oxidative stress and inflammatory injury in retinal tissues and serves as a primary pathogenic factor in DR[28]. In the retina, oxidative stress contributes to increased basement membrane thickness, leading to hypoxia, apoptosis, BRB disruption, pathological inflammation, and aberrant angiogenesis[13]. The endogenous retinal antioxidant defense system consists of nonenzymatic antioxidants (*e.g.*, GSH) and antioxidant enzymes, which jointly maintain intracellular redox homeostasis[13]. Some studies on diabetic rat models have confirmed that the expression and activity of retinal antioxidant enzymes are markedly suppressed under hyperglycemic conditions[4,27], strongly indicating aggravated retinal oxidative stress in diabetic states. Excessive ROS generation not only induces lipid peroxidation, as shown by increased MDA production, but also disrupts endogenous antioxidant systems.

Additionally, ROS overaccumulation promotes mitochondrial injury, inflammation, apoptosis, and DNA fragmentation[30]. Nevertheless, antioxidant-based therapy can protect against retinal injury by reversing oxidative stress-induced damage and inhibiting inflammatory cascades[13]. Consistent with these therapeutic principles, our findings verified that ganoderic acid attenuates DR-associated BRB breakdown by suppressing microglia-mediated excessive inflammation and oxidative stress in the diabetic retina.

Mounting studies have demonstrated that ICAM-1 and VCAM-1 are implicated in the pathogenesis of DR[31,32]. As a typical microvascular complication of diabetes, DR is characterized by progressive retinal vascular injury. The involvement of ICAM-1 and VCAM-1 in DR is closely linked to their roles in promoting leukocyte adhesion and inflammation. Predominantly expressed on the surface of endothelial cells, ICAM-1 primarily facilitates the adhesion and migration of leukocytes to the vascular endothelium. Under diabetes-induced chronic low-grade inflammatory conditions, ICAM-1 facilitates leukocyte migration into retinal tissue, exacerbating local inflammatory responses and vascular damage. Similarly, VCAM-1 mediates the adhesion of monocytes and lymphocytes to the endothelium[10,31,33], and its overexpression is associated with vascular dysfunction, a typical pathological feature of DR. Collectively, the aberrant elevation of ICAM-1 and VCAM-1 triggers retinal leukostasis, characterized by excessive leukocyte adhesion and accumulation on the endothelial surface[32,34]. This pathological process further promotes BRB breakdown, allowing the infiltration of inflammatory cells and macromolecular proteins into retinal parenchyma, which is recognized as a critical event in the pathogenesis of DR[35]. In line with the above mechanisms, our results demonstrated that ganoderic acid treatment significantly downregulated ICAM-1 and VCAM-1 levels, further confirming its protective role against DR *via* inhibiting endothelial inflammatory injury.

The retina is particularly susceptible to oxidative stress, and exaggerated oxidative responses are well documented in the diabetic retinal microenvironment[27]. HO-1 and Nrf2 are major regulators of cellular defense mechanisms against oxidative stress and inflammation, playing an essential role in DR. HO-1 is a cytoprotective enzyme that mitigates oxidative damage by catalyzing the degradation of heme into biliverdin, carbon monoxide, and iron[13,36]. Biliverdin is then converted to the antioxidant bilirubin. As a master antioxidant transcription factor, Nrf2 regulates the expression of a broad spectrum of cytoprotective and antioxidant genes. Under physiological basal conditions, Nrf2 is stably sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), which mediates Nrf2 ubiquitination and subsequent proteasomal degradation to maintain low basal Nrf2 levels. In contrast, diabetic oxidative stress induced by excessive ROS disrupts the binding interaction between Nrf2 and Keap1, thereby stabilizing Nrf2 and facilitating its nuclear translocation. Upon nuclear accumulation, Nrf2 binds to antioxidant response elements in the promoter regions of target genes, leading to transcriptional upregulation of various cytoprotective and antioxidant enzymes. The activated Nrf2/HO-1 signaling cascade effectively offsets oxidative stress by increasing antioxidant capacity and reducing ROS accumulation[37,38]. Moreover, Nrf2 regulates the expression of many key antioxidant and phase II detoxifying enzymes, such as NAD(P)H quinone dehydrogenase 1 (NQO1), CAT, HO-1, and SOD. Additionally, Nrf2 signaling sustains the synthesis and recycling of GSH, a key endogenous antioxidant. Collectively, these downstream molecular mechanisms constitute a robust cellular defense system to mitigate retinal oxidative injury under diabetic conditions[13].

VEGF is a potent vasoactive cytokine and key regulator of retinal vascular remodeling, which drives pathological angiogenesis, BRB disruption, and abnormal endothelial proliferation in DR[37]. Prior reports have demonstrated that VEGF levels are significantly elevated in the retina, plasma and vitreous humor of diabetic patients with retinopathy compared to individuals without retinopathy[38,39]. Józkowiak *et al.* found elevated VEGF levels in the ocular fluids of patients experiencing retinal ischemia[40]. In the present study, ganoderic acid administration significantly reduced VEGF levels, indicating its capacity to suppress pathological angiogenesis and alleviate DR-associated microvascular damage.

The study has several limitations. First, all experiments were performed using a rodent diabetic model; thus, the results cannot be directly extrapolated to human diabetic retinal injury. Second, the current results indicated that ganoderic acid exerted protective effects *via* modulation of antioxidant signaling, inflammatory responses, and apoptotic pathways; however, further mechanistic studies are needed to confirm direct regulatory relationships among these signaling cascades. Third, the long-term efficacy and biosafety of ganoderic acid intervention were not evaluated in this study. Finally, future studies incorporating multiple experimental models, optimized positive controls, prolonged intervention durations, and rigorous clinical trials are warranted to further validate the therapeutic potential and clinical translational value of ganoderic acid for the treatment of DR.

In summary, ganoderic acid ameliorated diabetes-associated retinal injury in the experimental rat model, as evidenced by improved retinal pathological alterations and mitigated oxidative stress, inflammatory responses, and retinal cell apoptosis. Collectively, these findings indicate that the protective effects of ganoderic acid against DR are closely associated with the modulation of antioxidant, inflammatory, and apoptotic signaling pathways. Nevertheless, the present study is limited to animal experiments, and the underlying molecular mechanisms, chronic toxicity, and long-term treatment efficacy of ganoderic acid in diabetic retinal injury remain to be further systematically explored and validated.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

SJZ was responsible for conceptualization, methodology, investigation, data curation and writing original draft. HCL contributed to methodology, investigation, data collection and formal analysis. QL was involved in data validation and analysis, visualization, and manuscript revision. LW contributed to conceptualization, supervision, project administration, funding acquisition and writing review and editing. All authors reviewed and approved the final version of the manuscript.

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