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Research Article

Effect of Glucose on Retinal Endothelial Cell Viability and VEGF Secretion

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Abstract

Previous studies have shown that in diabetic patients, there is an increase of retinal capillaries associated with the development of diabetic retinopathy in the eye. The objective of current study is to investigate the effect of glucose on retinal endothelial cell viability and VEGF secretion. 20,000 cells per well were treated without glucose or with 5.5mM (euglycemic), 18.5mM and 30mM (hyperglycemic) glucose for 24 hours. Viable cells were counted using Trypan blue dye exclusion method. ELISA was used to measure VEGF secretion from cells into the cell medium. The number of viable cells incubated with 5.5mM glucose (physiological control) increased by 53.7% after 24 hours. In comparison, cells treated with 18.5mM glucose decreased by 2.8% while cells treated with 30mM glucose decreased by 20% after 24 hours of incubation. Cells without glucose treatment (0mM control) decreased by 33.3%. In contrast to the decrease of viable cell numbers after treatment with high glucose, there is an increase in VEGF secretion (pg/mL) to the cell medium with increase in glucose concentration from 5.5mM to 0, 18.5, and 30mM. The amount of VEGF secreted per cell also increased with increasing glucose concentrations. 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 microaneursyms.

Keywords: Diabetic retinopathy; Glucose; Retina; Retinal endothelial cell; VEGF

Introduction

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 [2,3]. 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, glycemic control, and genetics [4,5]. In the United States, 1 out of 12 persons with diabetes mellitus, age 40 and older, has advanced vision threatening retinopathy [6]. Angiogenesis is a significant process in the development of diabetic retinopathy. A strong controller and promoter of angiogenesis, Vascular Endothelial Growth Factor (VEGF) is a specific mitogen that induces new blood vessel formation by targeting growth and differentiation of endothelial cells [7]. In diabetic patients, VEGF is excessively produced in the retina resulting in endothelial cell proliferation, tissue damage, fluid leakage, new blood vessel formation [8], and microaneurysms. The polymorphisms in the VEGF gene are associated with VEGF expression and diabetic retinopathy [9,10]. Previously, we reported high glucose changes cell viability and VEGF secretion in Müller cells, retinal Pericytes, and ARPE 19 cells [11-13]. We hypothesize that the viability of retinal endothelial cells and their secretion of VEGF is significantly impacted by changes in glucose concentration. The objectives of our current study is to compare the effects of high glucose on viability of endothelial cells and their VEGF secretion compared to no-glucose and physiological glucose controls. Our novel findings in Rhesus Monkey retinal endothelial cells may help us establish a model for further research in the diabetic, non-human primate eye.

Methods

Cell culture

Rhesus monkey retinal endothelial cells (Catalog number: CRL1780) transformed were propagated in T75 flasks using minimum essential media alpha (Life Technologies) supplemented with 10% FBS per manufacturer's instructions. Cells were maintained at 37°C + 5% CO_2 with medium changed every 48 hours. Confluent cells were harvested per manufacturer's instructions and plated at 20,000 per well (T_0). After cells were allowed to settle (an additional 24 hours), they were treated without glucose (hypoglycemic conditions; 0mM), physiological glucose (5.5mM), hyperglycemic glucose (18.5mM), or severe hyperglycemic conditions (30mM). Each condition was measured in triplicate. After 24 hours of treatment, the cell medium 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, per manufacturer's instructions, and prepared for trypan blue dye exclusion in order determine the viable cell number (see trypan blue dye exclusion method below).

Glucose treatments

Serum free medium was used for the hypoglycemic (0 mM) control. For 5.5, 18.5, and 30mM treatments, D-(+)-Glucose (Catalog Number: G7021; Sigma Aldrich) was added to and dissolved in serum

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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. Each treatment was filtered by 0.2 micron syringe filter and warmed to 37°C prior to introduction to the cells. D-mannitol was used as osmotic control.

Trypan blue dye exclusion method

Trypan blue dye exclusion method was used to determine the number of viable cells present at T_0 and after 24 hours glucose treatment. Cells were diluted 1:1 (volume: volume) using Trypan Blue (Corning, catalog number: 25-900-Cl) per manufacturer's instructions, before counting with a Neubauer hemocytometer.

hVEGF ELISA

Conditioned medium was harvested as noted above and kept at -20°C overnight for ELISA. Human VEGF ELISA (category number DVE00; R&D systems) was performed per manufacturer 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. BLAST database comparison of *Macaca mulatta* (Rhesus Monkey) VEGF to human VEGF is noted to be 99% identical.

Statistical analysis

Statistical analysis was performed by students T-test as well as using GraphPad prism software (Version 6.07). All data were expressed as mean ± SEM (n=3 for 0, 5.5 and 30mM and n=2 for 18.5mM). Mean differences between groups were calculated using One-way ANOVA (with Dunnett's multiple comparison Post-hoc Test); P- values < 0.05 were considered to be statistically significant.

Results

Concentration-dependent change in cell viability

At T_0 there were 20,000 viable cells in each well. Figure 1 shows RhREC growth under hypoglycemic conditions (0mM), physiological conditions (5.5mM), and at hyperglycemic conditions (18.5 or 30mM glucose) after a period of 24 hours. Cells exhibit elongated shape with morphology indicative of cells of smooth muscle origin. After 24 hours, the photomicrograph of cells treated with 5.5mM shows a large increase in confluence compared to T_0 (Figure 1). In comparison, images from cells treated with 0mM, 18.5, and 30mM show notable decreases compared to 5.5mM (physiological control). After 24 hours, cells treated with 5.5mM glucose increased to 30,741 from 20,000. Cells treated with 18.5mM decreased to 19,444, while cells treated with 30mM decreased to 15,926 respectively (Figure 2). Cells in 0mM glucose decreased to 13,333. A concentration dependent change was observed between 5.5mM and 18.5mM glucose as well as between 5.5mM glucose and 30mM glucose treatments (Figure 2). One-way ANOVA was significant for a glucose effect on cell viability (P≤0.00004). Dunnett's multiple comparison test suggests that change in glucose concentration from 5.5mM to18.5 and 5.5mM to 30mM result in a significant change in cell viability. D-mannitol (osmotic control) experiments showed no effect on endothelial cells (data not shown).

Concentration-dependent change in VEGF level in cell media

Figure 3 shows VEGF levels in cell medium after a 24 hour glucose treatment period. In comparison to VEGF levels in the cell medium from the 5.5mM treatment group, increasing the glucose concentration to 18.5 or 30mM resulted in a progressive increase in VEGF levels in the cell medium. This suggests higher glucose induces more VEGF synthesis and release from RhREC. However, RhREC grown in 0mM glucose conditions secreted a significantly higher amount of VEGF compared to 5.5mM glucose. When looking at VEGF secretion in pg/mL of cell medium after a 24 hour period, 146.3 pgVEGF/mL was observed in 0mM glucose treatment, 22.6 pgVEGF/mL in 5.5mM glucose, 28.0 pgVEGF/mL in 18.5mM glucose, and 37.6 pgVEGF/mL in 30mM glucose (Figure 3). One way ANOVA shows a significant glucose effect on VEGF secretion in the cell medium (P≤0.0052).

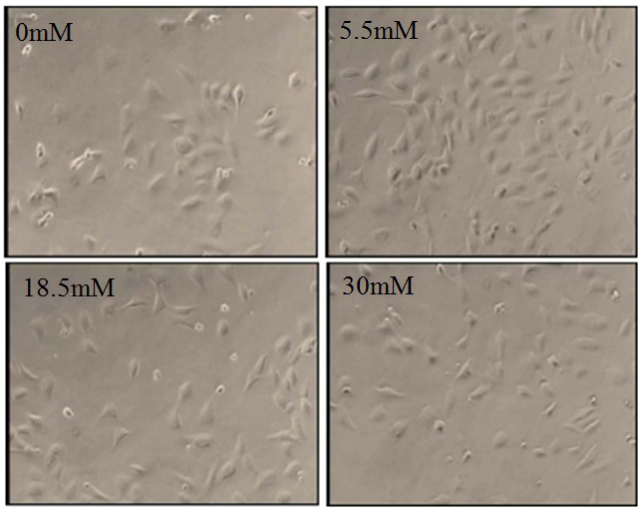

Figure 1: Photomicrographs of RhREC grown in 0mM, 5.5mM, 18.5mM and 30mM glucose for 24 hours.

Images from cells in 5.5mM show 75% confluence, a significant increase from T_0 (60%). In comparison, images from cells in 0mM and 30mM show 45% and 50% confluence, a significant decrease from T_0 density and a notable decrease in confluence was observed in cells incubated at 18.5mM. All images were taken at 100X magnification.

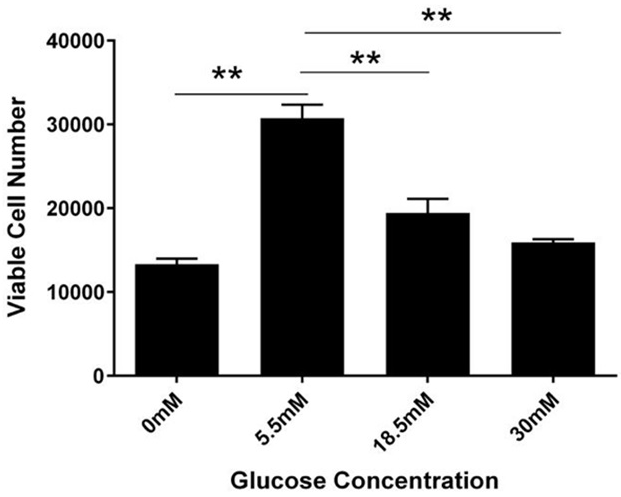

Figure 2: Concentration-dependent change in cell viability.

RhREC were plated at 20,000 per ml in a 24 well plate. After 24 hours of glucose treatment, there were 13,333 cells in 0mM, 30,741 cells in 5.5mM, 19,444 in 18.5mM and 15,926 in 30mM. One-way ANOVA determined the difference between groups was significant (F (3, 7) =50.48, P=0.00004). A Dunnett's multiple comparison test was performed using 5.5mM as control (**P<0.0001).

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

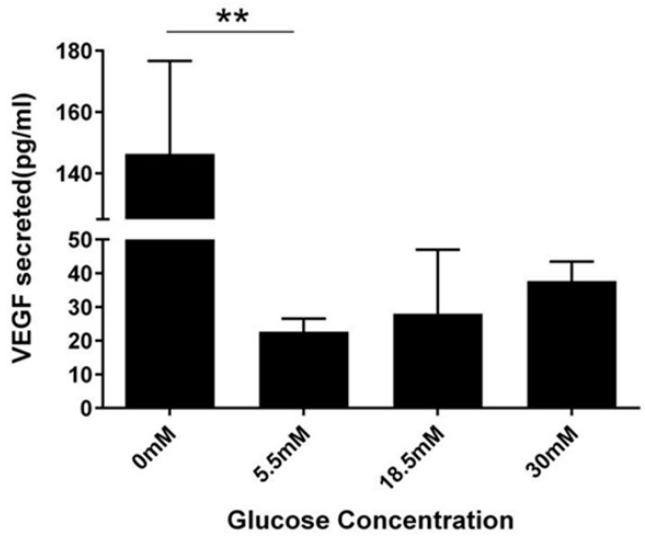

Figure 3: Concentration-dependent change in VEGF secretion per mL.

After 24 hour glucose treatment, VEGF amounts secreted into conditioned medium was measured for by ELISA. Cells in 0mM secreted 146.3pg/ml of VEGF, 22.6pg/mL in 5.5mM, 28pg/mL in 18.5mM, and 37.6pg/mL in 30mM. Results from One way ANOVA were significant (F (3, 7) =10.74, P=0.0052). A Dunnett's multiple comparison test was performed using 5.5mM as control (**P<0.05).

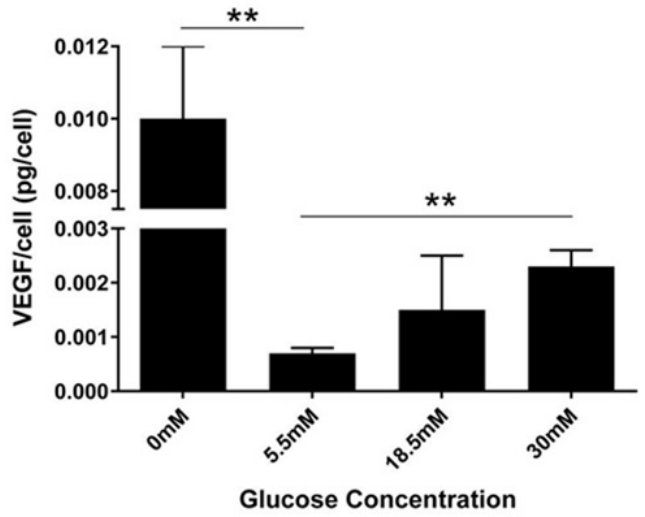

Figure 4: Concentration dependent change in VEGF secretion per cell.

VEGF/cell was calculated by dividing VEGF levels measured in cell medium with respective cell number. After 24 hours, cells treated with 0mM glucose secreted approximately 0.01pg/cell, 0.00073pg/cell in 5.5mM, 0.001pg/cell in 18.5mM, and 0.0023pg/cell in 30mM. Results from one way ANOVA showed significance (F (3, 7) =14.17, P=0.0023). A Dunnett's multiple comparison test was performed using 5.5mM as control (**P<0.05). T-Test showed significant difference (5% level) in the level of VEGF between the 5.5mM vs the 30mM treatment groups.

Discussion

In the present study, it was observed that changes in RhREC confluence and viability over a 24 hour period was dependent on glucose concentration. Physiological glucose significantly increased cell number but hyper- or hypoglycemic conditions induced a significant decrease in cell number (Figure 2). Our results validate the hypothesis that a change in glucose concentration induces change in the number of RhREC. It is of great interest to point out the 18.5mM glucose treatment did not result in a large change in cell number compared to T_0. When related to diabetic conditions in humans, it is possible that uncontrolled hyperglycemic conditions in excess of plasma levels of 18.5mM 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 (18mM, 33mM and 40mM) for a period of 5 days caused significant decreases in cell viability in primary human retinal pericytes [12]. When ARPE 19 cells were treated with high glucose (18mM) for 8 days there were significant increases in cell viability [13]. Retinal Müller cells treated with no glucose for 24 hours resulted in a decrease in cell viability, however high glucose (30mM) increased cell viability [11]. Previous studies on primary human retinal endothelial cells show that high glucose increased cell viability [14,15]. The differences in our findings compared to those published are likely due to inter- species differences (human vs rhesus monkey) as well as the transformation of the rhesus monkey retinal endothelial cells compared to the primary human line reported by our colleagues.

RhREC subjected to treatment with higher glucose concentrations resulted in a significant increase in VEGF levels in the conditioned media (Figure 3). Dunnett's multiple comparison test suggests a significant change between VEGF levels in RhREC treated with 0mM or 5.5mM as well.

In human synovial fibroblasts, treatment with 33mM glucose for a 24 hour period resulted in an increase in VEGF secretion from the cells into the medium [16]. In earlier reports, high glucose not only induced changes in viable cell number but also increased VEGF secretion in cell medium from human retinal pericytes and ARPE-19 [12,13]. Vellanki et al., 2016 showed that both high and low glucose treatments resulted in an increase in VEGF secretion into cell medium from rat and human müller cells [11]. Primary human retinal endothelial cells show that high glucose stimulates VEGF expression [14,15] and Human Umbilical Vein Endothelial Cells (HUVEC) treated with high glucose (25mM/L) resulted in a decrease in VEGF secretion in cell medium. This contrasting effect to our reports may be due to the difference in cell type and exposure time compared to our rhesus monkey cell line [17].

In the current study, the amount of VEGF secreted per cell increased significantly with changes in glucose concentration from 0, 18.5 or 30mM compared to physiological (5.5mM) conditions (Figure 4). These results are consistent with our previous findings in which increasing glucose concentrations to 18.5mM resulted in an increase in VEGF secretion per cell from both ARPE-19 and human retinal pericytes.

Our study observed that glucose deprived conditions induce a more pro-angiogenic response than high glucose conditions. It is not clear how VEGF affects RhREC proliferation. Further studies are required to determine the interactive effect of glucose, VEGF, RhREC proliferation, and VEGF levels inside the cell. It is possible that glucose may induce apoptosis of RhREC via a molecular mechanism

involving TGFβ similar to that reported earlier [18]. Overall, our data clearly suggests that glucose concentration has a significant effect on the viability of RhREC in culture as well as their secretion of VEGF. We plan to extend our current results to include longer time points and assays to better understand the mechanism of viability change as well as the changes in VEGF secretion. Nevertheless, such glucose effects on RhREC number and VEGF release are novel and important as they provide innovative approaches to investigate the cause of angiogenesis in diabetic retinopathy.

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