



Anti-inflammatory role of sesamin in STZ induced mice model of diabetic retinopathy



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ABSTRACT

Diabetic retinopathy (DR) is the common cause of diabetic vascular complications that leads to the blindness in the working age population throughout the world. Free radicals mediated oxidative stress and inflammation play a significant role in pathophysiology of DR. To find a new and safe drug to treat DR is still challenging and for that purpose the natural compounds may be therapeutic agents. Here we show that sesamin (SES), which is the main component of sesame seed and its oil, and has been reported as potent antioxidant and neuroprotective, could be a therapeutic agent in DR. In the present study, we investigated protective effect of SES in Streptozotocin (STZ) induced DR in mice. The mice were divided into three groups (Control, DR and DR+SES) for the study. After two weeks post-diabetic establishment, mice were treated with SES (30 mg/kg BW, i.p, alternate day) for four weeks. Mice body weight and blood glucose level were measured from each group. The microglial activation of retina was determined by immunohistochemistry analysis by using Iba-1 as a microglia marker. Retinal mRNA levels of Iba-1, tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS) and Intercellular Adhesion Molecule 1 (ICAM-1) were examined by qRT-PCR. The level of iNOS protein expression was examined by immunoblotting. Together these data demonstrate that SES treatment lowered the progression of diabetic retinal injury by: 1) decreasing blood glucose level, 2) suppressing microglia activation, 3) reducing retinal TNF- α and ICAM-1 levels and 4) quenching iNOS expression. In conclusion, the results suggest that SES treatment may be of therapeutic benefit in reducing the progression of DR by ameliorating hyperglycemia and inflammation in diabetic retina.

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1. Introduction

Diabetic retinopathy (DR) is known to be the most common microvascular complication of diabetes and a main cause of preventable blindness among working adults (Abu El-Asrar et al., 2013). The entire neurovascular unit of the retina may be affected by diabetes as a consequence of gradual neurodegeneration, gliosis, neuro-inflammation, compromise of vascular blood-retinal barrier (BRB), edema, angiogenesis,

Abbreviations: DR, diabetic retinopathy; TNF- α , tumor necrosis factor- α ; ICAM-1, Intercellular Adhesion Molecule 1; Iba1, ionized calcium-binding adapter molecule 1; iNOS, inducible nitric oxide synthase; STZ, streptozotocin.

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and eventual fibrosis (Gologorsky et al., 2012). An increasing body of literature is reporting a positive association between DR and vascular inflammatory reactions (Zhang et al., 2011). Several mechanisms have been hypothesized to explain appearance of low-grade chronic inflammation in the diabetic retina. These include: a direct effect of hyperglycemia, dyslipidemia, advanced glycation end products, hypertension, endoplasmic reticulum stress, systemic inflammation, pro-inflammatory cytokines, oxidative stress, and the subsequent up-regulation of leukocyte adhesion molecules (Tang and Kern, 2011). Under these pathological conditions, normally quiescent microglial cells become activated. Microglia are sensitive to smallest changes in the environment as they continuously survey the retina for signs of disease and cell damage. Thus, it is generally presumed that microglia initiate neuro-inflammation and other glial cells respond to and amplify these responses (Saijo and Glass, 2011). Reactive oxygen species and pro-inflammatory mediators release by activated microglia lead ultimately to vascular occlusion, tissue ischemia, and neuronal cell death (Elsherbiny et al., 2013a).

The current approach of DR treatment based on anti-inflammatory, anti-angiogenesis drugs, vitrectomy, and laser photocoagulation is effective but exhibits adverse side effects too (Cheung et al., 2010). Therefore, targeting biochemical and molecular changes in the diabetic retina by safe and potential therapeutic agents is needed to control the development and progression of DR.

Sesamin (SES) is a phytonutrient of lignans class, and one of the major components of sesame seed and its oil (Bournival et al., 2012b). SES has been found to have various health beneficial effects, including protection against oxidative stress (Baluchnejadmojarad et al., 2013; Cao et al., 2015; Chen et al., 2015; Hou et al., 2014; Su et al., 2014), anti-inflammation (Fukunaga et al., 2014; Lee et al., 2009; Li et al., 2016; Phitak et al., 2012; Qiang et al., 2016), anti-hypertension (Matsumura et al., 1998; Zhao et al., 2015), hypocholesterolemic (Hirata et al., 1996) and hepatocyte-protecting effects (Akimoto et al., 1993). Further, in the brain, SES was found to quench excess generation of free radicals in different brain injury models (Cheng et al., 2006; Park et al., 2015). SES was also reported to exhibit antioxidant and anti-inflammatory effects in the murine microglial cell line BV-2 (Jeng et al., 2005), rat primary microglia cells (Hou et al., 2003) and neuronal cells (Hsieh et al., 2011). Interestingly, it has been shown that SES inhibits activation of p38-MAPK and NF- κ B signaling pathways in prostate cancer cells (Xu et al., 2015). In this consistency, we previously demonstrated neuroprotective effect of sesame seed oil and SES in different models of brain injury (Ahmad et al., 2006, 2012; Khan et al., 2010). The present study was therefore designed to explore the beneficial effect of SES on STZ-induced DR in mice.

2. Materials and methods

2.1. Materials

Streptozotocin (STZ) was purchased from (Sigma, MO). Sesamin (SES) was procured from (Sabinsa Corporation, USA). All the chemicals used were AR and molecular grade.

2.2. Animals and treatment

Animal surgery and experiments were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, National Institutes of Health Publication No. 80-23), Georgia Regents University (GRU), Augusta, GA and King Abdulaziz University, Jeddah, KSA guidelines. C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME; 45 in number; ten-weeks-old; weight ranging 25–30 g) were used for this study. I.P injection was given to animals with vehicle or freshly prepared streptozotocin in 0.01 mol/L sodium citrate buffer, pH 4.5 (45 mg/kg) after 4 h fast each day for 5 consecutive days. Diabetes was confirmed by fasting blood glucose levels >250 mg/dL.

After the establishment of diabetes (2 weeks), the diabetic and non-diabetic mice were randomly divided into three subgroups: vehicle-treated control (12 mice), vehicle-treated diabetic (13 mice) and SES-treated diabetic subgroups (12 mice; 30 mg/kg intraperitoneally dissolved in 0.1% DMSO, alternate day). Four weeks after treatment, the mouse retina was isolated and snap frozen in liquid nitrogen and stored at -80°C . The mRNA level and protein expression were analyzed by Quantitative Real Time-PCR (qRT-PCR) and Western blot, respectively. Frozen eye sections were prepared for histology analysis.

2.3. Measurement of blood glucose

Blood glucose meter (OneTouch UltraEasy, USA) was used to monitor blood glucose level.

Table 1

The primer sets used for the detection of mouse genes by RT-PCR.

Gene	Primer sequence (5'–3')	Accession number
Iba-1	GTCTTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC	NM_019467
TNF- α	CCCTCACACTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ICAM-1	CGCTGTGCTTTGAGAAGTGTG ATACACGGTGATGGTAGCGGA	NM_010493
iNOS	ACA TCG ACC CGT CCA CAG TAT CAG AGG GGT AGG CTT GTC TC	Primer Bank ID 6754872
GAPDH	CAT GGC CTC CAA GGA GTAAGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

2.4. Quantitative real time-PCR

Total RNA was isolated from mouse retina following manufacturer's instructions (SV Total RNA Isolation kit, Promega, Madison, WI) and RNA quality was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). Reverse transcription to cDNA was carried out using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). Using appropriate primers (Table 1), 50 ng of cDNA was amplified in each qRT-PCR (Bio-Rad iCycler, ABgene reagents, Fisher scientific). Average of GAPDH and 18S RNA was used as the internal control for normalization.

2.5. Quantitative Western blot analysis

Mouse retinas were homogenized in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, supplemented with 40 mM NaF, 2 mM Na_3VO_4 , 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at $12,000 \times g$ at 4°C for 30 min. Protein concentration was determined by DC Protein Assay (Bio-Rad, Hercules, CA). 100- μg protein was loaded on SDS-PAGE (4–20% gradient gel, Pierce, Rockford, IL) and subsequently transferred to PVDF membrane and probed with specific antibodies. iNOS protein was detected on the membrane as previously described (Ahmad et al., 2012). The same membrane was re-probed with actin as internal control. Quantification of immunoreactivity was done by densitometry (Image J software, NIH).

2.6. Immunostaining

Briefly, frozen eye sections of 7 μm thickness were fixed with 4% PFA for 20 min at RT and washed 3 times with $1 \times$ PBS. After washing, eye sections were blocked with Dako serum-free protein blocker and incubated overnight at 4°C with primary antibody (anti-ICAM-1 antibody with 1:100 dilution; Santa Cruz Biotechnology, CA; Cat No. sc-107). Sections were then briefly washed with $1 \times$ PBS and incubated with secondary antibody (FITC green; 1:500). Stained sections were examined under the fluorescent microscope. Specificity of the reaction was confirmed by omitting the primary antibody, or by using non-immune IgG. Iba-1 staining was done by immunohistochemistry. Frozen retinal sections were fixed and treated with 0.3% H_2O_2 followed by the Mouse immunoglobulin Blocking Solution (M.O.M, Vector Laboratories, Burlingame, CA). Sections were incubated with Iba-1 antibody (1:100; Wako Pure Chemical, Wako, TX) for 16 to 20 h at room temperature followed by M.O.M. biotinylated anti-mouse Ig reagent (1:250). DAB (3,3'-diaminobenzidine) was used as substrate for color development. The pictures were taken under the microscope.

2.7. Statistics

Data were analyzed by GraphPad PRISM software. The results are expressed as mean \pm SD. Differences among experimental groups were evaluated by analysis of variance (one-way ANOVA), and the significance of differences between groups was assessed by the posthoc test (Newman–Keuls multiple comparison). Significance was defined as $P < 0.05$.

3. Results

3.1. Effect of sesamin (SES) on body weight and blood glucose levels

Diabetes caused a significant increase of blood glucose level and significant reduction of body weight as compared to control group. Treatment with antioxidant compound sesamin (SES) significantly decreased blood glucose level and increased body weight of mice as compared to diabetic mice (Fig. 1A, B). These results demonstrated that the use of SES might ameliorate glucose level and body weight in diabetic mice.

3.2. Sesamin (SES) ameliorated retinal microglia activation in diabetic mice

Retinal microglia are known to release cytokines upon activation and earlier findings suggest that excess release of cytokines activates MAP Kinase pathway that leads to inflammatory gene activation. Therefore, the role of microglia activation is one of the main targets in diabetic retinopathy research. To test the possible mechanisms by which SES could attenuate retinal injury in diabetic mice, we investigated the effect of SES treatment on microglia activation by measuring Iba1 (activated microglia marker) expression level. We observed increased of Iba1 immunostaining in retinal section of diabetic mice as compared with control group. However, Iba1 staining was significantly reduced in the SES-treated diabetic mice as compared with vehicle-treated diabetic mice (Fig. 2A). Further, the mRNA level of Iba1 was significantly reduced in the retinas of SES-treated diabetic mice as compared with vehicle-treated diabetic mice (Fig. 2B). These data indicate that SES treatment may reduce microglia activation in diabetic retina.

3.3. Sesamin (SES) treatment attenuated retinal inflammation in diabetic mice

Next, we examined the effect of SES on inflammatory markers in diabetic mice retina. To show the results, we examined the mRNA levels of TNF- α and ICAM-1 in retina. The mRNA levels of TNF- α and ICAM-1 were markedly increased in the retinal samples of 22-weeks old

diabetic mice as compared with control group, as revealed by qRT-PCR (Fig. 3A, B). In addition, ICAM-1 immunostaining was significantly increased in diabetic retina as compared to control group (Fig. 4A,B). The SES treatment reduced the mRNA levels of TNF- α and ICAM-1 in treated mice as compared to diabetic group. Results exhibit that SES treatment may reduce the release of inflammatory cytokines in diabetic retina.

3.4. Sesamin (SES) reduced diabetic induced up-regulation of iNOS

Scientific evidences have shown that iNOS plays an important role in diabetic retinal injury and is known to generate reactive nitrogen species. Diabetic mice showed a significant increase in mRNA and expression levels of iNOS as compared with control group. SES-treated diabetic mice showed decreased iNOS mRNA and protein levels as compared with vehicle-treated diabetic mice (Fig. 5A,B).

4. Discussion

Diabetic retinopathy (DR) is a microvascular complication of diabetes that affects the blood vessels of retina, leading to vision loss (Ahmad et al., 2012; Elsherbiny et al., 2013a,b). Chronic hyperglycemia is believed to play the primary role in the pathogenesis of retinal damage in DR. The biochemical mechanisms associated with hyperglycemic-induced DR are multifactorial (Hong et al., 2013; Khan et al., 2010). There is considerable published evidence that both inflammation and oxidative stresses are potential biochemical mechanisms in hyperglycemic-induced cell damage (Ahmad et al., 2006; Baluchnejadmojarad et al., 2013; Bournival et al., 2012a; Elsherbiny et al., 2013a,b; Mitsushashi et al., 2013; Zheng et al., 2015). In this study, the results demonstrate that the treatment of antioxidant compound SES inhibits the progression of diabetic retinopathy through hypoglycemic, anti-inflammatory and antioxidant mechanisms. Further, SES treatment helps in lowering of blood glucose levels of treated mice as compared to untreated diabetic group. Our results are in accordance with other findings where it has been shown that hypoglycemic activity of SES in type 2 diabetic animal models through attenuating insulin resistance (Hong et al., 2013; Song et al., 2012). In addition, another study suggested that SES treatment has protective effect against STZ induced damage in pancreatic β cells through antioxidant mechanisms (Shen et al., 2013).

Microglia is resident immune cells in the retina. Under pathological conditions, microglia becomes activated and participates in immune and inflammatory reaction (Ahmad et al., 2013, 2014a; Kumar et al., 2013). Microglial activation has been recognized as a neuropathological characteristic of diabetic retina, which leads to the release of soluble cytotoxins that contribute to neuronal and vascular cell death and ultimately diabetic retinopathy (Hong et al., 2013). However, several

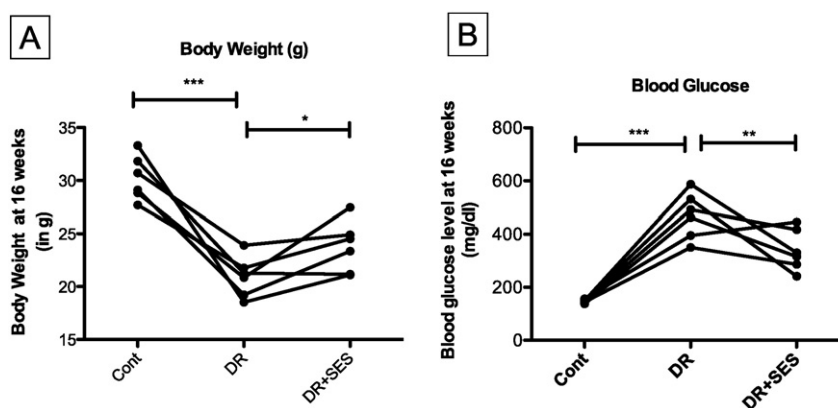


Fig. 1. Effect of sesamin (SES) treatment on body weight (g) and blood glucose level in DR. A–B) Cont; control group, DR; diabetic retinopathy group, SES; sesamin treated diabetic group. Data shown are the mean \pm SD (n = 6). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

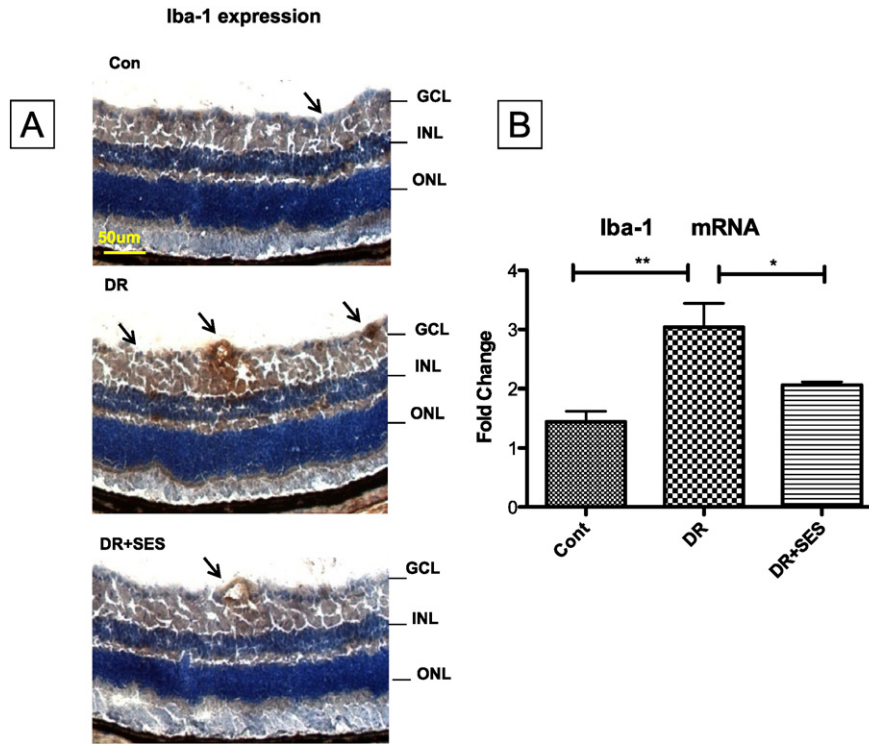


Fig. 2. Sesamin (SES) treatment ameliorated retinal microglia activation in diabetic mice. A) Effect of SES on Iba-1 expression in the diabetic mouse retina determined by immunohistochemistry, Scale bar: 50 μ m, GCL; ganglion cell layer, INL; inner nuclear layer, ONL; outer nuclear layer. B) Determined by RT PCR analysis; GAPDH and 18S were used as reporter genes. The results represent the means \pm SD of fold changes calculated using expression level, normalized to the level of the control non-diabetic mice ($n = 6$). Data shown are the mean \pm SD. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

studies have reported a dramatic increase of the number and activity of microglia in experimental diabetic retinopathy (Kettenmann et al., 2011; Lei et al., 2012). Consistent with earlier studies, we previously reported similar changes in morphology and antigen-expression patterns of retinal microglia cells during diabetes and traumatic optic neuropathy condition (Ahmad et al., 2014a; Elsherbiny et al., 2013a; Zeng et al., 2008). In this study, we investigated the effect of SES on microglia activation and found that the SES treatment significantly decreased the expression of Iba-1, a marker of microglia activation as compared to untreated diabetic group. That indicates that SES may inhibit microglia activation in DR.

TNF- α is robust marker of inflammation that exhibited potential role in diabetic-induced retinal injury. Earlier studies have been shown that

TNF- α levels are increased in the diabetic retina (Chen et al., 2002; Fulzele et al., 2015; Ibrahim et al., 2011a,b; Krady et al., 2005). In the present study, SES treatment reduced TNF- α mRNA levels in retinas of diabetic mice as compared to untreated group. It was reported that TNF- α contributes to blood retinal barrier breakdown in DR by many mechanisms including: mediating death/apoptosis of retinal neuronal and endothelial cells (Elsherbiny et al., 2013b) and mediating leukostasis in the retinal vasculature (Behl et al., 2008). Thus, reducing TNF- α mRNA level by SES may have vascular benefits in DR.

Leukocyte adhesion to retinal vasculature is involved in pathophysiology of DR and resulted in blood-retinal barrier breakdown, capillary occlusion, endothelial cell injury and death, suggesting a significant role of adhesion molecules in pathogenesis of DR (Jousen et al.,

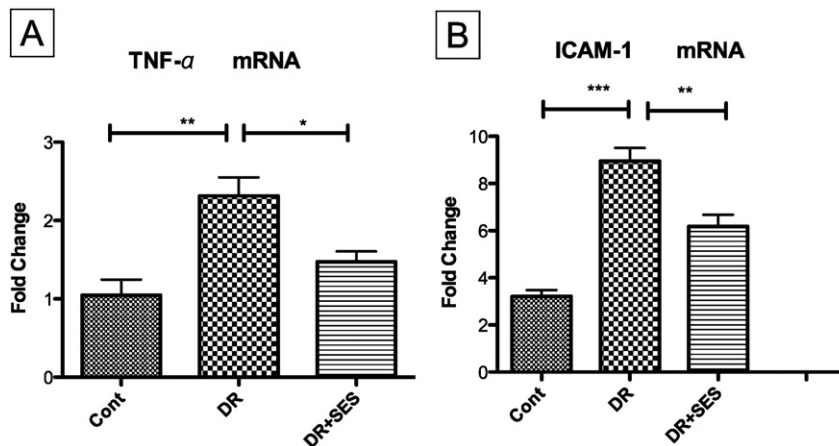


Fig. 3. Sesamin (SES) treatment attenuated retinal inflammation in diabetic mice. A) Effect of SES on TNF- α mRNA levels in the diabetic mouse retina. B) Effect of SES on ICAM-1 mRNA levels in the diabetic mouse retina. GAPDH and 18S were used as reporter genes. The results represent the means \pm SD of fold changes calculated using expression level, normalized to the level of the control non-diabetic mice ($n = 6$). Data shown are the mean \pm SD. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

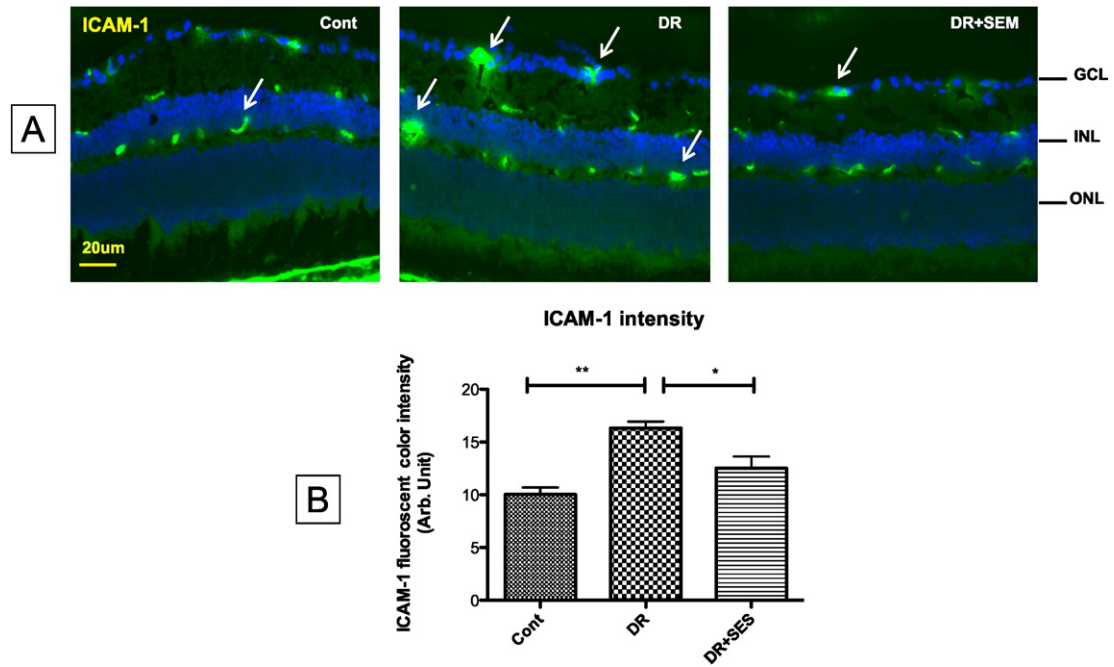


Fig. 4. Effect of sesamin (SES) treatment on ICAM-1 expression in the diabetic mouse retina. A) ICAM-1 was determined by immunofluorescence staining. Scale bar: 20 µm, GCL; ganglion cell layer, INL; inner nuclear layer, ONL; outer nuclear layer. B) ICAM-1 fluorescent color intensity. Data shown are the mean ± SD (n = 6). *P < 0.01, **P < 0.001, ***P < 0.0001.

2002). Of these adhesion molecules, ICAM-1 was shown to be of fundamental importance to the recruitment and migration of leucocytes at inflammation sites (Joussen et al., 2009). Our results suggest that ICAM-1 mRNA and expression levels in diabetic retina were decreased by SES treatment. Previous study demonstrated a similar effect of SES on TNF-α-induced ICAM-1 expression levels in endothelial cells through attenuating extracellular signal-regulated kinase/p38 pathway (Vinores et al., 2007).

iNOS is cytokine-inducible nitric oxide (NO) synthase that synthesizes NO from L-arginine. It has been shown that iNOS plays a crucial role in retinal neovascular disease and retinal degeneration (Serra et al., 2012). Evidence suggested that iNOS levels in the diabetic retina are elevated due to extensive production by endothelial cells and pericytes

(Noda et al., 2012). In diabetic retinal endothelium, iNOS was reported to be the main enzyme involved in NO mediated up-regulation in ICAM-1 expression and subsequent leukostasis (Wu et al., 2010). Moreover, superoxide anions can react with NO to produce the potent oxidant peroxynitrite, which has been implicated in the development of diabetic retinal injury (Sennlaub et al., 2002). In addition, iNOS was reported to play a crucial role in retinal apoptosis in ischemic proliferative retinopathy (Serra et al., 2012). However, selective iNOS inhibition is able to inhibit vitreal neovascularization and to protect the hypoxic retina from degeneration (Kowluru et al., 2003). Further, many reports point to the inhibitory effect of SES on iNOS activation in various animal models (Baluchnejadmojarad et al., 2013; Kowluru, 2003; Lahaie-Collins et al., 2008; Leal et al., 2007; Lei et al., 2012; Sennlaub et al., 2001). In the

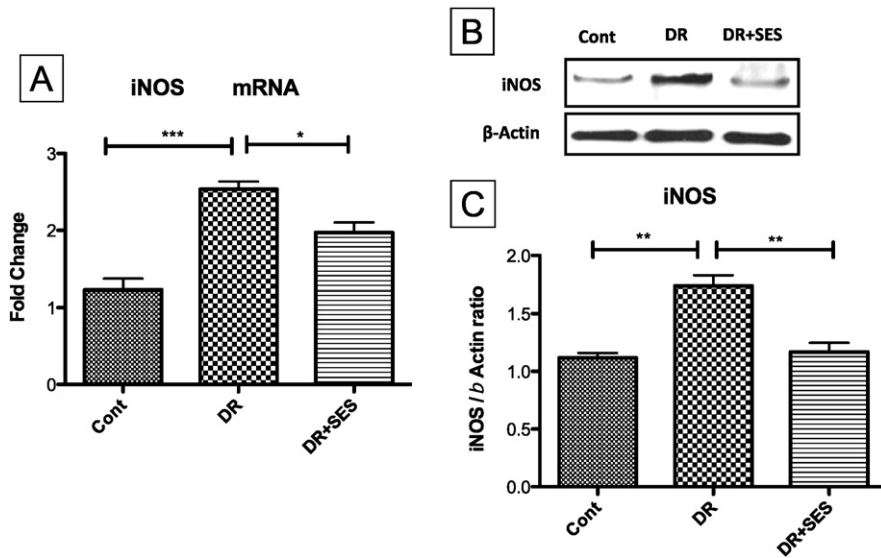


Fig. 5. Sesamin (SES) treatment reduced iNOS levels in diabetic mouse retina. A) Retinal expression of iNOS measured by RT-PCR. GAPDH and 18S were used as reporter genes. The results represent the means ± SD of fold changes calculated using expression level, normalized to the level of the control non-diabetic mice (n = 4–6). B–C) Representative Western blots and densitometry analysis of retinal iNOS expression (n = 6). Data shown are the mean ± SD. *P < 0.01, **P < 0.001, ***P < 0.0001.

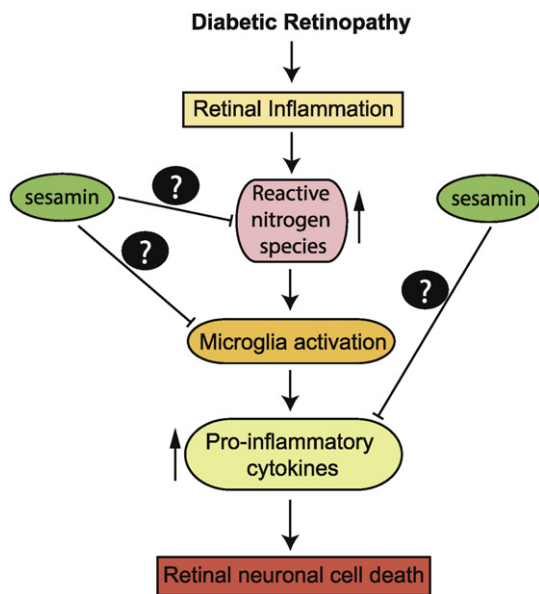


Fig. 6. Hypothetical mechanism of neurodegeneration in diabetic retinopathy and its attenuation by sesamin treatment.

present investigation, we hypothesized that SES treatment would have a similar effect in diabetic retina. In contrast, the results of our study indicated a decrease in iNOS mRNA and protein levels in treated diabetic mice as compared with untreated group, which suggests that SES has a protective role in diabetic retina that may be attributed to its inhibitory effect on synthesis of tissue damaging NO.

In conclusion, we found that SES treatment significantly reduced the expression levels of inflammatory markers, blood glucose level and increased body weight. This study is an agreement with our earlier reports where we have reported that sesame oil and its active constituents SES may ameliorate oxidative stress and inflammation in neurodegenerative disorders (Ahmad et al., 2006, 2012, 2014b; Khan et al., 2010). Interestingly, one of the recent studies has shown that SES prevents impairment of skeletal muscle mitochondrial function in diabetic mice model (Takada et al., 2015). Overall, this study demonstrates that SES treatment may be useful in attenuating the progression of diabetic retinopathy by hypoglycemic and anti-inflammatory mechanisms, and here we have shown a graphical presentation (Fig. 6) to understand the beneficial role of SES in DR.

Conflict of interest

There is no conflict of interest with any person or institute.

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