The effects of anti-vascular endothelial growth factor agents on human retinal pigment epithelial cells under high glucose conditions

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Abstract

● **AIM: To investigate the effects of high glucose levels and anti-vascular endothelial growth factor (VEGF) agents (bevacizumab, ranibizumab and aflibercept) on retinal pigment epithelium (RPE) cells.**

● **METHODS: ARPE-19 cells were cultured at different glucose levels (5.5 mmol/L, 25 mmol/L, and 75 mmol/L). Cell viability was evaluated by MTT assay at 3d after treatment with D-glucose. Cell migration ability was measured by wound healing assay at 3d. A cell death detection kit was used to assess apoptosis at 3 and 14d. Cell proliferation was assessed by EdU assay at 3d. The culture medium was treated with anti-VEGF agents at clinically relevant concentrations. The experiment was then repeated at a different glucose level.**

● **RESULTS: The viability and migration of ARPE-19 cells were significantly decreased in the presence of 75 mmol/L as compared to 5.5 mmol/L glucose. The percentage of TUNEL-positive cells was significantly increased and the proliferative potential was decreased with 75 mmol/L compared to 5.5 mmol/L glucose. There were no significant differences in the results between 25 mmol/L and 5.5 mmol/L glucose. In the presence of 75 mmol/L glucose, the groups treated with anti-VEGF showed decreased cell viability and proliferation and increased apoptosis. However, there were no significant differences between the anti-VEGF groups.**

● **CONCLUSION: High glucose level decreases the viability, wound healing ability, and proliferation of RPE cells, while increasing apoptosis. Furthermore, anti-VEGF agents interfered with the physiological functions of RPE cells under high-glucose conditions, accompanied by decreases in cell viability and proliferation.**

INTRODUCTION

Diabetic retinopathy is one of the main ocular complications associated with diabetes mellitus (DM). Diabetic macular edema (DME) is a frequent manifestation of diabetic retinopathy characterized by swelling of the retina due to leakage of fluid from the blood vessels. Macular edema is a common cause of vision loss in patients with diabetic retinopathy[1-3].

Anti-vascular endothelial growth factor (VEGF) therapy is the current treatment of choice for DME[4-5]. Several clinical trials have demonstrated that intravitreal injection of anti-VEGF agents can improve visual acuity in patients with DME[6-9]. Although many patients experience an improvement in vision with anti-VEGF therapy, a recent report described adverse effects after 2y of anti-VEGF therapy that suggested the possibility of damage to the retinal pigment epithelium (RPE) and photoreceptors from "off-target" effects of VEGF neutralization[10]. A number of different anti-VEGF agents are currently in clinical use, *i.e.* ranibizumab, bevacizumab, and the recently approved aflibercept. These anti-VEGF molecules differ not only in their VEGF binding affinity, but also in their molecular structure[11]. These molecular differences may result in different effects on retinal cells[12-13].

Several studies investigated and compared the effects of anti-VEGF agents on RPE cells. Anti-VEGF agents are taken up and stored by RPE cells for at least 7d. The presence of intracellular anti-VEGF agent impairs the phagocytic function of RPE cells, and has also been shown to impair the wound healing capacity of PRE cells[14-15]. However, most studies on the effects of anti-VEGF agents on RPE cells were performed only at normal glucose levels, and there has been no such research at high glucose levels.

In this study, we investigated the effects of high glucose level and treatment with anti-VEGF agents under conditions of high glucose level on RPE cells with regard to toxicity, wound healing ability, apoptosis, and proliferation.

MATERIALS AND METHODS

Cell Culture The human RPE cell line, ARPE-19, was obtained from the American Type Culture Collection (Manassas, Virginia, USA). ARPE-19 cells were cultured in Dulbecco's Modified Eagle's Medium containing 4 mmol/L L-glutamine, nutrient mixture (Invitrogen, Carlsbad, CA, USA) without fetal bovine serum (FBS), 5 mmol/L D-glucose, 100 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen) at 37 ℃ under an atmosphere of 5% CO_2. The culture medium was changed for fresh medium every third day. Upon reaching confluence, cultures were passaged by dissociation in 0.05% trypsin (Gibco-Life Technologies, Roseville, MD, USA) in 0.1% phosphate-buffered saline (PBS) at pH 7.4.

To evaluate the functional changes in human RPE cells under high-glucose conditions, the cultures were treated with D-glucose at final concentrations of 25 and 75 mmol/L and compared to cultures treated with 5.5 mmol/L D-glucose as controls. Cells were maintained in fresh medium for 2h prior to induction of high-glucose stress. Mannitol (27.5 mmol/L) was used to balance the different concentrations to exclude the potential effects of hyperosmotic stress[16].

Anti-vascular Endothelial Growth Factor Treatment of Cultured Human Retinal Pigment Epithelium Cells Confluent human RPE cells cultured in the presence of different D-glucose concentrations were treated with diluted bevacizumab (250 µg/mL) (Avastin; Roche, Basel, Switzerland), ranibizumab (125 µg/mL) (Lucentis; Genentech, South San Francisco, CA, USA), or aflibercept (500 µg/mL) (Eylea; Regeneron Pharmaceuticals, Tarrytown, NY, USA) for various times (3 or 14d) depending on the respective experiment. Further amounts of anti-VEGF agents were not added when medium was changed.

Cell Viability Assay Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay, as described previously with some modifications[17]. Briefly, the cells were seeded at a density of 2×10^5 cells/mL in 96-well plates and allowed to attach to the wells overnight. Then, cultured cells were treated with different concentrations of D-glucose (5.5 mmol/L, 25 mmol/L, 75 mmol/L) for 3d at 37 ℃. After 3d, the cells were incubated for an additional 4h in 10 µL of (5 mg/mL) MTT (Sigma-Aldrich) at 37 ℃ in a humidified 5% CO_2 atmosphere. The supernatant was subsequently removed, and MTT crystals were dissolved in 100 µL/well dimethyl sulfoxide (DMSO). Thereafter, optical density at 570 nm was read using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Treatment was performed with different anti-VEGF agents at clinically relevant concen-

trations (bevacizumab, 250 µg/mL; ranibizumab, 125 µg/mL; aflibercept, 500 µg/mL) in the presence of different glucose levels. The anti-VEGF agents were added together when cultured cells were treated with different concentrations of D-glucose. Experiments were repeated seven times. The percentage of cell viability was calculated as (OD of treated samples/OD of control)×100.

Wound Healing Assay Human RPE cells (2×10^5) were plated into the two wells of a culture insert (Cat. Number 80206; Ibidi GmbH, Martinsried, Germany) located at the center of a 35-mm culture dish. After 24h, when the cells had reached 90% confluency, the culture insert was removed to reveal the wound gap and the cells were washed with PBS to remove detached cells. The PBS was then replaced with fresh medium with three different glucose concentrations (5.5 mmol/L, 25 mmol/L, or 75 mmol/L). Immediately after removal of the culture inserts, baseline photographs were taken at a magnification of ×100 with a digital camera attached to an inverted microscope (Axio Observer A1;Carl Zeiss, Göttingen, Germany). Seventy-two hours later, another picture was taken at the same coordinates. The same set of experiments was repeated to evaluate the effects of each anti-VEGF agent on ARPE-19 cells. Bevacizumab, ranibizumab, or aflibercept was added to the ARPE-19 cells immediately after replacing fresh medium with different glucose concentrations. Experiments were repeated four times. For further quantitative analyses, the gap size of the wound was measured using Image J software (National Institutes of Health, Bethesda, MA, USA), and the percentage coverage of the wound was evaluated. Complete coverage was defined as 100%.

Cell Death Assay Human RPE cell death was assessed by the *in situ* terminal deoxynucleotidyl-transferase-mediated dUTP digoxigenin nick end labeling (TUNEL) assay. TUNEL staining was carried out using an *in situ* cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. After washing the confluent cell layers with PBS, fresh culture media with different glucose concentrations (5.5 mmol/L, 25 mmol/L, and 75 mmol/L) were replaced. The cell layers were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20min at 4 ℃ on day 3 or 14 after treatment. The cells were washed with PBS three times and incubated in permeabilization solution (0.1% Triton® X-100 and 0.1% sodium citrate) for 2min on ice. After washing with PBS, labeling reaction was performed with TUNEL reaction mixture. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 µg/mL; Sigma-Aldrich). The number of TUNEL-positive cells was calculated and photographs were taken using a fluorescence microscope at ×100 magnification. The experiment was repeated utilizing different anti-VEGF agents with different glucose levels. Experiments were repeated six times.
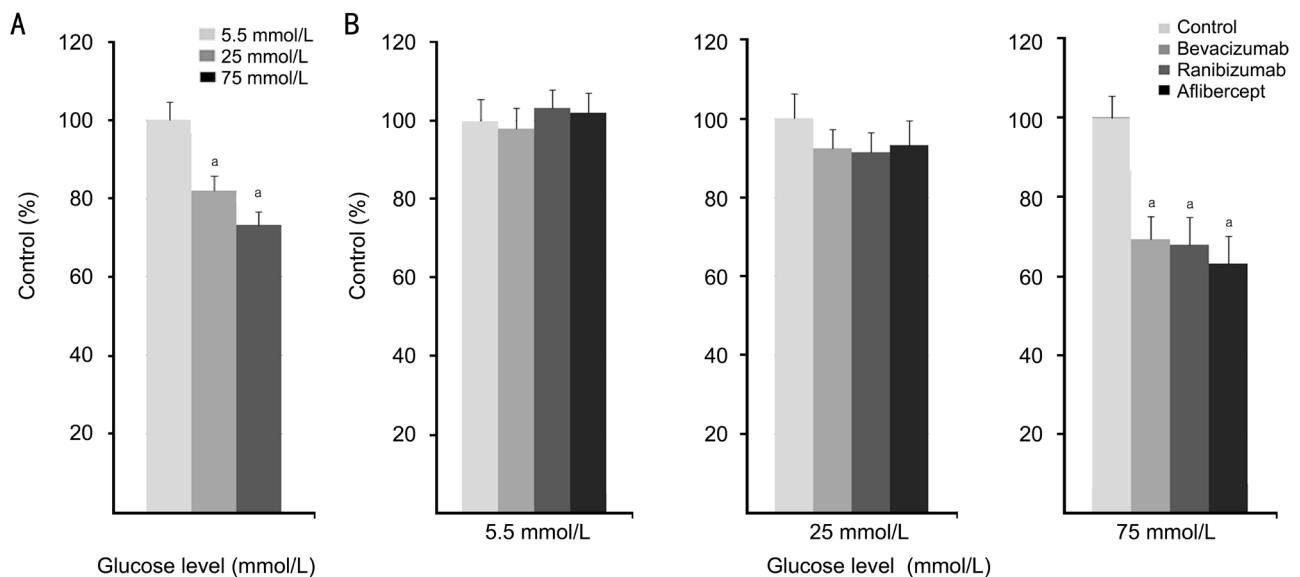
Figure 1 Cell viability with the effects of anti-VEGF agents at different concentrations of D-glucose after 3d Confluent ARPE-19 cells at the second or third passage were treated with anti-VEGF agents at clinically relevant concentrations for 3d. The toxicity was evaluated using a MTT assay (n=7). A: Cell viability decreased significantly in the presence of 25 mmol/L and 75 mmol/L D-glucose compared with 5.5 mmol/L D-glucose; B: The viability of cells treated with anti-VEGF agents was not significantly different compared to controls at 5.5 mmol/L or 25 mmol/L D-glucose. However, at 75 mmol/L D-glucose, cell viability treated with anti-VEGF agents was lower than that in controls. There was no difference between the anti-VEGF groups. [a]P <0.05.

EdU Assay Cellular proliferative activity was evaluated in ARPE-19 cells treated with different concentrations of D-glucose (5.5 mmol/L, 25 mmol/L, 75 mmol/L). On day 3, ARPE-19 cells were pulsed with 5 μmol/L EdU for 2h, fixed with 4% PFA, and then washed three times with PBS. The azide/alkyne cycloaddition reaction was performed according to the manufacturer's protocol using a Click-iT® EdU Alexa Fluor® 647 Imaging Kit (Invitrogen). The samples were then evaluated under a fluorescence microscope (detection at 647 nm), and the number of EdU-positive cells was calculated and photographed using a fluorescence microscope at ×100 magnification. The experiment was repeated with different anti-VEGF agents in the presence of different glucose concentrations. Experiments were repeated six times.

Statistical Analysis Data are presented as mean±SD. Each experiment was repeated independently at least four times. Significance was evaluated by Mann-Whitney U test. In all analyses, P<0.05 was taken to indicate statistical significance. Analyses were carried out using SPSS for Windows software (ver. 20.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Cell Viability The cell viability decreased significantly in the presence of 25 mmol/L (82.9%±3.8%) and 75 mmol/L (73.8%±3.3%) compared to 5.5 mmol/L glucose (P<0.05), but there was no significant difference in cell viability between 25 mmol/L and 75 mmol/L glucose (Figure 1A). The viability of cells treated with any of the anti-VEGF agents was not significantly different from controls in the presence of 5.5 mmol/L or 25 mmol/L glucose. However, at 75 mmol/L glucose, the viability of cells treated with anti-VEGF (bevacizumab 69.3%±5.1%, ranibizumab 68.1%±6.3%, aflibercept 63.2%±6.9%) showed significant decreases (P<0.05). There were no differences between groups treated with the different anti-VEGF agents (Figure 1B).

Wound Healing Wound healing assay determines the migratory and proliferative activities of cells. Here, we evaluated the effects of glucose on wound healing in cultured ARPE-19 cells. In this assay, the effects of 25 mmol/L and 75 mmol/L glucose were compared with normal glucose level (5.5 mmol/L). Wound healing activity of cells cultured with high-glucose medium was significantly decreased compared with normal glucose conditions (wound closure, 14.8%±4.6% at 75 mmol/L *vs* 26.3%±3.7% at 5.5 mmol/L, P<0.05). There was no significant difference between 5.5 mmol/L and 25 mmol/L (25.2%±4.2% at 25 mmol/L, P=0.21).

The effects of three different anti-VEGF agents on wound healing were also evaluated at each glucose concentration. No significantly cumulative adverse effects on wound healing were detected with the addition of each anti-VEGF agent to the culture medium (Figure 2).

Cell Death Assay No differences were observed on day 3 among all groups (data not shown). However, the apoptosis percentage after treatment with 75 mmol/L glucose (the number of TUNEL positive cells/field, 29.2±5.3; the ratio of TUNEL positive cells/DAPI positive cells, 4.8%±0.5%) was significantly higher than that in the presence of 5.5 mmol/L D-glucose on day 14 (the number of TUNEL positive cells/field, 16.3±3.2; the ratio of TUNEL positive cells/DAPI positive
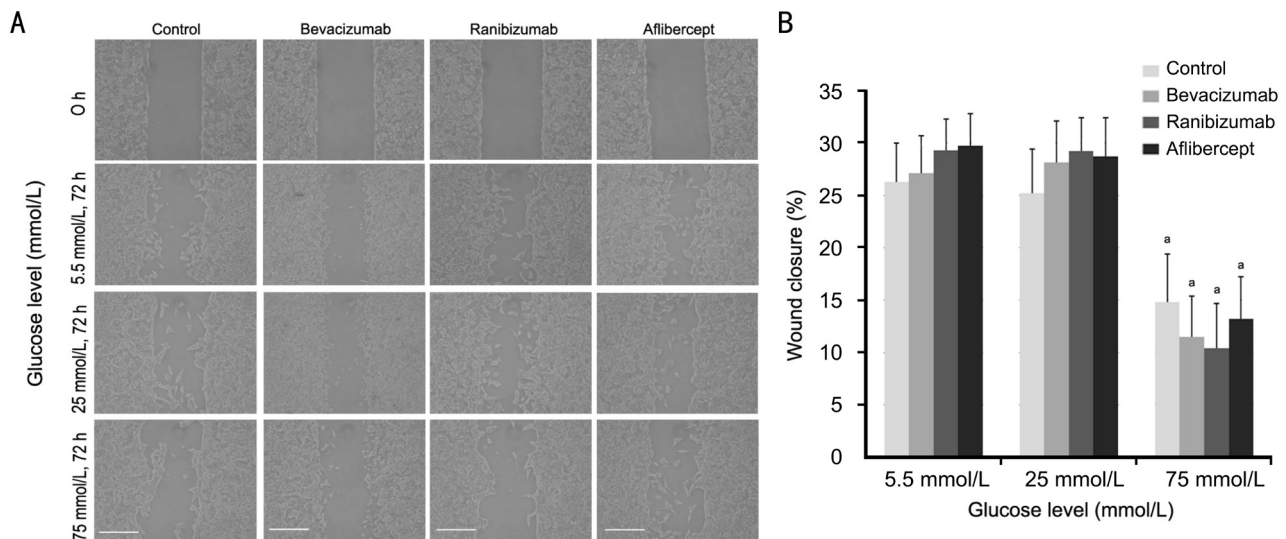
Figure 2 The wound healing assay in cells treated with anti-VEGF agents at different concentrations of D-glucose after 3d The migration ability was determined using a wound healing assay in confluent RPE cell layers. Microscopic bright field photographs were taken and the precise coordinates were reassessed after 72h of anti-VEGF treatment at clinically relevant concentrations. A: Scratch; B: Quantification (*n*=4). At 75 mmol/L D-glucose, all groups showed significantly decreased wound healing compared with 5.5 mmol/L D-glucose and 25 mmol/L D-glucose. However, there was no significant difference between 5.5 mmol/L and 25 mmol/L D-glucose. None of the anti-VEGF treatment groups showed significant effects on wound healing compared to controls at any D-glucose concentration. Scale bars represent 100 μm. [a]*P* <0.05.

cells, 3.2%±0.3%; *P*<0.05). There was no significant difference in apoptosis percentage between 25 mmol/L D-glucose (the number of TUNEL positive cells/field, 18.3±4.5; the ratio of TUNEL positive cells/DAPI positive cells, 3.2%±0.3%), and 5.5 mmol/L D-glucose (Figure 3A, 3C). On day 14, there was no significant difference in the percentage of TUNEL positive cells between any of the anti-VEGF groups and controls at all D-glucose levels (Figure 3B, 3D).

Cell Proliferation After Treatment The proliferation percentage in the presence of 75 mmol/L D-glucose (the number of EdU positive cells/field, 20.1±6.2; the ratio of EdU positive cells/DAPI positive cells, 4.2%±1.2%) was significantly lower than that with 5.5 mmol/L D-glucose (the number of EdU positive cells/field, 87.4±5.5; the ratio of EdU positive cells/DAPI positive cells, 8.3%±0.9%; *P*<0.05). There was no significant difference in proliferation percentage between 25 mmol/L and 5.5 mmol/L D-glucose in all groups (Figure 4A, 4C). There was no significant difference in proliferation percentage between anti-VEGF agents and controls in the presence of 5.5 mmol/L and 25 mmol/L D-glucose. However, the proliferation percentages of cells treated with the anti-VEGF agents were lower than that in the control cultures in the presence of 75 mmol/L D-glucose (the number of EdU positive cells/field: bevacizumab, 10.2±7.3; ranibizumab, 15.2±7.9; aflibercept, 16.4±7; the ratio of EdU positive cells/ DAPI positive cells: bevacizumab, 1.8%±0.9%; ranibizumab, 2.8%±1.1%; aflibercept, 2.9%±1.2%; *P*<0.05). Furthermore, the bevacizumab group was significantly lower than that in the other groups treated with ranibizumab or aflibercept (*P*<0.05; Figure 4B, 4D).

DISCUSSION

Using *in vitro* studies, 5.5 mmol/L D-glucose corresponded to fasting plasma D-glucose levels of subjects without type-1 DM. However, plasma D-glucose levels of diabetic patients vary, depending on the ability to regulate D-glucose levels. Chen *et al*[18] investigated the effects of high D-glucose levels on secreted proteomes in cultured RPE cells, reporting that 25 mmol/L D-glucose corresponded to plasma levels 2h after a meal in diabetic patients and 100 mmol/L D-glucose corresponded to plasma levels 2h after a meal in uncontrolled diabetic patients. Thus, in the present study, 5.5 mmol/L D-glucose treatment of cultured ARPE-19 cells was used to mimic normal controls, and 25 and 75 mmol/L D-glucose treatment of cultured ARPE-19 cells was used to mimic diabetic retinopathy.

Here, we investigated the effects of high glucose levels and anti-VEGF agents on RPE cells with regard to toxicity, wound healing ability, apoptosis, and proliferation. RPE cells showed reduced viability, wound healing ability, and proliferation, along with increased apoptosis, in the presence of glucose at 75 mmol/L as compared to 5.5 mmol/L. However, there were no significant differences in these results between 25 and 5.5 mmol/L glucose except cell viability.

There is some controversy regarding whether anti-VEGF agents are toxic to RPE cells, with most investigators concluding that they show no toxicity[12,19-22]. Our data were consistent with these findings, as anti-VEGF agents in the presence of 5.5 or 25 mmol/L glucose showed no toxic effects on ARPE-19 cells after 3d in MTT assay. However, the viability of RPE cells decreased when glucose level was high
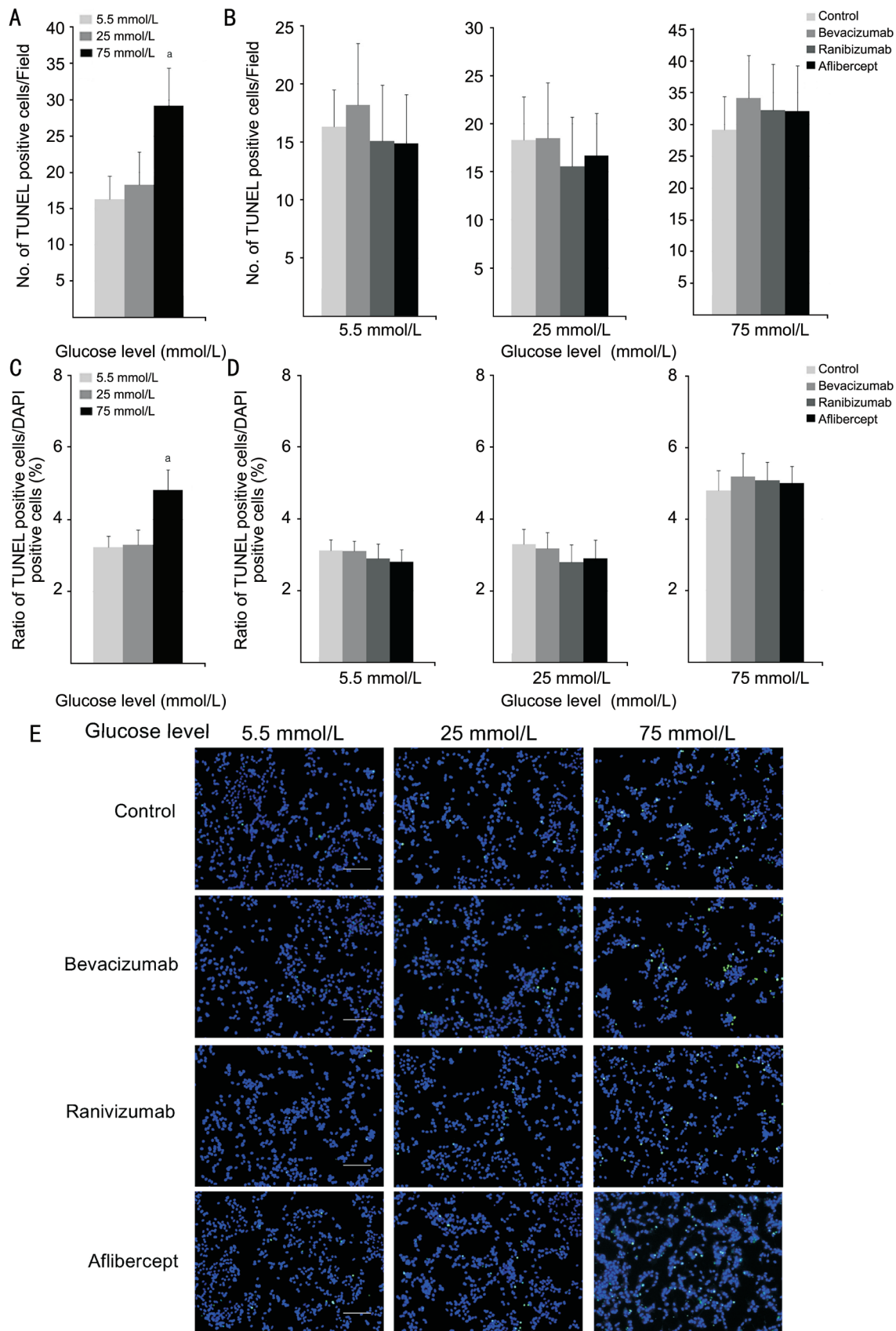
Figure 3 Apoptosis was assessed using the TUNEL assay in anti-VEGF-treated cells in the presence of different concentrations of D-glucose after 14d The percentages of apoptotic cells were calculated (*n*=6). A, C: At 14d, the percentage of apoptosis in the presence of 75 mmol/L D-glucose was significantly higher than that at 5.5 mmol/L D-glucose (*P*<0.05); B, D: There was no significant difference in the percentage of apoptosis between the anti-VEGF groups and controls at any D-glucose concentration; E: The green cells indicate TUNEL-positive apoptotic cells and the blue cells indicate DAPI-positive cells. The number of apoptotic cells increased in the presence of 75 mmol/L D-glucose compared with the presence of 5.5 mmol/L D-glucose at 14d ([a]*P*<0.05, Mann-Whitney *U* test). Scale bars represent 40 μm.
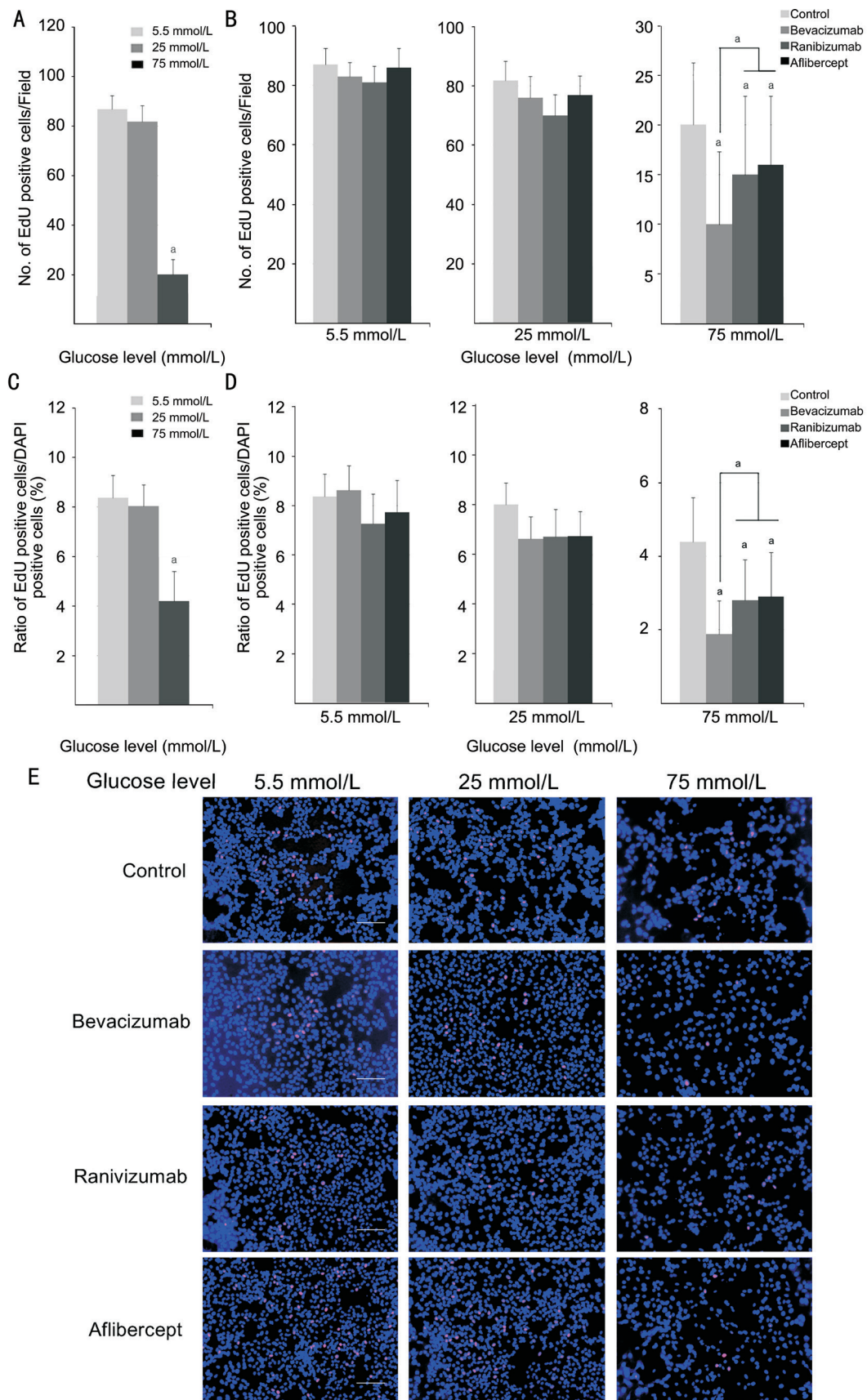
Figure 4 EdU proliferation assay of the effects of anti-VEGF treatment at different concentrations of D-glucose after 3d The percentages of cell proliferations were calculated (n=6). A, C: The proliferation in the presence of 75 mmol/L D-glucose was significantly lower than that at 5.5 mmol/L D-glucose. There was no significant difference in proliferation between 25 mmol/L and 5.5 mmol/L D-glucose; B, D: There was no significant difference in cell proliferation between the anti-VEGF groups and controls at 5.5 mmol/L or 25 mmol/L D-glucose. However, at 75 mmol/L D-glucose, cell proliferation was lower in the anti-VEGF treatment groups compared with controls. Furthermore, the group treated with bevacizumab showed significantly lower proliferation than the other groups treated with ranibizumab or aflibercept; E: The red cells indicate EdU positive RPE cells and the blue cells indicate DAPI positive cells. The number of proliferating cells decreased after treatment with 75 mmol/L D-glucose compared with 5.5 mmol/L D-glucose. Scale bars represent 40 μm. [a]P<0.05.

(75 mmol/L). Especially, the viability of cells treated with anti-VEGF agents was lower than that in the control group with 75 mmol/L glucose. We hypothesized that if hyperglycemia results in RPE cell damage, the cells would be more susceptible to the toxic effects of anti-VEGF agents. The results of TUNEL assay also showed that anti-VEGF agents did not significantly alter the apoptosis rate of RPE cells compared to controls at both 5.5 and 25 mmol/L glucose. However, a tendency toward an increased apoptosis rate was seen in anti-VEGF agent treatment groups compared to controls at the high glucose level of 75 mmol/L.

The wound healing ability of the RPE cells decreased in the presence of a high glucose level (75 mmol/L). However, the clinically relevant concentrations of anti-VEGF agents did not exhibit significant effects on wound healing compared to controls at any glucose level examined. Klettner et al[12] reported that aflibercept and bevacizumab significantly decreased wound healing ability at clinically relevant concentrations. In the present study, D-glucose was used instead of fetal calf serum to investigate the effects of different glucose levels on RPE cells. Therefore, proliferation induced by hormones such as IGF-1 and cytokines associated with cell proliferation in fetal calf serum may have affected the previous results. For example, at the clinically relevant concentrations used in the previous study[12], the cells showed approximately 70%-80% wound closure at 24h. In the present study, however, 20% wound closure was observed at 72h. These discrepancies were considered to be due to differences in the methods used between the two studies. However, both studies showed the potential toxicity of anti-VEGF agents. It is may be clinically relevant that high glucose level decreased wound healing capability, viability, and proliferation of RPE. Laser therapy induces wound healing reactions in RPE cells, which is considered to be part of the therapeutic effect[23-24]. Therefore, changes in retinal function should be observed carefully in diabetic retinopathy patients undergoing laser therapy, especially in combination with intravitreal anti-VEGF injection.

Spitzer et al[25] investigated the effects of bevacizumab and ranibizumab on proliferation of ARPE-19 cells; in these experiments, only bevacizumab at 0.3 mg/mL (clinically relevant dose) showed a significant inhibitory effect on proliferation. Our results suggested that the proliferation of ARPE-19 cells treated with bevacizumab in the presence of 75 mmol/L glucose was lower compared to those treated with ranibizumab or aflibercept. However, our experiment was focused on glucose level control and anti-VEGF treatment under in vitro conditions. The clinical efficacy involves multiple factors in addition to direct inhibition of cell proliferation, such as drug half-life, biostability, and retinal penetration. In addition, the levels of inflammatory cytokines (i.e. NO, sIL-2R, IL-8

and TNF-α levels) in patients with diabetic retinopathy were increased and RPE cells of diabetic retinopathy patients would behave differently under normal conditions[26]. Furthermore, anti-VEGF agents were used at clinically relevant concentrations with reference to the intravitreal concentration immediately after injection. However, these may not reflect the actual concentrations reached in the RPE cells. Thus, no direct conclusions can be drawn from our results regarding which of the three drugs may be more beneficial, or whether they are equally efficacious, in patients with DME or macular edema.

A limitation of our study was the small sample size. The present study showed that high D-glucose levels decreased the physiological functions of RPE cells, and that anti-VEGF agents at clinically relevant concentrations in the presence of high D-glucose concentrations decreased cell viability and proliferation, and induced an increase in apoptosis. To strengthen the conclusions, a larger sample size is suggested. Even though a small sample size was analyzed, we believe that our study is important because it showed that anti-VEGF agents interfered with the physiological functions of RPE cells under high-glucose conditions.

ACKNOWLEDGEMENTS

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