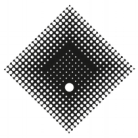
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ORIGINAL ARTICLE, MEDICINE

The Impact of Hyperglycemia on VEGF Secretion in Retinal Endothelial Cells

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Background: Diabetic retinopathy is a serious sight-threatening complication which is manifested by excessive angiogenesis in diabetic patients.

Aim: We hypothesize that cultured Rhesus monkey retinal endothelial cells (RhRECs) respond to high glucose with a change in cell proliferation and vascular endothelial growth factor (VEGF) secretion.

Materials and methods: In our study, 20 000 cells per well were treated without glucose or with 5.5 mM, 18.5 mM and 30 mM glucose for 24 hours. Viable cells were counted using trypan blue dye exclusion method. VEGF concentrations were measured in cell media by ELISA method.

Results: The number of viable cells incubated with 5.5 mM glucose increased significantly by 53.7% after 24 hours. In comparison, the number of viable cells decreased by 2.8% at 18.5 mM of glucose and by 20.4% at 30 mM of glucose after 24 hours of incubation. In contrast to this effect of glucose on the number of viable cells, a significant increase in VEGF levels (pg/mL) in the cell media with a glucose concentration of 0 mM compared to 5.5 mM of glucose was found. VEGF secretion in cell medium with 18.5 and 30 mM of glucose increased non-significantly in comparison with euglycemic levels.

Conclusion: Our results show that viability of retinal endothelial cells and VEGF release are highly responsive to changes in glucose concentration. Such glucose-induced changes in retinal endothelial cells may negatively impact the integrity of the microvasculature in the diabetic retina leading to angiogenesis and microaneursym.

BACKGROUND

Diabetes mellitus is a metabolic disorder characterized by high levels of glucose in the blood (hyperglycemia). Diabetes is highly prevalent in developing countries and is estimated to affect approximately 439 million people by 2030[1], suggesting that diabetes is a global health problem. This chronic disease has long term effects on kidneys, nerves, the heart, and other major organs. One such complication affects the eye and is known as diabetic retinopathy (DR). DR is one of the leading causes for blindness which affects approximately 93 million people worldwide. The key determinants in development of DR are diabetic duration and glycemic control.[2] In the United States, 1 out of 12 persons with diabetes mellitus, age 40 and older, have advanced vision threatening retinopathy.[3]

Neurodegeneration is an initial process in the development of DR. Oxidative stress, hypoxia, high levels of glutamate, the induction of the renin-angiotensin system, and the elevated levels of receptor for advanced glycation endproducts are important factors included in the retinal neurodegeneration caused by diabetes mellitus.[4] Somatostatin,

pigment epithelium-derived factor, and erythropoietin are neuroprotective peptides that play an essential role in retinal homeostasis, but they are decreased in the eye in diabetic patients. On the contrary, insulin-like growth factor 1 and vascular endothelial growth factor (VEGF) are key proteins involved in the development of new capillaries and finally cause damage of neurons in the retina. Thus the angiogenesis is a significant process in the development of diabetic retinopathy. VEGF, a strong controller and promoter of angiogenesis, is a specific mitogen that induces new blood vessel formation by targeting growth and differentiation of endothelial cells.[5] In many diabetic patients, VEGF is excessively produced in retina resulting in the proliferation of endothelial cells, new blood vessel formation, thus contributing to abnormalities such as microaneurysms[6], fluid leakage, and tissue damage.

In the last years, the knowledge about the pathogenesis of retinal neurodegeneration including the function of growth factors has grown; however, follow-up research in this field is needed to clarify the basic processes that contribute in retinal neurodegeneration and its connections with damage of the capillary blood vessels. Based on the above motioned results, we hypothesize that changes in glucose concentration will significantly impact the viability of retinal endothelial cells and their VEGF secretion.

MATERIALS AND METHODS

Cell Culture

Rhesus monkey retinal endothelial cells (transformed) were purchased from American Type Culture Collection (ATCC) (Catalog number: CRL 1780). Minimum Essential media Alpha (supplemented with 10% FBS, purchased from Life Technologies Co., Carlsbad, CA, USA) was used to revive cells in a T75 flask. The cells were stored in a 37°C incubator with 5% CO_2 and the media changed every 48 hours. Confluent cells were harvested as per manufacturer's instructions and plated at 20 000 per well (T0). After cells were allowed to settle (24 hours), they were treated without glucose (hypoglycemic conditions; 0 mM), physiological glucose (5.5 mM), hyperglycemic glucose (18.5 mM), or severe hyperglycemic conditions (30 mM). Each condition was measured in triplicate. After 24 hours of treatment, the cell media was collected and stored at -20°C (to be later used for cytokine analysis by ELISA). Cells were then harvested from each well using trypsin EDTA as per manufacturer's instructions and prepared for trypan blue dye exclusion in order to determine the viable cell number (see trypan blue dye exclusion method below).

Glucose Treatments

Serum free media was used for the hypoglycemic (5.5 mM) condition. For 5.5, 18.5, and 30 mM treatments, D-(+)-Glucose (Catalog Number: G7021; Sigma Aldrich) was added to and dissolved in serum free medium at least one hour in advance before being introduced to the cells. Serial dilutions were necessary to derive the specific concentration for each treatment group.

Trypan Blue Dye Exclusion Method

Trypan blue dye exclusion method was used to determine number of viable cells present at T0 and after 24 hours glucose treatment. The cells were diluted 1:1 (volume: volume) using Trypan Blue (Corning, catalog number: 25-900 Cl) according to manufacturer's instructions, before counting using a Neubauer's hemocytometer.

hVEGF ELISA

Conditioned medium was harvested as noted above and kept at -20ºC overnight for ELISA. Human VEGF ELISA (Category Number DVE00; R&D Systems) was performed as per manufacturer's instructions and analyzed using a DYNEX MRXII plate reader equipped with Revelation software or BIORAD microplate reader with Manager Software. Data was quantified in comparison to VEGF standards.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (Version 6.07). All data were expressed as mean ± SEM (n=3 for 0, 5.5 and 30 mM and n=2 for 18.5 mM). Mean differences between groups were calculated using One-way ANOVA (with Dunnett's Multiple Comparison Post-hoc Test). $P<0.05$ was considered to be statistically significant.

RESULTS

Concentration-dependent changes in cell viability

At T0 (the beginning of experiment), 20 000 viable cells in each well were used in experiment. **Fig. 1** shows RhRECs growth under hypoglycemic conditions (0 mM), physiological conditions (5.5 mM), and at hyperglycemic conditions (18.5 or 30 mM of glucose) after a period of 24 hours. Cells exhibited elongated shape with morphology indicative of the cells of smooth muscle origin. Image of

cells in 5.5 mM of glucose shows large increase in confluence from T0 **(Fig. 1)**. In comparison, images from cells in 0 mM and 30 mM show large decreases in confluence and a notable decrease was also observed in 18.5 mM of glucose. After 24 hours of glucose treatment, the cells in 5.5 mM of glucose increased from 20 000 to 30 741 cells (increase by 53.7%) and the cells in 18.5 mM and 30 mM decreased to 19 444 or 15 926 respectively (decrease by 2.8% and 20.4%) **(Fig 2)**. Cells in 0 mM of glucose decreased from the initial number to 13 333 (decrease by 33.3%). A concentration dependent significant changes were observed when we compared the physiological glucose conditions (5.5 mM) with the cells incubated in hyperglycemic (18.5 mM and 30 mM) and hypoglycemic conditions (0 mM). One-way ANOVA was significant for a glucose effect on cell viability ($P \leq 0.00004$). Dunnett's multiple comparison test suggests that the change in glucose concentration from 5.5 mM to 0, 18.5, and 30 mM results in significant changes in cell viability **(Fig. 2)**.

Concentration-dependent changes in VEGF levels in cell media

Fig. 3 shows VEGF levels in cell medium after a 24 hour period of glucose treatment. In comparison to VEGF levels in cell media of cells grown in euglycemic glucose of 5.5 mM, increasing glucose concentration of 18.5 or 30 mM resulted in a progressive increase in VEGF levels in the cell medium. This result points out to fact that the higher glucose induced more VEGF synthesis and release from RhRECs. However, RhRECs grown in 0 mM glucose conditions secreted a significantly higher amount of VEGF compared to 5.5 mM of glucose ($P \leq 0.0052$). The VEGF levels produced by RhRECs found in the media were as follows: 146.3 pg/ml (0 mM of glucose), 22.6 pg/ml (5.5 mM), 28.0 pg/ml (18.5 mM), and 37.6 pg/ml (30 mM) **(Fig. 3)**.

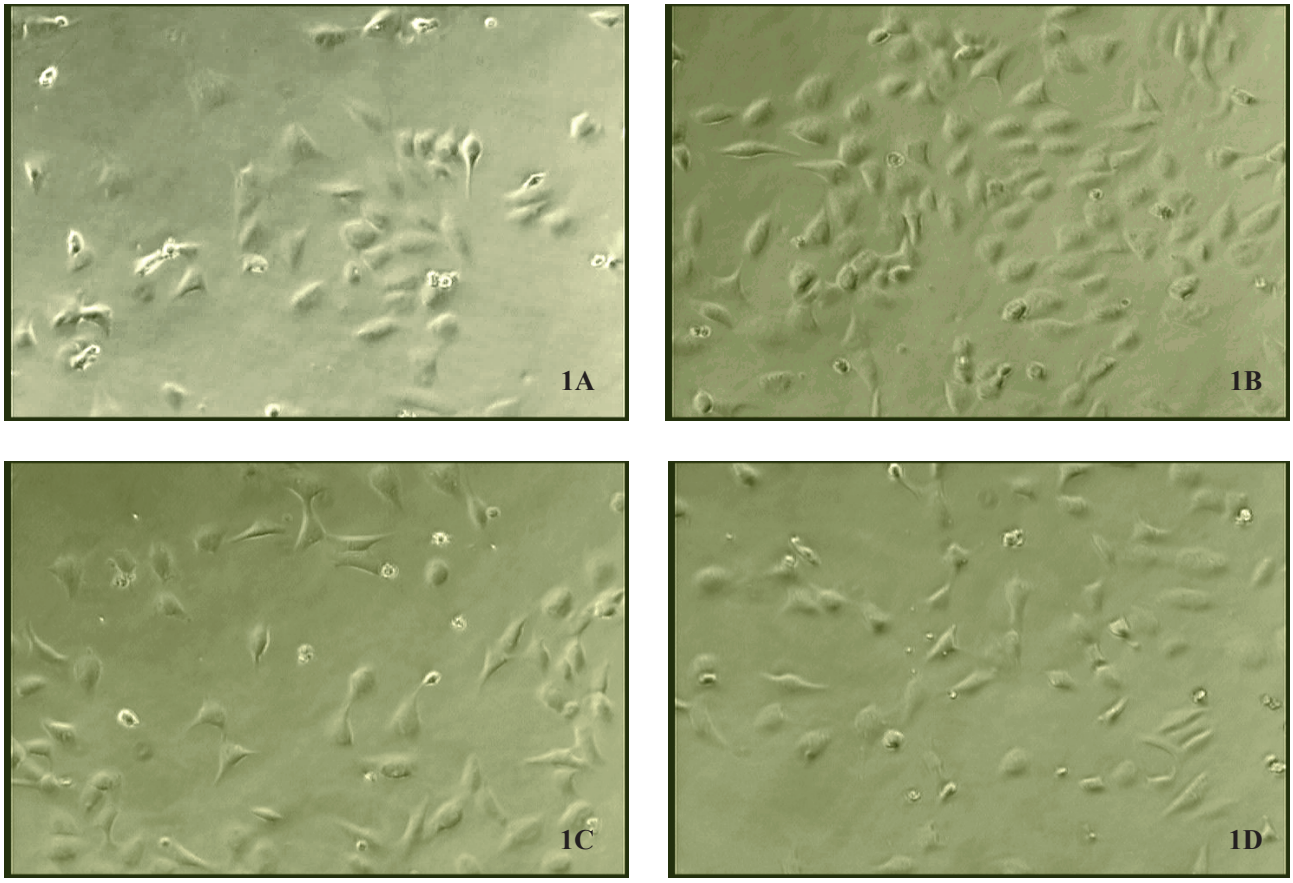

Figure 1. Photomicrographs of RhRECs grown in 0, 5.5, 18.5, and 30 mM glucose for 24 hours.
Images from cells in 5.5 mM **(Fig. 1B)** show 75% confluence, a significant increase from T0 (60%). In comparison, images from cells in 0 mM **(Fig. 1A)** and 30 mM **(Fig. 1D)** show 45% and 50% confluence, a significant decrease from T0 density and a notable decrease in confluence was observed in cells incubated at 18.5mM **(Fig. 1AC)**. All images were taken at 100x magnification.

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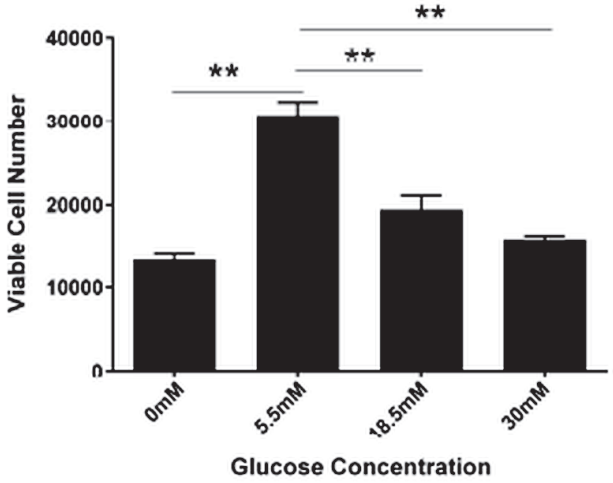

Figure 2. Concentration-dependent change in cell viability. RhRECs were plated at 20 000 per ml in a 24 well plates. After 24 hours of glucose treatment, the changes in the number of viable cells in medium were found: 13 333 cells in 0 mM, 30 741 cells in 5.5 mM, 19 444 cells in 18.5 mM, and 15 926 cells in 30 mM. One-way ANOVA determined significant differences between groups (F/3.7/=50.48, P=0.00004). A Dunnett's multiple comparison test was performed using 5.5 mM as control (**P<0.0001).

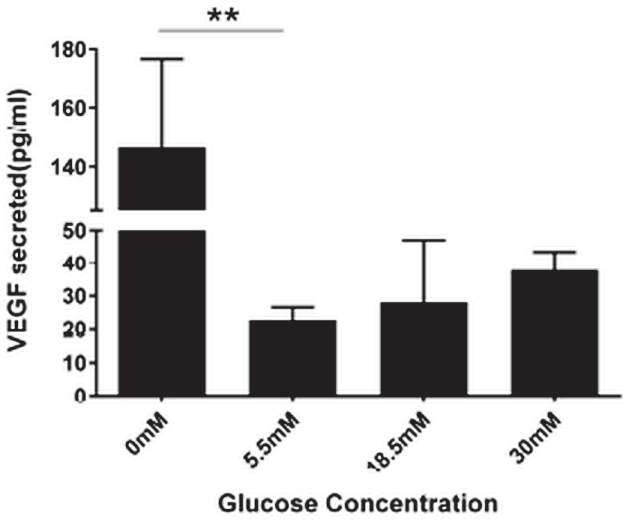

Figure 3. Concentration-dependent change in VEGF secretion per mL. After 24-hour glucose treatment, VEGF amounts secreted into conditioned medium was measured by ELISA. RhRECs secreted 146.3 pg/ml of VEGF in cell media with 0 mM of glucose, 22.6 pg/mL (5.5 mM), 28 pg/mL (18.5 mM), and 37.6 pg/mL (30 mM). One-way ANOVA analysis found a significant difference (F/3,7/=10.74, P=0.0052). A Dunnett's multiple comparison test was performed using 5.5 mM as control (**P<0.05).

The VEGF levels per cell (pg/cell) were calculated by dividing the VEGF levels in cell medium with the respective cell number. After 24 hours treatment, each cell in 5.5 mM glucose secreted 0.00073 pg of VEGF per cell, which was doubled when the glucose concentration was increased to 18.5 mM (0.0015 pg/cell) and tripled when glucose concentration was further increased to 30 mM (0.002 pg/cell). A 13-fold increase was observed in cells grown in 0 mM glucose (0.01 pg/cell). One way ANOVA showed a highly significant glucose effect on VEGF secretion (pg/cell; P≤0.00023) **(Fig. 4)**.

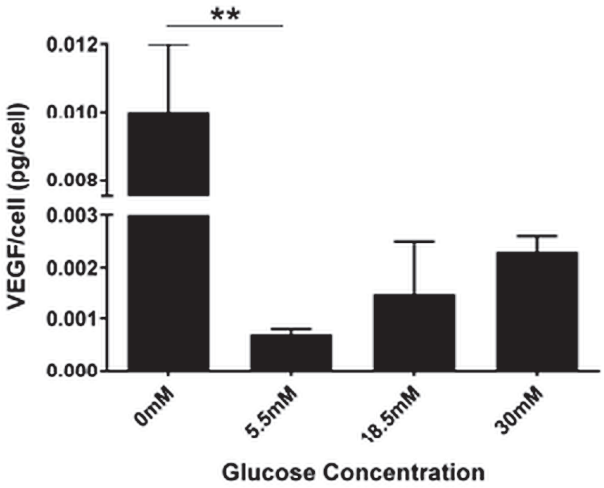

Figure 4. Concentration dependent changes in VEGF secretion per cell. VEGF secretion per cell was calculated by dividing VEGF levels measured in cell medium with the respective cell number. After 24 hours of glucose treatment, the changes in VEGF secretion per cell were found: 0.01 pg/cell (in 0 mM of glucose), 0.00073 pg/cell (5.5 mM), 0.001 pg/cell (18.5 mM), and 0.0023 pg/cell (mM). Results from one-way ANOVA showed significant difference (F/3,7/=14.17, P=0.0023). A Dunnett's multiple comparison test was performed using 5.5 mM as control (**P<0.05).

DISCUSSION

In the present study, we have observed the changes in cell confluence and viability of RhRECs over a 24-hour period which were dependent on glucose concentration. Physiological glucose significantly increased cell number but hyper- or hypoglycemic conditions induced a significant decrease in cell number **(Fig. 2)**. Our results validate the hypothesis that a change in glucose concentration induces change in the number of RhRECs. It is of great interest to point out the 18.5 mM glucose treatment did not

result in a large change in cell number compared to T0. When related to diabetic conditions in humans, it is possible that uncontrolled hyperglycemic conditions in excess of plasma levels of 18.5 mM may result in a large reduction in retinal endothelial cells leading to complications such as angiogenesis and microaneurysms. We have previously reported that treatment of high glucose (18 mM, 33 mM and 40 mM) for a period of 5 days caused significant decreases in cell viability in primary human retinal pericytes.[7] When ARPE 19 cells were treated with high glucose (18 mM) for 8 days, it resulted in a significant increase in cell viability.[8]

This study clearly showed that changes in glucose concentrations in RhRECs resulted in a significant increase in VEGF levels in the conditioned media **(Fig. 3)**. Dunnett's Multiple Comparison Test suggests that the significant change is due to difference between VEGF level in RhRECs treatment with 0 mM or 5.5 mM. The amount of VEGF secreted per cell increased significantly with changes in glucose concentration from 0, 18.5 or 30 mM compared to physiological conditions **(Fig. 4)**. These results are consistent with our previous findings where increasing glucose concentrations of 18.5 mM caused an increase in VEGF secretion per cell in both ARPE-19 cells and human retinal pericytes. In human synovial fibroblasts, increasing glucose concentration in cell medium to 33 mM glucose for a 24-hour period caused an increase in VEGF secretion by the cells into the medium.[9] Earlier reports showed that high glucose induced changes in cell number of human retinal pericytes and ARPE-19 cells which were accompanied with increased VEGF secretion in cell media.[7,8] Furthermore, human umbilical vein endothelial cells (HUVEC) treated with high glucose (25 mM/L) resulted in a decrease in VEGF secretion in cell media.[10] In another study, both high glucose and low glucose treatment resulted in increase in VEGF secretion in cell media of rat and human Müller cells (Tsin et al., unpublished observations). More recent results of Velanki et al.[11] demonstrated that human (MIO-M1) and rat (rMC-1) Müller cells (glial cells of the retina) are highly responsive to changes in glucose concentrations. 30 mM compared to 5.5 mM significantly increased cell viability in both cell lines but induced a significant decrease in VEGF secretion per cell in rMC-1 only (5.5 mM and 30 mM in comparison with 0 mM). In both MIO-M1 and rMC-1, the amount of VEGF secreted per cell increased by about 100% when glucose was changed from 5.5 to 0 mM but decreased slightly (17% in MIO-M1 and 11% in rMC-1) when glucose was increased from 5.5 to 30 mM. According to the authors, mechanisms remain unknown.

In the present study, it was observed that glucose deprived conditions induce more proangiogenic response than high glucose conditions. It is not clear how VEGF affects RhRECs proliferation. Further studies are required to determine the interactive effect of glucose, VEGF, and RhRECs proliferation. It is possible that glucose may induce apoptosis of RhRECs via a molecular mechanism involving TGFβ similar to that reported earlier.[12] Moreover, Jiang et al.[13] demonstrated that eNOS and PKC signaling are included in the regulation of VEGF levels in diabetic retina. In another study, Joyal et al.[14] summarized that impaired glucose entry into photoreceptors results in a dual (lipid and glucose) fuel shortage and a reduction in the levels of the Krebs cycle intermediate α-ketoglutarate (α-KG). Low α-KG levels promotes stabilization of hypoxia-induced factor 1a and secretion of VEGF-A by starved Vldlr(-/-) photoreceptors, leading to neovascularization. The authors concluded that dysregulated lipid and glucose photoreceptor energy metabolism might be a driving force in several retinal diseases including DR.

CONCLUSION

Overall, our data clearly suggests that glucose concentration has a significant effect on viability of RhRECs in culture as well as their secretion of VEGF. Nevertheless, such glucose effects on RhRECs cell number and VEGF release are novel and potentially important and thus may provide innovative approaches to investigate the pathology of angiogenesis in diabetic retinopathy.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Воздействие гипергликемии на секрецию VEGF в эндотелиальных клетках сетчатки

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Введение: Диабетическая ретинопатия является серьёзным угрожающим жизни осложнением, которое проявляется с избыточным ангиогенезом у больных сахарным диабетом.

Цель: Мы высказываем гипотезу, что эндотелиальные клетки сетчатки лабораторных макаков-резус (RhRECs) реагируют на высокие уровни глюкозы изменением клеточной пролиферации и секрецией фактора сосудистого роста эндотелия (ФСРЭ).

Методы: В нашем исследовании 20 000 клеток на лунку планшета были обработаны без глюкозы или с 5.5 mM, 18.5 mM и 30 mM глюкозы в течение 24 часов. Количество жизнеспособных клеток было установлено при помощи метода исключения красителя Trypan blue. ФСРЭ концентрации были измерены в клеточной среде при помощи метода ELISA.

Результаты: Количество жизнеспособных клеток, инкубированных 5.5 mM

Дата публикации: 27 июня 2017

Ключевые слова: VEGF, глюкоза, диабетическая ретинопатия, эндотелиальная клетка сетчатки, сетчатка глаза

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глюкозы, увеличилось на 53.7 % через 24 часа. Для сравнения, количество жизнеспособных клеток уменьшилось на 2.8 % при 18.5 mM глюкозы и на 20.4 % при 30 mM глюкозы после 24-часовой инкубации. В отличие от данного эффекта глюкозы на количество жизнеспособных клеток, было установлено значительное повышение ФСРЭ уровней (pg/mL) в клеточной среде с концентрацией глюкозы 0 мМ по сравнению с 5.5 mM глюкозной концентрацией. ФСРЭ секреция в клеточной среде с 18.5 и 30 mM концентрацией глюкозы повысилось незначительно по сравнению с нормальным уровнем.

Заключение: Наши результаты показывают, что жизнеспособность эндотелиальных клеток сетчатки и освобождение ФСРЭ проявляют высокую степень чувствительности на изменения в концентрации глюкозы. Установленные изменения, индуцированные глюкозой, у эндотелиальных клеток сетчатки могут повлиять отрицательным способом на целостность микрососудов диабетической сетчатки, что приведёт к ангиогенезу и микроаневризме.