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ORIGINAL ARTICLE

Minimization of the Risk of Diabetic Microangiopathy in Rats by *Nigella sativa*

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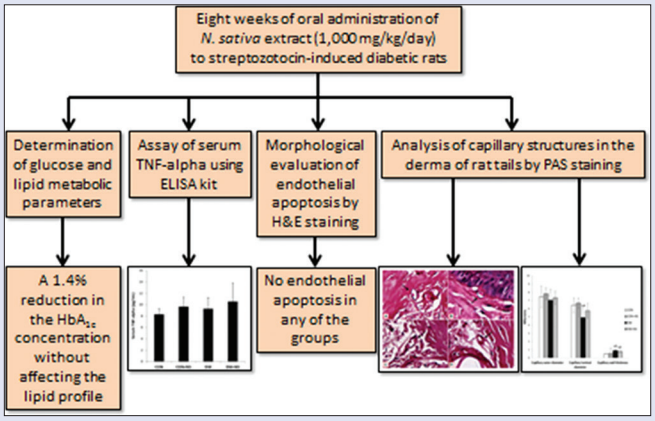
ABSTRACT

Background: Microangiopathy is a chronic diabetic complication resulting from metabolic derangements, oxidative stress, and increased pro-inflammatory cytokine production. *Nigella sativa* Linn. is used as an herbal medicine that exerts hypoglycemic, antilipidemic, anti-inflammatory, and antioxidant effects. **Objective:** To examine the effects of *N. sativa* extract on cutaneous microvascular changes in diabetic rats. **Materials and Methods:** Sprague-Dawley rats were randomly assigned into the following four groups: Untreated and *N. sativa*-treated normal controls and untreated and *N. sativa*-treated rats with streptozotocin-induced diabetes. A cold-pressed *N. sativa* extract was then orally administered (1000 mg/kg/day). After 8 weeks of treatment, the glucose, glycosylated hemoglobin (HbA_{1c}), tumor necrosis factor-alpha (TNF-α), insulin levels, and lipid profile were determined in cardiac blood. Dermal capillary wall thickness was measured in tail skin sections stained with periodic acid-Schiff. Endothelial apoptosis was morphologically evaluated by hematoxylin and eosin staining. **Results:** Diabetes significantly reduced the circulating insulin and low-density lipoprotein levels and caused elevations in the glucose, HbA_{1c}, and triglyceride levels, accompanied by a slight increase in total cholesterol levels and no change in the high-density lipoprotein and TNF-α levels. Capillary basement membrane thickening and a decreased capillary luminal diameter despite no evidence of endothelial cell apoptosis were also observed. *N. sativa* treatment of diabetic rats reduced the mean HbA_{1c} concentration by 1.4%, enlarged the capillary lumens, and tended to attenuate dermal capillary basement membrane thickening without affecting the lipid profile or TNF-α level. **Conclusion:** Our results indicate that *N. sativa* may be used to minimize the risk of diabetic microangiopathy, potentially due in part to its glycemic control activity.

Key words: Capillary basement membrane, diabetic microangiopathy, endothelial apoptosis, glycosylated hemoglobin, *Nigella sativa* Linn.

SUMMARY

- Diabetes causes dermal capillary basement membrane thickening and a decreased capillary luminal diameter
- *Nigella sativa* treatment of diabetic rats enlarged the capillary lumens and tended to attenuate dermal capillary basement membrane thickening
- *N. sativa* treatment of diabetic rats reduced the mean glycosylated hemoglobin concentration by 1.4%, which exceeds the necessary reduction previously described to decrease the risk of diabetic microangiopathy, without affecting the lipid profile or tumor necrosis factor-alpha level
- *N. sativa* improves rat diabetic microangiopathy, potentially due in part to its glycemic control activity.



Abbreviations used: H and E: Hematoxylin and eosin, HbA1c: Glycosylated hemoglobin, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, PAS: Periodic acid-Schiff, STZ: Streptozotocin, TNF-α: Tumor necrosis factor-alpha.

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INTRODUCTION

Diabetic microangiopathy is a chronic complication characterized by functional and morphological alterations of the microvasculature, including endothelial dysfunction, endothelial cell apoptosis, and capillary basement membrane thickening. These alterations result in ischemic injury, especially to the retina, glomerulus, nerve, and skin.[1,2] The pathogenetic mechanisms of diabetic microangiopathy involve diabetes-associated metabolic imbalances, including hyperglycemia, dyslipidemia, and insulin resistance. These imbalances induce oxidative stress, which in turn stimulates the synthesis of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins.[3,4] TNF-α is considered a key mediator in the development of diabetic microangiopathy because it triggers the inflammatory process, induces endothelial cell apoptosis,[5] and upregulates leukocyte adhesion molecules.[6] In addition, the accumulation of advanced glycation end products secondary to chronic hyperglycemia results in thickening of the capillary basement membrane.

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To mitigate the risk of diabetic microangiopathy, several studies have suggested the improvement of glycemic control and plasma lipid levels, inhibition of oxidative stress, and suppression of inflammation and TNF-α production.[6-9] For example, a reduced risk has been found to be associated with a 1% reduction in the glycosylated hemoglobin (HbA_{1c}) concentration.[7]

Nigella sativa Linn. is an herbaceous plant belonging to the family Ranunculaceae and is native to the Mediterranean region and parts of Asia, including India, Sri Lanka, and Thailand. It has been used in traditional medicine for the treatment of many conditions, including diabetes. In addition to its glucose-lowering effect,[10,11] *N. sativa* has been found to exert antilipidemic,[12,13] antioxidant,[14,15] and anti-inflammatory effects.[16] Furthermore, it prevents oxidative stress in streptozotocin (STZ)-induced diabetic rats.[17] *In vitro*, *N. sativa* and its active ingredient thymoquinone also inhibit eicosanoid generation[18] and modulate TNF-α production.[19,20] Moreover, thymoquinone therapy has been reported to improve renal morphology and functions in diabetic nephropathy in rats.[21] Thus, we hypothesized that this plant may be used to ameliorate alterations of the skin microvasculature in the diabetic state. To our knowledge, this is the first report of the effects of *N. sativa* on diabetic microangiopathy in rats.

The purpose of this study was to investigate the effects of *N. sativa* on serum TNF-α levels, capillary basement membrane thickening, and endothelial apoptosis in the skin of type 1 STZ-induced diabetic rats. The results of this study provide basic knowledge that can be used for further optimization of the prevention and treatment of microvascular complications in patients with diabetes mellitus.

MATERIALS AND METHODS

Chemicals and reagents

Cold-pressed *N. sativa* extract was obtained from Sungsomboon Co., Ltd., (Lopburi, Thailand). STZ was obtained from Sigma Chemicals (Saint Louis, MO, USA). A rat insulin enzyme immunoassay kit was obtained from SPI-Bio (Montigny Le Bretonneux, France). A TNF-α rat ELISA kit was obtained from Abcam (Cambridge, UK). An Accu-Chek® Advantage system was obtained from Roche (Mannheim, Germany).

Rat preparation

Male Sprague-Dawley rats weighing 180–200 g were purchased from the National Laboratory Animal Center of the Salaya Campus of Mahidol University in Nakhon Pathom, Thailand. All experiments were carried out in accordance with the Animals in Research: Reporting *In Vivo* Experiments guidelines[22] and the Guide for the Care and Use of Laboratory Animals of the National Research Council of Thailand. The experimental protocol was approved by the Committee of Animal Care of the Faculty of Medicine of Chulalongkorn University. The rats were housed at 25°C under a 12-h light-dark cycle and fed standard rat chow and water *ad libitum*. The animals were acclimatized to the laboratory environment for 7 days prior to the experiment.

Experimental design

The rats were randomly assigned to a normal control or diabetic group. Diabetes was induced by intravenous injection of STZ (55 mg/kg) (Sigma Chemicals, St. Louis, MO, USA) into the tail vein after 8–10 h of fasting. At the same time, the control rats were injected with an equal volume of citrate buffer solution. At 48 h after STZ injection, the glucose concentration in the tail blood was measured using a glucometer (Accu-chek® Advantage; Boehringer Mannheim, Mannheim, Germany). STZ rats with a blood glucose concentration of >200 mg/dL were diagnosed with diabetes mellitus and recruited for the study.

On the following day, the normal control and diabetic rats were randomly stratified into the following four subgroups: (1) Untreated normal rats (CON, $n = 5$), (2) normal rats treated with *N. sativa* (CON + NS, $n = 5$), (3) untreated diabetic rats (DM, $n = 5$), and (4) diabetic rats treated with *N. sativa* (DM + NS, $n = 6$). The rats in the CON + NS and DM + NS groups were orally administered 1000 mg/kg/day of a cold-pressed *N. sativa* extract (Sungsomboon Co., Ltd., Lopburi, Thailand), once daily for 8 weeks. The rats in the CON and DM groups were administered sterile water equal to the volume per dose of *N. sativa*.

At the end of the study, the fasting (8–10 h) tail blood glucose concentration was measured. The next day, after a 12-h fast, the rats were euthanized with an overdose of intraperitoneal thiopental sodium (60 mg/kg). Blood samples were obtained via cardiac puncture for subsequent analysis of metabolic parameters and the TNF-α level. The rat tails were cut off approximately 1–1.5 cm from the tip of the tail and immediately fixed in formalin. Rat tail skin sections were prepared according to standard pathology laboratory procedures and stained with hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS). The H and E-and PAS-stained slides were then digitally scanned for further evaluation of endothelial cell apoptosis and morphological examination of dermal capillaries, respectively.

Determination of glucose and lipid metabolic parameters

Blood samples were collected in ethylenediaminetetraacetic acid tubes for the HbA_{1c} assay and in anticoagulant-free tubes for determination of the serum insulin level, lipid profile, and TNF-α level. The serum was separated from blood cells by centrifugation and stored at − 20°C. The HbA_{1c} level and lipid profile were assessed within 12 h of blood collection.

Blood glucose was measured in the tail blood using a glucometer (Accu-chek® Advantage; Boehringer Mannheim, Mannheim, Germany) as previously described. The HbA_{1c} level was determined by the turbidimetric immunoinhibition method (Bangkok RIA Laboratory Co., Bangkok, Thailand). The insulin level was determined using a rat insulin enzyme immunoassay kit (SPI-Bio, Montigny Le Bretonneux, France). The lipid profile, including the total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride levels, was determined by an enzymatic colorimetric method (Bangkok RIA Laboratory Co., Bangkok, Thailand).

Determination of serum tumor necrosis factor-alpha level

The TNF-α concentration in the serum was assayed using a TNF-α rat ELISA kit (Abcam, Cambridge, UK).

Morphological assessment of capillary endothelial cell apoptosis

Capillary endothelial cell apoptosis was morphologically evaluated by examination of H- and E-stained slides in a blinded manner. H and E-stained apoptotic cells are characteristically shrunken, with a condensed cytoplasm and pyknotic and fragmented nuclei.[23]

Analysis of capillary basement membrane thickness and capillary luminal diameter in the dermis

Capillaries were stained with PAS for visualization of their basement membranes. The basement membrane thickness and luminal diameter of the dermal capillaries were analyzed using Aperio ImageScope software, version 12.1.0.5029 (Aperio Technologies, Vista, CA, USA). All

the dermal capillaries that were cut in cross section and had a continuous basement membrane were evaluated. The luminal and outer diameters of each capillary were measured along the same axial line. Capillary wall thickness was calculated by subtracting the luminal diameter from the outer diameter and then dividing the resulting value by 2, and the thickness is expressed in microns.

Statistical analysis

The results are presented as means ± standard deviation. Data were analyzed by analysis of variance followed by Duncan's *post hoc* test using SPSS version 22 (IBM Corp., Armonk, NY, USA). Significant differences were considered at $P < 0.05$.

RESULTS

Glucose and lipid metabolic parameters

The metabolic parameters of the normal and diabetic rats following 8 weeks of oral administration of *N. sativa* extract are presented in Table 1. The rats in both the DM and DM + NS groups exhibited a significant decrease in the insulin level and significant increases in the glucose and HbA_{1c} levels compared with rats in the CON and CON + NS groups. There were no significant differences in the glucose metabolic parameters between the DM and DM + NS groups. However, the DM + NS rats tended to have a lower glucose concentration, and the HbA_{1c} level was approximately 1.4% lower in the DM + NS rats than in the DM rats.

The lipid profiles did not differ between the DM and DM + NS groups. These two diabetic groups exhibited significant elevations in the triglyceride levels and a significant reduction in LDL-C compared with the CON or CON + NS groups. The total cholesterol levels were greater in the DM and DM + NS groups than in the CON group, but these increases were not significant. No significant difference in HDL-C was noted among all groups.

Serum tumor necrosis factor-alpha level

The serum TNF-α levels in the CON, CON + NS, DM, and DM + NS groups were 8.32 ± 1.00, 9.67 ± 1.64, 9.31 ± 1.92, and 10.57 ± 3.22 pg/mL, respectively, and they did not significantly differ among the groups [Figure 1].

Evaluation of endothelial cell apoptosis

No visible signs of endothelial apoptosis were observed in any of the rats.

Structural changes in dermal capillaries

Figure 2a-d show the histological findings for the capillary structures in the derma of rat tails from the CON, CON + NS, DM, and DM + NS groups, respectively.

The average outer diameters of the capillaries did not differ among the CON, CON + NS, DM, and DM + NS groups (7.45 ± 1.32, 7.82 ± 0.73, 7.05 ± 1.35, and 7.34 ± 0.82 μ, respectively). As depicted in Figure 3, the capillary luminal diameters were comparable among the CON, CON + NS, and DM + NS groups (6.43 ± 0.92, 6.69 ± 0.63, and 5.75 ± 0.92 μ, respectively), and they were all larger than the average diameter of the DM group (4.92 ± 1.19 μ). Capillary wall thickness tended to be greater in the DM group than in the DM + NS group (0.91 ± 0.21 and 0.80 ± 0.30 μ, respectively), but this difference was not significant. Both diabetic groups had significantly greater capillary wall thicknesses compared with those of the CON and CON + NS groups (0.51 ± 0.02 and 0.55 ± 0.06 μ, respectively).

DISCUSSION

The results of the present study show that treatment of diabetic rats with *N. sativa* causes slight reductions in blood glucose and HbA_{1c} levels, but no changes in insulin levels, lipid metabolic parameters, or serum TNF-α levels. In contrast, *N. sativa*-treated normal rats had significantly lower insulin and LDL-C levels. The present findings also demonstrated capillary basement membrane thickening and a decreased capillary luminal diameter despite no evidence of endothelial cell apoptosis in the dermis of rat tail skin after 8 weeks of diabetic induction. Interestingly, *N. sativa* exhibited beneficial effects on cutaneous diabetic microangiopathy.

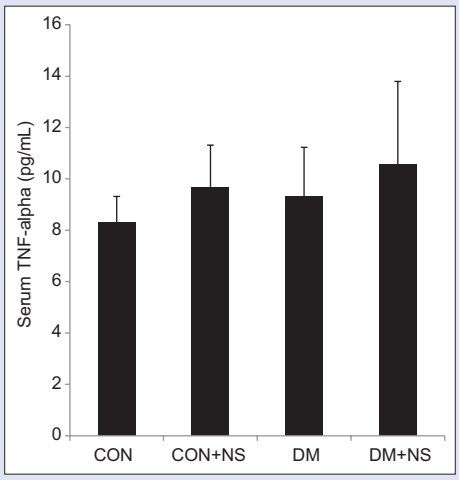

Figure 1: Serum tumor necrosis factor-alpha levels in normal and diabetic rats after 8 weeks of oral administration of a *Nigella sativa* extract; CON: Untreated normal control group; CON + NS: Normal rats treated with *Nigella sativa* extract; DM: Untreated diabetic rats; and DM + NS: Diabetic rats treated with *Nigella sativa* extract. The values are expressed as means ± standard deviation. There were no significant differences among all groups

Table 1: Circulating levels of glucose and lipid metabolic parameters in normal and diabetic rats following 8 weeks of oral administration of *Nigella sativa* extract

Metabolic parameters	Experimental groups			
	CON	CON+NS	DM	DM+NS
Blood glucose (mg/dL)	83.00±4.69	77.60±5.03	425.20±89.35[a,b]	388.00±58.23[a,b]
HbA_{1c} (%)	4.04±0.05	3.92±0.13	9.76±1.35[a,b]	8.38±0.56[a,b,c]
Insulin (ng/mL)	1.25±0.82	1.81±1.12	0.13±0.03[a,b]	0.11±0.02[a,b]
Total cholesterol (mg/dL)	82.40±9.29	77.60±6.54	90.40±14.47	93.33±9.14[b]
Triglyceride (mg/dL)	66.60±17.16	63.60±16.09	225.60±42.95[a,b]	238.17±97.37[a,b]
LDL-C (mg/dL)	13.80±3.19	9.60±1.67[a]	4.80±1.92[a,b]	4.33±2.42[a,b,c]
HDL-C (mg/dL)	67.80±9.01	66.00±4.42	68.60±12.82	65.00±13.39

The values are expressed as mean±SD [a]$P<0.05$ compared with CON; [b]$P<0.05$ compared with CON+NS; [c]$P<0.05$ compared with DM. SD: Standard deviation; HbA_{1c}: Glycosylated hemoglobin; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol

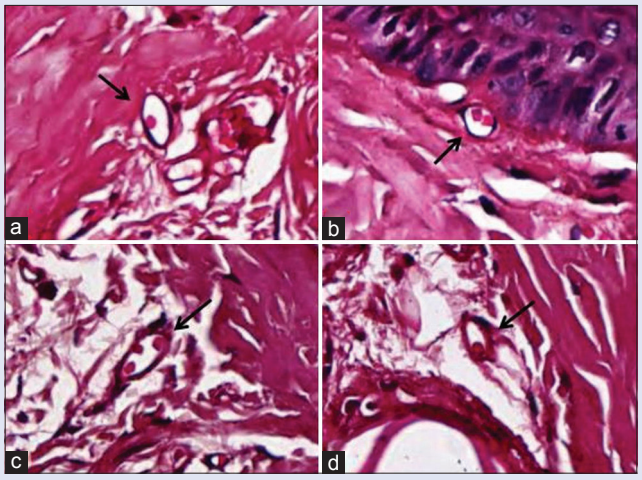

Figure 2: Photomicrographs of dermal capillaries (arrows) in the tails of (a) untreated normal rats, (b) normal rats treated with *Nigella sativa*, (c) untreated diabetic rats, and (d) diabetic rats treated with *Nigella sativa* after an 8-week experimental period. Periodic acid-Schiff staining (×40)

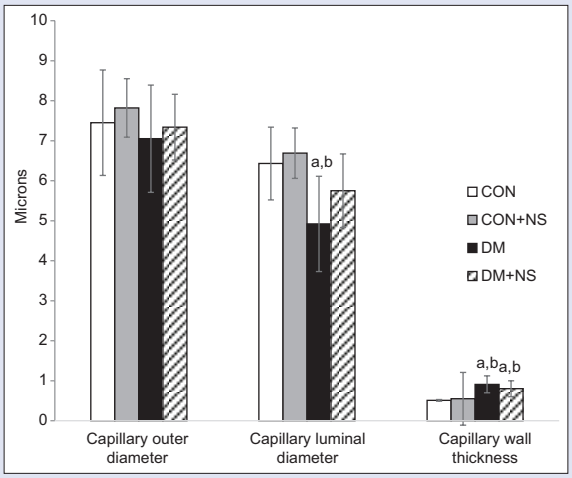

Figure 3: Structural analysis of dermal capillaries in the tails of normal and diabetic rats following 8 weeks of oral administration of *Nigella sativa* extract; CON: Untreated normal control group; CON + NS: Normal rats treated with *Nigella sativa* extract; DM: Untreated diabetic rats; and DM + NS: Diabetic rats treated with *Nigella sativa* extract. The values are expressed as means ± standard deviation $^{a}P < 0.05$ compared with CON; $^{b}P < 0.05$ compared with CON + NS

Consistent with the pathophysiology of diabetes, the untreated diabetic rats in the present study presented disturbances in carbohydrate, lipid, and lipoprotein metabolism, as evidenced by hyperglycemia and hypertriglyceridemia, as well as a tendency toward an increase in total cholesterol levels. These manifestations were consequences of the insulin deficiency caused by pancreatic beta cell damage induced by a single high dose of STZ, mimicking the effects of type 1 diabetes. People with insulin deficiency present decreased glucose utilization and increased lipolysis compared with nondiabetic individuals. Elevated plasma levels of free fatty acid as a result of increased lipolysis subsequently enhance hepatic triglyceride synthesis, causing hypertriglyceridemia. Consistent with earlier reports, the LDL-C levels in diabetic rats were reduced;[24,25] however, they have been shown to be increased in some studies investigating diabetes.[26,27] Thus, given that LDL production and catabolism are defective in diabetes,[28] the variations in the levels of LDL observed in these studies may be influenced by the rate of these two processes.

The present finding that *N. sativa* administration improves HbA_{1c} levels without any elevations in serum insulin levels in diabetic rats is in agreement with a previous study demonstrating that *N. sativa* has anti-diabetic activities, but does not increase insulin levels.[13] A possible explanation for these findings is that *N. sativa* has an insulin-sensitizing activity that is manifested via activation of the mitogen-activated protein kinase and phosphokinase B intracellular signal transduction pathways,[13] activation of the AMP-activated protein kinase signaling pathway, and an increase in the concentration of muscle glucose transporter 4.[29] Another explanation is that *N. sativa* has extra-pancreatic effects[10] and the ability to decrease hepatic gluconeogenesis.[11] Alternatively, the anti-diabetic effect of *N. sativa* may be derived from its insulinotropic activity leading to the stimulation of insulin release by Langerhans islets,[30,31] which results in increased plasma insulin levels.[30] Moreover, *N. sativa* enhances beta cell survival in rats with STZ-induced diabetes[32] through the antioxidant and anti-inflammatory activities of this plant,[33,34] among which the latter is mediated by the inhibition of pancreatic cyclooxygenase 2 mRNA expression.[34]

In addition to its hypoglycemic effect, *N. sativa* has been reported to have lipid-lowering properties in normal rats,[12,13,35] STZ-induced diabetic rats,[36] and patients with type 2 diabetes.[37] Buriro and Tayyab reported that *N. sativa* seed oil (30 mg/kg/day) administered for 24 weeks to normal rats fed a high-fat diet resulted in reductions in triglyceride, total cholesterol and LDL-C levels, and increases in HDL-C levels.[38] The mechanisms underlying the antilipidemic effect of *N. sativa* involve its antioxidant activity and suppression of hepatic HMG-CoA reductase, which is a key enzyme in cholesterol synthesis.[39] The present findings show that *N. sativa* lowers only LDL-C levels in normal rats and has no beneficial effects on the lipid profile in diabetic rats. We propose that the effects of this plant on lipid and lipoprotein metabolism may be influenced by the dose and duration of the treatment.

Diabetes mellitus is an inflammatory disease associated with enhanced production of pro-inflammatory cytokines including TNF-α, which is a principal mediator in endothelial apoptosis.[6] Jain *et al.* delineated a significant increase in serum TNF-α levels over a period of 7 weeks in rats with STZ-induced diabetes (65 mg/kg), demonstrating increases in blood glucose levels of up to 600 mg/dL and in HbA_{1c} levels of greater than 15%.[4] However, according to Kalantarinia *et al.*, TNF-α is undetectable in the sera of STZ rats after 21 days of diabetic induction, whereas the levels in renal interstitial fluid are elevated as early as day 5.[40] Similarly, in the present study, neither the untreated diabetic rats nor those treated with *N. sativa* extract displayed any increases in serum TNF-α levels or evidence of endothelial apoptosis in the dermal capillaries. Therefore, the duration and degree of the glycemic imbalance are likely factors determining the magnitude and/or onset of increases in the levels of serum TNF-α. Notably, histologic examination of apoptosis by H and E staining cannot detect early apoptotic changes that occur prior to the morphological changes characteristic of apoptotic cells,[23] which is a limitation of this study. Therefore, further quantitative assessments of endothelial cell apoptosis are warranted.

Metabolic abnormalities in diabetes also cause impaired turnover of the vascular wall, leading to abnormal vascular remodeling and subsequently, thickened capillary walls, which is a characteristic feature of diabetic microangiopathy. Thickening of the capillary basement membrane can result in progressive occlusion of capillaries and subsequent ischemic injury due to luminal narrowing, such as that observed in patients with diabetic foot ulcer.[41] In animal experimental models, basement membrane thickening of glomerular and retinal capillaries can be observed after 4 and 6 months of diabetic induction, respectively.[42,43]

A study involving transmission electron microscopic examination of capillaries in the derma of diabetic rats after 21 days of STZ injection indicated that these capillaries undergo ultrastructural changes, including endothelial cell swelling, narrowing of the capillary lumen, basement membrane thickening, and fusion of mitochondrial cristae.[44]

Using the PAS technique, this is the first study to quantitatively demonstrate a significant increase in capillary wall thickness together with a decrease in the luminal capillary diameter in diabetic rat tail skin within 8 weeks of diabetes induction. Daily oral supplementation of the rats with *N. sativa* extract throughout the study period increased the luminal size to a value comparable to that of the normal controls and tended to decrease the capillary wall thickness. These findings may partly explain the results of a previous study demonstrating improvements in altered renal hemodynamics in response to *N. sativa* administration in diabetic rats.[45] The present study revealed that *N. sativa* lowered the mean HbA_{1c} level by 1.4%, which exceeds the necessary reduction previously described by the United Kingdom Prospective Diabetes Study to decrease the risk of diabetic microangiopathy.[7] Therefore, this amelioration of dermal capillary basement membrane thickening in diabetic rats treated with *N. sativa* extract is suggested to be a consequence of its glycemic control activity. Although *N. sativa* did not cause any significant reductions in the lipid profile or serum TNF-α level in diabetic rats, its potential antilipidemic and anti-inflammatory activities cannot be excluded. An evaluation of various dosages of *N. sativa* and an experimental period longer than 8 weeks may be required to verify these activities.

Further investigations are suggested to explore the effects of *N. sativa* on basement membrane thickness and the luminal diameters of dermal capillaries via other mechanisms involving transforming growth factor-beta, fibronectin, and oxidative stress.

CONCLUSION

The development of diabetic microangiopathy is known to be related to chronic hyperglycemia, dyslipidemia, oxidative stress, and increased TNF-α production. In this study, 8 weeks of oral administration of *N. sativa* extract to STZ-induced diabetic rats reduced the HbA_{1c} concentration by >1%, enlarged the capillary lumens, and tended to attenuate dermal capillary basement membrane thickening, without ameliorating the lipid profile or serum TNF-α level. Our results indicate that *N. sativa* may be used to minimize the risk of cutaneous diabetic microangiopathy, possibly at least partly due to its glycemic control activity.

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Conflicts of interest

There are no conflicts of interest.

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