

<b>Original Article</b>	<b>Protective effect of simvastatin on induced diabetic retinopathy and endothelial progenitor cells in adult male albino rat, histological and immunohistochemical study</b> <i>Eman M. Sadek, Lamiaa I. Abd El Fattah, Zeinab M. El Maadawi and Manal A. Abd-El Mohsen</i> <i>Department of Histology, Faculty of Medicine, Cairo University</i>
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### ABSTRACT

**Background and Objectives:** Diabetic retinopathy (DR) is one of the main causes of blindness and there is no available treatment for complete cure. This study was planned to evaluate the potential protective effects of simvastatin in a rat model of DR with special emphasis on endothelial progenitor cells (EPCs).

**Methods:** Thirty adult male albino rats were equally divided into: Group I (control group), Group II (diabetic group) and Group III (diabetic/simvastatin treated group). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) 50 mg/Kg /rat in 20 adult male albino rats. Simvastatin was administered as 20 mg/Kg/rat 48 hours after STZ injection via oral route. Body weight and blood glucose levels were measured weekly for 1 month. Flowcytometry was used to detect circulating endothelial progenitor cells (EPCs). Retinal specimens were processed for hematoxylin and eosin staining as well as CD31 and caspase 3 immunohistochemistry. Morphometric analysis included measurement of retinal thickness, number of ganglion cells, number of CD31 positive cells and area percent of caspase 3 positive immune-reaction. All results were statistically analyzed.

**Results:** Flowcytometry showed that simvastatin was able to increase the percentage of circulating EPCs in group III compared to group II. Group II showed histological features of DR, CD 31 immuno-reaction was comparable to control and caspase 3 immuno-reaction increased significantly compared to control. Group III showed improved features of DR, significant increase in CD 31 and decrease in caspase 3 immuno-reactivity.

**Conclusions:** These results suggest that simvastatin has protective effects on DR and might be considered as promising therapeutic agent for it.

**Key Words:** diabetic retinopathy - endothelial progenitor cells– statins - STZ

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### INTRODUCTION

Diabetic retinopathy (DR), as a prevalent complication of diabetes mellitus (DM), is a leading cause of reduced visual acuity and acquired blindness in working-age adult

population in both developed and developing nations (*Si et al., 2013*).

Diabetic retinopathy is classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) (*Tarr et*

*al., 2013*). Currently, NPDR is the focus of study and is also the best phase for drug intervention, because when it comes into PDR, the probability of blindness increases significantly and the possibility of recovery reduces greatly (*Cavallerano et al., 2012 and Gardner et al., 2011*).

Patients with DR usually receive combinations of treatment options including systemic control of blood glucose and blood pressure, surgery, laser photocoagulation and intravitreal injection of steroid or anti-vascular endothelial growth factor agents (*Jo et al., 2010 and Antonetti et al., 2012*). Although laser therapy has shown partial therapeutic effect on DR, the current treatments for DR are far from satisfactory (*Si et al., 2013*).

The reduction and dysfunction of circulating blood endothelial progenitor cells (EPCs) has been extensively reported in both type I and type II diabetic patients (*Liu et al., 2010*). Specifically in the retina, correction of this dysfunction could treat early and intermediate stages of vasodegeneration to enhance vessel repair, reverse ischemia and prevent progression to the late stages of DR. Interestingly, EPCs might also participate in pathological neovascularization (*Bhatwadekar et al., 2010*).

Simvastatin is a hypolipidemic drug that belongs to the class of pharmaceuticals called statins. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis (*Hassan et al., 2011*). Statins have been described extensively for the treatment of hyperlipidemia since 1987. However, their pleiotropic properties beyond cholesterol reduction have gained increasing attention over the past decade (*Ko et al., 2011*).

The present study was designed to detect the potential protective effect of simvastatin on experimentally induced DR in adult male albino rats as well as its possible effect on EPCs; monitored by flowcytometry, histological, immunohistochemical and morphometric studies.

## MATERIAL AND METHODS

### A-Material

#### a) Drugs:

- Simvastatin (ZOCOR) was supplied by Global Napi Pharmaceutical – Egypt under license from: Merk & Co.Inc., Whitehouse Station, N.J., USA in the form of 10 mg tablets.

- Streptozotocin (STZ) was purchased from Sigma Company (St. Louis, Mo, USA) in a powder form as 1 gm vial.

#### b) Animals:

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

Group I (control group): It included 10 rats which received equivalent amount of citrate buffer only by intraperitoneal injection. Then they received distilled water orally everyday in milliliters equivalent to simvastatin. They were sacrificed with groups II & III (4 weeks after receiving distilled water).

#### Experimental Group:

Diabetes was experimentally induced in 20 rats at the same time. Diabetic rats were further divided into:

Group II (diabetic group): It included 10 diabetic rats that received distilled water orally daily in milliliters equivalent to simvastatin. They were sacrificed after 4 weeks of receiving distilled water.

Group III (diabetic/simvastatin treated group): It included 10 diabetic rats which received simvastatin orally daily. They were sacrificed 4 weeks after administration of simvastatin.

## ***B-Methods***

### ***1) Induction of diabetes:***

Diabetes was induced in rats by a single intraperitoneal injection of Streptozotocin (STZ), freshly dissolved in citrate buffer (0.1 M, pH 4.5), at a dose of 50 mg/kg body weight (Mansouri *et al.*, 2011). After injection, animals had free access to food and water. The animals were allowed to drink 5% glucose solution overnight to overcome hypoglycaemic shock, as STZ can induce fatal hypoglycemia as a result of massive pancreatic insulin release (Ramachandran *et al.*, 2011 and Ghosal and Mandal, 2013).

STZ is a chemical substance specifically toxic to pancreatic  $\beta$ -cells. When injected into adult rats, STZ causes type I DM with severely elevated blood glucose level (Kulkarni *et al.*, 2012).

Rats with blood glucose concentrations  $\geq 250$  mg/dl, 48 h after STZ injection, were considered diabetic and included in this study. Diabetic rats received subcutaneous insulin (0-4 units) (Humulin-N; Eli Lilly & Co., Indianapolis, IN) twice a week to maintain body weight and maximize survival rate. Insulin was given based on the stability of body weight. The tolerated maximum difference in body weight between diabetic and nondiabetic rats was 40% (Navaratna *et al.*, 2007 and Zheng *et al.*, 2009).

For rats in all groups, body weight and blood glucose levels were measured weekly.

### ***2) Administration of simvastatin:***

Simvastatin was received via gastric gavage after being dissolved in distilled water for each rat of group III at a dose of 20 mg/kg body weight per rat where it was first administered 48h after injection of STZ and was continued everyday for 4 weeks (Zhang and Yan, 2012).

### ***3) Laboratory investigations:***

Blood samples were collected from the retro orbital vein of the left eye and random blood glucose level was measured for all rats to select the rats proved to be diabetic after STZ injection. Moreover, blood glucose level was measured weekly for all rats and the measurements were

done at the Biochemistry Department, Kasr Al Ainy Faculty of Medicine, Cairo University.

Before scarification of rats, the percentage of circulating blood EPCs was quantified by flowcytometry (Coulter Epics xl-BECKMAN coulter) at Flowcytometry Unit, Clinical Pathology Department, Kasr Al Ainy hospital where two cellular markers were detected as follows (Yoder *et al.*, 2007):

- CD34 antibody that detects stem cells of several lineages.
- CD133 antibody which primarily recognizes immature EPCs.

### ***4) Histological study:***

The specimens (right eyeballs) dissected from all rats were fixed in 10% buffered formalin solution for 24-48 hours, dehydrated in ascending grades of ethanol and embedded in paraffin. Serial sections of 7  $\mu$ m thickness were cut and subjected to the following stains:

a) Hematoxylin & eosin (H&E) stain to evaluate morphological changes in DR and the possible effects of simvastatin (Kiernan, 2001).

b) Immunohistochemical staining (Bancroft and Gamble, 2008) using the following primary antibodies:

- CD31 as an endothelial progenitor cell marker (Tian *et al.*, 2010). It was supplied as concentrated rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, sc-1506-R) and it was used at dilution 1:300.

- Caspase 3 to detect apoptosis (Yüksel *et al.*, 2014). It was supplied as pre-diluted ready-to-use rabbit polyclonal antibody (Lab Vision Corporation Laboratories, Fremont, CA, USA, RB-1197-R7).

Histostain SP kit (LAB\_SA system, Zymed Laboratories Inc, SF, USA, 95-9643) was used as detection system. Mayer's haematoxylin was used for counter staining of nuclei while positive immunoreaction appears as brown cytoplasmic deposits. Negative control was included in which primary antibody was omitted.

### 5) Morphometric & Statistical analysis:

Morphometric study was done using Leica Qwin 500 LTD image analyzer (Cambridge UK). All measurements were done in five stained sections per stain obtained from each animal. Retinal thickness and mean number of ganglion cells were measured in H & E stained sections using the distance and count parameters respectively, in the interactive measurements menu. From each section, 10 random non overlapping fields were examined using x10 objective lens. Mean number of CD31 immunopositive cells was detected in 10 random non overlapping fields per CD 31 immunostained section using x10 objective lens. Mean area percent of caspase 3 was measured in 10 random non overlapping fields per section using the binary mode with x40 objective lens.

All measurements were expressed as mean  $\pm$  standard deviation and significant differences between groups were evaluated using Student T test and Analysis of Variance test (ANOVA) in which P value of less than 0.05 was considered to be statistically significant (Emsley *et al.*, 2010).

## RESULTS

No loss of rats was incurred due to death throughout the experiment.

I- Measurements of Blood Glucose Levels in the Studied Groups (Table1)

Statistical analysis revealed no significant difference in the mean blood glucose values between all groups before STZ injection (at base line). The mean values were  $81.7 \pm 3.89$ ,  $81.52 \pm 3.00$  and  $79.64 \pm 2.22$  for control and groups II and III respectively.

At 4 weeks, the mean values of blood glucose levels for all experimental groups showed significant increase ( $P < 0.05$ ) when compared to the control value ( $79.97 \pm 1.88$ ). The values recorded were  $314.27 \pm 10.04$  and  $315.02 \pm 5.08$  for groups II and III, respectively.

II- Measurements of Body Weights in the Studied Groups (Table 1)

Statistical analysis revealed significant reduction ( $P < 0.05$ ) in body weight measurements in experimental groups when compared to the control at 4 weeks. The values reported were  $231.2 \pm 9.98$ ,  $156 \pm 5.16$  and  $157 \pm 8.23$  for control, groups II and III, respectively.

III- Measurements of Circulating Blood EPCs Levels in the Studied Groups at the Time of Scarification (Table 2)

The mean percentage of circulating blood EPCs levels was significantly decreased in groups II and III when compared to control group. The detected values were  $0.22 \pm 0.042$ ,  $0.06 \pm 0.028$  and  $0.16 \pm 0.026$  for control group and groups II and III respectively. Meanwhile, the mean percentage of circulating blood EPCs levels was significantly increased in group III when compared to group II ( $P < 0.05$ ).

### IV- Histological Results

Examination of H&E stained sections of rat retina of the control group exhibited normal histological architecture, demonstrating well organized retinal layers formed of the photoreceptor layer appeared as an outer lightly stained part (outer segment) and inner deeply stained part (inner segment). The outer nuclear layer exhibited darkly stained nuclei arranged into multiple regular rows of photoreceptors nuclei. The outer plexiform layer formed fibrillar acidophilic band. The inner nuclear layer appeared thinner with larger and paler nuclei than those of outer nuclear layer. The inner plexiform layer also appeared reticular but thicker than outer plexiform layer. The ganglion cell layer showed larger paler nuclei than those of other nuclear layers representing ganglion cells (Fig.1A).

Retinal sections of group II revealed histopathological features of diabetic retinopathy. Disorganized retinal layers with the appearance of multiple empty spaces inbetween the nuclei of outer and inner nuclear layers. Cavities within photoreceptor layer were detected along with disruption of outer plexiform layer. Retinal sections also exhibited apparently reduced number of ganglion cells nuclei with shrunken

darkly stained nuclei with the appearance of clear areas inbetween. Dilated congested blood vessel in outer plexiform layer was also detected (Fig. 1B).

Sections in the retina of group III showed apparently normal histological structure comparable to control. More organized retinal layers, few small empty spaces inbetween nuclei of inner nuclear layer were observed. Cells in ganglion cell layer were more densely packed and not widely separated as compared to group II (Fig.1C). Occasional spindle shaped nuclei could be detected within ganglion cell layer in some sections (Fig.1C).

#### ***V-Immunohistochemical results***

##### ***-CD31 immunostaining***

Retinal sections of control group showed CD 31 immuno-positive cells in inner nuclear layer and ganglion cell layer. CD31 positive immunoreactivity in blood vessel wall was also detected in ganglion cell layer (Figs.2A&2B). Sections of group II were comparable to that of control regarding the distribution of CD 31 immunopositive cells (Figs.2C&2D). Retinal sections of simvastatin treated group (group III) demonstrated many CD 31 immuno-positive cells within inner nuclear layer, inner plexiform layer and ganglion cell layer (Figs. 2E&2F). Cells with spindle shaped nuclei could be observed in inner plexiform layer and ganglion cell layer (Figs. 2E&2F).

##### ***-Caspase 3 immunostaining***

Examination of retinal sections of control group revealed negative caspase 3 immunoreactivity in all retinal layers (Fig. 3A). Retinal sections of group II showed positive cytoplasmic immunoreactivity for caspase 3 in many cells of the outer nuclear layer, inner nuclear layer and ganglion cell layer (Figs. 3B&3C). Sections of group III demonstrated positive cytoplasmic caspase 3 immuno

reactivity in some cells of the inner nuclear layer and ganglion cell layer. The immunoreactivity was apparently decreased as compared to group II (Figs. 3D&3E).

#### ***VI- Morphometric results***

##### **a-Mean Retinal Thickness: (Table 3)**

A statistically significant decrease ( $P < 0.05$ ) in retinal thickness was recorded in group II as compared to that of control and group III. The values were  $233.52 \pm 19.88$ ,  $118.37 \pm 13.90$  and  $219.52 \pm 15.10$  for control, group II and group III respectively. No statistically significant difference was noticed between control and group III.

##### **b- Mean number of Ganglion Cells: (Table 3)**

A statistically significant reduction ( $P < 0.05$ ) in the mean number of ganglion cells in group II when compared to that of control and group III was detected. The reported values were  $19.3 \pm 1.86$ ,  $11.3 \pm 2.41$  and  $17.6 \pm 1.43$  for control, group II and group III respectively. No statistically significant difference was noticed between control and group III.

##### **c- Mean Number of CD 31 Immuno-Positive Cells: (Table 3)**

Mean number of CD 31 immuno-positive cells of group III ( $4.3 \pm 1.16$ ) showed a significant increase ( $P < 0.05$ ) when compared to control ( $2.0 \pm 0.47$ ) and group II ( $2.2 \pm 0.63$ ). There was no significant difference between control group and group II.

##### **d-Mean Area Percent of Caspase 3 Immunoreactivity: (Table 3)**

Statistical significant increase in the mean area percent of caspase 3 immunoreaction ( $P < 0.05$ ) was detected in group II ( $1.54 \pm 0.40$ ) when compared to group III ( $0.62 \pm 0.27$ ). Sections of control group revealed negative caspase 3 immunoreactivity in all retinal layers and were excluded from statistical analysis.

**Table 1:** Mean blood glucose level and Mean body weight in control and experimental groups.

	Group I	Group II	Group III
Mean blood glucose level at baseline (mg/dl)	81.7± 3.89	81.52± 3.00	79.64± 2.22
Mean blood glucose level at 4 weeks (mg/dl)	79.97±1.88	314.27±10.04*	315.02±5.08*
Mean body weight at 4 weeks (gm)	231.2±9.98	156±5.16*	157±8.23*

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

**Table 2:** Mean percentage of circulating blood EPCs.

Groups	Mean percentage of circulating blood EPCs
Group I	0.22±0.042
Group II	0.06± 0.028*
Group III	0.16± 0.026*#

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

# Significantly different from the corresponding value of group II at  $P < 0.05$ .

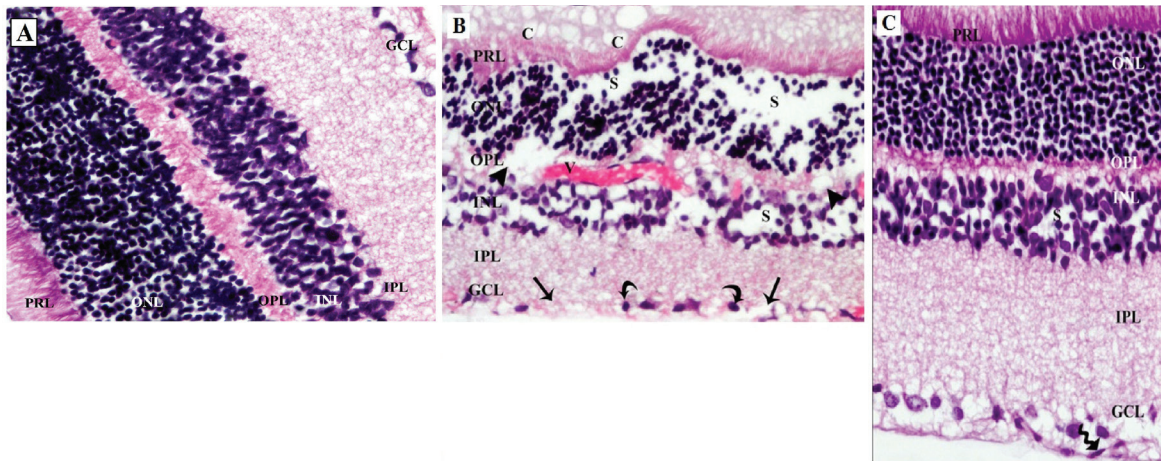
**Table 3:** Mean retinal thickness, mean number of ganglion cells, mean number of CD31 immuno-positive cells and mean area percent of caspase 3 in control and experimental groups

	Group I	Group II	Group III
Mean retinal thickness (µm)	233.52± 19.88	118.37±13.90*•	219.52±15.10
Mean number of ganglion cells	19.3±1.86	11.3± 2.41*•	17.6±1.43
Mean number of CD31 immuno-positive cells	2.0± 0.47	2.2±0.63	4.3± 1.16*
Mean area percent of caspase 3	0.00±0	1.54± 0.40•	0.62± 0.27

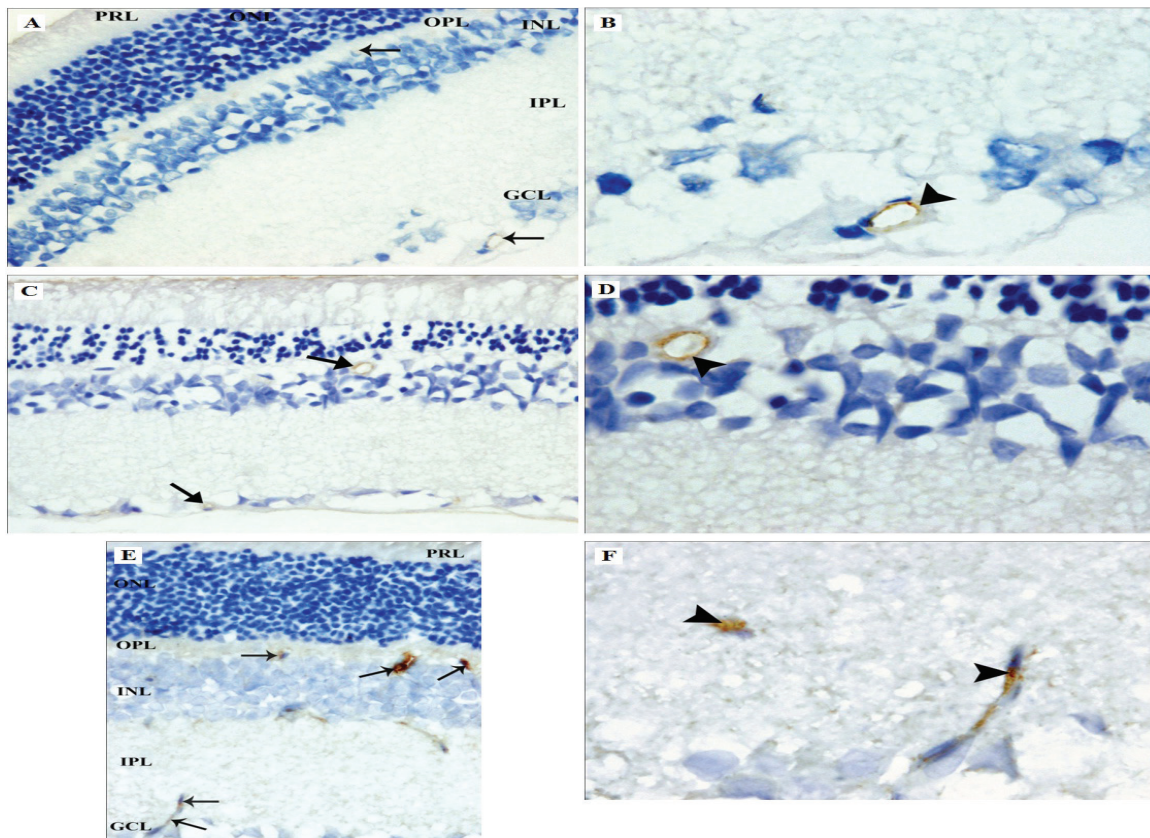
\* Significantly different from the corresponding value of the control group at  $P < 0.05$ .

• Significantly different from the value of group III at  $P < 0.05$ .

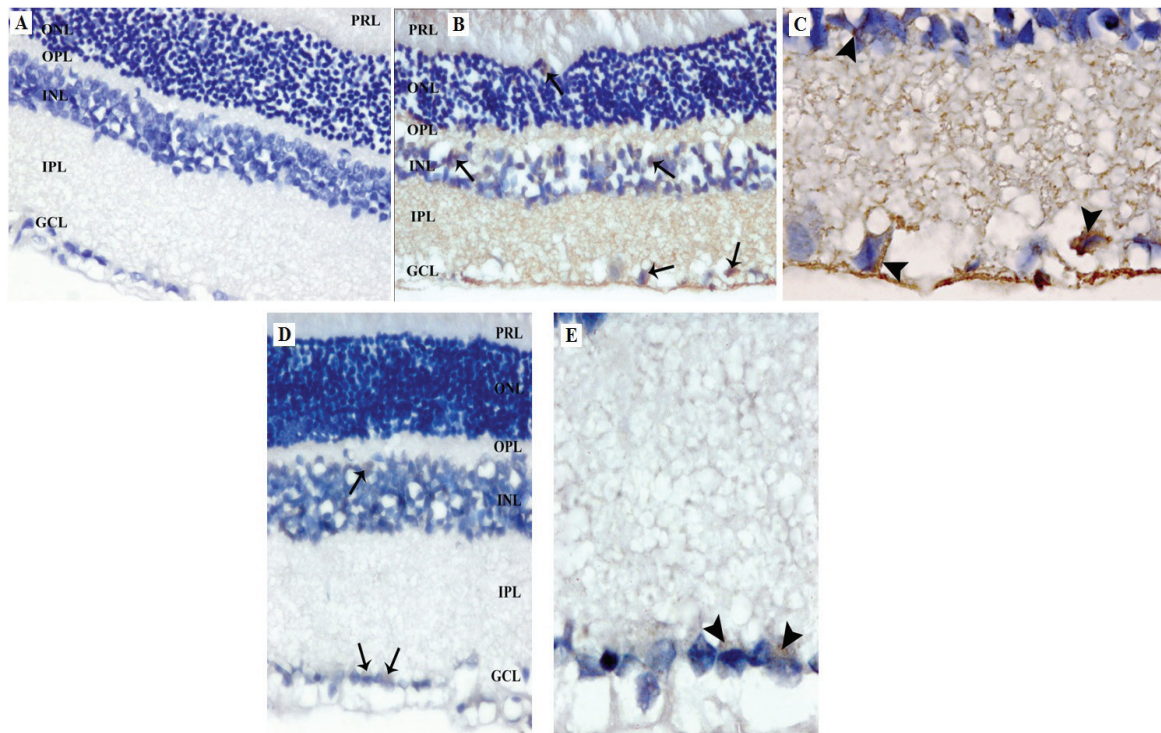




**Fig. 1:** (Retina, H & E, x400) (A) Group I (control group) showing well organized photoreceptor layer PRL. Outer nuclear layer (ONL) formed of small darkly stained nuclei. Outer plexiform layer (OPL) is forming a fibrillar acidophilic band. Inner nuclear layer (INL) consists of larger paler nuclei than that of ONL. Inner plexiform layer (IPL) is formed of thick acidophilic band. Ganglion cell layer (GCL) is formed of continuous layer of larger paler nuclei. (B) Group II (untreated), showing multiple cavities (C) within PRL, empty spaces (S) inbetween nuclei of ONL and INL, disruption (arrowheads) of OPL, and dilated congested vessel (V) in OPL. Apparently reduced number of ganglion cells with shrunken darkly-stained nuclei (curved arrows) can be observed with clear areas (arrows) inbetween. (C) Group III (treated), showing apparently normal histological architecture with few small empty spaces (S) inbetween nuclei of INL and closely separated cells in GCL as compared to group II. Spindle shaped nucleus (wavy arrow) is detected within GCL.



**Fig. 2:** (Retina, Anti CD 31 immunostaining) (A) Group I (control group) showing positive immune reaction for CD 31 (arrows) in INL and GCL. (x400) (B) Higher magnification showing positive brown cytoplasmic immune reaction for CD 31 (arrowhead) in blood vessel wall in GCL. (x1000) (C) Group II (untreated), showing CD 31 immuno-positive cells (arrows) in INL and GCL apparently comparable to control. (x400) (D) Higher magnification showing positive cytoplasmic CD 31 immune reaction (arrow head) in INL. (x1000) (E) Group III (treated), showing multiple CD 31 immuno-positive cells (arrows) in INL, IPL and GCL. Spindle shaped nuclei can be observed in GCL and IPL. (x400) (F) Higher magnification showing positive CD 31 cytoplasmic immune reaction (arrow heads) for in IPL and GCL. (x1000)



**Fig. 3:** (Retina, Anti caspase 3 immunostaining) (A) Group I (control group) showing negative immune reaction for caspase 3 in all retinal layers. (x400) (B) Group II (untreated), showing positive immune reaction for caspase 3 (arrows) in some cells of ONL, multiple cells of INL and the remaining cells of GCL. (x400) (C) Higher magnification showing positive caspase 3 cytoplasmic immune reaction (arrow heads) in INL and GCL. (x1000) (D) Group III (treated) showing positive caspase 3 immune reaction (arrows) in INL and GCL with apparent decrease in caspase 3 immunoreactivity compared to group II.(x400) (E) Higher magnification showing positive caspase 3 cytoplasmic immune reaction (arrow heads) in GCL. (x1000)

## DISCUSSION

The present study was designed to evaluate the potential protective effect of simvastatin on experimentally induced diabetic retinopathy (DR) in adult male albino rats as well as its possible effect on endothelial progenitor cells (EPCs). Therefore, the percentage of circulating EPCs was detected using flowcytometry with CD34 and CD133 double-labeling according to previous reports (Liu *et al.*, 2010, Lara-Hernandez *et al.*, 2010 and Li *et al.*, 2012). The mean percentage of circulating blood EPCs was found to significantly decrease 4 weeks after induction of diabetes when compared to control group, a finding that is in agreement with Zhang and Yan (2013) and Westerweel *et al.* (2013).

The initial reduction in circulating EPCs in DR can be attributed to different mechanisms (Busik *et al.*, 2009; Kang *et al.*, 2009; Avogaro *et al.*, 2011 and Westerweel *et al.*, 2013). Explanations would include consumptive loss of EPCs due to increased endothelial damage

with decreased cell proliferation and shortened survival. Also, defective EPCs mobilization might occur in response to ischaemia with disturbed bone marrow microenvironment.

In the current work, significant increase in the mean percentage of circulating blood EPCs was detected in the group that received simvastatin compared to the untreated group which correlates with previous studies (Walter *et al.*, 2002 and Wenzel *et al.*, 2008). This finding can be attributed to the capability of statins to mobilize and differentiate EPCs from the bone marrow (Dimmeler *et al.*, 2001; Hirstov *et al.*, 2003 and Yu and Feng, 2008). Moreover, statins are found to increase the adhesion of EPCs (Wenzel *et al.*, 2008) and it was reported that EPCs in diabetics are characterized by reduction of their adhesiveness (Tepper *et al.*, 2002).

The molecular mechanisms that control the modulatory effect of simvastatin on circulating EPCs have been vigorously explained. First, among the several kinds of statins, simvastatin



has been proven to be one of the most effective reagents to regulate the Notch signaling pathway which plays a key role in differentiation of multiple cell lineages (Xu *et al.*, 2009).

Second, it was demonstrated (Spyridopoulos *et al.*, 2004) that statins have a protective effect on telomere biology. Statins induce expression of a telomere repeat-binding factor 2 (TRF2) in cultured EPCs. Removal of TRF2 triggers apoptosis or senescence. Moreover, statins enhanced migratory activity of EPCs, depending in part on the induction of TRF2.

Statins might also reduce the consumptive loss of EPCs through preserving retinal vasculature. This hypothesis was based on the findings of Al-Shabrawey *et al.* (2013) who stated that hyperglycemia-induced oxidative stress and pro-inflammatory pathways lead to apoptosis of pericytes and endothelial cells. As well, it was stated (Moon *et al.*, 2011) that simvastatin has been shown to be a potent antioxidant in vitro and in vivo suppressing production of reactive oxygen species (ROS). Concomitantly, simvastatin could reduce apoptosis of retinal capillary cells. More recently, it was reported (Yiu and Tse, 2014) that statins increase the number and function of circulating EPCs through increasing the bioavailability of nitric oxide (NO).

In this study, histological examinations of H&E stained sections revealed morphological alterations in diabetic retina 4 weeks after induction of diabetes. This is in parallel with Tombran-Tink *et al.* (2012) who reported that DR rat model of STZ-induced diabetes showed functional abnormalities reported by electroretinograph at 2 weeks after induction of diabetes. Moreover, neural apoptosis and glial reaction was detected 4 weeks after starting diabetes (Tombran-Tink *et al.*, 2012).

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

In the current study, reduced retinal thickness

was confirmed by morphometric measurements which revealed statistically significant reduction in diabetic non-treated group compared to control. These findings were in line with the results of previous studies (Zhang *et al.*, 2008 and Saberi and Gholami, 2012).

The aforementioned findings can be explained by progressive loss of retinal neurons due to diabetes induced apoptosis of neural and vascular cells leading to reduction in retinal thickness (Barber *et al.*, 2011 and Enzoly *et al.*, 2014). Moreover, it was suggested that scaffolding of the retina may be compromised leading to disruption and disorganization of retinal layers (Smith *et al.*, 2008). Müller cells, which have a major role in maintaining retinal structural integrity and organization, might also be implicated (Du *et al.*, 2004 and Zhan *et al.*, 2008).

Apoptosis of retinal neurons, which are further phagocytosed by glial cells, may also contribute to the appearance of empty spaces (Marc *et al.*, 2008). In addition, retinal edema contributes to separation between retinal cells due to dysfunction of blood retinal barrier and accumulation of fluid in the inner and outer retinal plexiform layers (Zhang *et al.*, 2014).

Dilated congested blood vessels were detected in DR in the current study. The same finding was cited in other research (Saint-Geniez and D'Amore, 2004 and Ozdemir *et al.*, 2014 a) reporting that early changes in retinal vasculature include vessel dilatation and vascular engorgement. This can be explained by retinal ischemia and tissue hypoxia with subsequent release of vascular endothelial growth factor (VEGF) leading to dilatation and congestion of blood vessels (Li Calzi *et al.*, 2010 and Ozdemir *et al.*, 2014 b).

In the present study, further histopathological findings were reported in the ganglion cell layer such as clear spaces in-between ganglion cells. Quantitative morphometric results of H&E stained sections showed statistically significant reduction in the number of ganglion cell nuclei in diabetic untreated group compared to control. Additionally, suggestive features of cell death and apoptosis were observed such as shrunken darkly-stained nuclei, a finding that was further

confirmed by caspase 3 immunohistochemistry. These results are consistent with previous reports (Yang *et al.*, 2009; Kusari *et al.*, 2010; Feng *et al.*, 2013 and Yüksel *et al.*, 2014).

In the present study, histopathological examination and morphometric analysis revealed that simvastatin was able to delay the onset and improve morphological derangements of DR through preserving retinal thickness and number of ganglion cells. The improvement noticed with simvastatin administration was in accordance with previous studies which demonstrated that treatment with simvastatin (El-Azab *et al.*, 2011) and atorvastatin (El-Hossary *et al.*, 2011), respectively, produced improvement of DR. It was mentioned that statins protected the retina against oxidative stress and the accelerated apoptosis of retinal capillary cells in diabetic rats (Kowluru and Kanwar, 2009). Moreover, it was stated that simvastatin could be useful in the treatment of early DR by inhibiting vascular permeability in the diabetic retina (Miyahara *et al.*, 2004).

In the present study, some sections exhibited spindle shaped nuclei within ganglion cell layer sometimes traversing the inner plexiform layer in treated group. This finding might be indicative of new vessel formation (Gong *et al.*, 2013) where mobilized EPCs migrate to ischemic retina in order to differentiate to endothelial cells for revascularization. Concomitantly, they might be resident endothelial cells that proliferate and start new vessel formation in response to ischemia.

Therefore, we used CD31 immunohistochemistry as a marker for EPCs and in turn to determine angiogenesis. CD31 (PECAM-1) is a transmembrane glycoprotein which has been recognized for its angiogenic role and is used primarily as a specific marker of EPCs (Wilson *et al.*, 2007 and Gopal *et al.*, 2014). It is noteworthy to mention that CD133 is not used to detect homing EPCs in tissues as its immuno-expression is lost after leaving circulation (Deng *et al.*, 2011).

The mean number of CD 31 immuno-positive cells of treated group showed significant increase compared to control and untreated group. However, there was no significant

difference between control and untreated group which is in accordance with Gong *et al.* (2013) who reported that there was no much difference between normal and STZ-diabetic rat retinas up to 2 months after induction of diabetes.

Similar to our findings, it was reported (Zhang and Yan, 2012) that CD31-positive cells increased noticeably in simvastatin treated diabetic group compared with non-treated diabetic group. This was attributed to the ability of simvastatin to mobilize EPCs in order to regulate retinal pericytes and endothelial cells, thus promoting more angiogenesis for retinal blood vessel repair (Zhang and Yan, 2012). Furthermore, CD31 immunohistochemistry findings were supported in the current study by flowcytometric results as circulating CD34/CD133 positive EPCs were higher in treated group compared to the untreated one.

Subsequently, in order to detect retinal apoptotic cells, caspase 3 immunohistochemistry was done as it plays an important role during apoptosis (Song *et al.*, 2012). In the present study, induction of diabetes increased caspase 3 immunoreactivity which is in accordance with other studies (Kim *et al.*, 2013 and Szabadfi *et al.*, 2014). Quantitative morphometric results revealed significant increase of caspase 3 immunoreactivity in untreated group compared to control & treated group.

Multiple factors are implicated in induction of apoptosis in DR such as hyperglycemic-induced oxidative stress (Abu El-Asrar *et al.*, 2007 and Kern and Barber, 2008), dysfunction of the blood retinal barrier (Huang *et al.*, 2012) and Müller cell dysfunction (Vujosevic and Midena, 2013).

Meanwhile, the current study revealed that simvastatin decreased caspase 3 expression in DR. Ali *et al.* (2011) stated that treatment with statins blocked cell death in diabetic retinas which can be attributed to the pleiotropic effects of statins, including antioxidant effects and inhibition of GTP-binding proteins.

## CONCLUSION

Eventually, the ability of simvastatin to alleviate DR could be referred to several

interacting mechanisms. Improving retinal vasculature and concomitantly eliminating retinal ischemia through its effect on EPCs is one mechanism. In addition, direct neuroprotective effect that would preserve retinal cells from apoptosis can also be attributed. Having such protective effects, simvastatin could be used as a novel pharmacological modality for prophylaxis against DR.

## CONFLICTS OF INTEREST

There are no conflicts of interest.

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## التأثير الوقائي للسيمفاستاتين على إعتلال الشبكية السكري المستحدث و الخلايا الاصلية البطانية في ذكر الجرذ الابيض البالغ, دراسه هستولوجيه وهستوكيميائية مناعيه

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### ملخص البحث

**الخلفية:** إعتلال الشبكية السكري (DR) هو واحد من الأسباب الرئيسية للعمى، وليس هناك علاج متاح للشفاء الكامل. تم التخطيط لهذه الدراسة لتقييم الآثار الوقائية المحتملة للسيمفاستاتين في نموذج إعتلال الشبكية السكري في الجرذ مع التركيز بشكل خاص على الخلايا الاصلية البطانية (EPCS).

**المواد و الطرق:** تم تقسيم ثلاثون من ذكور الجرذان البيضاء البالغه بالتساوي إلى: المجموعة I (المجموعة الضابطة)، المجموعة الثانية (مجموعة مرضى السكري) والمجموعة الثالثة (مجموعة السكري المعالجة بالسيمفاستاتين). تم استحداث مرض السكري عن طريق حقن 50 مجم لكل كجم لكل جرذ من الستربتوزوتوسين (STZ) داخل الغشاء البريتوني في 20 من ذكور الجرذان البيضاء البالغه. تم إعطاء السيمفاستاتين بجرعة 20مجم / كجم / فأر 48 ساعة بعد حقن الSTZ عن طريق الفم. تم قياس وزن الجسم ومستويات السكر في الدم أسبوعيا لمدة 1 شهر. وقد استخدم التدفق الخلوي للكشف عن الخلايا الاصلية البطانية (EPCS). تم تجهيز عينات الشبكية للصبغ بالهيماتوكسيلين والايوسين والصبغ الهستوكيميائي المناعي لكل من سى دى 31 وكاسباز 3. إشملت الدراسات المورفومترية القياسات التالية: سمك الشبكية ومتوسط عدد الخلايا العقدية في المقاطع المصبوغة بالهيماتوكسيلين والايوسين و متوسط عدد الخلايا المناعية الإيجابية ل سى دى 31 و متوسط نسبة مساحة التفاعل المناعي للكاسباز 3. تم خضوع كل القياسات للتحليل الإحصائي.

**النتائج:** أظهر الفلوسيتومتري أن سيمفاستاتين تمكن من زيادة نسبة الخلايا الاصلية البطانية EPCS في المجموعة الثالثة مقارنة مع المجموعة الثانية. وأظهرت المجموعة الثانية الخصائص النسيجية لإعتلال الشبكية السكري و كان التفاعل المناعي ل سى دى 31 مماثل للمجموعه الضابطة. وقد ارتفع التفاعل المناعي لكاسباز 3 بشكل ملحوظ بالمقارنة بالمجموعه الضابطة. وأظهرت المجموعة الثالثة تحسن في مظاهر إعتلال الشبكية السكري DR، زيادة ملحوظه في التفاعل المناعي للسى دى 31 وانخفاض ملحوظ في التفاعل المناعي لكاسباز 3. كان الغشاء الفخدى المنتسح مكونا من عدة حزم كولاجينية رفيعة و مستعرضة القطع. أثبتت الدراسة الكيميائية النسيجية المناعية للغشائين غياب ألياف العضلات الإرادية و ظهر ذلك عن طريق التفاعل المناعي السلبي.

**الخلاصة:** تشير هذه النتائج إلى أن سيمفاستاتين له آثار وقائية على إعتلال الشبكية السكري و لذلك يمكن اعتباره عامل علاجي واعد.