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Anti-angiogenic effect of deguelin on choroidal neovascularization

Jeong Hun Kim, Jin Hyoung Kim, Young Suk Yu,

Kyu Hyung Park, Hye Jin Kang, Ho-Young Lee, Kyu-Won Kim

Department of Ophthalmology, College of Medicine, Seoul National University & Seoul Artificial Eye Center Clinical Research Institute, Seoul National University Hospital, Seoul, Korea (Kim JH, Kim JH, Yu YS); [2]Department of Ophthalmology, College of Medicine, Seoul National University & Bundang Seoul National University Hospital, Seong-nam, Korea (Park KH); [3]NeuroVascular Coordination Research Center, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea (Kang HJ, Yu YS); [4]Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA (Lee HY)

- Running title: **Inhibition of choroidal neovascularization by deguelin**

- Correspondence should be addressed to Dr. Young Suk Yu, Department of Ophthalmology, College of Medicine, Seoul National University & Seoul Artificial Eye Center Clinical Research Institute, Seoul National University Hospital, Seoul 151-744, Republic of Korea, Phone: 82-2-2072-3492; Fax: 82-2-741-3187; E-mail: ysyu@snu.ac.kr

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Abstract

Age-related macular degeneration is the leading cause of blindness in elderly. choroidal neovascularization (CNV) leads to severe vision loss in patients of AMD. Previously, we have demonstrated that deguelin, isolated from plants in the Mundulea sericea family, is a chemo-preventive agent. This study is to evaluate the anti-angiogenic effect of deguelin on CNV. The toxicity of deguelin was evaluated through MTT assay in HUVECs as well as histological examination and TUNEL staining in the deguelin-injected retina. Anti-angiogenic activity of deguelin was evaluated by *in vitro* tube formation assay of human umbilical vein endothelial cells (HUVECs) and *in vivo* angiogenesis of chick chorioallantoic membrane (CAM). In C57BL/6 mice with laser-induced CNV, deguelin or PBS was injected intravitreously. CNV lesions were examined by fluorescence angiography and vessel counting in cross sections. Deguelin showed no effect on cell viability of HUVECs and no retinal toxicity in a concentration range of 0.01-1μM. Deguelin effectively inhibited *in vitro* tube formation of HUVECs and *in vivo* angiogenesis of CAM. Interestingly, deguelin significantly reduced CNV and its leakage in mouse model of laser-photocoagulation-induced CNV. Our data suggests that deguelin is a potent inhibitor of CNV and may be applied in the treatment of other vaso-proliferative retinopathies such as retinopathy of prematurity and diabetic retinopathy.

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Introduction

Angiogenesis occurs with the imbalance between positive and negative factors that keep angiogenesis. Under certain conditions such as hypoxia or inflammation that activate angiogenic factors, the balance may shift to angiogenesis (Folkman, 2006). Stimulation of endothelial cells with angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) induce endothelial cells to produce and export a plethora of proteases, such as matrix metalloproteases and plasminogen activators to digest the basement membrane and to allow endothelial cells to invade the surrounding tissue. Endothelial cells proliferate and migrate to form a sprout (Risau, 2003). Pathological angiogenesis in the eye is the most common cause of blindness in all age groups (Aiello *et al.*, 1994). Particularly, age-related macular degeneration (AMD) is the leading cause of blindness over the age of 55 years in developed countries (Klein *et al.*, 1995). Although the detailed mechanism was not elucidated as yet, choroidal neovascularization (CNV) leads to severe vision loss in patients of AMD (Macular Photocoagulation Group, 1991). CNV invades the subretinal space by the vessel proliferation from the choroidal vessels following the rupture of Bruch's membrane. The new vessels are fragile and leaky inducing hemorrhages or exudation damaging to photoreceptor cell function. On the other hand, these proliferating vessels induce the formation of fibrovascular scar, which result in the irreversible damage to the retinal function and likely blindness (Gehrs *et al*., 2006). Since excessive proliferation of vascular cells is the essential mechanism of CNV, it is

reasonable to directly ameliorate the proliferating vascular endothelial cells. The most common model of CNV is created by the laser-photocoagulation-induced rupture of Bruch's membrane, which stimulates proliferation of vascular endothelial cells in pre-existing choroidal capillary networks (Miller *et al*., 1986).

Rotenoids, compounds from the flavonoid family, have chemo-preventive activity by inhibiting NADH:ubiquinone oxidoreductase activity and by suppressing steady-state mRNA levels and enzymatic activity of 12-*O*-tetradecanoylphorbol 13-acetateinduced ODC (Gerhäuser *et al*., 1995; Fang and Casida, 1998). The rotenoid, deguelin, was found in several plant species, including *Mundulea sericea* (*Leguminosae*) (Gerhäuser *et al*., 1997). We have demonstrated that deguelin inhibits cyclooxygenase-2 expression and phosphatidylinositol 3-kinase (PI3K)/Akt-mediated signaling pathways and that contributes to its anti-proliferative effects (Chun *et al*., 2003; Lee, 2004; Lee *et al*., 2004; Lee *et al*., 2005). However, we also found that deguelin reduced the expression of Hsp90-binding proteins, including HIF-1α protein and induced the degradation of HIF-1α protein independent of the reactive oxygen species and PI3K-Akt pathways (Oh *et al*., 2007).

In the present study, we demonstrate that deguelin inhibits *in vitro* angiogenesis of human umbilical vein endothelial cells (HUVECs) and *in vivo* angiogenesis of CAM without cytotoxic effect, and significantly reduces laser-induced CNV in a mouse model of AMD without significant retinal toxicity. In addition to the anti-proliferative activity of deguelin to cancer cells (Chun *et al*., 2003; Lee, 2004; Lee *et al*., 2004; Lee *et al*., 2005; Oh *et al*., 2007), we herein suggest that deguelin, a

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new angiogenesis inhibitor, may have a therapeutic potential in the treatment of CNV of AMD as well as in other vaso-proliferative retinopathies such as retinopathy of prematurity (ROP) and diabetic retinopathy.

Methods

Animals

C57BL/6 mice were purchased from Samtako (Korea). Care, use, and treatment of all animals in this study were in strict agreement with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Cell culture

HUVECs were maintained in a gelatin-coated dish at 37 □ in a humidified atmosphere of 5 % CO2/95 % air in M199 medium with 20 % fetal bovin serum, 100 units/ml penicillin, 100 g/ml streptomycin, 3ng/ml basic fibroblast growth factor and 1ml heparin. HUVECs used in this study were taken from passages 6 to 7.

Preparation of Deguelin

Deguelin was manufactured from the natural product rotenone (Sigma-Aldrich, Milwaukee, WI, USA) via four steps, as previously described (Anzenveno, 1979). The final product was more than 98 % pure. Deguelin was stored at a stock concentration of 1mM in a nitrogen tank.

Cell viability assay

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-

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diphenyltetrazolium bromide (MTT) assay. HUVECs ($1x10^5$ cells) were plated in 96 well plates and cultured overnight. Cells were treated with deguelin (0.01~10 μM) for 48 hrs. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate and DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

Deguelin was intravitreously injected to 7- to 8-week-old female C57BL/6J mice. The mice were sacrificed at 3 days after 0.1 μM deguelin injection, and eyes were enucleated. Enucleated globes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h, and embedded in paraffin. TUNEL staining was performed with a kit (ApopTag Fluorescein Green; Intergen, Purchase, NY, USA), according to the manufacturer's instructions. TUNEL-positive cells were evaluated in randomly selected fields at ×400 magnification viewed under fluorescein microscopy (BX50, OLYMPUS, Japan).

Tube formation assay

Tube formation was assayed as described previously (Min *et al*., 2007). HUVECs (1×10^5 cells) were inoculated on the surface of the Matrigel, and treated with 0.1

μM deguelin or VEGF (20 ng/ml) for 18 h. The morphological changes of the cells and tubes formed were observed under a microscope and photographed at ×200 magnification. Tube formation was quantified by counting the number of connected cells in randomly selected fields at ×200 magnification (Carl Zeiss, Chester, VA, USA), and dividing that number by the total number of cells in the field.

Chick chorioallantoic membrane (CAM) assay

Three-day-old fertilized eggs were incubated at 37 □, and a window was made after the extraction of 3~4 ml ovalbumin. After 2 days of incubation, a thermanox coverslip (Nunc, NY, USA) covered with deguelin (0.1 μM) was applied on the CAM of individual embryos. After 48 h, the intralipose was injected into the chorioallantois of the embryos, and CAMs were evaluated (Carl Zeiss, Chester, VA, USA).

Western Blotting

Cell extracts were prepared and aliquots (10~30 μg protein) were loaded onto 9 % gel and transferred to protein nitrocellulose membrane. The membrane was incubated in blocking buffer (5 % skim milk in PBS-T) at room temperature. Then the filter was incubated with anti-VEGF antibody (1:2000, Santa Cruz) at 4 ℃ for overnight and washed with 0.1 % Tween 20 in PBS (PBS-T) three times every 10 min, followed by incubation with anti-rabbit polyclonal antibody at room temperature for 1 h. Immunoreactive bands were visualized using

chemiluminescent reagent.

Laser-photocoagulation-induced CNV

7- to 8-week-old female C57BL/6J mice were anesthetized, and the pupils were dilated with 1% tropicamide (Alcon Laboratories Inc., Forth Worth, TX, USA). Three burns of 831-nm diode laser photocoagulation (75 μm spot size, 0.1-second duration, 120 mW) were delivered to each 3, 6, 9, and 12 o'clock position of 2 disc diameters from optic disc by using indirect head set delivery system of a photocoagulator (OcuLight; Iridex, Mountain View, CA, USA) and a handheld +78 diopter lens. The bubbling or pop sensing with laser photocoagulation was considered as the successful rupture of Bruch's membrane.

To assess the anti-angiogenic activity of deguelin, the mice were injected intravitreously with 0.1 μM deguelin in 1μl PBS on the tenth day after laser photocoagulation, when maximum CNV began. These experiments were repeated at least 25 times.

Qualitative assessment of CNV by fluorescein angiography

At 14 days after laser photocoagulation, deeply anesthetized mice were perfused through the tail vein with high molecular weight (MW=500,000) fluorescein conjugated dextran (Sigma–Aldrich Ltd., St. Louis, MO, USA) dissolved in PBS. After 1 h perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 4 hours. The eyeballs were dissected, flat-mounted in Dako mounting medium

(DakoCytomation, Glostrup, Denmark), and viewed by fluorescein microscopy (BX50, OLYMPUS, Japan) at a ×100 magnification.

Quantitative assessment of CNV by counting vessels from subretinal fibrovascular membrane

At 14 days after laser photocoagulation, the eyes were removed, fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer for 24 h, and embedded in paraffin. Sagittal sections of 4-5 μm, each 10 μm apart, were cut through the center of laser-photocoagulation site. The sections were stained with hematoxylin and eosin to assess CNV via light microscopy (Carl Zeiss, Chester, VA, USA). Any vessels from subretinal fibrovascular membrane were counted in 5 sections from each laser-photocoagulation site by two independent observers blind to treatment (Kim JH and Yu YS). The average was calculated for one hundred sites of each group. There were at least 25 animals in each group.

Statistical Analysis

Statistical differences between groups were evaluated with the Student unpaired t-test (two-tailed). Mean ± SD is shown. $P \leq 0.05$ was considered significant.

Results

Effect of deguelin on the viability of HUVECs

To investigate cytotoxic effect of deguelin on HUVECs, MTT assay was carried out in various concentrations of deguelin (0~10μM). The viability of HUVECs was not affected up to 1 μM deguelin (Figure 1). 0.1μM deguelin, effective therapeutic concentration to inhibit VEGF expression in our previous report (Oh *et al*., 2007), did not affect the viability of HUVECs.

Retinal toxicity of deguelin

Retinal toxicity to intravitreal injection of 1 μM deguelin (10 times of effective therapeutic concentration of deguelin) was evaluated through histological examination and TUNEL assay. As demonstrated in figure 2, the retina was of normal thickness and all retinal layers were clear without any cell inflammation in the vitreous, retina, or choroid. Compared to control, TUNEL positive cells were not increased with deguelin injection.

Effect of deguelin on tube formation of HUVECs

To investigate the effect of deguelin on the angiogenic phenotype of tube formation *in vitro* assay, VEGF was used as an angiogenic factor. VEGF induced the formation of extensive capillary-like networks of HUVECs cultured on 2-D Matrigel matrix, and this effect was almost completely inhibited by co-treatment

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with deguelin (Figure 3A). VEGF stimulated tube formation of HUVECs approximately 1.3-fold, and this effect was abolished by deguelin (Figure 3B).

Effect of deguelin on *in vivo* angiogenesis of CAM

Consistent with inhibition of tube formation, *in vivo* angiogenesis of CAM was blocked by deguelin (Figure 4A). Deguelin inhibited in vivo angiogenesis to about 70 % (Figure 4B).

Effect of deguelin on VEGF expression in HUVECs and laser-photocoagulation-induced CNV

Based on our previous data of inhibition of VEGF expression (Oh *et al*., 2007), we injected deguelin intravitreously with 0.1μM deguelin in 1μl PBS on 10 days after laser photocoagulation when active neovascularization occurs. We confirmed that 0.1μM deguelin dramatically reduces VEGF expression in HUVECs (Figure 5A). To visualize CNV, fluorescein angiography using fluorescein conjugated dextran was performed. Retinas from control mice subjected to laser photocoagulation showed CNVs with diffuse leakage on the laser photocoagulation site (Figure 5B). In contrast, retinas from deguelin-treated mice showed significantly reduced CNV and its leakage (Figure 5C). To quantify CNV, vessels from subretinal fibrovascular membrane were counted in a masked fashion. The vessels were evaluated as the mean number per section found in 5 sections per laser-photocoagulation site. Retinas from control mice demonstrated multiple CNVs

(Figure 5D), whereas retinas from deguelin-treated mice showed significantly fewer neovascular lumens (Figure 5E). We found that deguelin-injected groups had significant decrease of CNV compared to controls (Figure 5F).

Discussion

We have demonstrated that deguelin has anti-proliferative activity to cancer cells via regulation of cyclooxygenase-2 expression and phosphatidylinositol 3-kinase (PI3K)/Akt-mediated signaling pathways (Chun *et al*., 2003; Lee, 2004; Lee *et al*., 2004; Lee *et al*., 2005). Recently, we also found out that deguelin reduces VEGF expression through the destabilization of HIF-1α protein. Interestingly, this anti-angiogenic activity of deguelin was independent on reactive oxygen species and PI3K-Akt pathways (Oh *et al*., 2007).

Because deguelin is derived from rotenone, which can inhibit NADH: ubiquinone oxidoreductase in mitochondrial oxidative phosphorylation (Fang and Casida, 1998), it is possible that deguelin induces side effects in cardiovascular, respiratory, and nervous system. However, we never observed major toxicity in the mice at the therapeutically effective dose (Lee *et al*., 2005; Oh *et al*., 2007). In terms of the mechanism of action deguelin may be different from that of rotenone to inhibit tubulin polymerization (Marshall and Himes, 1978; Srivastava and Panda, 2007), which could be one reason that deguelin is safer. However, it is also possible that the reason of no grave toxicity in deguelin may be in degree, which means that deguelin may be a weaker inhibitor. In this study, deguelin showed no effect on the viability of HUVEC cells and no retinal toxicity up to 1μM which is equivalent to 10 times of effective dose (0.1 μM) to CNV.

The blood vessel growth in CNV correlates with the expression of VEGF, bFGF, and their receptors (Edelman and Castro, 2000). At the concentration to effectively

reduce VEGF expression in cancer cells and that inhibit tumor growth (Oh *et al*., 2007), deguelin reduces VEGF expression in HUVECs (Figure 5A), and inhibits *in vitro* tube formation (Figure 3) as well as *in vivo* chorioallantoic membrane of chick embryo (Figure 4). Choroidal neovascularization and vascular leakage are two important factors that induce serious visual loss in AMD. In the present study, we presented the inhibitory effect of deguelin on choroidal neovascularization. Deguelin reduced the incidence of clinically significant vascular leakage in an experimental model of CNV. This was consistent with histologic findings of significantly less number of CNV in deguelin-treated group. Based on these results, it is possible that deguelin may attenuate laser-photocoagulation-induced CNV through a direct anti-angiogenic effect without cytotoxic effect in the therapeutic range.

Given the well-documented anti-proliferative effect on HUVECs and anti-angiogenic effect on CAM and CNV in the present study, we suggest that deguelin could be a new anti-angiogenic agent to CNV, which is from rotenone. Furthermore, deguelin may be also applied to other vaso-proliferative retiniopathies such as ROP and diabetic retinopathy.

Acknowledgments

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Footnotes

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Legends for Figures

Figure 1. Effect of deguelin on the viability of HUVECs. Various concentrations of deguelin (0~10 μM) were treated on HUVECs and cells were incubated for 2 days. Cell viability was measured by MTT assay. Each value represents means ± SE from three independent experiments (*P<0.05).

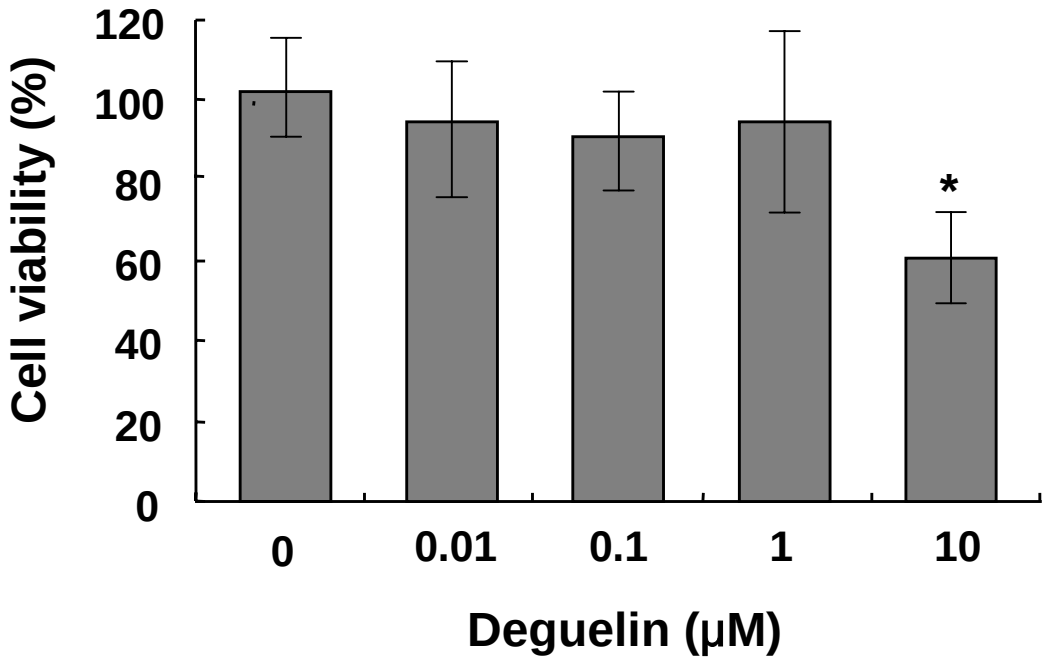
Figure 2. Retinal toxicity of deguelin. 1μM deguelin was intravitreously injected, and the globes were enucleated 3 days after treatment. The retina was normal without any inflammatory cells in the vitreous, retina, or choroid. Scale bars, 50 μm.

Figure 3. Effect of deguelin on VEGF-induced tube formation of HUVECs. (A) Each Figure is representative ones from three independent experiments. (B) The basal tube formation of HUVECs that were left in serum free media was normalized to 100 %, respectively. Each value represents means ± SE from three independent experiments (*P<0.05).

Figure 4. Effect of deguelin on *in vivo* angiogenesis of CAM. (A) Each Figure is representative ones from three independent experiments. Sector form indicates the position of thermanox with PBS or 0.1 μM deguelin. (B) Each value to indicate Inhibition of capillary formation represents means ± SE from three independent experiments (*P<0.05).

Figure 5. Effect of deguelin on VEGF expression in HUVECs and laser-photocoagulation-induced CNV. (A) 0.1 μM deguelin dramatically reduces VEGF expression in HUVECs. Wholemount preparation from control (B) and 0.1 μM deguelin-treated (C) mice subjected to laser-photocoagulation-induced CNV was performed after I hour perfusion of fluorescein conjugated dextran, respectively. Circles indicate CNVs in the laser-photocoagulation site. Hematoxylin-stained cross-sections prepared from control (D) and 0.1 μM deguelin-treated (E) mice subjected to CNV, respectively. Arrows show the new vessels growing from choroidal vessels. (F) Data in each column are the mean ± SD values from 100 sites of 25 mice (*P<0.05). Scale bars, 50 μm (D, E).

Figure 1.



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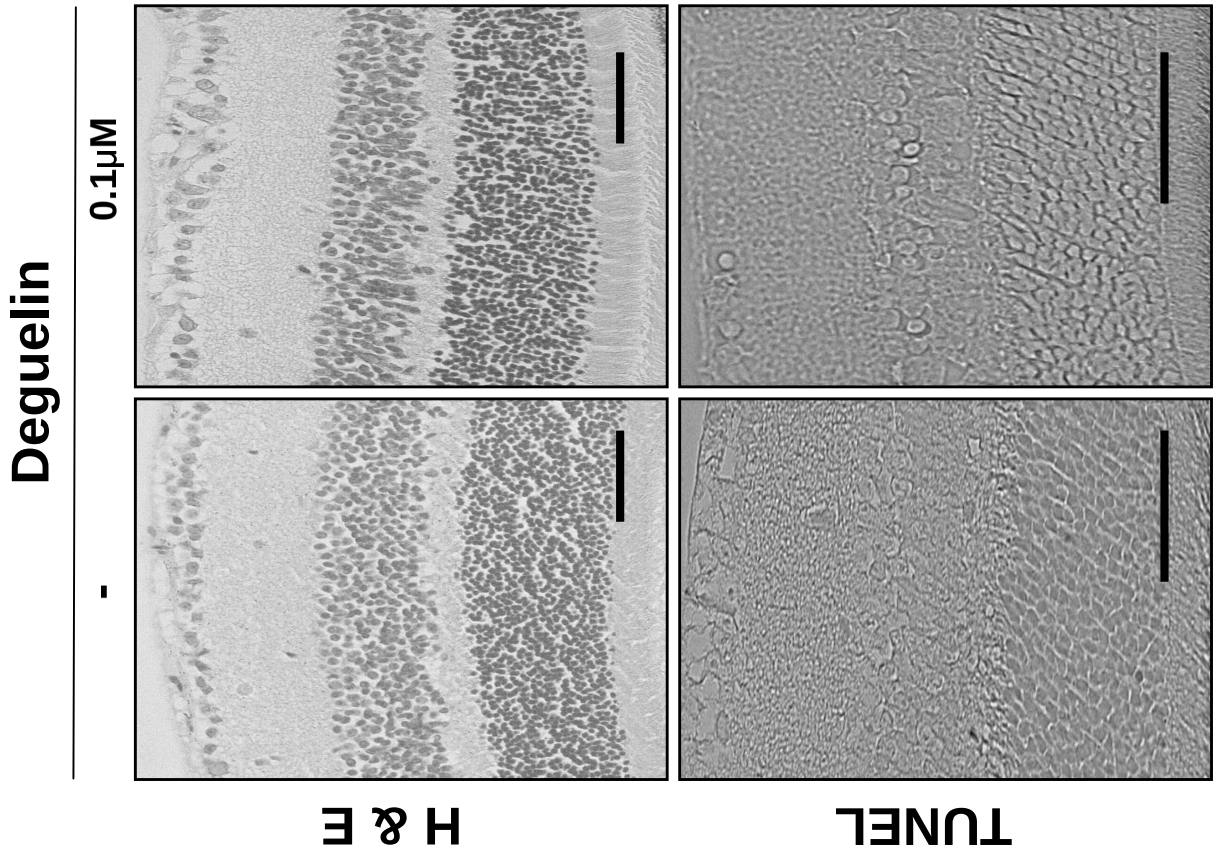

Figure 2.

Figure 3.

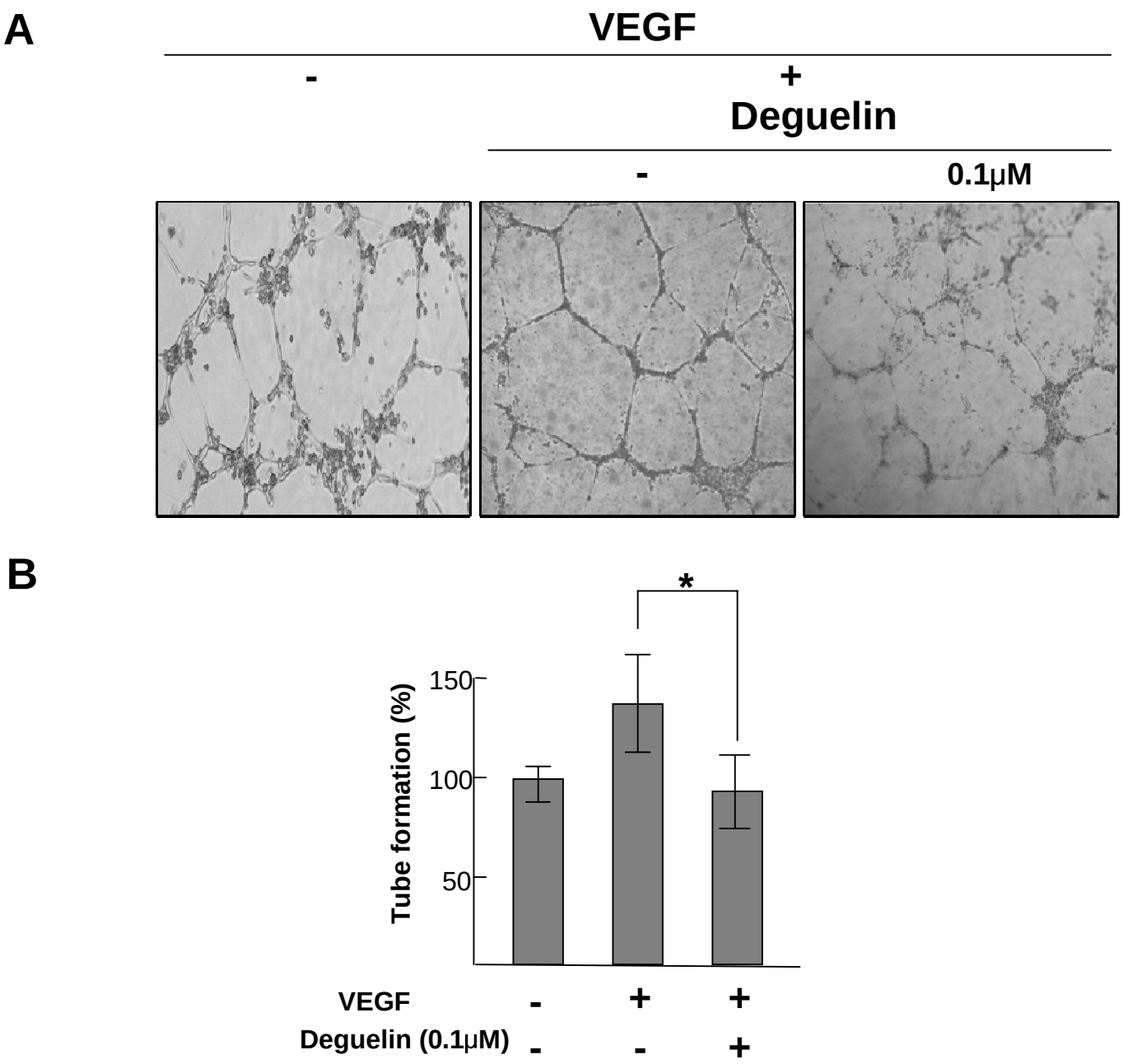

Figure 4.

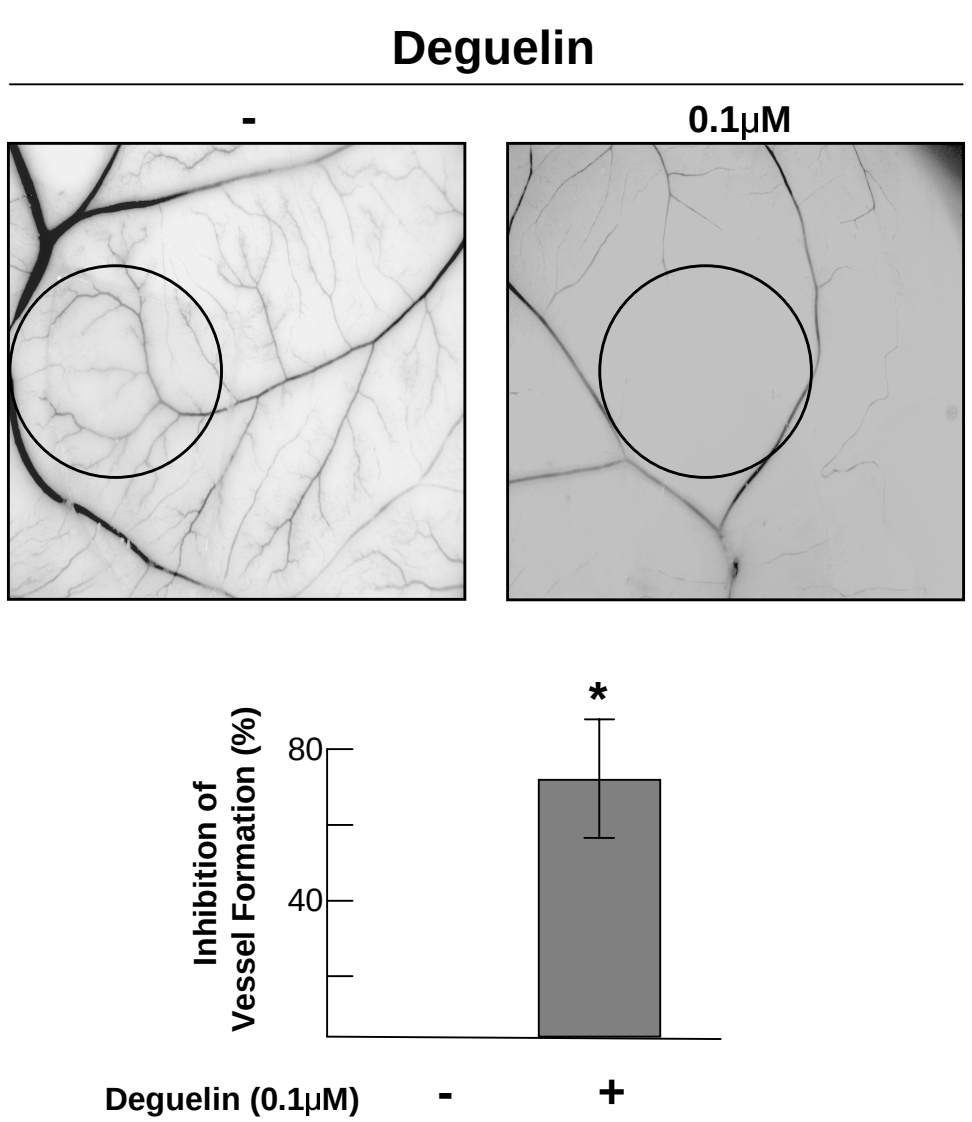

Figure 5.