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Fangchinoline Ameliorates Diabetic Retinopathy by Inhibiting Receptor for Advanced Glycation End-Products (RAGE)-Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF- κ B) Pathway in Streptozotocin (STZ)-Induced Diabetic Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Diabetic retinopathy (DR) is a macrovascular complication that occurs in diabetic patients. Conventional treatments for the management of DR have many limitations. Thus, the present investigation evaluated the protective effect of fangchinoline against diabetic retinopathy (DR).

Material/Methods: DR was induced by streptozotocin (STZ; 60 mg/kg; i.p.) and rats were treated with fangchinoline 1, 3, and 10 mg/kg for 16 weeks. DR was confirmed by determining the concentration of advanced glycation end-products (AGEs) and morphology of retinal tissues. Parameters of oxidative stress and expression of inflammatory cytokines and receptor for advanced glycation end-products (RAGE) in the retinal tissue were determined by Western blot assay and reverse transcription polymerase chain reaction (RT-PCR). Moreover enzyme-linked immunosorbent assay (ELISA) was used to determine the apoptosis index and activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in the retinal tissues.

Results: Our study reveals that the concentration of glycosylated hemoglobin (HbA1c) and glucose in the plasma and AGEs in the retinal tissue were significantly reduced in the fangchinoline group compared to the DR group. Moreover, treatment with fangchinoline attenuated the altered retinal morphology and expression of inflammatory mediators and RAGE in the retinal tissues of DR rats. There was a significant ($p < 0.01$) decrease in oxidative stress, activity of NF- κ B, and apoptosis index in the fangchinoline group compared to the DR group of rats.

Conclusions: Our investigation shows that fangchinoline attenuates the apoptosis of retinal cells in STZ-induced diabetic retinopathy rats by inhibiting the RAGE/NF- κ B pathway.

MeSH Keywords: **Apoptosis • Diabetic Retinopathy • Interleukin-17 • Oxidative Stress**

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Background

Diabetic retinopathy is a chronic complication that occurs in diabetes mellitus [1]. DR is characterized by neovascularization, ischemia, changes in the retinal blood flow, and dysfunction of endothelial cells [2]. Mild symptoms are observed in early stages of DR, and untreated DR develops into advance stage that can result in blindness [3]. Moreover, uncontrolled hyperglycemia leads to many chronic diabetic complications, including DR. The pathogenesis of DR involves the accumulation of glucose in the retinal cells and activation of several biochemical pathways involved in it [4]. There are several inflammatory mediators involved in the process of inflammation induced due to hyperglycemia. The literature reveals that edema and neovascularization of the retina occurs due to altered levels of VEGF, as it is an angiogenic and proinflammatory mediator [5]. Moreover, the progression of DR involves activation of leukocytes and adhesion molecules [6]. There are other pathways, such as uncontrolled hyperglycemia, that promote the formation of AGEs and induction of apoptosis, as well as expressions of inflammatory mediators and enhanced production of reactive oxygen species due to the interaction between AGEs and receptors for AGEs (RAGE) [7]. Thus, the

RAGE/AGE pathway has become a target for the development of molecules for the treatment of DR.

In the last few decades, molecules from the herbal sources have shown promise in the management of metabolic disorders, including DR [8]. Fangchinoline is chemically an alkaloid isolated from *Stephania tetrandra* S. [9] and is reported to have anti-diabetic and anti-inflammatory activity [9,10]. The literature reveals that fangchinoline attenuates the expression of IL-6, IL-1, and TNF- α , and inhibits the activity of cyclooxygenase [11]. Fangchinoline also has strong radical scavenging, hypotensive, anticancer, antioxidant, and antithrombosis activity [12–14]. Thus, the present investigation assessed the protective effect of fangchinoline in diabetic retinopathy.

Material and Methods

Animal

Male Wistar rats weighing 180–200 g were procured from the Fourth Military Medical University, China. All the rats were acclimatized at a 12-h light-dark cycle and a temperature of 23–25°C.

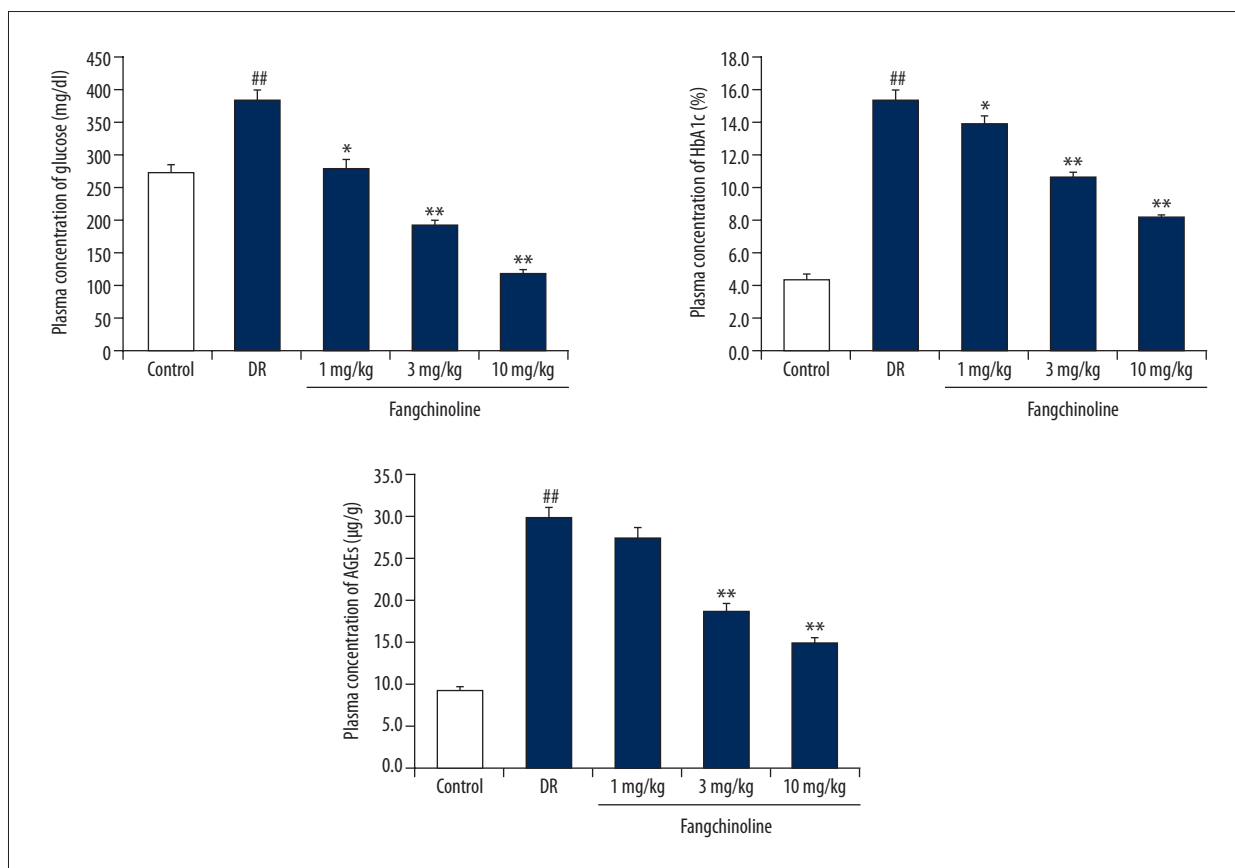


Figure 1. Fangchinoline attenuates the biochemical parameters such as plasma concentration of HbA1c and glucose and AGEs in the retinal tissues of DR rats. Mean \pm SEM (n=10), ^{##} p<0.01 than control group; ^{*} p<0.05, ^{**} p<0.01 than DR group.

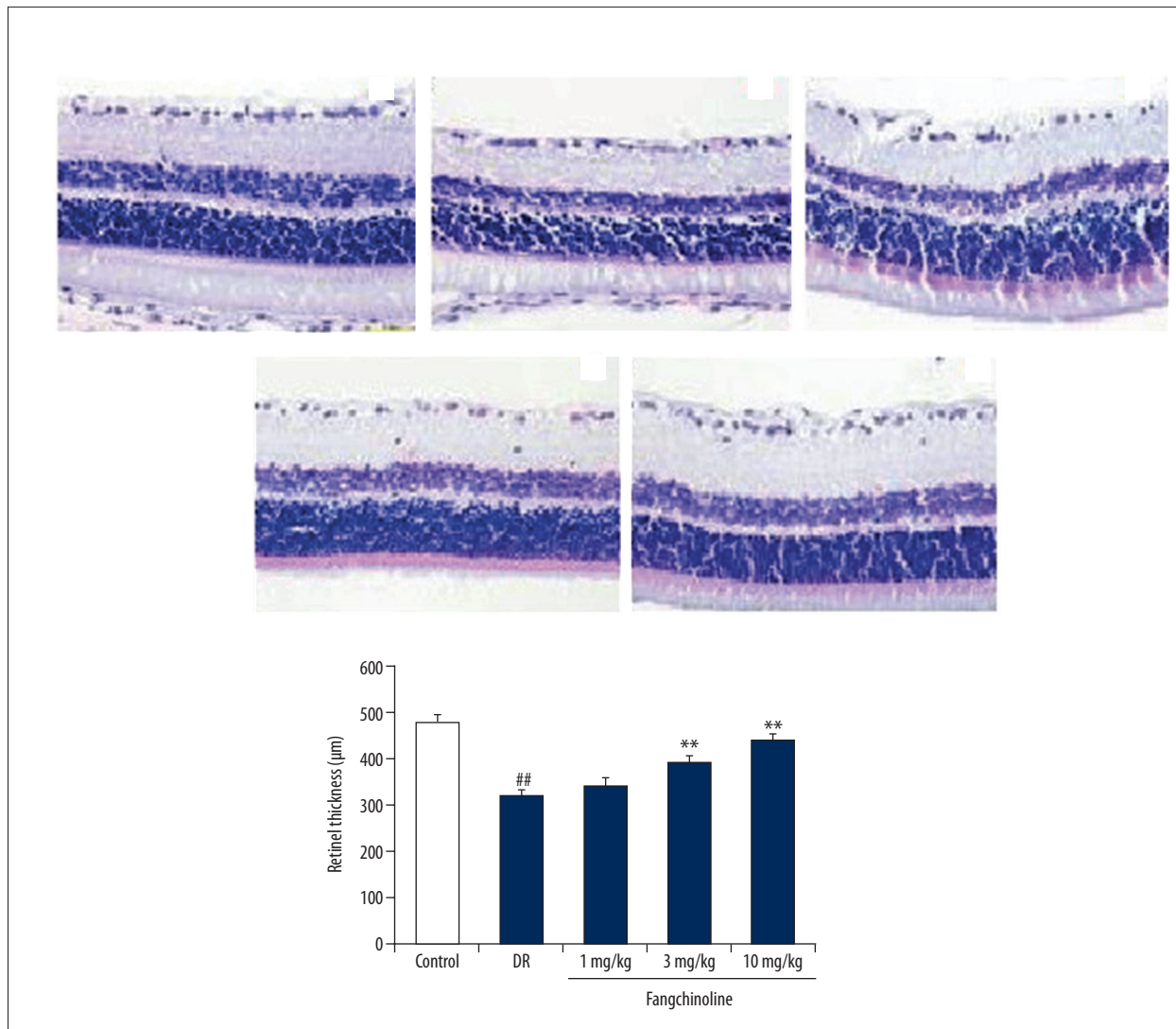


Figure 2. Fangchinoline attenuates morphology of retinas of DR rats. Mean \pm SEM (n=10), ^{##} p<0.01 than control group; * p<0.05, ^{**} p<0.01 than DR group.

Animals had free access with tap water and standard chow diet. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Affiliated Zhongshan Hospital of Dalian University, China (IACUC/AZH-DU/2017/13) and the study followed the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use.

Chemicals

STZ and fangchinoline were procured from Sigma Aldrich (USA). ELISA kits for the determination of HbA1c and AGEs were procured from Thermo Fisher Scientific (China). Primary antibodies of VEGF, IL-1 β , IL-6, TNF- α , RAGE, and GAPDH were procured from Abcam (USA).

Induction of diabetic retinopathy

All the animals were separated into 2 groups: as control group (n=6) receiving vehicle and a diabetic group (n=40) receiving intraperitoneal (i.p.) injection of STZ (60 mg/kg). Blood glucose level was determined for the confirmation of diabetes. Animals were separated into 4 different groups (n=10): a DR group receiving saline for 16 weeks, and fangchinoline 1, 3, and 10 mg/kg groups receiving 1, 3, and 10 mg/kg fangchinoline p.o. for 16 weeks.

Determination of biochemical parameters

Blood was collected from all animals and plasma was separated out at 2000 rpm at 4°C for 10 min. Blood glucose level was determined by using their diagnostic kit, and glycosylated

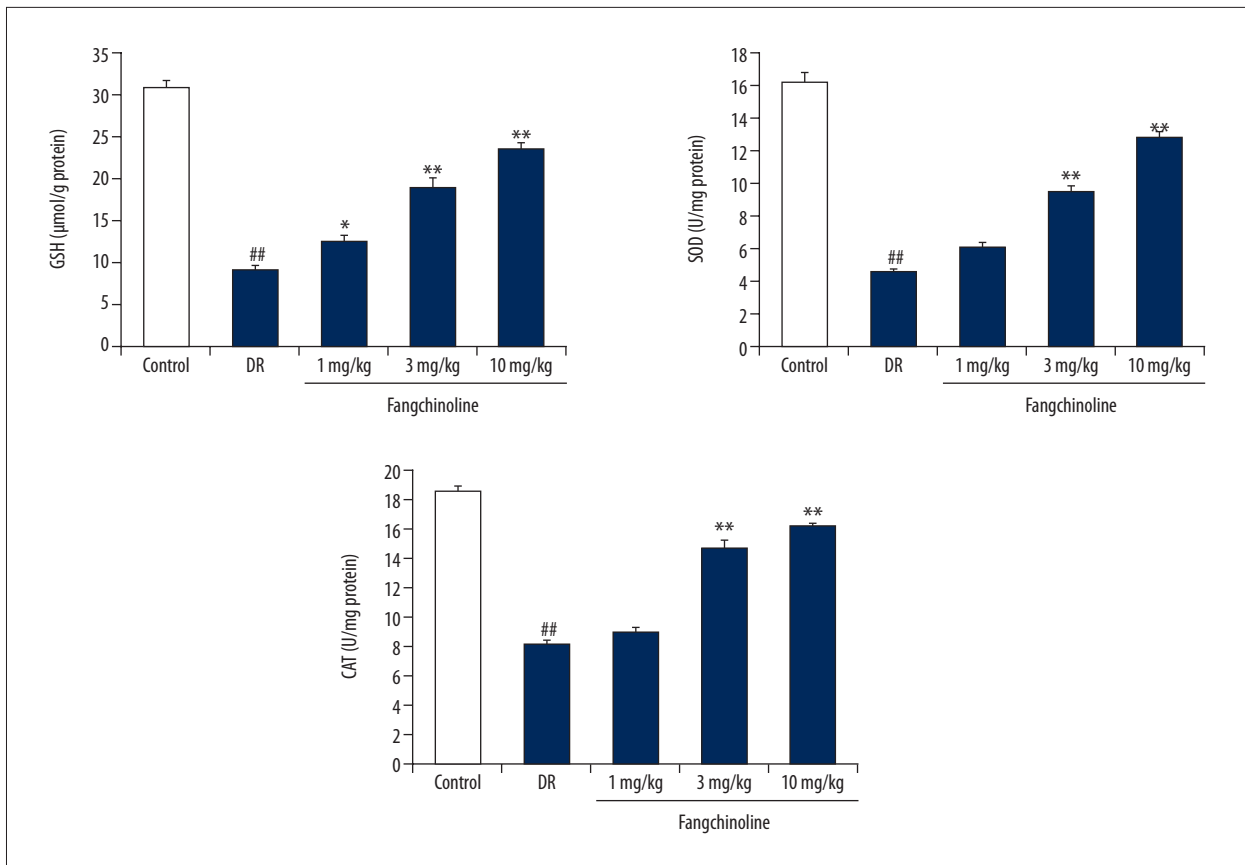


Figure 3. Fangchinoline attenuates the oxidative stress parameters in the tissue homogenate of retinas of DR rats. Mean \pm SEM (n=10), ## p<0.01 than control group; * p<0.05, ** p<0.01 than DR group.

hemoglobin (HbA1c) levels were determined by using ELISA kits according to the instructions given by the manufacturer.

Determination of AGEs in the retinal tissues

AGEs level was determined in the isolated eye of all animals, and protease inhibitor cocktail contained ice-cold RIPA buffer was used to lyse the retina, followed by centrifuging at 10 000 rpm for 15 min at 4°C. Bio-Rad DC protein assay kit was used to estimate the protein content in the collected supernatant, and the level of AGEs was determined using an ELISA kit.

Determination of retinal morphology

Xylene was used to deparaffinized 4-µm sections of the eye and dehydrated with gradient concentrations of ethanol (70%, 96%, and 100%). Tissue sections were stained with HE stain and distance from the inner limiting membrane to the outer limiting membrane was measured as retinal thickness. All observations were made using a digital trinocular microscope.

Determination of parameters of oxidative stress

Markers of oxidative stress, including activity of CAT and SOD enzymes and level of glutathione (GSH), were determined in the retinal tissue homogenate by using their respective kits according to the manufacturer's instructions.

RT-PCR

Trizol reagent was used to extract the total RNA from retinas, and agarose gel electrophoresis was used to verify the integrity of RNA by ethidium bromide. Random hexamer primers (8.5 µg/ml) and 1 µg of total RNA/sample were heated for 5 min at 65°C and quenched on ice for the reverse transcriptase reaction. The mixture was combined with 500 µmol/L each of dCTP, dTTP, and dATP, 5 mmol/L MgCl₂, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 10 mmol/L DTT, dGTP, 40 units of RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen), and 100 units SuperScript III reverse transcriptase (Invitrogen). A GeneAmp 9700 Thermal Cycler was used to treat the sample with DNase for 20 min at 37°C and then at 4°C. Quantitative real-time PCR was used to quantify the mRNA on a Fluorescent Temperature Light Cycler 480. Primers of VEGF, IL-6, IL-1β, TNF-α, RAGE, and

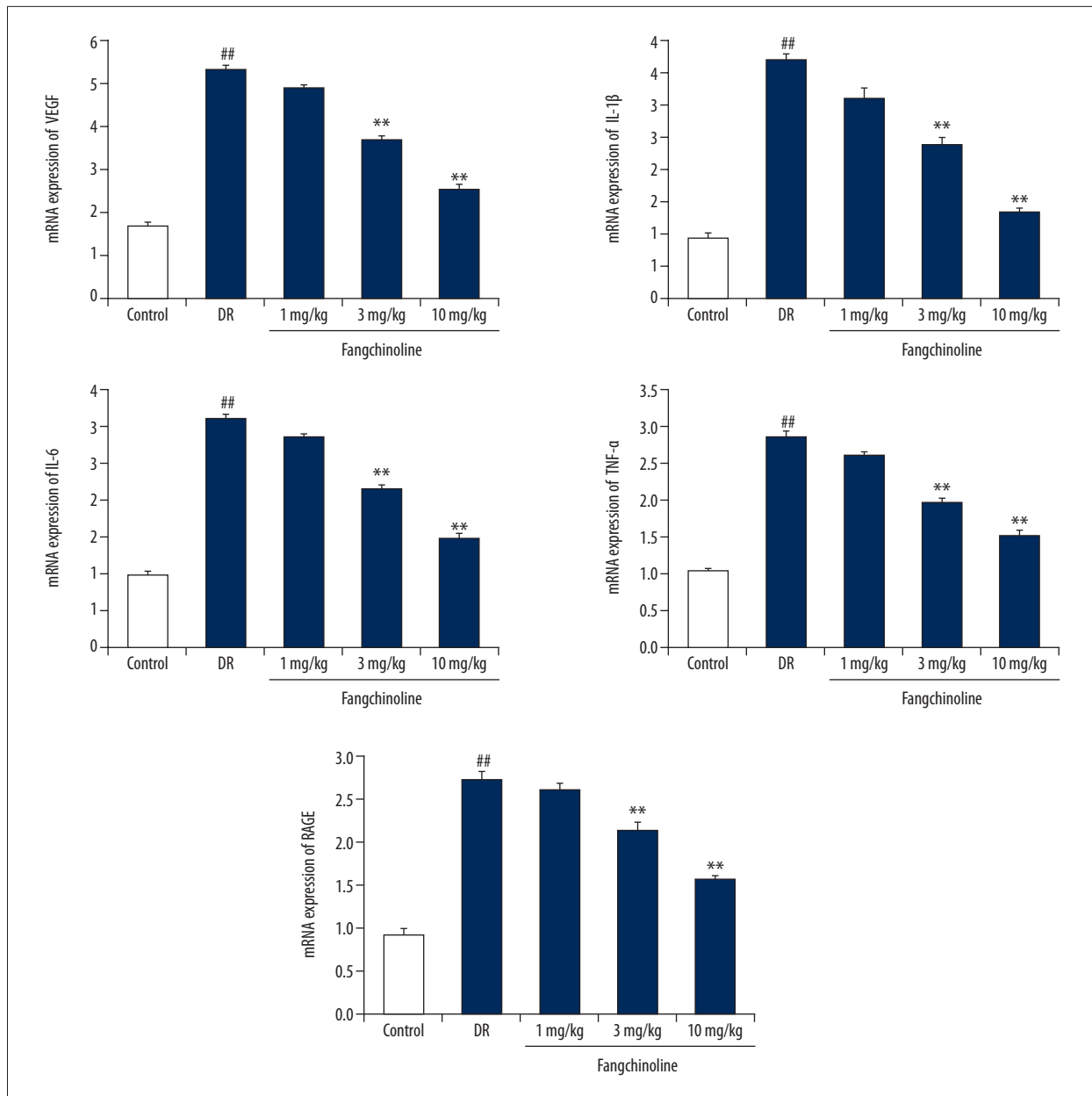


Figure 4. Fangchinoline attenuates the mRNA expression of proinflammatory mediators and RAGE in the retinas of DR rats. Mean \pm SEM (n=10), ## p<0.01 than control group; * p<0.05, ** p<0.01 than DR group.

β -actin were used, and Primer Express Software version 2.0 System was used to design the primers. The cycling protocol was 95°C for 5 min, then at 95°C for 5 s for 45 cycles, followed by 58°C for 15 s, and 20 s at 72°C. Expression of mRNA level was determined in comparison to β -actin mRNA levels by delta-delta Ct method.

Determination of activation of NF- κ B

A nuclear extraction kit was used for extraction of retinal nuclei and activity of NF- κ B was estimated by NF- κ B p65 transcription

factor assay kit as per the instructions of the manufacturer. Oligonucleotide was incubated with retinal nuclear extracts (20 μ g) containing NF- κ B and then monoclonal and secondary antibody directed against the NF- κ B p65 subunit. Quantification of reactions was determined at 450 nm.

Determination of apoptosis

Cytosolic histone-associated DNA fragmentation was detected using an ELISA kit for cell death detection. In sandwich ELISA, cytosolic retina extract was used as an antigen and a Dynex

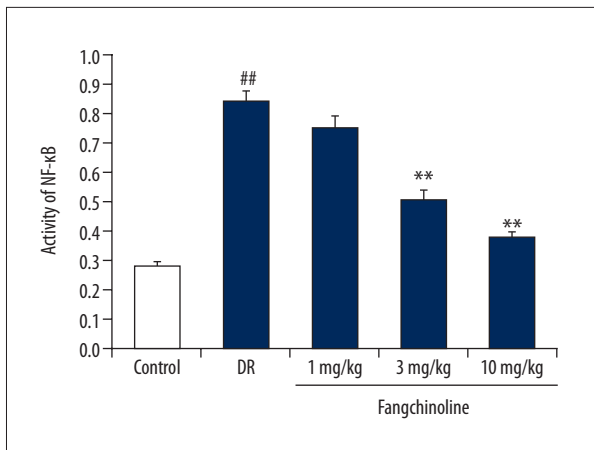


Figure 5. Fangchinoline attenuates the activity of NF-κB in the retinas of DR rats. Mean ± SEM (n=10), ## p<0.01 than control group; * p<0.05, ** p<0.01 than DR group.

MRX plate reader used to detect the color change at 405 nm. The level of cellular death was considered the apoptotic index.

Western blot assay

Retinal tissue homogenate was centrifuged at 14 000 RPM for 2 min at 4°C with 80 μl of Nonidet P-40 (10%). A Bio-Rad protein assay kit was used to determine the concentration of tissue protein. Sodium dodecyl sulfate-polyacrylamide gel at concentrations of 8%, 12%, and 15% was used to separate the tissue protein by gel electrophoresis, and nitrocellulose membrane-containing proteins were blocked with milk solution (5%) for 60 min. The cellular membrane was incubated with primary antibodies VEGF (1: 500 dilution; Santa Cruz Biotechnology, USA), IL-1β (1: 500 dilution; Santa Cruz Biotechnology, USA), IL-6 (1: 500 dilution; Santa Cruz Biotechnology, USA), TNF-α (1: 500 dilution; Santa Cruz Biotechnology, USA), RAGE (1: 500 dilution; Santa Cruz Biotechnology, USA), and GAPDH (1: 500 dilution; Santa Cruz Biotechnology, USA) overnight at 4°C. Membranes were incubated with peroxidase-conjugated secondary antibodies, and ATTO Densitograph software was used to determine the optical density of bands.

Statistical analysis

All data are expressed as means ± SEM (n=10) and the statistical analysis consisted of a one-way analysis of variance (ANOVA). Post hoc comparisons of means were carried out with Dunnett's post hoc test using GraphPad Prism software (ver. 6.1; San Diego, CA, USA). P values <0.05 were considered to indicate statistical significance.

Results

Fangchinoline attenuates the biochemical parameters

Figure 1 shows the effect of fangchinoline on the plasma concentration of HbA1c and glucose and AGEs in the retinal tissues of DR rats. Plasma concentrations of HbA1c and glucose and AGEs in the retinal tissues were lower in the DR group than in the control group. Treatment with fangchinoline reduced the biochemical parameters of plasma concentration of HbA1c and glucose and AGEs in the retinal tissues of DR rats.

Fangchinoline attenuates retinal morphology

Figure 2 shows the effect of fangchinoline on the retinal morphology of DR rats. The arrangement of the retinal layer, nuclear layer, ganglion cells, and layer of nerve fiber were normal in the control group of rats, but rats in the DR group had atrophy in layers of ganglionic cells and nerve fibers. Treatment with fangchinoline attenuated altered retinal morphology in STZ-induced diabetic retinopathic rats. Moreover, retinal thickness was enhanced in the fangchinoline-treated group but not in the DR group.

Fangchinoline attenuated oxidative stress

Figure 3 shows the effect of fangchinoline on the oxidative stress in the retinal tissues of DR rats. In DR rats, the level of GSH was reduced and activity of enzymes CAT and SOD also were lower in the retinas of the DR group than in the control group. We found that fangchinoline attenuates the altered level of markers of oxidative stress in DR rats.

Fangchinoline attenuates the mRNA expression of proinflammatory mediators and RAGE

Figure 4 shows the effect of fangchinoline on the mRNA expression of inflammatory mediators and RAGE in the tissue homogenate of retinas of DR rats. In DR rats, mRNA expression of RAGE and proinflammatory mediators was higher than in the control group. Treatment with fangchinoline attenuated the altered mRNA expression of VEGF, TNF-α, IL-1β, IL-6, and RAGE in the tissue homogenate of retinas of DR rats.

Fangchinoline attenuates the activity of NF-κB

Figure 5 shows the effect of fangchinoline on the activity of NF-κB in tissue homogenate of retinas of DR rats, showing that activity of NF-κB was higher in the DR group than in the control group. Moreover, fangchinoline treatment reduced the activation of NF-κB in DR rats.

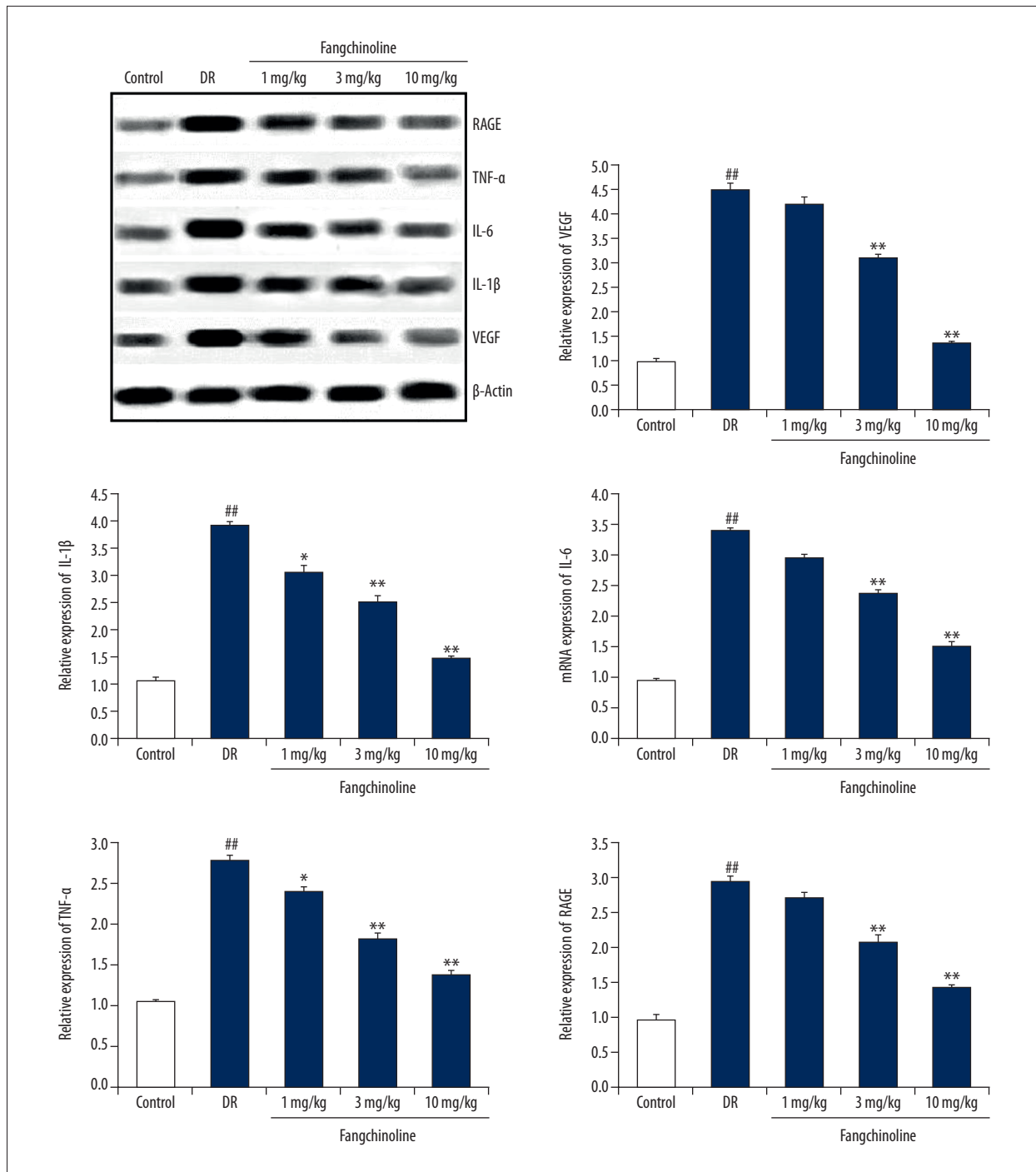


Figure 6. Fangchinoline attenuates the expression of proinflammatory mediators and RAGE protein in the retinas of DR rats. Mean \pm SEM (n=10), ^{##} p<0.01 than control group; ^{*} p<0.05, ^{**} p<0.01 than DR group.

Fangchinoline attenuates the expression of proinflammatory mediators and RAGE

Expression of VEGF, IL-6, IL-1 β , TNF- α , and RAGE protein was significantly (p<0.01) higher in the retinas of the DR group than in the control group. There was significantly (p<0.01)

lower expression of VEGF, IL-6, IL-1 β , TNF- α , and RAGE protein in the retinas of the fangchinoline-treated group than in the DR group of rats (Figure 6).

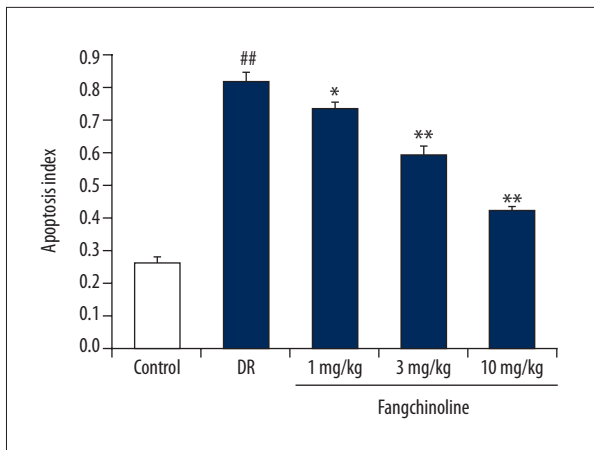


Figure 7. Fangchinoline attenuates the apoptosis index in the retinas of DR rats. Mean \pm SEM (n=10), ## p<0.01 than control group; * p<0.05, ** p<0.01 than DR group.

Fangchinoline attenuates the apoptosis index

Fangchinoline affected the apoptosis index in retinas of DR rats, as shown by ELISA. There was significantly ($p<0.01$) more retinal cell death as shown by the apoptosis index in the DR group than in the control group. However, treatment with fangchinoline attenuated the enhanced level of apoptosis in the retinas of DR rats (Figure 7).

Discussion

Uncontrolled diabetes results in several complications. Fangchinoline is reported to have antidiabetic activity, and this is supported by the present investigation. Diabetic retinopathy is one of the major complications of diabetes, in which retinal damage is caused by reduction of retinal thickness [15]. Moreover, loss of vision in DR occurs due to this disarrangement of retinal structure. The present investigation reveals that treatment with fangchinoline attenuates the altered thickness of the retinal layer and morphology in retinas of DR rats.

Production of AGEs is enhanced due to non-enzymatic glycation of protein by glucose [16]. The literature reveals that localization of RAAGE and AGEs in the retinal tissues promotes the production of cytokines due to altered protein function [17]. Moreover, apoptosis of neuronal cells in the retina is associated with AGEs [18]. Our results suggest that the concentration of AGE and expression of RAGE and cytokines is significantly

enhanced in the retinas of DR rats compared to control rats. A study found that treatment with selective AGE inhibitor attenuates DR [19], and treatment with fangchinoline significantly reduces ($p<0.01$) the concentration AGEs and expression of RAGE compared to the DR group of rats.

The pathogenesis of DR suggests that inflammatory response contributes to the development of DR [20]. In DR, the blood-retinal barrier becomes disturbed, which enhances the permeability of the retina and angiogenic cytokines such as VEGF that contributes to it [21]. Moreover, inflammatory cytokines such as interleukins and TNF- α have proven roles in transmigration of leukocytes from the blood vessels. All these events contribute to the development of DR. Our results show that expression of inflammatory cytokines such as VEGF, IL-6, IL-1 β , and TNF- α are significantly reduced in the retinas of the fangchinoline-treated group compared to the DR group.

Apoptosis and immune and inflammatory responses are regulated by the NF- κ B pathway and activation of NF- κ B occurs due to enhanced expression of RAGE and concentration of AGEs [22]. Activation of NF- κ B was reported in diabetes, which migrates the p65 in the ganglionic cells, pericytes, and endothelial cells of the retina [23]. Our study also reveals that treatment with fangchinoline attenuates the enhanced activity of NF- κ B in the retinas of DR rats.

Conclusions

We found that fangchinoline attenuates the apoptosis of retinal cells in STZ-induced diabetic retinopathy. Fangchinoline reduces the expression of AGE and RAGE and thereby inhibits the activation of inflammatory cytokines in the retinal tissues of DR rats. Fangchinoline has shown therapeutic potential and could be used clinically for the management of DR.

Acknowledgement

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Conflict of interest

None.

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