• Basic Research •

Protective effects of zingerone on the retina in diabetic rats

Bahadir Utlu[1], Betül Dertsiz Kozan[2], Serkan Yildirim[3]

[1]Ophthalmology, Erzurum Regional Training and Research Hospital, University of Health Sciences, Erzurum 25150, Türkiye
[2]Ophthalmology, Gazi Yasargil Training and Research Hospital, Diyarbakır 0021, Türkiye
[3]Pathology, Atatürk University Faculty of Veterinary Medicine, Erzurum 25240, Türkiye
Correspondence to: Bahadir Utlu. Ophthalmology, Erzurum City Hospital, University of Health Sciences, Erzurum 25150, Türkiye. bahadirutlu@gmail.com
Received: 2025-07-24 Accepted: 2025-09-16

Abstract

● **AIM:** To investigate the effects of zingerone (ZO) on the retina in diabetic rats.

● **METHODS:** A total of 70 rats were randomly selected and divided into seven groups [diabetic group (Dm+; *n*=10), diabetic+metformin group (Dm+Met; *n*=10), diabetic+ZO25 group (Dm+ZO25; *n*=10), diabetic+ZO50 group (Dm+ZO50; *n*=10), diabetic+metformin group+ZO 50 Group (Dm+Met+ZO50; *n*=10)]. Diabetes was induced by streptozotocin (STZ), and metformin and two different doses of ZO were administered *via* gavage. Retinal tissues were evaluated by histopathological and immunohistochemical analyses.

● **RESULTS:** In diabetic rats, severe retinal inflammation, tissue necrosis, and increased tumor necrosis factor-α (TNF-α) expression were observed. ZO administration reduced these effects in a dose-dependent manner. Protective effects of metformin alone were limited, and no synergistic benefit was observed in ZO+Met groups. Administration of 50 mg/kg ZO to non-diabetic rats caused no retinal toxicity. Additionally, elevated 8-OHdG and c-Jun N-terminal kinase (JNK) expressions in diabetic retinopathy models were significantly reduced by ZO treatment.

● **CONCLUSION:** ZO can markedly reduce the pathological effects of the retina in a diabetic rat model.

● **KEYWORDS:** zingerone; retina; diabetic retinopathy; rat

DOI:10.18240/ijo.2026.03.04

Citation: Utlu B, Kozan BD, Yildirim S. Protective effects of zingerone on the retina in diabetic rats. *Int J Ophthalmol* 2026;19(3):455-461

INTRODUCTION

The unique vascularization of the retina provides essential molecules to the inner and outer retinal layers, which are necessary for the conversion of light into chemical energy and its perception[1]. In addition, the retinal vascular structure functions as a barrier, known as the blood-retinal barrier, which is crucial for the regulation and maintenance of the retinal microenvironment. This barrier is formed by tight junctions of endothelial cells on the inner side and retinal pigment epithelial cells on the outer side. Preservation of the barrier's integrity depends on healthy pericytes and astrocytes surrounding the endothelial cells[2].

Diabetic retinopathy (DR) is a microangiopathy secondary to chronic hyperglycemia. Elevated glucose levels lead to vascular defects by affecting the morphophysiology of retinal vascular cells, beginning with astrocyte and pericyte damage[3]. Dysfunction of these vascular cells, resulting in disruption of the blood-retinal barrier, represents the initial step in a cascade leading to ischemia. Chronic hyperglycemia ultimately results in increased vascular permeability, ischemia, arteriolar hyalinosis, neovascularization, and, depending on exposure duration, vision loss[4]. To understand the progression of blood-retinal barrier damage in diabetic patients, it is essential to elucidate the cellular mechanisms underlying retinal vascular cell dysfunction, particularly in endothelial cells, astrocytes, and pericytes. Although retinal microvascular damage involves multiple biochemical alterations in early stages, clinical manifestations appear later, with changes in the retinal neurovascular unit and its cellular components. Previous studies indicate that inflammation and oxidative stress constitute the primary cellular pathologies in the development of DR. DR is considered a low-grade inflammatory condition[5]. Studies have shown that patients treated with salicylic acid for rheumatoid arthritis have a lower incidence of DR, and proinflammatory cytokines are elevated in the diabetic retina in animal models[6]. Although anti-vascular endothelial growth factor (anti-VEGF) therapy, which targets the primary

pathogenic mechanism of diabetes and underpins many treatment modalities, often fails to achieve desired outcomes in some patients, this supports the theory that other inflammatory mediators, particularly elevated in DR, may contribute. Accumulating evidence demonstrates that inflammatory factors such as interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) are elevated in the vitreous and retina of diabetic patients[7].

Redox reactions at the cellular level are necessary to maintain cellular viability. Oxidative stress, a common cytopathic outcome of excessive reactive oxygen species (ROS) production and compromised antioxidant defenses, plays a critical role in DR pathogenesis. Hyperglycemia induces oxidative stress through accumulation of advanced glycation end products and increased flux through the polyol and hexosamine pathways. This creates a positive feedback loop that exacerbates diabetes-related metabolic abnormalities. Furthermore, hyperglycemia-mediated epigenetic modifications suppress the antioxidant system and disrupt redox balance. Excessive accumulation of oxidative products leads to mitochondrial damage, apoptosis, inflammation, and increased lipid peroxidation in retinal cells, which are particularly rich in polyunsaturated lipids[8].

Zingerone (ZO), a phenolic compound derived from ginger, possesses a range of pharmacological properties that have attracted attention due to its therapeutic potential. Studies have shown that ZO exhibits various biological activities, particularly in the context of inflammation and metabolic regulation. Emerging evidence suggests that ZO can significantly reduce proinflammatory cytokine levels by inhibiting key signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells and peroxisome proliferator-activated receptor[9-10]. This finding supports its role as an anti-inflammatory agent, which is highly relevant given the connection between chronic inflammation and metabolic diseases. Similarly, Rehman *et al*[11] all provided evidence that ZO can improve diabetic nephropathy by controlling oxidative stress and inflammation, highlighting its potential in managing diabetic complications.

In parallel, metformin is also known for its anti-inflammatory properties, which significantly contribute to its therapeutic efficacy in diabetes management. It primarily acts *via* activation of AMP-activated protein kinase (AMPK), improving insulin sensitivity and promoting glucose uptake in peripheral tissues[12]. Metformin also attenuates inflammatory signaling pathways, thereby reducing hyperglycemia and associated complications[13]. Regarding metabolic effects, ZO has been reported to activate AMPK similarly to metformin, improving glycemic control in animal models[11-12]. Furthermore, ZO's impact on lipid metabolism involves inhibition of enzymes involved in gluconeogenesis, which resembles the glucose-lowering effects exhibited by metformin[12]. Additionally, this compound shows promise in activating mechanisms that affect adipocyte lipolysis, contributing to anti-obesity effects that are particularly important for individuals with metabolic syndrome[9,14].

While the metabolic effects of ZO present new therapeutic opportunities, metformin's well-established role as a first-line treatment for type 2 diabetes is supported by extensive clinical evidence demonstrating its safety and efficacy[13]. Moreover, metformin has been rigorously studied in long-term applications and is associated with improved cardiovascular outcomes, whereas this aspect is still under investigation for ZO. Nevertheless, the potential of ZO, highlighted by its antioxidant and anti-inflammatory properties advantageous in preventing diabetes-related complications, aligns with current research trends toward the use of natural compounds in therapy[15-16].

ZO was included in studies to compare the established effects of metformin with those of ZO and to investigate potential synergistic effects. While metformin's comprehensive clinical validation has made it a cornerstone in diabetes treatment, ZO offers a novel and potentially complementary approach. Identifying targets associated with mitigating the effects of cellular and molecular mechanisms that lead to retinal vascular dysfunction will lay the groundwork for developing new therapeutic strategies for more effective management of retinopathy. In this context, the indirect effects of inflammation and cellular-level changes associated with oxidative stress appear as multiple potential therapeutic targets for DR. The primary aim of current studies is to investigate the antioxidant and anti-inflammatory effects of ZO on these pathways.

MATERIALS AND METHODS

Ethical Approval This study was conducted in accordance with the Declaration of Helsinki. Ethics Committee approval was obtained from Erzurum Atatürk University Animal Experiments Local Ethics Committee with its decision dated 27.12.2018 and numbered 233.

Chemicals, Animals, and Experimental Design In this study, 70 adult male Sprague-Dawley rats of the same weight, obtained from Atatürk University Medical Experimental Application and Research Center, were used. The rats included in the study were randomly divided into 7 experimental groups as shown in Table 1.

Streptozotocin (STZ) is an antineoplastic known to cause pancreatic cell destruction. It is commonly used to create a diabetic animal model when administered at high doses in research[12]. In our study, a 50 mg/kg single dose of STZ (Sigma, USA) was administered at pH: 4.5 in cold citrate buffer and intraperitoneally at a dose of approximately 0.2 mL. ZO (Sigma-Aldrich Cat No: W312401) was formulated in 2 doses

Table 1 Study groups

Study groups	Treatment
DM-group (*n*=20)[a]	-
Control group (Cont)	
ZO50 group (*n*=10)	50 mg/kg ZO *via* gavage once at the same time of the day, for 6wk
DM+treatment groups (*n*=50)[b]	
Diabetic group (*n*=10)	-
Diabetic+metformin group (*n*=10)	100 mg/kg metformin *via* gavage once at the same time of day, for 6wk
Diabetic+ZO25 group (*n*=10)	25 mg/kg ZO *via* gavage once at the same time of the day, for 6wk
Diabetic+ZO50 group (*n*=10)	50 mg/kg ZO *via* gavage once at the same time of the day, for 6wk
Diabetic+metformin group+ZO 50 group (*n*=10)	50 mg/kg ZO and 100 mg/kg metformin, *via* gavage, once a day at the same time of day with 12h in between, for 6wk

[a]Intraperitoneal saline; [b]A single dose of 50 mg/kg streptozotocin (STZ) solution was administered intraperitoneally. DM: Diabetes Mellitus; ZO: Zingerone; ZO50: ZO-treated group (50 mg/kg dose); ZO25: ZO-treated group (25 mg/kg dose).

of 25 and 50 mg/kg by diluting in saline to be given to rats in the treatment group. The formulations were administered daily by gastric gavage to the rats in the treatment group. In addition, Metformin (Glifor 1000 mg) at a dose of 100 mg/kg was administered to the rats by gavage at the same time every day for effect and synergy control in 2 groups. On day 40, rats were anesthetized with sevoflurane, pupils were dilated using tropicamide 0.5% drops, and fundus examination was conducted with an indirect ophthalmoscope. Retinal tissues were then collected and preserved in 10% formalin for histopathological and immunohistochemical analyses. Rats were then sacrificed and rat retinal tissues were preserved in formaldehyde (10% formalin solution, room temperature) for immunohistochemical staining and microscopic examination.

Histopathological Examination After sacrifice, the tissues were kept in formaldehyde solution and embedded in paraffin blocks after routine procedures. 4 μm sections were taken from each block and the preparations were stained with haematoxylin-eosin (HE) and evaluated under a light microscope (Olympus Bx 51, Japan). The findings were classified as absent (-), very mild (+), mild (++), moderate (+++) and severe (++++). For immunohistochemical (IHC) examination, an antigen retrieval buffer was used for one-step dehydration and antigen retrieval of paraffin-embedded tissue sections. It was diluted 100-fold with distilled water before use. Paraffin-embedded preparations were placed in the buffer, covered with plastic wrap and boiled in the microwave for 10min. After the sections were cooled and washed in citrate buffer, the IHC protocol was started.

The tissues were then subjected to 2 different formulations for both classical and double immunofluorescence examination. In the first one, TNF-α (52B83; Sc-52746), a rat monoclonal antibody, was incubated (1h) after being diluted and instilled at a 1/100 dilution. DAB chromogen was used as chromogen in the tissues. The stained sections were examined by light microscopy (Zeiss Axio Germany). In the second one, 8-hydroxyguanosine (8-OHdG; Sc-66036), a monoclonal antibody, was diluted to 100 parts percent and incubated (1h). Anti-mouse IgG (AB6785), an immunofluorescence secondary antibody (FITC), was applied as a secondary marker and kept in the dark. Then, c-Jun N-terminal kinase (JNK) 1/3 (Sc-514539), a polyclonal antibody as the second primary antibody, was diluted 100-fold percent and incubated (1h). Texas red (sulforhodamine 101:ab6719), an immunofluorescence secondary antibody (FITC), was applied as a secondary marker and kept in the dark. Then, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI: D1306) was diluted in 200 parts the prepared solution was dripped on the sections and the sections were kept in the dark and the sections were covered with coverslips. The stained sections were examined under a fluorescence attachment microscope (Zeiss Axio Germany). In order to determine the intensity of positive staining from the images obtained as a result of IHC and immunofluorescence staining, 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program by three different pathologists at the same level of expertise, who did not know which procedure was applied to which subject. Similar results were included in the study.

Statistical Analysis SPSS 13.0 program was used for statistical analysis of the tissue examination results. Duncan's test was used for comparison between groups. Non-parametric Kruskal-Wallis test was used to determine the group interaction and Mann Whitney *U* test was used to determine the differences between groups. The data were statistically defined as mean±standard deviation (SD) for area%. In the study, one-way ANOVA followed by the Tukey test was performed for statistical analyses between groups. As a result of the test, $P<0.05$ was accepted as significant and the data were presented as mean±SD.

RESULTS

Histopathological examination of retinal tissues showed that the tissue structures were in their natural appearance in the

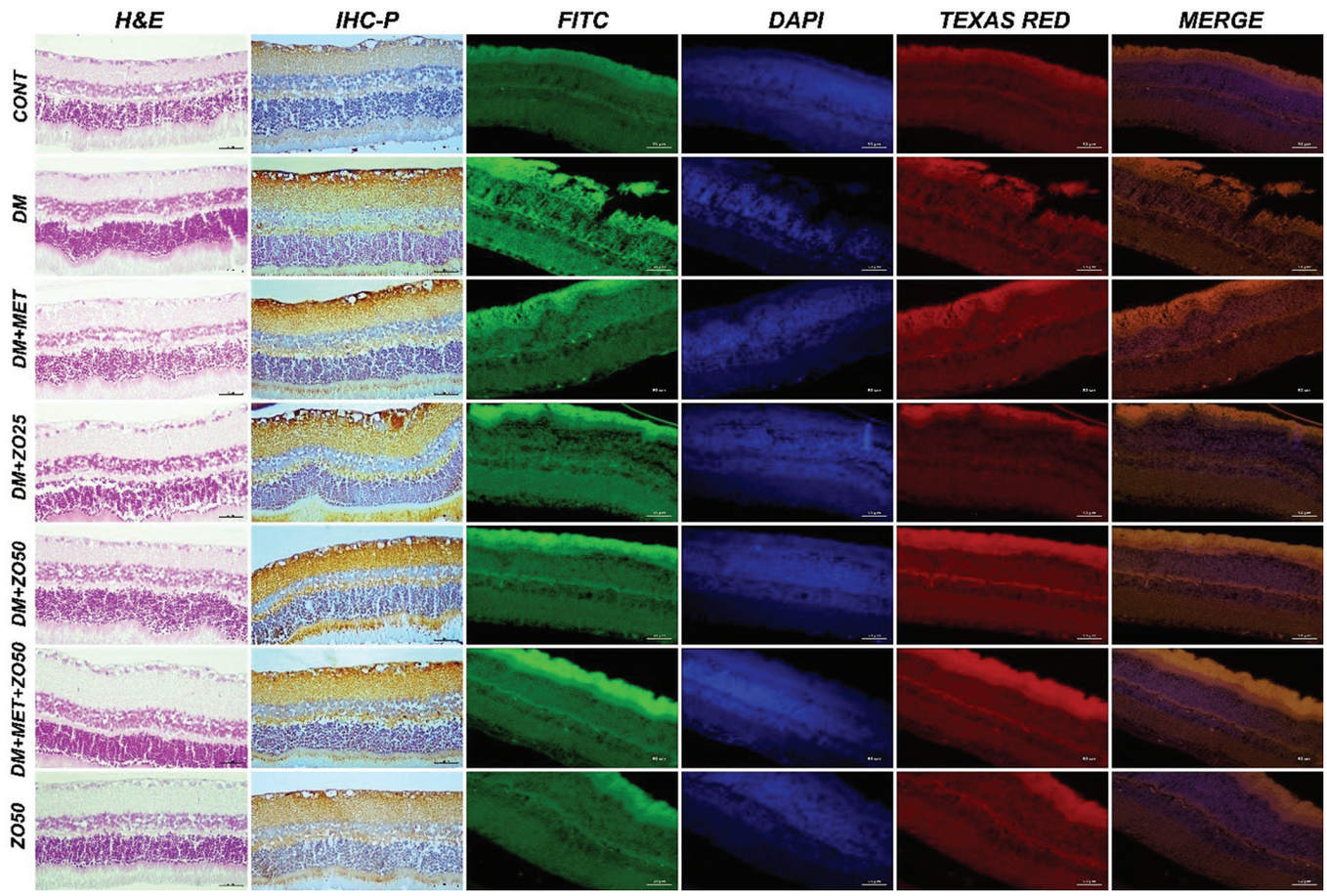

Figure 1 Images of all groups after histopathological and immunohistochemical staining The irregularity of neuroretinal tissues, especially in the DM+group, is noteworthy. HE: Hematoxylin and Eosin; IHC-P: Immunohistochemistry-peroxidase; FITC: Fluorescein isothiocyanate; DAPI: 4',6-diamidino-2-phenylindole; TEXAS RED: Texas red fluorescent dye; DM: Diabetes mellitus; ZO50: Zingerone-treated group (50 mg/kg dose) ; ZO25: Zingerone-treated group (25 mg/kg dose); MET: Metformin.

control group, whereas severe degenerative and necrotic changes in retinal vascular endothelial cells and intense irregularities in neuroretinal layers were detected in the DM+ group. In the DM+Met group, moderate degeneration and mild necrosis in retinal vascular endothelial cells and mild irregularities in neuroretinal layers were detected. In the DM+ZO25 group, high-severity degeneration moderate necrosis in endothelial cells, and moderate irregularity in neuroretinal layers were observed. In the DM+ZO50 group, moderate degeneration and mild necrosis in retinal vascular endothelial cells and mild irregularity in neuroretinal layers were observed. Finally, the DM+Met+ZO50 group showed very mild degeneration in retinal vascular endothelial cells and mild irregularities in neuroretinal layers. When these findings were compared with the DM+ group, the differences between them were statistically significant (P<0.05). In addition, it was observed that the neuroretinal tissues in the ZO50 group, in which toxicity was examined, had a normal histological structure (Figure 1). To detect DNA damage and necrosis in retinal tissues, 8-OHdG and JNK antibody necrosis markers were used. The results and statistical evaluations are shown in (Figure 2). In general, inflammatory effects were detected in all retinal layers. However, it was observed that the inner layers were more affected by inflammatory cells and retained more dye in IHC staining.

DISCUSSION

According to the results of our study, severe retinal inflammation and tissue necrosis were observed in diabetic rats. In diabetic rats supplemented with ZO, the inflammatory effect was found to decrease in a dose-dependent manner. Retinal tissues were less affected in diabetic rats treated with metformin; however, the expected synergistic protective effect was not observed in the groups receiving ZO+Met. Daily administration of 50 mg/kg ZO to non-diabetic rats did not cause any retinal toxicity. Our findings further demonstrate that retinal TNF-α expression levels were significantly increased in diabetic rats, and ZO prevented these increases in a dose-dependent manner. Notably, marked intracytoplasmic 8-OHdG expression was detected in the endothelial cells of DR rats, and administration of ZO at 25 and 50 mg/kg significantly improved 8-OHdG expression. Moreover, intracytoplasmic JNK expression in the vascular endothelial cells of DR rats was markedly elevated,

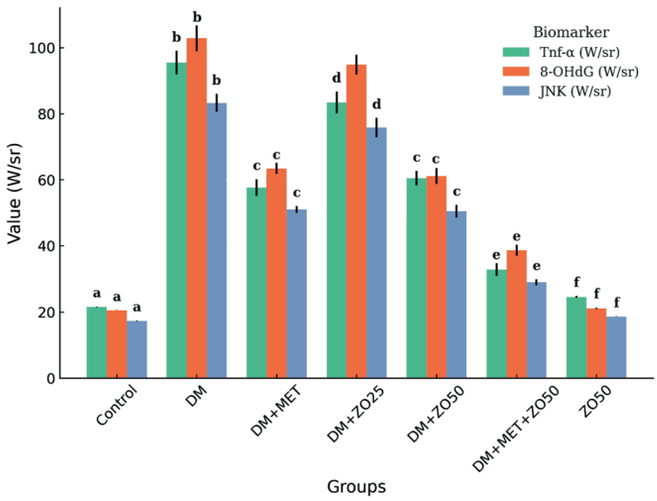

Figure 2 Immunohistochemical and immunofluorescent staining data and statistical analysis results in retinal tissues. Data are expressed as mean±SD unless otherwise stated. [a,b,c,d,e,f]Statistically significant differences (P<0.05). W/sr: Unit of light intensity. Black vertical lines represent SD. SD: Standard deviation; DM: Diabetic group; ZO50: Zingerone-treated group (50 mg/kg dose); ZO25: Zingerone-treated group (25 mg/kg dose); MET: Metformin.

and both 25 and 50 mg/kg doses of ZO significantly prevented the increase in JNK expression.

Chronic hyperglycemia, the main pathogenic factor in DR, activates alternative glucose metabolism pathways, producing advanced glycation end products, oxidative stress, and inflammatory responses that disrupt retinal integrity[17]. Activation of the alternative pathway also triggers various proinflammatory cytokines, and the resulting inflammation leads to astrocyte-pericyte damage and endothelial cell dysmorphology, ultimately disrupting the retinal barrier. Barrier disruption increases vascular permeability and edema in the neuroretinal tissue. Additionally, endothelial cell damage induces microvascular occlusions, resulting in retinal ischemia, intraretinal microvascular abnormalities, and neovascularization[18].

In a diabetic rat model, increased retinal vascular permeability was associated with ischemia and retinal leukostasis. Although the exact cause of retinal leukostasis is not fully understood, it is thought to be related to chronic hyperglycemic conditions and an increase in intercellular adhesion molecule-1 secretion as a marker of retinal inflammation[19]. Another study reported elevated levels of IL-6, IL-8, and TNF-α in the vitreous of patients with DR, suggesting that these inflammatory cytokines may contribute to pathological events in the vitreous[20]. Furthermore, cyclooxygenase-2 (COX-2), an enzyme regulating prostaglandin production, is upregulated in the diabetic retina, and COX-2 inhibitors have been shown to prevent tissue damage[21]. Evidence indicates that inflammation is increased in diabetes. TNF-α, a proinflammatory cytokine secreted primarily by activated macrophages during inflammatory conditions, regulates immune function and mediates the inflammatory response[22]. TNF-α can inhibit insulin signaling and is closely associated with hyperinsulinemia and insulin resistance[23]. TNF-α has been implicated in various intraocular inflammatory disorders, including macular edema and DR, with elevated levels observed in DR[24].

ZO, a phytochemical derived from the reverse aldolization of 6-gingerol found in fresh ginger, exhibits multiple therapeutic effects. Studies have shown that ZO inhibits arachidonic acid metabolism *via* cyclooxygenase and leukotriene pathways and possesses analgesic and anti-inflammatory properties [14]. Mehrzadi *et al*[25] reported that ZO dose-dependently decreased COX-2 enzyme, prostaglandin E2, TNF-α, and IL-1β levels in tissues. Singh *et al* [26] demonstrated that ZO prevented TNF-α upregulation in diabetic rats through its anti-inflammatory activity. Our findings align with the literature, showing significant increases in retinal TNF-α expression in diabetic rats, which were mitigated by ZO in a dose-dependent manner. Histopathologically, ZO also reduced neuroretinal layer disorganization in a dose-dependent fashion, likely due to its anti-inflammatory effects.

Under physiological conditions, the production of ROS is natural and inevitable, and certain levels are necessary for many physicochemical processes. Cells employ enzymatic and non-enzymatic defense systems to maintain antioxidant balance. However, disruption of redox homeostasis in favor of free radicals induces oxidative stress. Hyperglycemia contributes to oxidative stress *via* multiple mechanisms. Accumulation of glycation end products can cause occlusion in the retinal vasculature, leading to ischemia, which activates various intracellular signaling pathways. These processes result in hypoxia, cytosolic ROS production, and a decrease in antioxidant defenses. Finally, defects in protein and nucleic acid glycation and mitochondrial glycation perpetuate ROS formation in a vicious cycle. Hyperglycemia also triggers oxidative stress through activation of the polyol and hexosamine pathways and overexpression of protein kinases, promoting the development of DR[27]. Recent studies highlight elevated ROS levels in the diabetic retina and their role in neuroretinal tissue damage[28].

Oxidative DNA damage occurs due to covalent cross-linking and single or double strand breaks induced by ROS attacks on DNA molecules[28]. 8-OHdG, an oxidized derivative of deoxyguanosine, is a major product of DNA oxidation[29]. Intracellular 8-OHdG expression increases in response to elevated ROS. Lee *et al*[30] reported significantly higher retinal 8-OHdG levels in STZ-induced diabetic rats compared to control and treated groups. Deliyanti *et al*[31] observed a

significant increase in 8-OHdG expression in the retinal tissue of DR rats. In our study, marked intracytoplasmic 8-OHdG expression was detected in endothelial cells of DR rats, and administration of ZO at 25 and 50 mg/kg significantly improved 8-OHdG expression.

The JNK pathway, activated by proinflammatory cytokines, is part of the mitogen-activated protein kinase superfamily, involved in apoptosis, proliferation, and DNA damage repair[32]. It has been suggested as a molecular target for treating neurodegeneration in diseases such as diabetic neuropathy[33]. Fukuda *et al*[34] reported that in STZ-induced hyperglycemic rats lacking functional JNK, blood glucose levels and hyperglycemia-induced apoptosis were reduced. Abo El Gheit *et al*[35] observed increased JNK activation in STZ-induced DR in rats. In our study, intracytoplasmic JNK expression in vascular endothelial cells of DR rats was significantly elevated, and ZO at both 25 and 50 mg/kg markedly prevented this increase.

Significant histopathological changes are observed in the retinas of diabetic rats. It has been reported that the integrity of retinal layers is disrupted and irregular in diabetic rats[36]. The findings of our study are consistent with the literature, showing degeneration and necrosis in retinal vascular endothelial cells and irregularities in the neuroretinal layer in diabetic rats.

Administration of ZO was found to significantly reduce these histopathological changes in retinal tissue and prevent tissue damage. ZO exerts a protective effect in the neuroretinal tissue through its anti-inflammatory and anti-apoptotic activities, suggesting its potential as a therapeutic agent that affects the underlying mechanisms of DR.

Although oral administration of ZO through diet or supplementation may play a protective role in the retina, its hydrophobic nature and low bioavailability limit its clinical application. Pharmacokinetic studies have shown that the solubility of hydrophobic drugs like ZO can be enhanced using nanomicellar drug delivery systems[37]. Nanomicelles are widely used to transport poorly water-soluble drugs in the body, and encapsulated ZO has been shown to have increased oral absorption. Therefore, dietary or supplemental use of ZO may provide potential benefits in preventing retinal damage in diabetic patients.

This study has several limitations. First, experiments were conducted solely on STZ induced diabetic rat models, which limits the direct generalization of the findings to human DR. ZO was administered only short-term; therefore, its long-term effects and safety profile in chronic DR were not evaluated. Additionally, electrophysiological measurements, such as electroretinography, were not performed, which restricts a full understanding of ZO's effects on retinal function. The complex interactions between retinal vasculature and neuroretinal cells were also only partially examined. Finally, the bioavailability of ZO is low, and the doses used in the laboratory setting may not produce the same effects in clinical applications. These limitations should be carefully considered when interpreting the results.

In conclusion, a thorough understanding of the pathophysiology of DR and the effects of oxidative stress and inflammation on the development of DR will provide opportunities for new therapeutic targets. Current therapeutic principles are severely limited by factors such as long-term side effects, costs, and variability in therapeutic efficacy. Due to its proven anti-inflammatory and antioxidant effects, ZO will come to the fore as a retina-protective molecule in the treatment of DR. The study evaluated the effect of ZO in this context, and also provided the opportunity to compare the proven benefits of Metformin on retinal tissues and to evaluate its possible synergistic effect with ZO.

ACKNOWLEDGEMENTS

Authors' Contributions: Utlu B: clinical data collection, writing-original draft, manuscript revision, management and editing; Kozan BD: statistical analysis and editing; Yildirim S: formal analysis and editing; All authors reviewed the manuscript.

Conflicts of Interest: Utlu B, None; **Kozan BD,** None; **Yildirim S,** None.

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