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ORIGINAL PAPER

Effects of neferine on retinal tissue in experimental diabetic rat model

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Abstract

Purpose To investigate vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen (PCNA) immunoreactivities, as well as apoptosis and oxidative stress levels in Streptozotocin (STZ)-induced diabetic rats, and determine how neferine affected these parameters.

Methods Thirty-five male Sprague Dawley rats were divided into five groups of seven. Fasting blood glucose was measured 72 h after diabetes mellitus (DM) induction in 21 rats using 60 mg/kg STZ dissolved in 0.4 ml (0.1 M) sodium-citrate buffer (pH:4.5), with values > 250 mg/dl considered diabetic. **Group 1** received no treatment. **Group 3** (healthy rats) received daily intraperitoneal (IP) 4 mg/kg neferine. Following DM induction: **Group 2** (sham) received daily IP 0.25 ml/kg 0.9% normal saline; **Group 4** received single IP 0.01 mL (2.5 mg/kg) bevacizumab, followed by daily IP 0.25 mL/kg 0.9% normal saline; and **Group 5** received daily IP 4 mg/kg neferine. Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in serum and ocular tissue homogenates were evaluated using ELISA. TUNEL method was used for determining apoptosis and immuno-histochemical staining for PCNA and VEGF immunoreactivities.

Results Group 5 had significantly higher TAC and lower TOS in serum and ocular tissue homogenates than Group 4 ($p < 0.05$). Despite significantly lower VEGF levels and apoptosis ($p < 0.05$), there was no significant change in PCNA immunoreactivity in Group 5 ($p > 0.05$).

Conclusions DM was associated with lower TAC, higher TOS and apoptotic cells, as well as VEGF and PCNA immunoreactivities in the retina. Neferine altered parameters other than PCNA in the opposite direction, demonstrating reductive effects on DM.

Keywords Diabetic retinopathy · Neferine · Proliferating cell nuclear antigen · Rat · Total antioxidant capacity · Total oxidative stress

Introduction

Diabetes mellitus (DM) is a major global health issue expected to affect one out of every three people by 2050, posing a physical, psychosocial, and financial

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burden. It is a progressive multi-systemic disease characterized by chronic hyperglycemia caused by the absence, deficiency, or decreased effect of endogenous insulin on tissues [1]. Diabetes mellitus may cause macrovascular, microvascular, as well as neuropathic complications, primarily affecting the eyes, kidneys, and heart. Microangiopathy and accompanying neuropathy affecting retinal capillaries, arterioles, and venules characterize diabetic retinopathy (DR), one of the most common DM-induced ocular complications, and one of the leading causes of global blindness in adults of working age [2].

Neferine is a bioactive alkaloid derived from a green seed embryo (Lianzixin) of *Nelumbo nucifera*, alternatively known as sacred lotus, an aquatic perennial plant in the *Nelumbonaceae* family [3–5]. It has been used largely as a food source throughout Asia, and its therapeutic effects have been explained in Ayurvedic and Traditional Chinese Medicine. It has anti-inflammatory [6, 7], antioxidant, anti-diabetic, antihypertensive [8], antiarrhythmic, anticoagulant [9, 10], vascular smooth muscle relaxant, as well as anticancer properties [11–13]. In addition to its beneficial effects in DM-related cardiovascular disease treatment [14], neferine inhibits inflammatory molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8, boosts antioxidant capacity, and reduces or prevents oxidative damage in healthy cells [6]. Following ingestion, neferine is rapidly distributed throughout the body, with the liver being the most effective at metabolizing it [15].

The purpose of the present study was to investigate vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen (PCNA) immunoreactivities, as well as apoptosis and oxidative stress levels in Streptozotocin (STZ)-induced diabetic rats, and determine how neferine affected these parameters.

Materials and methods

This experimental rat study, which was approved by Firat University's Animal Research and Ethics Committee with the approval number and date 169–18.09.2019, was carried out at the Firat University Experimental Research Center and the University Faculty of Medicine Pathology and Biochemistry laboratories.

Experimental rats and nutrition

The Firat University Experimental Research Center provided 35 adult male Sprague Dawley rats aged 8–10 weeks, which were observed in the animal laboratory for 12 h (07:00–19:00) in the light and 12 h (19:00–07:00) in the dark, with the environment temperature kept constant at 22–25 °C. Rats were fed in specially designed cages, and their bottoms were cleaned daily. Their weights were recorded at the start (baseline) and end of the experiment (final). All rats were fed the same standard rat chow and were given add libitum water and food intake.

Diabetes mellitus induction

A single dose of 60 mg/kg STZ (Streptozotocin, Zanosar, Pharmacia, France) was dissolved in 0.4 ml (0.1 M) sodium-citrate buffer (pH: 4.5) and administered intraperitoneally to 21 rats using 26-gauge insulin injector to induce DM. Blood was drawn from the tail vein after 72 h, and fasting blood glucose was measured with a glucometer device, Chemstrip bG (Bio-Dynamics, Boehringer Mannheim, Indianapolis, IN) between 8 and 10 a.m. after 8–10 h of fasting. Diabetic rats were defined as having fasting blood glucose levels > 250 mg/dl.

Experimental rat groups

Thirty-five male Sprague Dawley rats weighing 200–250 g were divided into five groups of seven. While **Group 1 (Control group)** received no treatment, normal healthy rats in **Group 3** (*Neferine-treated non-DM rats*) received daily intraperitoneal 4 mg/kg neferine throughout the experiment. Following DM induction: **Group 2 (Sham group, non-treated DM rats)** received daily intraperitoneal 0.25 ml/kg 0.9% normal saline throughout the experiment; **Group 4** (*Bevacizumab-treated DM rats*) received a single intraperitoneal 0.01 mL (2.5 mg/kg) bevacizumab, followed by daily intraperitoneal 0.25 mL/kg 0.9% normal saline throughout the experiment; and **Group 5** (*Neferine-treated DM rats*) received daily intraperitoneal 4 mg/kg neferine throughout the experiment.

Sample collection

At the end of the 4th week of the experiment, all rats were weighed before being decapitated under anesthesia with intraperitoneal 75 mg/kg Ketamine plus 10 mg/kg Xylazine. Blood samples were drawn from each rat and placed in tubes containing K3-EDTA for biochemical analysis. Plasmas from blood samples were separated after 10-min centrifugation (Heraeus Biofuge Stratos; Kendo Laboratory Products, Osterode-German) at 3000 rpm. The ocular tissue samples were then quickly removed and homogenized for 4 min at 16,000 rpm in a 0.01 M Phosphate buffer (w:v; 1:3) solution under standard conditions. The homogenate was centrifuged for 10 min at 5000 rpm, and the supernatants were separated and examined right away.

Total antioxidant capacity and total oxidative stress

The enzyme-linked immunosorbent assay (ELISA) method was used to measure TAC and TOS levels in serum and supernatant. The Lowry method [16], which is based on the formation of a blue color by the Folin-Phenol reagent of the proteins in alkaline medium, was used to determine the protein levels in the supernatants.

The TAC levels in plasma and supernatant were determined using the rat TAC ELISA kit (Sunred Biotechnology Company, Catalog No; 201–11–2672) as directed by the manufacturer. Absorbances were read spectrophotometrically at 450 nm on an EPOCH 2 (BioTek Instrument, Inc, USA) microplate reader. The plasma results were expressed as U/ml, while the supernatant results were expressed as U/mg protein. The kit's assay range was 0.2–60 U/ml, and sensitivity was 0.175 U/ml. The intra-Assay coefficient of variability (CV) was $<9\%$, and the inter-Assay CV was $<11\%$.

The TOS levels in plasma and supernatant were determined using the rat TOS ELISA kit (Sunred Biotechnology Company, catalog no; 201-1-1669) as directed by the manufacturer. Absorbances were read spectrophotometrically at 450 nm on an EPOCH 2 (BioTek Instrument, Inc, USA) microplate reader. The plasma results were expressed as nmol/ml, while the supernatant results were expressed as nmol/mg protein. The kit's assay range was 0.1–20 nmol/ml, and sensitivity was 0.073 nmol/ml. The intra-Assay CV was $<8\%$, and the inter-Assay CV was $<10\%$.

Histological study

The ocular tissue samples were fixed in a 10% formaldehyde solution before being washed in tap water and undergoing a series of routine histological follow-ups. They were then embedded in paraffin blocks, from which sections with a thickness of 4–6 µm were taken.

Immuno-histochemistry

Polylysine slides were loaded with 4–6-µm thick sections from paraffin blocks. Deparaffinized tissue samples were rinsed with a graded alcohol series before being boiled in a citrate buffer solution at pH 6.0 in a microwave oven (750 W) for $7+5$ min for antigen retrieval. The samples were allowed to cool for about 20 min after boiling before being washed with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA) for 3×5 min and incubated with a hydrogen peroxide block solution for 5 min to inhibit endogenous peroxidase activity (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA).

Following a 5-min Ultra V Block (TA-125-UB, Lab Vision Corporation, USA) solution application to prevent background paint, the samples were incubated for 60 min at room temperature in a humid environment with VEGF (Vascular endothelial growth factor, E2611, Spring Bioscience, USA) and PCNA (Monoclonal Mouse Anti-Proliferating Cell Nuclear Antigen, M0879, Dako, Baltimore, MD, USA) primary antibodies diluted at 1/200. This procedure was followed by 3×5-min PBS sample washing, and 30-min incubation with secondary antibody (Biotinylated Goat Anti-Poliyvalent (Anti-mouse/rabbit IgG), TP-125-BN, Lab Vision Corporation, USA) at room temperature in a humid environment. Again, this procedure was followed by 3×5-min PBS sample washing and 30-min incubation with Streptavidin Peroxidase (TS-125-HR, Lab Vision Corporation, USA) in a humid environment at room temperature before returning to PBS. 3-amino-9-ethyl carbazole (AEC) Substrate plus AEC Chromogen solution (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) was dripped onto the tissue samples and washed with PBS

simultaneously after the image signal was obtained under the light microscope. The ocular tissue samples counterstained with Mayer's hematoxylin were rinsed in PBS and distilled water before being sealed with an appropriate closure solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA). They were then examined under an Olympus BX51 microscope, and the Olympus DP Controller was used for photographing (2002–2006 Olympus Corporation).

A histoscore, which is prevalence x severity, was created based on the prevalence (0.1: < 25%, 0.4: 26–50%, 0.6: 51–75%, 0.9: 76–100%) and severity (0: None, + 0.5: Very little, + 1: Little, + 2: Moderate, + 3: Severe) of immunoreactivity in staining.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) procedure

Polylysine slides were loaded with 4–6-µm thick sections from paraffin blocks as previously explained. The cells undergoing apoptosis were identified using the ApopTag plus Peroxidase in Situ Apoptosis Detection kit (Chemicon, Cat No: S7101, USA), as directed by the manufacturer. The ocular tissue samples that had been deparaffinized with Xylene were washed with PBS following rinsing with graded alcohol series. These samples were incubated for 10 min with 0.05% proteinase K, followed by incubation with 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase activity. After a PBS wash, the samples were incubated for 6 min with Equilibration Buffer before being incubated for 60 min with a working solution (70 µl Reaction Buffer plus 30% TdT Enzyme) at 37 °C in a humid environment. The samples were then treated with Anti-Digoxigenin-Perosidase for 30 min after being in Stop/Wash Buffer for 10 min. Diaminobenzidine substrate was used to visualize apoptotic cells. Appropriate closure solution was used to seal sections that had been counterstained with Harris hematoxylin. Examination and photographing of the samples were performed using Leica DM500 microscope (Leica DFC295). The TUNEL staining was used to determine whether cells were normal or apoptotic. Nuclei stained blue with Harris hematoxylin were considered normal, while cells with brown nuclear staining were considered apoptotic. Normal and apoptotic cells were counted in randomly selected areas of the sections at a magnification of 10. An apoptotic index (AI) was calculated by dividing apoptotic cells by total (normal plus apoptotic cells) cells in statistical analyses.

Statistical analysis

The SPSS version 22 program was used for statistical analysis. The data were determined as mean ± standard deviation. The groups were compared using one-way ANOVA and the post hoc Tukey tests. $P < 0.05$ values were considered statistically significant.

Results

Clinical findings

While Groups 1 and 3 had significantly higher final body weights than baseline ($p < 0.05$), Groups 2, 4, and 5 had significantly lower final body weights ($p < 0.05$) (Fig. 1).

Blood glucose

The baseline blood glucose levels in Groups 2, 4, and 5 were significantly higher than in Group 1 ($p < 0.05$). However, no significant differences were found between Groups 1 and 3 or among Groups 2, 4, and 5 ($p > 0.05$ for all). The final blood glucose levels in Groups 2 were significantly higher than in Group 1 ($p < 0.05$), but significantly lower than in Group 5 ($p < 0.001$). There were also significantly lower levels in Group 5 than in Group 4 ($p < 0.05$). No statistically significant differences existed between Groups 1 and 3 or 2 and 4 ($p > 0.05$ for both) (Fig. 2).

Total antioxidant capacity

There were no significant differences between Groups 1 and 3 or 2 and 4 in terms of serum and ocular tissue sample homogenate TAC levels. There was, however, a significant decrease in Group 2 when compared to Group 1 ($p < 0.006$), and a significant increase in Group 5 when compared to Groups 2 and 4 ($p < 0.005$ for both) (Fig. 3).

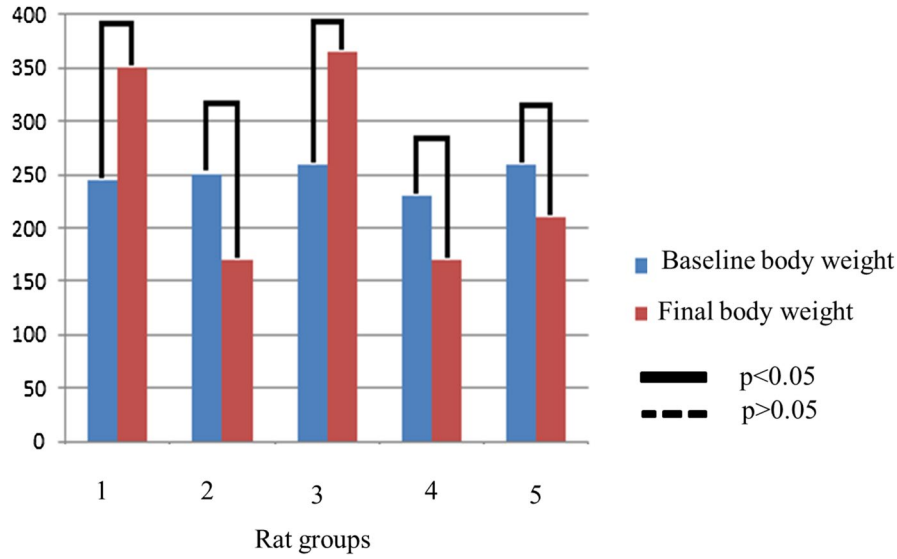

Fig. 1 Graphical representation of baseline versus final body weights (gram). Group 1: Control group; Group 2: Sham (Non-treated DM rats); Group 3: Neferine-treated non-DM rats; Group 4: Bevacizumab-treated DM rats; Group 5: Neferine-treated DM rats

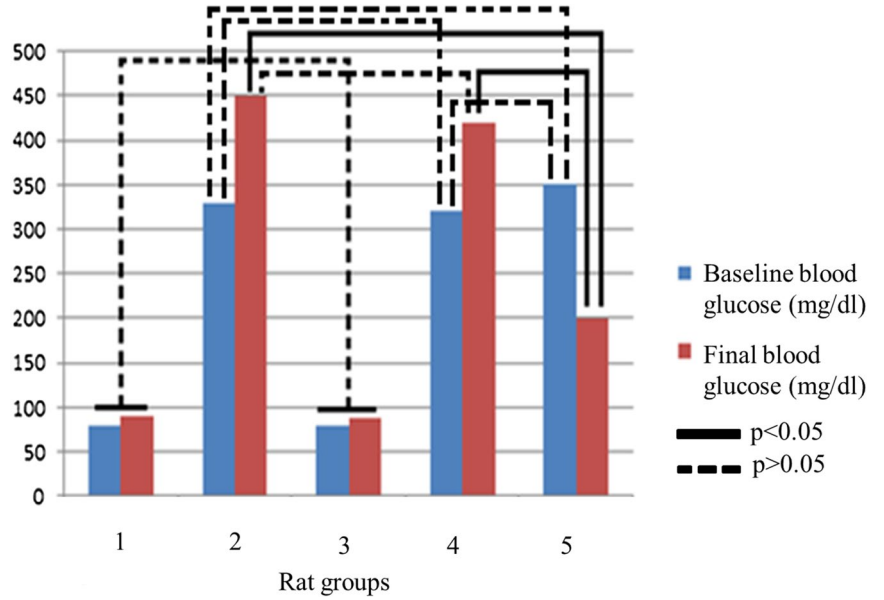

Fig. 2 Graphical representation of baseline versus and final blood glucose levels (mg/dl). Group 1: Control group; Group 2: Sham (Non-treated DM rats); Group 3: Neferine-treated non-DM rats; Group 4: Bevacizumab-treated DM rats; Group 5: Neferine-treated DM rats

Total oxidative stress

There were no significant differences between Groups 1 and 3 or 2 and 4 in terms of serum and ocular tissue sample homogenate TOS levels. There was, however, a significant increase in Group 2 when compared to Group 1 ($p < 0.001$), and a significant decrease in Group 5 when compared to Groups 2 and 4 ($p < 0.05$ for both) (Fig. 4).

TUNEL method

A light microscopic examination of TUNEL staining for determining apoptotic cells revealed TUNEL positivity in the retinal ganglion cell layer. This positivity did not differ statistically significantly between Groups 1 and 3, nor between Groups 4 and 5. However, the difference was significant between Groups 1 and 2 ($p < 0.001$). Compared to Groups 4 and 5,

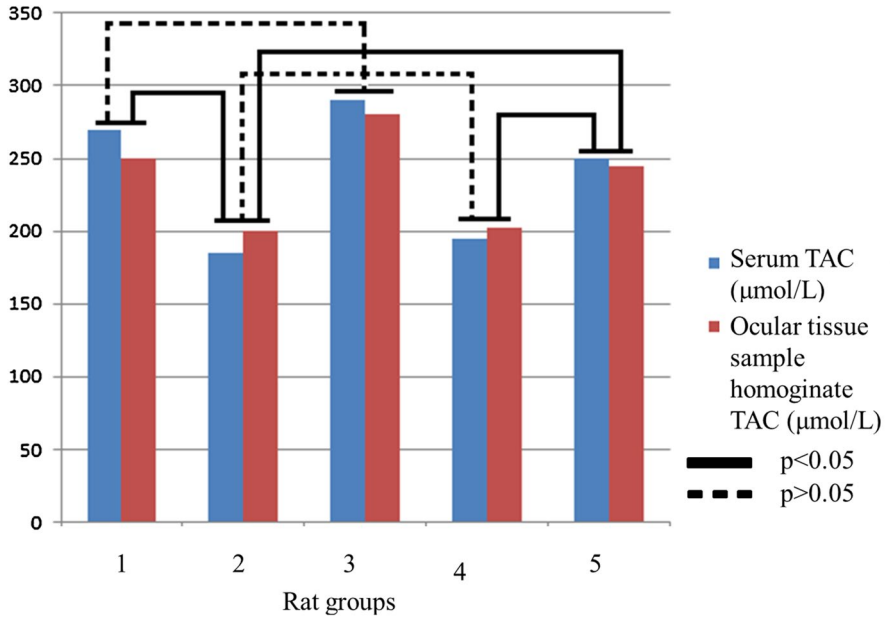

Fig. 3 Graphical representation of baseline versus final serum and ocular tissue sample homogenate total antioxidant capacity (TAC) levels (µmol/L). Group 1: Control group; Group 2: Sham (Non-treated DM rats); Group 3: Neferine-treated non-DM rats; Group 4: Bevacizumab-treated DM rats; Group 5: Neferine-treated DM rats

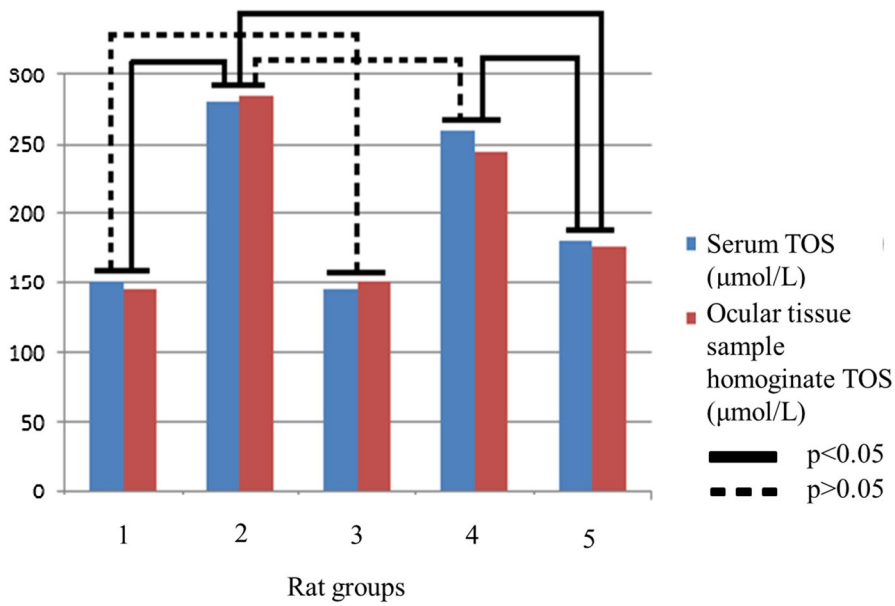

Fig. 4 Graphical representation of baseline versus final serum and ocular tissue sample homogenate total oxidative stress (TOS) levels (µmol/L). Group 1: Control group; Group 2: Sham (Non-treated DM rats); Group 3: Neferine-treated non-DM rats; Group 4: Bevacizumab-treated DM rats; Group 5: Neferine-treated DM rats

Group 2 had significantly lower TUNEL positivity ($p < 0.001$ for both) (Fig. 5 (A–F)).

VEGF Immunoreactivity

The light microscopic examination of immunohistochemical staining revealed VEGF immunoreactivity in the retinal ganglion cell layer as highlighted with cytoplasmic brown staining. VEGF immunoreactivity did not differ significantly between Groups 1 and 3, nor between Groups 4 and 5. In comparison to Group 2, Groups 1 ($p < 0.001$), 4 ($p < 0.005$) and 5 ($p < 0.005$) had significantly lower VEGF immunoreactivities (Fig. 6 (A–F)).

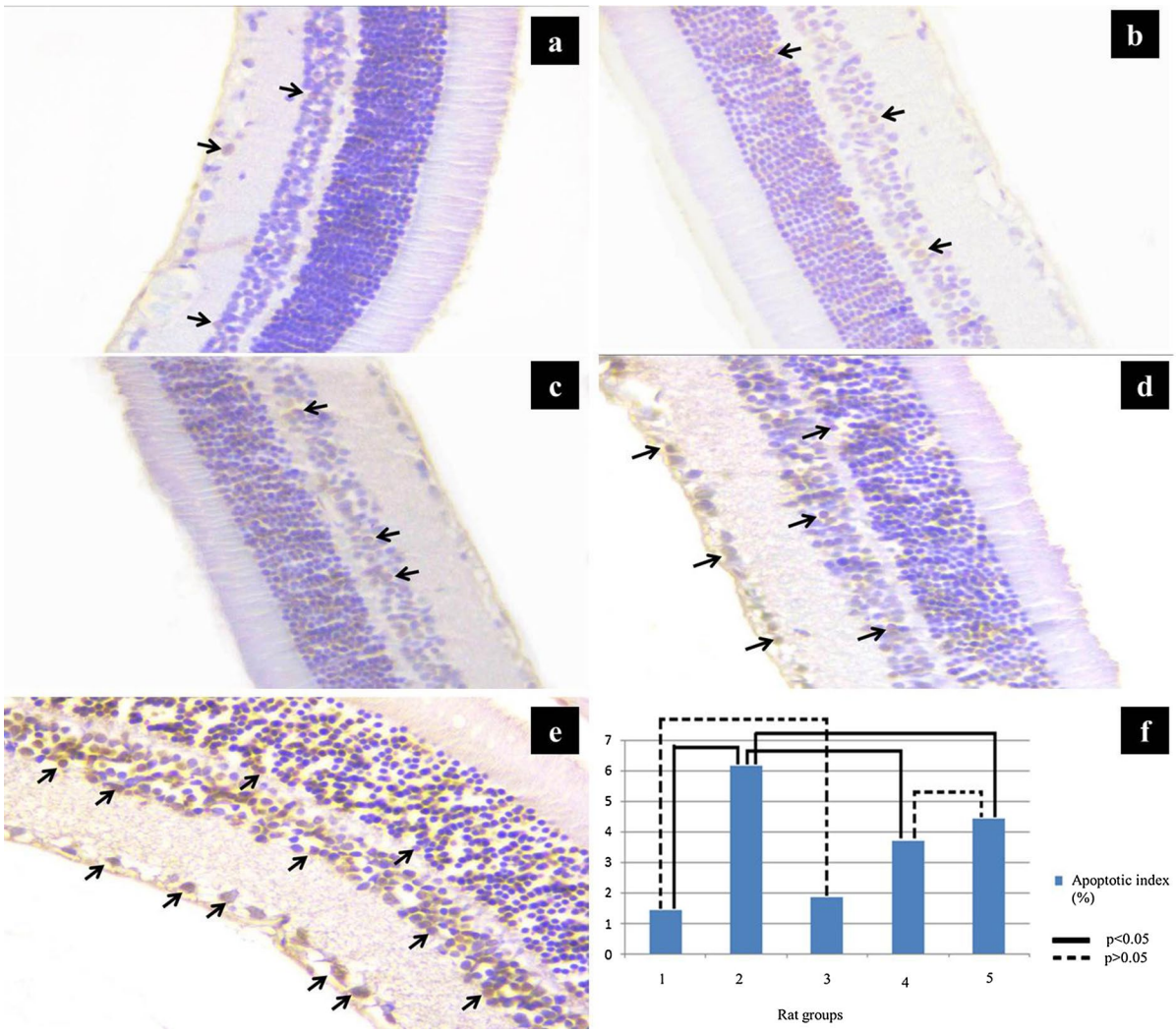

Fig. 5 A light microscopic examination of TUNEL staining for apoptotic cells reveals relatively increasing TUNEL positivity in the retinal ganglion cell layer in experimental rats from (**A**) Groups 1 (Control group); (**B**) Group 3 (Neferine-treated non-DM rats); (**C**) Group 2 (Sham, Non-treated DM rats); (**D**) Group 4 (Bevacizumab-treated DM rats); and (**E**) Group 5 (Neferine-treated DM rats). (**F**) Non-treated DM rats had a higher percentage of apoptotic index in comparison to the other groups

PCNA immunoreactivity

The light microscopic examination of immune-histochemical staining revealed PCNA immunoreactivity in the retinal ganglion cell layer as highlighted with brown nuclear staining. PCNA immunoreactivity did not differ significantly between Groups 1 and 3, nor between Groups 4 and 5. Despite significantly higher PCNA immunoreactivity in Group 2 when compared to Group 1 ($p < 0.011$), no significant differences were observed when compared to Groups 4 and 5 (Fig. 7 (A–E)).

Discussion

Chronic hyperglycemia causes production of reactive oxygen species and low-grade inflammation, which leads to retinal pigment epithelium (RPE) apoptosis and, eventually, DR progression. There may also be

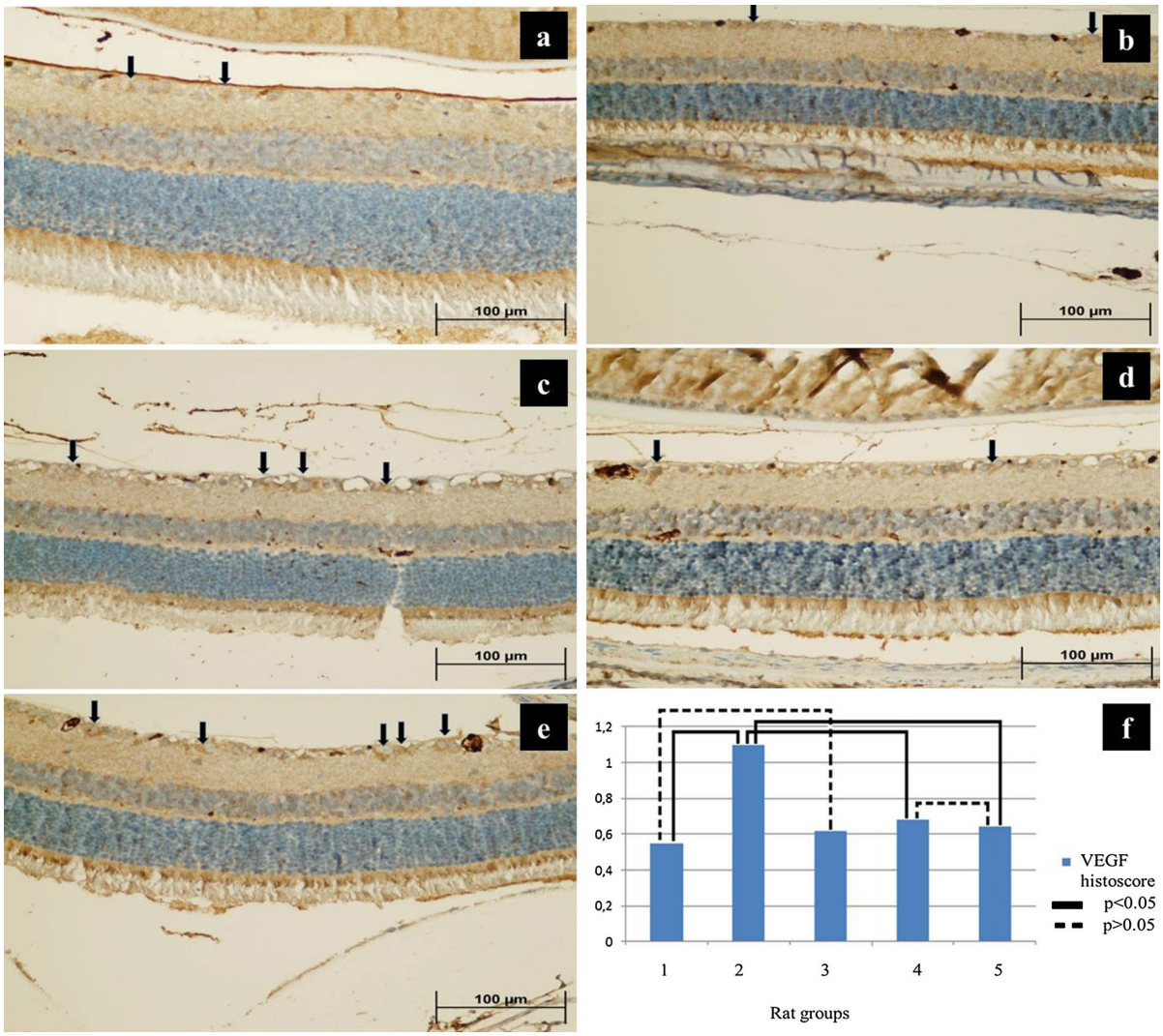

Fig. 6 A light microscopic examination of immune-histochemical staining reveals VEGF immunoreactivity in the retinal ganglion cell layer as highlighted with cytoplasmic brown staining in experimental rats from (**A**) Groups 1 (Control group); (**B**) Group 3 (Neferine-treated non-DM rats); (**C**) Group 4 (Bevacizumab-treated DM rats); (**D**) Group 5 (Neferine-treated DM rats); and (**E**) Group 2 (Sham, Non-treated DM rats). (**F**) Overall, non-treated DM rats had higher VEGF immunoreactivity than the other groups

sub-endothelial AGE accumulation, resulting in vascular structural changes, increased arteriosclerosis, and activation of intracellular signaling pathways, giving rise to oxidative stress and inflammation [17]. Aside from inflammation, oxidative stress is thought to be the most common mechanism in DR etiology [18], causing damage or dysfunction that persists long after blood glucose level has been stabilized. Thus, novel anti-oxidative stress and anti-inflammatory treatment strategies are required. Current treatment alternatives, such as insulin therapy, oral anti-diabetes medications, and glycemic control, are insufficient to halt the progression of DR-induced microvascular complications.

Neferine has long been used for various therapeutic purposes. However, with the introduction of cutting-edge technologies and research advancements, this bioactive alkaloid has been thoroughly investigated to provide experimental evidence of its exceptional and efficacious therapeutic potential. It is

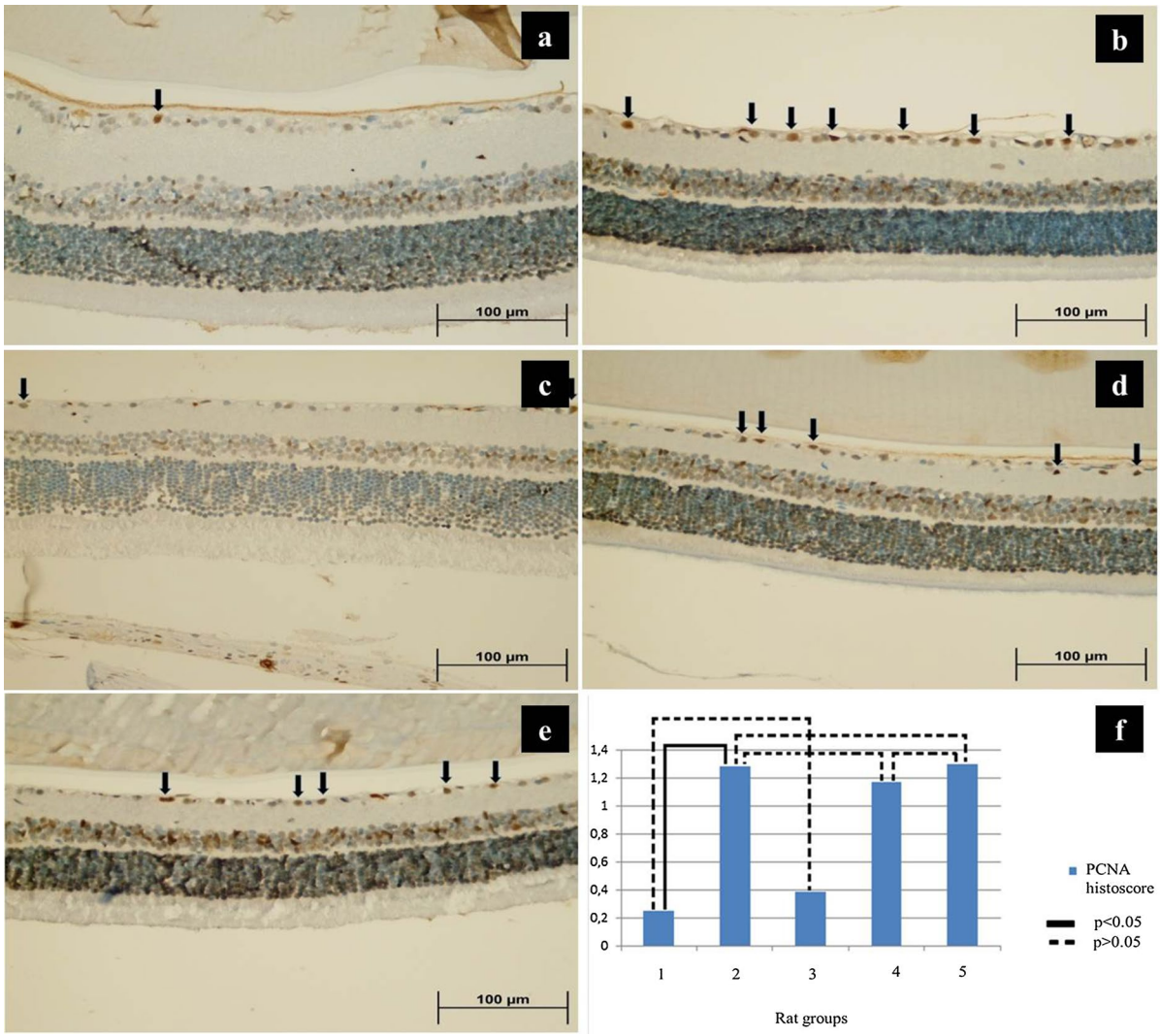

Fig. 7 A light microscopic examination of immune-histochemical staining reveals proliferating cell nuclear antigen (PCNA) immunoreactivity in the retinal ganglion cell layer as highlighted with brown nuclear staining in experimental rats from (**A**) Groups 1 (Control group); (**B**) Group 3 (Neferine-treated non-DM rats); (**C**) Group 4 (Bevacizumab-treated DM rats); (**D**) Group 5 (Neferine-treated DM rats); and (**E**) Group 2 (Sham, Non-treated DM rats). As shown in a graphical representation F, non-treated DM rats had significantly higher PCNA immunoreactivity than rats in control rats, but no significant differences relative to bevacizumab- and neferine-treated DM rats

analogous to a valuable asset with myriad therapeutic and preventive uses. If properly utilized, it can potentially lead to a plethora of new curative ingredients with minimal adverse effects.

Several studies have revealed that neferine is involved in many biological activities [19]. It is effective in treating DM and its complications, with its anti-diabetic property thought to be mediated by attenuation of phosphorylation upregulations of p-ERK, p-p38, and p-Smad2/3 caused by chronic hyperglycemia [20]. Further, neferine has anti-obesity properties and has been reported to lower fasting blood glucose, insulin levels, TNF-α and triglyceride levels, as well as increase insulin sensitivity, implying that this alkaloid bioactive agent could be a potent anti-diabetic agent comparable to rosiglitazone, an insulin sensitizer used in type 2 DM treatment [21]. Compared to non-treated type 2 DM rats,

neferine-treated type 2 DM rats had significantly lower fasting blood glucose, blood pressure, total cholesterol, triglycerides, and insulin levels, as well as significantly higher high-density lipoprotein (HDL) levels [22]. Correspondingly, in the present study, neferine-treated DM rats had significantly lower blood glucose than non-treated DM rats or bevacizumab-treated DM rats. However, blood glucose did not differ significantly between bevacizumab-treated DM rats and non-treated DM rats. This corroborates prior reports that neferine could be effective in controlling blood glucose.

It has been reported that DR patients have higher serum oxidative stress products and lower antioxidant enzyme activities than healthy controls, indicating a possible link between high TOS and low TAC levels and DR progression [23]. Among other things [8], neferine has been shown to have cytoprotective properties against hypoxia-induced cytotoxicity, inflammation, and oxidative stress [6]. It has also been identified as a potential therapeutic and preventive agent for Alzheimer's disease and other oxidative stress-related diseases [24]. Likewise, the present study revealed significantly higher apoptosis in the retinal tissues of DM rats compared to non-DM rats, which we believe was due to DM-induced oxidative stress, which also decreased significantly after neferine administration. Further, DM rats had significantly higher TOS levels than non-DM rats, which could be explained by the fact that hyperglycemia increases glucose auto-oxidation, lipid peroxidation, glycosylated proteins, and thus free radical formation. Furthermore, compared to non-treated DM rats, significantly lower apoptosis in neferine-treated DM rats could be attributed to neferine's antioxidant effect, as evidenced by significantly increased TAC and significantly decreased TOS levels in the latter group. Significantly higher TAC and lower TOS levels in neferine- versus bevacizumab-treated DM rats also indicate that neferine has greater antioxidant capacity than bevacizumab, indicating the neferine efficacy in reducing DM-induced oxidative stress.

Neferine has been shown to suppress hyperglycemia-induced human umbilical vein endothelial cell apoptosis, suggesting a potential role for this agent in the treatment of diabetic vasculopathy [25]. It also inhibits the PI3K/Akt pathway as well as NFκB, which can help prevent diabetic vasculopathy [26]. Higher TUNEL positivity in DM rats found in the present study could be interpreted as evidence that oxidative stress, which may lead to changes in intracellular calcium balances and mitochondrial membrane potential, could eventually induce apoptosis by causing mitochondrial and DNA damages, the results of which are consistent with the literature. Despite the lack of a significant difference in apoptosis between neferine- and bevacizumab-treated DM rats, it is widely assumed that neferine's anti-apoptotic activity could be useful in DM treatment, as evidenced by the significantly lower apoptosis in neferine-treated DM rats.

DM-induced retinal microvascular complications are exacerbated by VEGF, a vascular permeability factor that increases in tandem with neovascularization progression. Besides, high VEGF levels have been linked to blood-retina barrier breakdown and fibrovascular proliferation in DR patients [27]. As a result, VEGF has become an important therapeutic target in DR treatment. VEGF mRNA levels have been reported to be higher in DM rats, whereas non-DM rats have very low VEGF immunoreactivity. VEGF release has been found to be especially strong in retinal ganglion cells, as well as inner and outer nuclear layers [28]. Similarly, immuno-histochemical analysis in the present study revealed retinal VEGF immunoreactivities in non-treated DM rats' ganglion cell and nerve fiber layers relative to other retinal layers that was significantly higher than in non-DM rats. Compared to non-treated DM rats, neferine- and bevacizumab-treated DM rats had significantly lower VEGF immunoreactivity, but the difference between the latter groups was not significant. This could imply that neferine is as effective as bevacizumab, the current anti-VEGF treatment for DR. Furthermore, the present study's finding of decreased VEGF immunoreactivity is encouraging in terms of neferine's ability to prevent neovascularization and its secondary complications. Increased VEGF immunoreactivity in DM rats could be ascribed to oxidative stress and subsequent endothelial dysfunction [29]. Decreased retinal VEGF immunoreactivity in neferine-treated DM rats could also suggest that neferine may reduce endothelial dysfunction, the finding that supports its antioxidant and anti-angiogenic properties.

Many proteins involved in DNA synthesis, repair, and cell cycle control function by binding to PCNA, which is used to show the cell cycle rate and cell proliferation, as well as to differentiate and identify

proliferative cells from cells undergoing differentiation. The use of chimeric ribozyme for PCNA inhibition in a rabbit model of proliferative vitreoretinopathy resulted in decreased proliferation in RPE and fibroblast cells [30]. New treatment options for lowering PCNA levels detected in the retinas of patients with proliferative vitreoretinopathy are currently being investigated. Immuno-histochemical analysis revealed significantly higher retinal PCNA levels in non-treated DM rats versus control rats in the present study. Despite the fact that neferine has been revealed in past studies to have an anti-proliferative effect [11, 12], the present study did not find a significant effect of neferine on PCNA levels. This could be attributed to the short study duration and an insufficient dose of neferine for its anti-proliferative effect. PCNA levels in non-treated DM rats did not differ significantly from those in neferine- and bevacizumab-treated DM rats, nor did the difference between the latter groups. Long-term studies would be worthwhile to confirm the effect of neferine supplementation on PCNA levels.

Conclusions

Experimental DM induction resulted in lower retinal TAC, higher retinal TOS and apoptotic cells, as well as VEGF and PCNA immunoreactivities. Except for PCNA, all parameters changed in the opposite direction after neferine administration, which was found to be just as effective as bevacizumab. Neferine-treated DM rats had significantly lower blood glucose than non-treated DM rats. Long-term experimental animal models could back up this four-week rat model in elucidating the role of DM pathophysiological effects on the retinal tissue and confirming the effects of neferine supplementation in DR.

Author Contributions The authors all contributed to the study's conception and design. MC, HHG, and OA prepared the materials, collected data, and analyzed the results. MC wrote the first draft of the manuscript, and all authors provided feedback on previous drafts. The final manuscript was read and approved by all of the authors. MC = Mehmet CEVIK, HHG = Hamidu Hamisi GOBEKA, OA = Orhan AYDEMİR.

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Data Availability Data are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The author(s) declare(s) no conflict of interest.

Financial Interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials mentioned in this article.

Ethics Approval The Animal Research and Ethics Committee at Firat University approved this animal study with the approval number and date 169-18.09.2019

Consent to Participate and Consent for Publication My colleagues and I conducted the research and co-authored the manuscript. We have all approved the manuscript for submission and publication in your journal, and I am the corresponding author.

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