Effects of *Nigella sativa* L. seed oil on intima–media thickness and Bax and Caspase 3 expression in diabetic rat aorta

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Abstract

Objective: Hyperglycaemia is an important risk factor for the development and progression of the macrovascular and microvascular complications that occur in diabetes. The expression of apoptotic markers in the aortic medial layer of diabetic rats and the effects of N. sativa L. seed oil on the expression of these markers were investigated in this study.

Methods: Four-month-old adult female Wistar rats (n=21) were divided into 3 groups: Group 1, control; Group 2, diabetes and Group 3, diabetes+*N. sativa* L. seed oil. Group 3 received 0.2 mg/kg/day *N. sativa* L. seed (black cumin) oil intraperitoneally 6 days per week for 30 days. At the end of the experiment, abdominal and thoracic aortas of all animals were collected and fixed in 10% formalin solution. Then, 5-µm-thick sections were stained with Verhoeff–Van Gieson stain to evaluate Bax and Caspase 3 expression. Tunica intima–media thickness was measured using the stained sections.

Results: There were no significant differences in abdominal or thoracic aortic intima–media thickness among the 3 groups. However, there were significant differences in Bax and Caspase 3 expression in the tunica media of the thoracic and abdominal aortas between Group 1 and Group 2 ($p<0.05$) and between Group 2 and Group 3 ($p<0.05$) evaluated with the Kruskal–Wallis and Mann–Whitney U tests.

Conclusion: It is understood that *N. sativa* L. seed oil is effective against diabetes. *N. sativa* L. seed oil is a plant material and has value for further investigation to develop diabetes treatment strategies for preventing apoptosis in vascular structures. *(Anatol J Cardiol 2016; 16: 460-6)*

Keywords: diabetes, aorta, *Nigella sativa* L. seed oil, Bax, Caspase 3

Introduction

Systemic complications are the most important causes of morbidity and mortality in diabetes. Hyperglycaemia has an important role in the development and progression of vascular complications that occur in diabetes. These complications can be divided into 2 groups (1): macrovascular complications such as coronary artery disease, atherosclerosis and peripheral vascular disease and microvascular complications such as retinopathy, nephropathy and neuropathy (2).

Hyperglycaemia plays a role in the vascular complications of diabetes due to the direct effects of glucose and other sugars on proteins, including glycation and non-enzymatic glycosylation. The complex rearrangements and oxidative reactions that occur in glycation give rise to multiple reactive species known as advanced glycation end products (AGEs) (3), which accumulate in the vessel wall collagen and basement membrane, causing cross-linking of proteins, and show a variety of biological activities in vivo with ageing and glycaemia (4).

Elevated blood glucose levels result in increased AGEs production (5). AGEs are formed not only by the direct effects of sugars on proteins but also by various oxidative reactions (6). AGEs can form covalent bonds between molecules with collagen (7). Lumen narrowing is the most important feature of diabetic vessels and can occur as a result of subendothelial accumulation of plasma proteins such as albumin, low-density lipoprotein (LDL) and immunoglobulin G (IgG). These molecules can become lodged in the basement membrane by cross-linking of AGEs and collagen (4). The main causes of atherosclerosis are hyperlipidaemia and hypercholesterolaemia. Especially, LDL, very low-density lipoprotein and lipoprotein (a) are responsible for atherogenicity (8). It was reported previously that LDL particles are highly prone to oxidation in diabetes. Oxidised LDL particles contribute to the formation of atherosclerotic plaque by delivering cholesterol to macrophages (9). Previous reports have indicated increases in tunica intima–media thickness in both experimental diabetes (10) and in diabetes in human studies (11-13).

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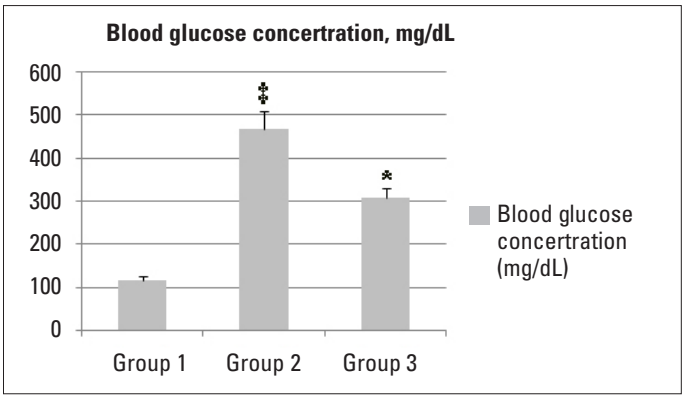

Figure 1. Comparison of blood glucose levels among the groups (mean±SE),
(‡ *P*<0.05 vs. Group 1; * *P*<0.05 vs. Group 2)

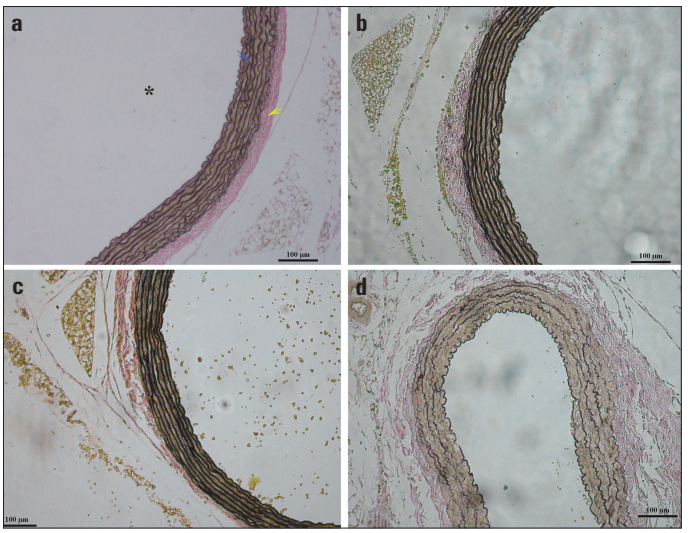

Figure 2. a-d. Representative Verhoeff–Van Gieson staining of abdominal aortic sections. (a) Control group, (b) Diabetes group, (c) Diabetes group, (d) Diabetes+Nigella sativa oil group. The blue arrow shows the tunica media, the long black lines indicate elastic laminae and the yellow arrow shows the tunica adventitia layer of the aorta. The asterisk (*) shows the lumen of the aorta

Two or more insulin injections daily must be given to control glycaemia in type 1 diabetes mellitus (14). It has been reported that insulin protects vascular smooth muscle cells from apoptosis in vitro (15) and that decreased insulin activity increases smooth muscle cell death in the arteries and can cause the formation of unstable plaque associated with diabetes (16). Diabetes has also been shown to cause apoptosis of vascular smooth muscle cells (17).

Apoptosis involves morphological and biochemical changes which appear to be triggered by the activation of a class of death proteases (caspases) (18). Among caspases; caspase-3 is a generally activated protease and has ability to cleavage numerous cellular proteins by catalyzing. Caspase-3 has a role in apoptotic chromatin condensation and DNA fragmentation in many cell types (19). Bcl-2 family proteins have effects on mitochondrial membrane and Bax is one of them and creates a channel which cytochrome c released from (20). The elevated oxidative stress seen in diabetic patients is associated with decreased cellular antioxidant defences (21, 22). The oxidative stress-dependent cellular changes in diabetic patients can be decreased by antioxidant supplementation (23, 24).

Medicinal plants are used to treat diabetes all around the world (25). *Nigella sativa* has been reported to exhibit antitumour, anti-cancer (26, 27), anti-fungal (28), anti-microbial (29) and anti-diabetic effects (23, 30). The biological activities of *N. sativa* seeds are due to thymoquinone, which is the main component of its essential oil (31).

Thymoquinone has been shown to ameliorate hypercholesterolaemic atherosclerosis, and this effect is associated with a decrease in serum lipid levels and reduction of oxidative stress (32). In rabbits fed a significantly cholesterol-enriched diet, addition of thymoquinone to the diet reduced elevated LDL-cholesterol (LDL-C) levels and thymoquinone was shown to repair early atherosclerotic lesions formed due to a diet high in cholesterol (33).

The present study was performed to investigate the expression of Caspase 3 and Bax in the aortic medial layer of diabetic rats and the effects of N. sativa L. seed oil on the expression of these markers. Tunica intima–media thickness was also measured.

Methods

The study protocol was approved by the Ethics Committee of Necmettin Erbakan University, Experimental Medicine, Research and Application Center, Konya, Turkey (2014-036).

Animals

Four-month-old adult female Wistar rats (n=21) weighing 250–300 g were used in this study in Necmettin Erbakan University. During the study, the rats were housed at a maximum of 5 per cage under conditions of 30%–40% relative humidity with a 12:12-h light/dark cycle at 20°C with food and water available ad libitum.

Streptozotocin (STZ; Sigma Aldrich Chemicals, St. Louis, MO) was used to induce experimental diabetes. STZ was dissolved in sodium citrate buffer (pH 4.5) and injected intraperitoneally (i.p.) in a single dose of 50 mg/kg into rats in the diabetes and diabetes+*N. sativa* L. seed (Origo) oil groups. *N. sativa* seed oil was obtained by cold pressing. Three days after STZ injection, blood glucose levels were measured using a glucometer (eBsensor; Visgeneer, Hsinchu, Taiwan), and animals with blood glucose levels of >270 mg/dL were considered as having diabetes (34).

The experimental animals were divided into 3 groups (n=7 per group).

Group 1 (control group): 0.2 mg/kg/day physiological saline i.p. 6 days per week for 30 days.

Group 2 (diabetes group): 3 days after STZ injection, 0.2 mg/kg/day physiological saline was injected i.p. 6 days per week for 30 days.

Group 3 (diabetes+*N. sativa* L. seed oil group): 3 days after STZ injection, 0.2 mg/kg/day i.p. *N. sativa* oil was injected 6 days per week for 30 days (19).

Table 1. Tunica intima–media thickness (mean±standard deviation)

	Group 1	Group 2	Group 3	*P*
AAIM	90.37±5.89	91.96±10.96	90.72±9.38	0.94
TAIM	94.11±6.54	96.09±7.7	93.26±3.82	0.67

AAIM - abdominal aortic intima–media thickness; TAIM - thoracic aortic intima–media thickness (*P*>0.05).

Table 2. Statistical values of abdominal aorta Bax staining (AA Bax)

Grup	n	Median	25%	75%	
Group 1	7	0	0	1	*P*<0.05
Group 2	7	2	2	2.25	
Group 3	7	1	1	1	

A significant difference in the Bax variable was detected between Group 1 and Group 2, between Group 1 and Group 3 and between Group 2 and Group 3 (0.002*, 0.032* and 0.01*, respectively).

Table 3. Statistical values of thoracic aorta Bax staining (TA-Bax)

Grup	n	Median	25%	75%	
Group 1	7	0	0	0	*P*<0.05
Group 2	7	2	2	3	
Group 3	7	1	1	1	

A significant difference in the Bax variable was detected between Group 1 and Group 2, between Group 1 and Group 3 and between Group 2 and Group 3 (0.001*, 0.01* and 0.003*, respectively).

Table 4. Statistical values of abdominal aorta Caspase 3 staining (AA-Cas3)

Grup	n	Median	25%	75%	
Group 1	7	0	0	1	*P*<0.05
Group 2	7	2	2	3	
Group 3	7	1	0	1	

A significant difference in the Caspase 3 variable was detected between Group 1 and Group 2 and between Group 2 and Group 3 (0.001* and 0.001*, respectively).

Table 5. Statistical values of thoracic aorta Caspase 3 staining (TA-Cas3)

Grup	n	Median	25%	75%	
Group 1	7	0	0	1	*P*<0.05
Group 2	7	2	2	3	
Group 3	7	1	0	1	

A significant difference in the Caspase 3 variable was detected between Group 1 and Group 2 and between Group 2 and Group 3 (0.001* and 0.001*, respectively).

Histology

The animals were euthanised with a ketamine and xylazine combination at doses of 50/10 mg/kg, and the thoracic and abdominal aortas of the rats in each group were removed and preserved in 10% formalin.

Paraffin sections were cut at a thickness of 5 µm and stained with Verhoeff–Van Gieson stain. The histological structure of the vessels was examined in these sections. Under light microscopy, the aortic tunica intima and media layers were measured together to determine tunica intima–media thickness. Intervals were measured commencing from the lumen tissue border and ending at the last elastic lamina of the adventitia border. The values were averaged over 10 measurements for each vessel. All sections were evaluated using a Nikon H5505 light microscope and DS-FI2 analysis system.

Immunohistochemistry

The sections were incubated with antibodies against Bax and Caspase 3 to examine apoptosis and divided into 4 groups according to the staining pattern (34).

0: No staining
+1: Weak staining
+2: Moderate staining
+3: Strong staining.

Statistical analysis

Aortic tunica intima–media thickness and blood glucose levels were compared by one-way analysis of variance with Tukey's test. The results of immunohistochemical analyses were compared using the Kruskal–Wallis test. Differences between pairs of groups were assessed using the Mann–Whitney U test (GraphPad Prism 6 Demo).

Results

N. sativa L. seed oil significantly decreased blood glucose levels (Fig. 1) (p<0.05). The control group showed normal histological structures. The tunica intima had a uniform structure beginning with the endothelium. Elastic fibres were organised into concentric lamellae, and circular smooth muscle cells were established between these lamellae (Fig. 2a).

Regular elastic laminae were observed in the diabetes group (Fig. 2b). Also, irregularities were observed in the elastic laminae of the vessel wall in 3 rats in the diabetes group (Fig. 2c). *N. sativa* L. seed oil ameliorated the elastic laminae of the tunica media (Fig. 2d).

There were no significant differences in abdominal and thoracic aortic intima–media thickness (AAIM and TAIM, respectively) between the groups (one-way analysis of variance with Tukey's test, p>0.05) (Table 1).

There were significant differences between the groups in terms of immunohistochemical staining as indicated by the Kruskal–Wallis test (Tables 2–5). Weak Bax (Fig. 3a) expression was observed in the control group and intense Bax expression was observed in the diabetic group (Fig. 3b) (Kruskal–Wallis test and Mann–Whitney U test, p<0.05). *N. sativa* L. seed oil significantly reduced Bax expression (Fig. 3c) in Group 3 compared with Group 2 (p<0.05) in the abdominal and thoracic aortic sections.

Caspase 3 expression was weak in the control group (Fig. 4a), although increased expression (Fig. 4b) was observed in the smooth muscle cells of the tunica media in the vessel wall (Kruskal–Wallis test and Mann–Whitney U test, p<0.05).

Caspase 3 expression were significantly reduced in Group 3 by the effect of *N. sativa* L. seed oil (Fig. 4c) (p<0.05) in the abdominal and thoracic aortic sections.

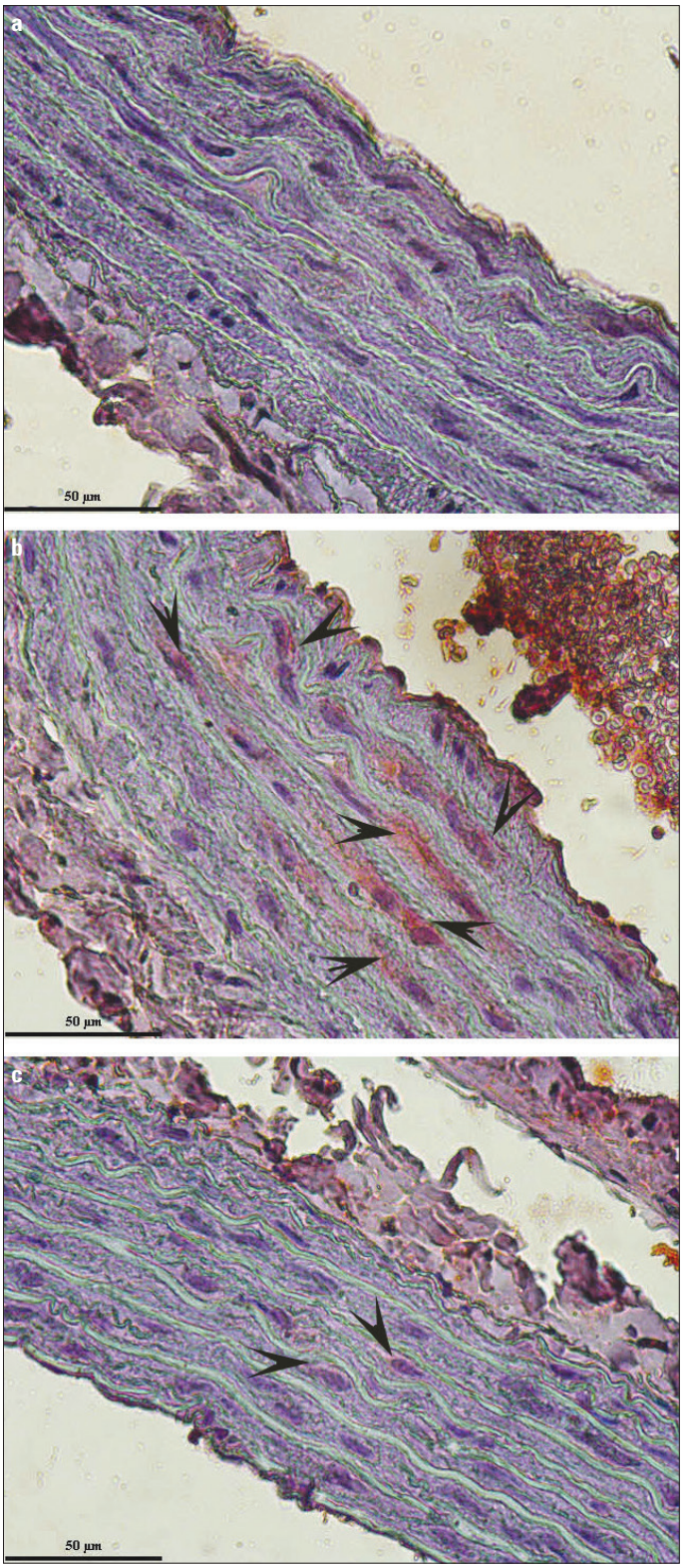

Figure 3. a–c. Representative staining for Bax in thoracic aortic sections. (a) Control group, (b) Diabetes group, (c) Diabetes+*Nigella sativa* oil group

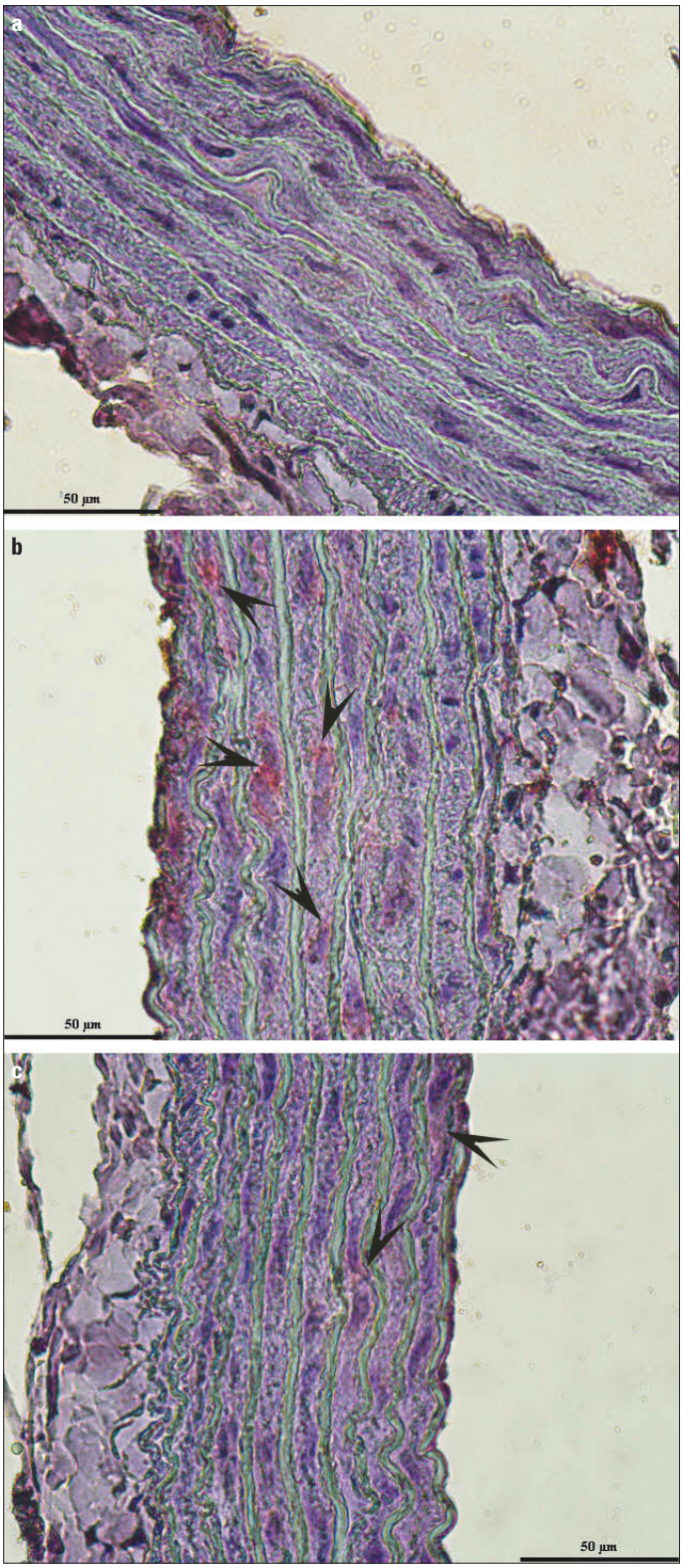

Figure 4. a–c. Representative staining for Caspase 3 in thoracic aortic sections. (a) Control group, (b) Diabetes group, (c) Diabetes+*Nigella sativa* oil group

Discussion

In our study, there were no significant differences in abdominal or thoracic aortic intima–media thickness among the 3 groups. However, there were significant differences in Bax and Caspase 3 expression in the tunica media of the thoracic and abdominal aortas between Group 1 and Group 2 ($p<0.05$) and between Group 2 and Group 3 ($p<0.05$).

There were no significant differences in tunica intima–media thickness among the groups in the present study. Consistent with these findings, a previous study indicated no significant difference in tunica intima–media thickness in 7-month-old obese male rats that had been hyperglycaemic for 5 months compared with controls (35). However, it was reported that the tunica media thickness was increased in male rats 6.5 months after induction of diabetes (36). In another study, diabetes for 10 weeks was reported to induce an increase in tunica media thickness in male rats (37). In contrast, 6.5-month-old male diabetic rats showed no increase in tunica media thickness after 60 days (10). Evaluation of intima–media thickness showed no significant differences between non-diabetic and diabetic groups in 3-month-old female rats (34). In our previous study, we found no significant increase over 60 days in tunica media or tunica intima thickness in female diabetic rats. These discrepancies in the literature were likely due to differences in the age and sex of the rats between the studies.

Cell death can contribute to changes in vascular proliferation and vascular disease with cell growth, migration and matrix transfer (38). Hyperglycaemia has been shown to induce apoptosis in many vascular cells, which is intimately involved in the initiation of diabetic pathology (39).

Hyperglycaemia increases the formation of free radicals in several ways. The occurrence of auto-oxidation in diabetes indicates that the capacity of glucose to enolise, resulting in the formation of an intermediate oxidising agent by reducing molecular oxygen. The reduced oxygen products formed in the auto-oxidation reaction are superoxide anion (O_2–), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2). The resulting free radical products damage lipids and proteins by crosslinking and fragmentation. Free radicals also accelerate the production of AGEs, which result in the formation of even more free radicals (40). H_2O_2 induces apoptosis in vascular smooth muscle cells, and insulin has an anti-apoptotic effect mediated by the Akt signalling pathway (41). AGEs and free radicals formed in diabetes have been reported to induce apoptosis (16).

AGEs increase apoptosis in retinal pericytes and impair retinal microvascular homeostasis (42). It has also been reported that AGEs impair glomerular homeostasis by increasing the apoptosis of glomerular mesangial cells and significantly increase the accumulation of Bax protein (43). In a previous study, the number of TUNEL-positive cells increased significantly in the tunica media of diabetic rat aorta, and continuing hyperglycaemia caused a reduction in smooth muscle cell content (35). It was reported that diabetes for 4 weeks increases apoptotic cell death in the tunica media of rat aorta. Severe and permanent hyperglycaemia can cause severe damage to the aortic wall (17).

In a previous in vitro study, addition of the cytokines IFN-γ, TNF-α and IL-1-ß to the culture medium was shown to induce Caspase 3 expression by translocation of Bax from the cytosol to the mitochondrial fraction, release of cytochrome C from the mitochondria and induction of apoptosis in aortic smooth muscle cells. Insulin blocks these changes and shows anti-apoptotic effects (16). In the present study, significantly increased Bax and Caspase 3 levels were seen in the smooth muscle cells of the tunica media layer of diabetic rats, indicating that hyperglycaemia triggers apoptosis in smooth muscle cells.

Kaleem et al. (44) have reported that the *N. sativa* seed extract has anti-diabetic activity and may be useful in controlling the complications of diabetes due to its antioxidant effects in experimental diabetic rats. In the present study, the levels of apoptotic markers were significantly decreased by *N. sativa* L. seed oil in the diabetes group.

Study limitations

At the end of 30 days of diabetes induction, there were no significant increases in AAIM and TAIM. Multiple parts of the aorta cannot be evaluated from the same animal, which is the major limitation of the present study. The anti-diabetic properties of *N. sativa* have been proven in the literature; therefore, Group 4 (only *N. sativa* L. seed oil) was not formed in the present study. However, studies examining the aorta in diabetes are very limited.

Conclusion

Further studies with larger numbers of tissue samples and histological sections from the aorta will clarify the discrepancies in the literature. It is understood that *N. sativa* L. seed oil has is effective against diabetes. *N. sativa* L. seed oil is a plant material and has value for further investigation to develop diabetes treatment strategies for preventing apoptosis in vascular structures.

Conflict of interest: None declared.

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