Simvastatin ameliorates vascular endothelial growth factor overexpression in a rat model of diabetic retinopathy: a histological and immunohistochemical study

Eman M. Sadek, Lamiaa I. Abd-El Fattah, Zeinab M. El Maadawi, Manal A. Abd-El Mohsen

Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence to Manal A. Abd-El Mohsen, MD, Department of Histology, Faculty of Medicine, Cairo University, Egypt;
Tel: +20 128 172 6043;
e-mail: dr.manal14@hotmail.com

Received 13 June 2016
Accepted 27 November 2016

Kasr Al Ainy Medical Journal
2017, 23:24–31

Background and objectives
Diabetic retinopathy (DR) is one of the main causes of vision loss. Treatment options during its early stages are not available. This study was designed to evaluate the potential protective effects of simvastatin on experimentally induced DR, with special emphasis on its possible modulatory effect on vascular endothelial growth factor (VEGF).
Materials and methods
Thirty adult male albino rats were divided equally into three groups: group I (the control group), group II (the diabetic group), and group III (diabetic/simvastatin-treated group). Diabetes was induced by intraperitoneal injection of streptozotocin at a dose of 50 mg/kg/rat in 20 adult male albino rats. Simvastatin was administered orally as 20 mg/kg/rat 48 h after streptozotocin injection. Blood glucose levels and body weight were measured. Retinal specimens were processed for hematoxylin and eosin staining and VEGF immunohistochemistry. Morphometric analysis included measurement of retinal thickness and area percentage of VEGF. All results were statistically analyzed using the Student *t*-test and analysis of variance test.
Results
The untreated group showed histological features of DR and increased VEGF immunoreactivity compared with controls. The simvastatin-treated group showed improved features of DR and decreased VEGF immunoreactivity.
Conclusion
These results suggest that simvastatin has protective effects against DR by eliminating VEGF overexpression and might be considered a promising therapeutic agent for it.

Keywords:
diabetic retinopathy, simvastatin, statins, streptozotocin, vascular endothelial growth factor

Kasr Al Ainy Med J 23:24–31

1687-4625

Introduction

Diabetic retinopathy (DR) remains a leading cause of vision loss, despite advances in diabetes care. The burden of DR is likely to increase as the evolving pandemic of type II diabetes progresses [1]. DR is usually classified into nonproliferative diabetic retinopathy and proliferative diabetic retinopathy [2].

Diabetic macular edema is the leading cause of blindness in diabetic patients. It involves the breakdown of the blood–retinal barrier, with increased vascular permeability [3,4]. In proliferative diabetic retinopathy, angiogenic-mediated response induced by vascular endothelial growth factor (VEGF) results in retinal neovascularization, which is followed by severe complications such as vitreous hemorrhage or tractional retinal detachment [5,6]. Current treatments for DR are indicated for advanced DR and have significant adverse effects. Therefore, new pharmacological treatments for the early stages of DR [1] and for prophylaxis against DR are needed.

Statin drugs, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are highly effective at reducing high blood cholesterol levels in patients at risk for hyperlipidemia [7]. It was revealed that statins have effects beyond their lipid-lowering effects, collectively known as pleiotropic effects [8].

The present study was designed to detect the potential protective effect of simvastatin on experimentally induced DR in adult male albino rats with special

 DOI: 10.4103/1687-4625.207186

reference to its possible modulatory effects on VEGF, monitored by histological, immunohistochemical, and morphometric studies.

Materials and methods

Materials

Drugs

(1) Simvastatin (Zocor) was supplied by Global Napi Pharmaceutical (2nd Industrial Zone, No.40, Street No.18, 6th of October City, Giza) (Egypt; under license from Merk & Co. Inc., Whitehouse Station, New Jersey, USA) in the form of 10 mg tablets.
(2) Streptozotocin (STZ) was purchased from Sigma Company (St Louis, Missouri, USA) in powder form as 1 g vial.

Animals

This study included 30 adult male albino rats, 180–200 (184±1.35) g body weight. They were randomly divided into three groups of 10 rats each. They were housed in hygienic stainless steel cages and kept in a clean well-ventilated room. They were fed standard chow diet and allowed free access to water. All procedures were carried out according to the guidelines of the Animal Ethical Committee of Kasr Al-Ainy Faculty of Medicine, Cairo University.

Group I (control group): It included 10 rats that received an equivalent amount of citrate buffer only by intraperitoneal injection. Thereafter, they received distilled water orally every day in milliliters equivalent to simvastatin. They were killed along with groups II and III after 12 weeks of receiving distilled water.

Group II: Diabetes was experimentally induced in 10 rats. They then received distilled water orally daily in milliliters equivalent to simvastatin. They were killed after 12 weeks of receiving distilled water.

Group III: Diabetes was experimentally induced in 10 rats. They then received simvastatin orally daily. They were killed 12 weeks after starting the administration of simvastatin.

Methods

Induction of diabetes

Diabetes was induced in rats by means of a single intraperitoneal injection of STZ freshly dissolved in citrate buffer (0.1 M, pH 4.5) at a dose of 50 mg/kg body weight [9]. Rats with blood glucose concentrations greater than and equal to 250 mg/dl, measured 48 h after STZ injection, were considered diabetic and included in this study. Diabetic rats received subcutaneous insulin (0–4 U) (Humulin-N; Eli Lilly & Co., Indianapolis, Indiana, USA) twice a week to maintain body weight and maximize survival rate [10,11]. For rats in all groups, blood glucose levels and body weight were measured weekly.

Adminstration of simvastatin

Simvastatin was dissolved in distilled water and 0.5 ml of it was given by means of gastric gavage at a dose of 20 mg/kg/rat starting 48 h after injection of STZ and was continued every day for 12 weeks (group III) [12].

Laboratory investigations

Blood samples were taken from the retro-orbital veins of the left eye and random blood glucose was measured (48 h after STZ injection) in all rats to select the rats who proved to be diabetic. It was also measured weekly in all rats at the Biochemistry Department (Kasr Al-Ainy Faculty of Medicine).

Histological study

Tissue specimens (right eyeballs) dissected from all rats were fixed in 10% buffered formalin solution for 24–48 h, dehydrated in ascending grades of ethanol, and embedded in paraffin. Serial sections of 7 μm thickness were cut and subjected to the following stains:

(1) Hematoxylin and eosin (H&E) stain to evaluate morphological changes in DR and possible effect of simvastatin [13].
(2) Immunohistochemical staining [14] for VEGF to detect its retinal expression as it plays a key role in the onset and development of DR [15]. It was supplied as prediluted ready-to-use rabbit polyclonal antibody (RB-222-R7; Lab Vision Corporation Laboratories, Fremont, California, USA).

Histostain SP kit (LAB_SA System, 95-9643; Zymed Laboratories Inc., San Francisco, California, USA) was used as the detection system. Mayer's hematoxylin was used for counterstaining. Negative control was included in which primary antibody was omitted.

Morphometric and statistical analysis

Morphometric study was carried out using Leica Qwin 500 LTD image analyzer (Leica, Cambridge, UK). All measurements were taken in five sections from both H&E and VEGF immunostained sections from each animal. Retinal thickness was measured in H&E-stained sections using the distance parameters, in

interactive measurements menu, where 10 random nonoverlapping fields were examined using ×10 objective lens. The mean area percentage of VEGF immunoreactivity was measured in 10 random nonoverlapping fields per section using binary mode with ×40 objective lens.

All measurements were expressed as mean±SD and significant differences between groups were evaluated using the Student's *t*-test and analysis of variance test in which *P*-value of less than 0.05 was considered statistically significant [16].

Results

Measurements of blood glucose levels in the studied groups

Before STZ injection (at baseline), the mean blood glucose levels in groups II and III revealed no statistically significant difference when compared with the control value (81.7±3.89). The values were 80.65±2.04 and 80.28±2.86 for groups II and III, respectively (Table 1).

At 4 weeks, the mean blood glucose levels in groups II and III showed significant increase ($P<0.05$) when compared with the control value (79.97±1.88). The values recorded were 313.07±7.16 and 314.57±7.39 for groups II and III, respectively.

At 8 weeks, the mean blood glucose levels in groups II and III showed significant increase ($P<0.05$) when compared with the control value (81.16±3.61). The values reported were 320.22±9.56 and 319.48±12.29 for groups II and III, respectively.

At 12 weeks, the mean blood glucose levels in groups II and III represented significant increase ($P<0.05$) when compared with the control value (79.54±2.27). The values detected were 325.69±9.77 and 327.23±10.00 for groups II and III, respectively.

Measurements of body weight in the studied groups

Statistical analysis revealed significant reduction ($P<0.05$) in body weight measurements in all groups when compared with the corresponding control values at 4, 8, and 12 weeks (Table 2).

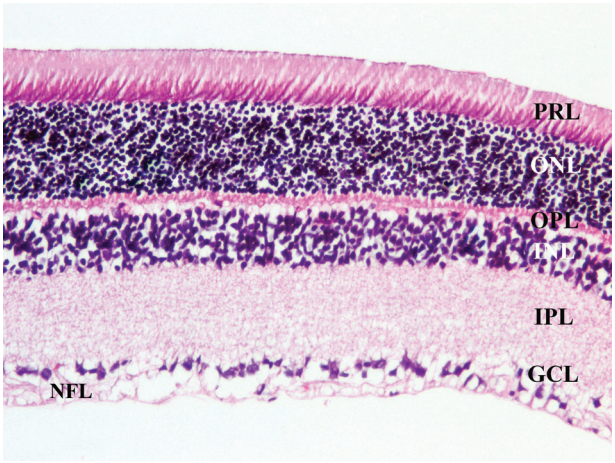
The values reported were 231.2±9.98 for control group at 4 weeks, whereas groups II and III reported 160.8±7.19 and 162.6±7.07, respectively. At 8 weeks the values demonstrated were 183.2±8.44 and 181±6.27 for groups II and III, respectively, and 271.5±12.7 for control. At 12 weeks the control group showed 320.6± 7.75, whereas values for groups II and III were 233.8±8.09 and 230.3±10.58, respectively.

The mean body weight measurements of groups II and group III showed no significant difference between them at 4, 8, and 12 weeks.

Histological results

Sections of the retina of the control group exhibited normal histological architecture, demonstrating well-organized retinal layers formed of the photoreceptor layer, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, and nerve fiber layer (Fig. 1).

Figure 1



Group I showing normal histological structure of well-organized retinal layers formed of photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL) (retina). Hematoxylin and eosin, ×200.

Table 1 Comparison between the mean values of blood glucose levels (mg/dl±SD) in the studied groups

Weeks	Group I	Group II	Group III
Baseline	81.70±3.89	80.65±2.04	80.28±2.86
4 weeks	79.97±1.88	313.07±7.16*	314.57±7.39*
8 weeks	81.16±3.61	320.22±9.56*	319.48±12.29*
12 weeks	79.54±2.27	325.69±9.77*	327.23±10.00*

*Significantly different from the corresponding value of the control group at $P<0.05$.

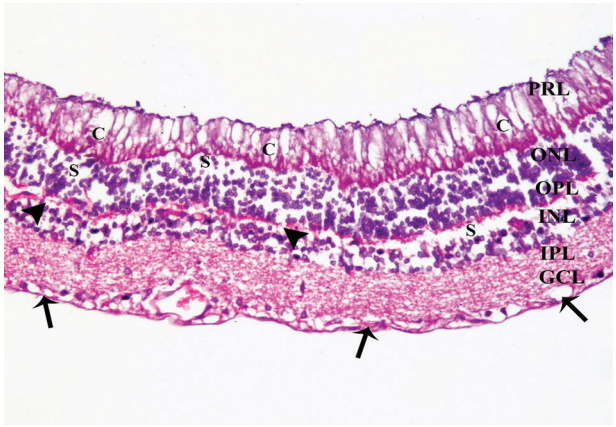
Table 2 Comparison between the mean values of body weight measurements (g±SD) in the studied groups

Weeks	Group I	Group II	Group III
4 weeks	231.2±9.98	160.8±7.19*	162.6±7.07*
8 weeks	271.5±12.7	183.2±8.44*	181.0±6.27*
12 weeks	320.6±7.75	233.8±8.09*	230.3±10.58*

*Significantly different from the corresponding value of the control group at $P<0.05$.

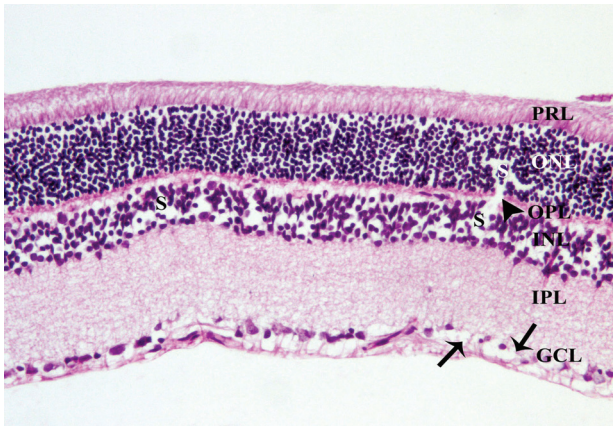
Sections of retina in group II (untreated) showed marked histological alterations of DR. Apparent marked reduction of retinal thickness and marked disorganization of retinal layers were detected. Sections also exhibited multiple empty spaces between the nuclei of the outer and inner nuclear layers. Multiple cavities within the photoreceptor layer were detected. Disruption of the outer plexiform layer was also demonstrated. Wide clear areas around ganglion cell nuclei were seen (Fig. 2).

Figure 2



Group II (untreated) showing apparent marked reduction of retinal thickness as compared with the control group, and marked disorganization of retinal layers with the appearance of empty spaces between the nuclei of outer nuclear layer (ONL) and inner nuclear layer (INL) (S). Multiple cavities within photoreceptor layer (PRL) are also detected (C). Disruption of outer plexiform layer (OPL) is seen (arrowheads). Clear areas (arrows) within ganglion cell layer (GCL) can be detected (retina). Hematoxylin and eosin, ×200. IPL, inner plexiform layer.

Figure 3



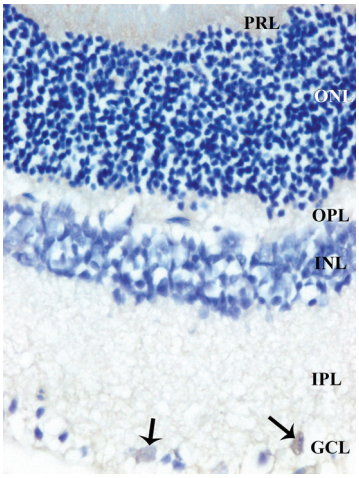
Group III (simvastatin treated) showing mild reduction of retinal thickness, with slight disorganization of retinal layers and the appearance of small empty spaces between the nuclei of outer nuclear layer (ONL) and inner nuclear layer (INL) (S). Focal areas of disruption of outer plexiform layer (OPL) could be observed (arrowhead). Some clear areas (arrows) within ganglion cell layer (GCL) can be detected (retina). Hematoxylin and eosin, ×200. IPL, inner plexiform layer; PRL, photoreceptor layer.

Examination of sections in the retina of group III (simvastatin treated) showed mildly reduced retinal thickness with mild disorganization of retinal layers. Small empty spaces between the nuclei of the outer and inner nuclear layers were observed. Disruption of the outer plexiform layer at certain points was also seen. Some clear areas within the ganglion cell layer were also detected (Fig. 3).

Vascular endothelial growth factor immunohistochemical results

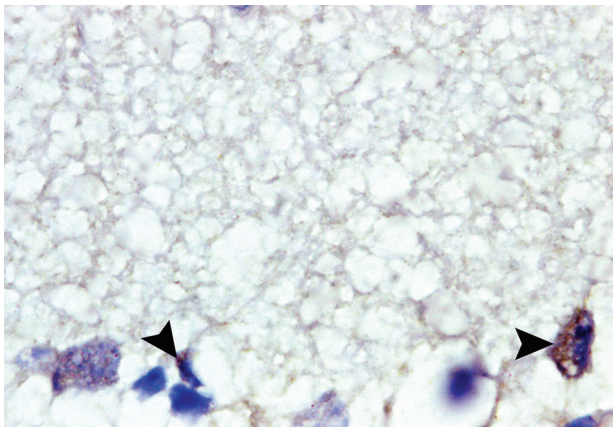
Retinal sections of group I (control group) revealed negative immunoreactivity for VEGF in almost all

Figure 4



Group I showing negative immunoreaction for vascular endothelial growth factor (VEGF) in almost all retinal layers. Some positive immunoreaction for VEGF (arrows) can be detected in scarce cells of ganglion cell layer (GCL). Anti-VEGF immunostaining, ×400. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; ONL, outer plexiform layer; PRL, photoreceptor layer.

Figure 5



Higher magnification of Fig. 4 showing positive immunoreaction for vascular endothelial growth factor (VEGF) (arrowheads) in cells of ganglion cell layer (GCL). The reaction appears as brown cytoplasmic deposits. Anti-VEGF immunostaining, ×1000.

retinal layers (Fig. 4). However, detection of some positive immunoreaction for VEGF was noted in a few cells in the ganglion cell layer (Figs 4 and 5).

Sections of the retina of group II showed markedly positive VEGF immunoreactivity in the photoreceptor layer. Many cells of the outer nuclear layer and inner nuclear layer exhibited positive immunoreactivity for VEGF (Fig. 6). Positive VEGF immunoreactivity in most cells of the ganglion cell layer was also demonstrated (Figs 6 and 7).

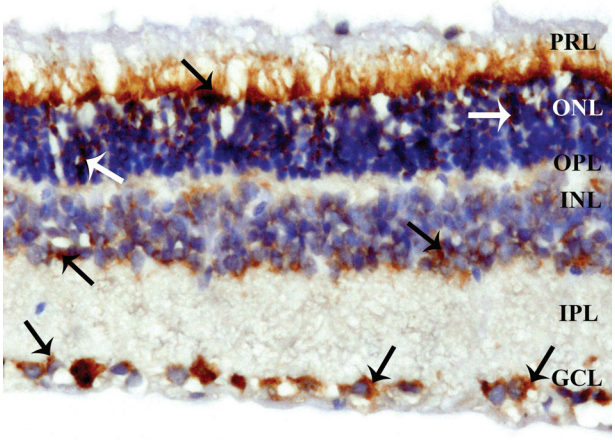
Retinal sections of group III showed positive immunoreaction for VEGF in the photoreceptor layer (Fig. 8). Some cells of the outer nuclear layer and some cells of the inner nuclear layer exhibited positive immunoreactivity for VEGF (Figs 8 and 9). Positive immunoreactivity in many cells of the ganglion cell layer was also detected (Figs 8 and 9). Apparent decrease in VEGF immunoreactivity in relation to group II was noticed.

Morphometric results

Mean retinal thickness

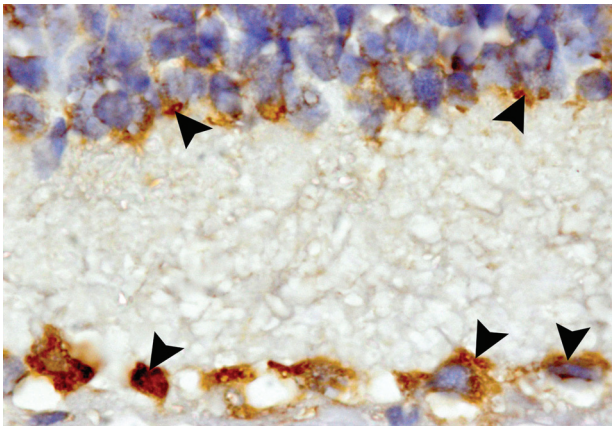
Thickness of the retina of groups II and III showed a statistically significant decrease ($P<0.05$) when compared with control values. The values reported for

Figure 6



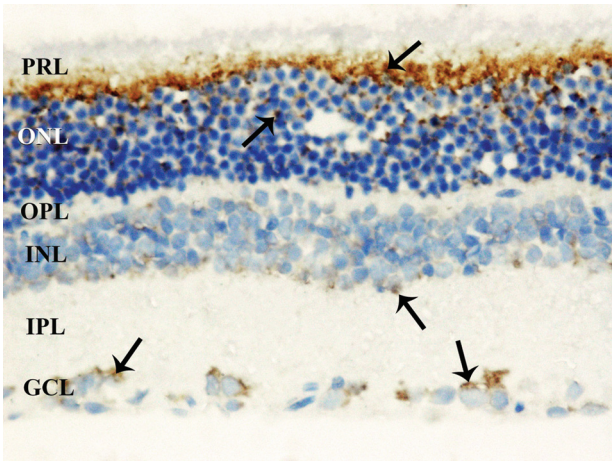
Group II (untreated) showing markedly positive immunoreaction for vascular endothelial growth factor (VEGF) (arrows) in photoreceptor layer (PRL), in many cells of outer nuclear layer (ONL) and inner nuclear layer (INL), and in most cells of ganglion cell layer (GCL). Anti-VEGF immunostaining, ×400. IPL, inner plexiform layer.

Figure 7



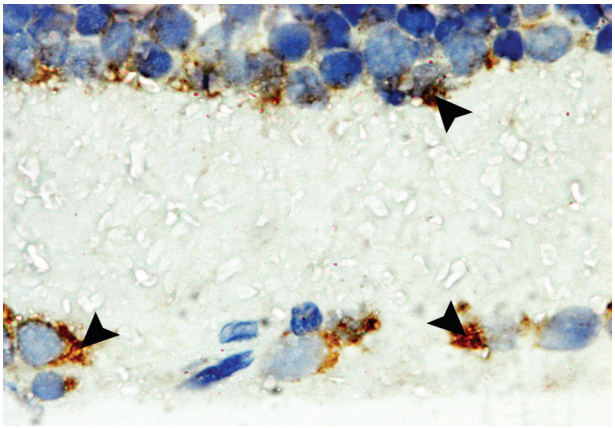
Higher magnification of Fig. 6 showing positive cytoplasmic immunoreaction for vascular endothelial growth factor (VEGF) (arrowheads) in cells of inner nuclear layer (INL) and ganglion cell layer (GCL). Anti-VEGF immunostaining, ×1000.

Figure 8



Group III (simvastatin treated) showing positive immunoreaction for vascular endothelial growth factor (VEGF) (arrows) detected in photoreceptor layer (PRL), some cells of outer nuclear layer (ONL) and inner nuclear layer (INL), and many cells of ganglion cell layer (GCL). Note the apparent decrease in VEGF positive immunoreactivity in relation to group II. Anti-VEGF immunostaining, ×400. IPL, inner plexiform layer.

Figure 9



Higher magnification of Fig. 8 showing the positive cytoplasmic immunoreaction for vascular endothelial growth factor (VEGF) in cells of inner nuclear layer (INL) and ganglion cell layer (GCL) (arrowheads). Anti VEGF immunostaining, ×1000.

groups II and III were 91.99±10.49 and 198.60±8.78, respectively, whereas the control value was 233.52 ±19.88. A statistically significant reduction in group II was noticed when compared with group III (Table 3).

Mean area percentage of vascular endothelial growth factor immunoreactivity

The mean area% of VEGF positive immunoreactivity in groups II and III showed statistically significant increase ($P<0.05$) when compared with controls. Controls reported a value of 0.37±0.11. The values recorded for groups II and III were 6.84±1.91 and 3.70 ±1.23, respectively. Statistically significant increase ($P<0.05$) was reported in group II when compared with group III (Table 3).

Discussion

The present study was designed to evaluate the potential protective effect of simvastatin on a rat model of DR with special consideration of its possible ameliorating effect on VEGF.

In the present work, histological changes of DR detected 12 weeks after induction of diabetes were in the form of apparent reduction of retinal thickness and disorganized retinal layers with the appearance of empty spaces between the nuclei of the outer and inner nuclear layers. Disruption of outer and inner plexiform layers was also demonstrated.

Reduced retinal thickness was confirmed by morphometric measurements, which revealed statistically significant reduction in the diabetic nontreated group compared with the control group. This was in line with the results of previous studies [17,18].

The previous findings can be explained by progressive loss of retinal neurons leading to reduction in retinal thickness. Similarly, Barber *et al.* [19] postulated that diabetes is implicated in apoptosis of neural and vascular cells in the retina. In addition, it was suggested that scaffolding of the retina may be compromised, leading to disruption and disorganization of retinal layers [20].

Table 3 Mean retinal thickness (μm±SD) and mean area percent of vascular endothelial growth factor immunoreactivity in the studied groups

Groups	Retinal thickness (mean±SD)	VEGF immunoreactivity (mean±SD)
Group I	233.52±19.88	0.37±0.11
Group II	91.99±10.49*,#	6.84±1.91*,#
Group III	198.60±8.78*	3.70±1.23*

VEGF, vascular endothelial growth factor. *Significantly different from the values of the control group at $P<0.05$. #Significantly different from the values of group III at $P<0.05$.

Apoptosis of retinal neurons, which are further phagocytosed by glial cells, may also contribute to the appearance of empty spaces [21]. In addition, retinal edema contributes to separation between retinal cells due to dysfunction of the blood–retinal barrier and accumulation of fluid in the inner and outer retinal plexiform layers [22].

Further histopathological findings were reported in the ganglion cell layer, such as clear spaces between ganglion cells. This may be explained by loss of retinal ganglion cells due to apoptotic death, followed by phagocytosis by glial cells. This was similar to what was proposed to explain the presence of empty spaces in both outer and inner nuclear layers.

In the current study, cavities within the photoreceptor layer were also detected, which coincides with the results of Hu *et al.* [23]. They mentioned that photoreceptor layer cavities were observed in histological examination in diabetic rats, attributing it to degenerative or apoptotic processes of the retina.

In the present work, detection of VEGF immunoexpression within the retina of the studied groups was also performed to further explore the process of vascular degenerative changes of diabetic retina. Although initially thought to be endothelial specific, VEGF has been shown to target a variety of nonvascular cells. VEGF is produced by retinal pigment epithelial cells, endothelial cells, ganglion cells, Müller cells, and smooth muscle cells. It is clear that the locally produced VEGF mediates neural protection. The absence of active vessel proliferation in the normal retina in the presence of endogenous VEGF may be due to the dose, localization, and/or the presence of opposing factors [24,25].

In the present study, VEGF was upregulated after induction of diabetes. VEGF expression in the control group was weak and localized to the ganglion cell layer, whereas in the diabetic untreated group it was more apparent in the ganglion cell layer, inner nuclear layer, and photoreceptor layer. The mean area percentage of VEGF positive immunoreactivity in the untreated diabetic group showed statistically significant increase when compared with control and simvastatin-treated diabetic groups.

These results are consistent with previous studies [15,26] that reported upregulation of VEGF expression in all retinal layers, particularly the inner nuclear layer and the ganglion cell layer, 4 weeks after STZ induction of diabetes. Furthermore, time-dependent increase in VEGF expression was

reported [27] where VEGF immunoreactivity in DR increased with prolongation of time. Meanwhile, it was reported that the retina of control rats expressed VEGF in the ganglion cell layer [28].

VEGF expression is regulated largely by hypoxia, but it also accumulates in the retina early in diabetes even before any retinal hypoxia is apparent [29]. Other causes, such as oxidative stress, may be implicated in stimulating VEGF release [6]. VEGF is an important mediator of increased vascular permeability in early DR [30]; hence retinal edema occurs.

In the present study, histopathological examination and morphometric analysis revealed that simvastatin was able to improve histopathological alterations of DR by preserving retinal thickness and organization of its different layers. The improvement noticed with simvastatin administration was in accordance with a previous study, which demonstrated that treatment with simvastatin [31] produced improvement in DR. It was mentioned that statins protected the retina against oxidative stress and the accelerated apoptosis of retinal capillary cells in diabetic rats [32].

Moreover, it was stated that simvastatin could be useful in the treatment of early DR by inhibiting vascular permeability in the diabetic retina [33]. In the current study, significant reduction in VEGF immuno-expression was noticed in the diabetic simvastatin-treated group in accordance with previous research [34]. This reduction in VEGF immunoexpression could be explained by the ability of simvastatin to reduce hyperglycemia-induced oxidative stress [35].

Therefore, the ability of simvastatin to improve DR could be referred to its ability to decrease VEGF expression leading to decreased vascular permeability and attenuated retinal edema. Having such protective effects, simvastatin might be used for prophylaxis or as a novel treatment option in the early stage of DR.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 Simo R, Hernandez C. Prevention and treatment of diabetic retinopathy: evidence from large, randomized trials. The emerging role of fenofibrate. Rev Recent Clin Trials 2012; 7:71–80.

2 Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. Lancet 2010; 376:124–136.

3 Midena E. Chapter 12: diabetes and retinal vascular disorders In: Vujosevic S, Midena E. Microperimetry and multimodal retinal imaging. 1st ed. Berlin, Heidelberg: Springer-Verlag; 2014. 119–128.

4 Yam JCS, Kwok AKH. Update on the treatment of diabetic retinopathy. Hong Kong Med J 2007; 13:46–60.

5 Barile GR, Pachydaki SI, Tari SR, Lee SE, Donmoyer CM, Ma W, *et al.* The RAGE axis in early diabetic retinopathy. Invest Ophthalmol Vis Sci 2005; 46:2916–2924.

6 Falcão M, Falcão-Reis F, Rocha-Sousa A. Diabetic retinopathy: understanding pathologic angiogenesis and exploring its treatment options. Open Circ Vasc J 2010; 3:30–42.

7 Dick M, Jonak P, Leask RL. Statin therapy influences endothelial cell morphology and F-actin cytoskeleton structure when exposed to static and laminar shear stress conditions. Life Sci 2013; 92:859–865.

8 Kostapanos MS, Milionis HJ, Elisaf MS. An overview of the extra-lipid effects of rosuvastatin. J Cardiovasc Pharmacol Ther 2008; 13:157–174.

9 Mansouri E, Panahi M, Ghaffari MA, Ghorbani A. Effects of grape seed proanthocyanidin extract on oxidative stress induced by diabetes in rat kidney. Iran Biomed J 2011; 15:100–106.

10 Navaratna D, McGuire PG, Menicucci G, Das A. Proteolytic degradation of VE-cadherin alters the blood-retinal barrier in diabetes. Diabetes 2007; 56:2380–2387.

11 Zheng Z, Chen H, Ke G, Fan Y, Zou H, Sun X, *et al.* Protective effect of perindopril on diabetic retinopathy is associated with decreased vascular endothelial growth factor to pigment epithelium-derived factor ratio. Involvement of a mitochondria-reactive oxygen species pathway. Diabetes 2009; 58:954–964.

12 Zhang W, Yan H. Simvastatin increases circulating endothelial progenitor cells and reduces the formation and progression of diabetic retinopathy in rats. Exp Eye Res 2012; 105:1–8.

13 Kiernan JA. Histological and histochemical methods: theory and practice. 3rd ed. London, New York, New Delhi: Arnold Publisher; 2001. pp. 111–162.

14 Bancroft JD, Gamble M. Theory and practice of histological techniques. 6th ed. China: Churchill Livingstone Elsevier; 2008. pp. 433–472.

15 Duan H, Huang J, Li W, Tang M. Protective effects of fufang xueshuantong on diabetic retinopathy in rats. Evid Based Complement Alternat Med. 2013; 2013:1–9.

16 Emsley R, Dunn G, White I. Mediation and moderation of treatment effects in randomized controlled trials of complex interventions. Stat Methods Med Res 2010; 19:237–270.

17 Zhang J, Wu Y, Jin Y, Ji F, Sinclair SH, Luo Y, *et al.* Intravitreal injection of erythropoietin protects both retinal vascular and neuronal cells in early diabetes. Invest Ophthalmol Vis Sci 2008; 49:732–742.

18 Saberi M, Gholami S. An investigation on the effects of the Aloe Vera extract on the thickness of the retina in male diabetic rats. Iran J Vet Res 2012; 13:296–302.

19 Barber AJ, Gardner TW, Abcouwer SF. The significance of vascular and neural apoptosis to the pathology of diabetic retinopathy. Invest Ophthalmol Vis Sci 2011; 52:1156–1163.

20 Smith SB, Duplantier J, Dun Y, Mysona B, Roon P, Martin PM, Ganapathy V. In vivo protection against retinal neurodegeneration by sigma receptor 1 ligand (+)-pentazocine. Invest Ophthalmol Vis Sci 2008; 49:4154–4161.

21 Marc RE, Jones BW, Watt CB, Vazquez-Chona F, Vaughan DK, Organisciak DT. Extreme retinal remodeling triggered by light damage: implications for age related macular degeneration. Mol Vis 2008; 14:782–806.

22 Zhang X, Zeng H, Bao S, Wang N, Gillies MC. Diabetic macular edema: new concepts in pathophysiology and treatment. Cell Biosci 2014; 4:27–40.

23 Hu CK, Lee YJ, Colitz CM, Chang CJ, Lin CT. The protective effects of *Lycium barbarum* and *Chrysanthemum morifolium* on diabetic retinopathies in rats. Vet Ophthalmol 2012; 15:65–71.

24 Saint-Geniez M, Maharaj AS, Walshe TE, Tucker BA, Sekiyama E, Kurihara T, *et al.* Endogenous VEGF is required for visual function: evidence for a survival role on Müller cells and photoreceptors. PLoS One 2008; 3:1–13.

25 Stewart MW. The expanding role of vascular endothelial growth factor inhibitors in ophthalmology. Mayo Clin Proc 2012; 87:77–88.

26 Du ZJ, Li P, Hui YN, Wang BR, Duan XL, Zhang R. Concomitant expression and combined localization of Ets-1 and VEGF in experimental diabetic rat's retina. Int J Ophthalmol 2009; 2:223–226.

27 Lu Y, Zhou N, Huang X, Cheng JW, Li FQ, Wei RL, Cai JP. Effect of intravitreal injection of bevacizumab-chitosan nanoparticles on retina of diabetic rats. Int J Ophthalmol 2014; 7:1–7.

28 Ueda H, Kashiwagi K, Iizuka Y. Vascular endothelial growth factor and its receptors expression in the rat eye. Acta Histochem Cytochem 2001; 34:329–335.

29 Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007; 2007:1–14.

30 Wu Y, Zuo Y, Chakrabarti R, Feng B, Chen S, Chakrabarti S. ERK5 contributes to VEGF alteration in diabetic retinopathy. J Ophthalmol 2010; 2010:1–11.

31 El-Azab MF, Mysona BA, El-Remessy AB. Statins for prevention of diabetic-related blindness: a new treatment option?. Expert Rev Ophthalmol 2011; 6:269–272.

32 Kowluru RA, Kanwar M. Translocation of H-Ras and its implications in the development of diabetic retinopathy. Biochem Biophys Res Commun 2009; 387:461–466.

33 Miyahara S, Kiryu J, Yamashiro K, Miyamoto K, Hirose F, Tamura H, *et al.* Simvastatin inhibits leukocyte accumulation and vascular permeability in the retinas of rats with streptozotocin-induced diabetes. Am J Pathol 2004; 164:1697–1706.

34 Lee SG, Kim JL, Lee HK, Ryu GW, Hur DY, Yun IH, *et al.* Simvastatin suppresses expression of angiogenic factors in the retinas of rats with streptozotocin-induced diabetes. Graefes Arch Clin Exp Ophthalmol 2011; 249:389–397.

35 Zheng Z, Chen H, Wang H, Ke B, Zheng B, Li Q, *et al.* Improvement of retinal vascular injury in diabetic rats by statins is associated with the inhibition of mitochondrial reactive oxygen species pathway mediated by peroxisome proliferator-activated receptor gamma coactivator 1 alpha. Diabetes 2010; 59:2315–2325.