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Anti-angiogenic effect of EGHB010, a standardized herbal formula of Paeoniae radix and Glycyrrhizae radix

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Abstract: EGHB010 is a standardized herbal formula of the rhizome mixture of *Paeonia lactiflora* Pallas and *Glycyrrhiza uralensis* Fisch. Neovascularization in the retina is a common pathophysiology of diabetic retinal microvasculopathy and exudative macular degeneration. In this study, we evaluated the inhibitory effects of EGHB010 on abnormal retinal angiogenesis in a hyperoxia-induced neovascular retinopathy model. Vascular endothelial growth factor (VEGF)-mediated vascular tube formation was assayed in human umbilical vascular endothelial cells (HUVECs). Experimental angiogenesis in the retinas was induced by exposing C57BL/6 pups to hyperoxic environment (75% oxygen) on postnatal day 7 (P7) and then returning them to normal oxygen pressure on P12. EGHB010 (50 and 100 mg/kg/day) was administered intraperitoneally for 5 days (P12 - P16). Retinal flat mounts were prepared to measure the extent of retinal neovascularization on P17. The incubation of HUVECs with EGHB010 (1-25 μg/mL) resulted in the inhibition of VEGF-mediated tube formation in a dose-dependent manner. EGHB010 at doses of 50 and 100 mg/kg/day inhibited the formation of retinal neovascular tufts by 31.15±2.28% and 59.83±2.92%, respectively. Together, our results indicate that EGHB010 is a potent anti-angiogenic agent and may have potential for the control of abnormal retinal vessel growth in patients with ischemic retinopathy.

Keywords: EGHB010, Paeoniae radix, Glycyrrhizae radix, retinal neovascularization, Shaoyao-gancao-tang

INTRODUCTION

Abnormal angiogenesis in the retinas is the most common cause of visual impairment and blindness in the elderly (aged > 65 years) (Jager *et al.*, 2008) and is a severe complication of retrolental fibroplasia, diabetic retinal microvasculopathy and exudative macular degeneration (Campochiaro, 2013).

Vascular endothelial growth factor (VEGF) is a well-known pro-angiogenic and vascular permeability factor and is a key mediator in the pathogenesis of these retinal diseases (Aiello, 1997). The use of VEGF antagonists to inhibit the VEGF signaling pathway was recently reported to suppress retinal neovascularization in several experimental animal models (Muranaka *et al.*, 2005) and human subjects (Eyetech Study, 2003). In numerous clinical trials, intravitreally injected anti-VEGF drugs, including bevacizumab, ranibizumab and aflibercept, notably suppressed neovascularization and stabilized vision loss (Campa & Harding, 2011; Frampton, 2013; Garcia-Layana *et al.*, 2015). However, the intravitreal injection of anti-VEGF agents presents the risk of adverse events (Diago *et al.*, 2009; Fintak *et al.*, 2008). Repeated intravitreal injections increased the incidence of ocular complications, including endophthalmitis, ocular inflammation, traumatic cataracts, intraocular pressure elevation, retinal detachment, and vitreous hemorrhage (Falavarjani & Nguyen, 2013). Thus, interest in the use of oral agents has been increasing (Honda *et al.*, 2010; Meredith *et al.*, 2015; Takahashi *et al.*, 2008).

EGHB010 is a standardized herbal formula of the rhizome mixture of *Paeonia lactiflora* Pallas and *Glycyrrhiza uralensis* Fisch (ratio of 2:1), which is also known as Shaoyao-gancao-tang (Shakuyaku-kanzo-to in Japanese; Jakyakgamcho-tang in Korean). This herbal formula has been used as an analgesic and anti-spasmodic agent (He *et al.*, 2001).

Recently, the total glycosides of *P. lactiflora* were reported to inhibit the proliferation, migration and tube formation of human vascular endothelial cells (Deng *et al.*, 2010). Licorice, the root of *Glycyrrhiza* species, inhibited angiogenesis in chronic inflammation (Kimura *et al.*, 1991). Based on these reports, we hypothesized that EGHB01 may have anti-angiogenic activity. To the best of our knowledge, no reports have described the inhibitory activity of EGHB010 on retinal pathogenic neovascularization. To elucidate this, we examined the anti-angiogenic activity of EGHB010 in an oxygen-induced ischemic retinopathy (OIR) model. We also investigated whether EGHB010 inhibited the VEGF-mediated endothelial cell tube formation.

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MATERIALS AND METHODS

Preparation of EGHB010

Standardized EGHB010 was provided by EYEGENE Co. Ltd. (Seoul, Korea). Paeoniae radix and Glycyrrhizae radix were purchased from CK herb store (Boeun, Chungcheongbukdo, Korea) and Gamcho Farming Association Corporation (Jecheon, Chungcheonbukdo, Korea), respectively. For the preparation of EGHB010, 200 kg of Paeoniae radix and 100 kg of Glycyrrhizae radix were weighed accurately and mixed. Distilled water (3,000 L) was added to the mixed herbs, which were extracted at 90°C for 8 h. The extract solution was filtered and concentrated to yield a 50 kg extract. The extract was then mixed with maltodextrin (120 kg) as a carrier and stirred to form an aqueous solution. Then, the mixture was subjected to spray-drying and filtered through a 400-mesh sieve to yield an extract powder of EGHB010 (140 kg). The contents of the major components in EGHB010 were quantified by high-performance liquid chromatography (HPLC) as described previously (Kim *et al.*, 2016).

Cell cytotoxicity

Cell cytotoxicity was examined using a MTS assay kit (Promega, Madison, WI, USA). Human umbilical vein endothelial cells (HUVECs, Korean Cell Line Bank, Seoul, Korea) were plated (1×10^4 cells/well) in a 96-well plate containing various concentrations of EGHB010 (1-100 μg/mL). Cell viability was determined at 24 h following incubation. The results of the MTS assay were evaluated by measuring absorbance using a microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 490 nm.

Tube formation assay

Ninety-six-well microplates were coated with 300 μL Matrigel (growth factor-reduced, BD Biosciences, San Jose, CA, USA). HUVECs were seeded at a density of 1×10^6 cells/well and treated with serum-free EGM-2 media (WelGENE, Inc., Daegu, Korea) containing EGHB010 (0-25 μg/mL) and recombinant human VEGF (20 ng/mL) for 17 h at 37°C. Capillary-like tube structures formed by HUVECs on the Matrigel were photographed with a DP71 digital camera (Olympus Corporation, Tokyo, Japan). Tube formation was determined by measuring the number of capillary tube branch points of the capillary-like structures per visual field. The experiments were repeated three times independently.

Experimental retinal neovascularization in OIR

Experimental retinal neovascularization was induced in C57BL/6 pups, as described previously (Lee *et al.*, 2013). Pups (postnatal day 7, P7) with nursing mother were maintained in 75% oxygen for 5 days and then returned to normal oxygen pressure on P12. The pups were randomly allocated to three groups of seven mice each as follows: (1) OIR mice; (2) OIR mice treated with EGHB010 (50 mg/kg body weight); and (3) OIR mice treated with EGHB010 (100 mg/kg body weight). EGHB010 was administered intraperitoneally for 5 days (P12-P16). The mice in the OIR group received an equal volume of the vehicle for 5 days. The care and use of the animals were approved by the Institutional Animal Care and Use Committee of Korea Institute of Oriental Medicine, Daejeon, Korea (IACUC approval no. 14-053). All animal experiments were performed in accordance with the IACUC approved protocol

Fluorescein-dextran angiography and isolectin staining for neovascular area analysis

At necropsy (P17), all mice were anesthetized by isoflurane inhalation. Fluorescein-dextran (10 mg/kg body weight, FD40, Sigma, MO, USA) in sterile PBS was directly injected into the heart. At 30 min after cardiac injection, the eyeballs were enucleated and placed in 4% paraformaldehyde for 1.5 h. The whole retinas were isolated and then mounted on microscope slides. The whole-mount retinas were observed using a fluorescence microscope (BX51, Olympus, Tokyo, Japan). The vas-obliterated area in the retina was measured using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). The neovascular tufts in the retina were stained with rhodamine-conjugated *Bandeiraea simplicifolia* isolectin B4 (Vector Laboratories Ltd., Burlingame, CA, USA). The neovascular areas labeled with isolectin B4 were examined using a fluorescence microscope (BX51, Olympus Corporation, Tokyo, Japan). The sizes of the neovascular tufts were calculated using the ImageJ program.

Real-time PCR

Frozen retinal samples were weighed and the total RNA was isolated using TRIzol solution (Invitrogen Inc., Waltham, MA, USA). Real-time RT-PCR was conducted according to a previously described protocol (Lee *et al.*, 2016). The primer sequences for VEGF and GAPDH were as shown in table 1. The mRNA levels of VEGF were determined using the Bio-Rad iQ5 software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

STATISTICAL ANALYSIS

Group data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test or an unpaired Student's *t*-test using Prism 6.0 software (Graphpad, CA, USA). A p-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

HPLC analysis of EGHB010

For the quality control (QC) test, the contents of the major compounds in EGHB010 were determined by HPLC analysis. The contents of paeoniflorin and glycyrrhizin in EGHB01 were 1.50 ± 0.13% and 0.51 ± 0.02%, respectively.

VEGF-mediated vascular tube formation

To investigate the cytotoxic effect of EGHB010 on HUVECs, we performed an MTS assay using various concentrations of EGHB010 (1-100 μg/mL). The viability of EGHB010-treated HUVECs was not affected up to concentrations of 100 μg/mL (fig. 1). Next, we examined whether EGHB010 could inhibit tube formation, an endothelial function crucial to angiogenesis, in human vascular endothelial cells. VEGF was used as an angiogenic factor. Treatment with EGHB010 inhibited the formation of extensive capillary-like networks of HUVECs in a dose-dependent manner (fig. 2).

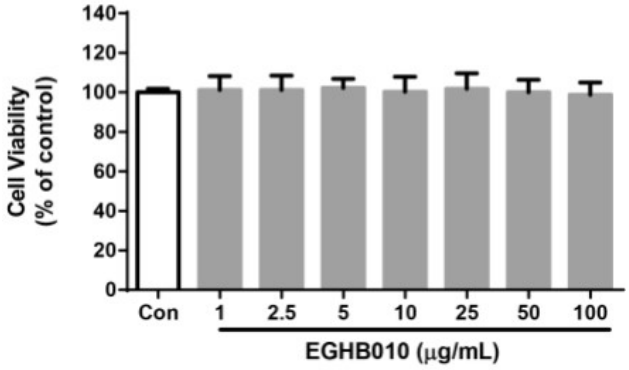

Fig. 1: Effects of EGHB010 on the viability of HUVECs. The viability of HUVECs was determined by MTS assay. Data are expressed as percentage of control. Data are expressed as mean ± SEM, n=4.

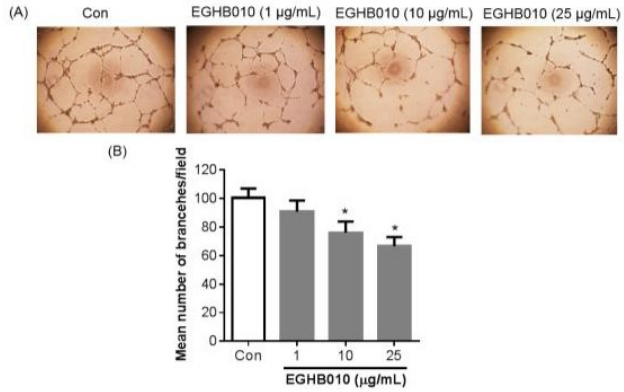

Fig. 2: EGHB010 inhibits tube formation in HUVECs. (A) Human vascular endothelial cells were treated with serum-free media containing HUVECs (0-25 μg/mL) with recombinant human VEGF (20 ng/mL) for 17 h. Tube formation on Matrigels was observed with a microscope. (B) The bar graph represents the quantification of tube formation. Data are expressed as mean ± SEM, n=4, *p<0.01 vs. control.

EGHB010 inhibits retinal neovascularization in OIR

The mice subjected to ischemic retinopathy showed vascular loss with non-perfused areas and abnormal angiogenesis. Newly formed neovascular tufts were visualized by immunofluorescence staining with isolectin B4. OIR mice treated with EGHB010 exhibited a significant decrease in these retinal vascular changes that occur during proliferative retinopathy. As presented in fig. 3, treatment with EGHB010 did not significantly alter vascular loss. However, EGHB010 inhibited the formation of neovascular tufts by 31.15±2.28% and 59.83 ± 2.92% at concentrations of 50 and 100 mg/kg/day, respectively (fig. 4). These results indicated that EGHB010 treatment significantly reduced the size of neovascular tufts.

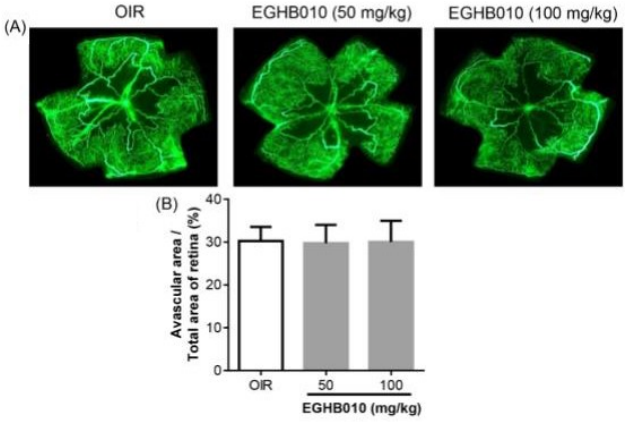

Fig. 3: Effects of EGHB010 on vascular obliteration of the central retina in OIR mice. (A) The retinal blood vessels were visualized via fluorescein angiography using FITC-dextran. (B) The quantification results are expressed as the percentage of the central nonperfused area within the total retinal area. The bar graph values represent the mean ± SEM, n = 7, *p < 0.05 vs. OIR mice.

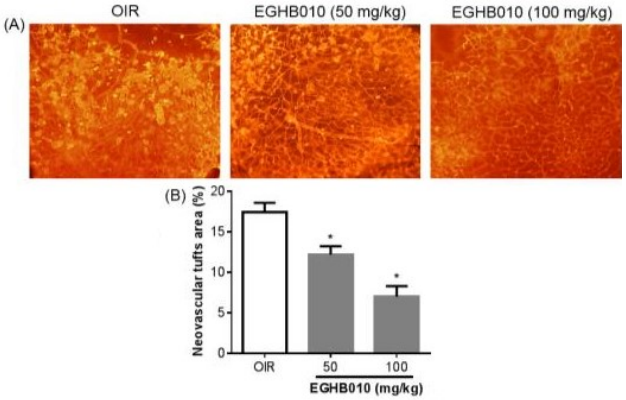

Fig. 4: Effects of EGHB010 on retinal neovascularization in OIR mice. (A) The retinal neovascular tufts were visualized using isolectin B4 staining. (B) Quantification results are expressed as neovascular tufts on the retina surface. The bar graph values represent the mean ± SEM, n = 7, *p<0.05 vs. OIR mice.

EGHB010 downregulates VEGF mRNA expression

To examine the changes in VEGF expression in the retina, we measured the expression levels of VEGF mRNA using real-time PCR. As predicted, the VEGF mRNA levels were markedly decreased by EGHB010 during ischemic retinopathy compared with that of the OIR group (fig. 5).

Table 1: Primer sequences for real-time PCR analysis

Genes	Primers	Sequences
VEGF	Forward	5′-TCCTCCTATCTCCACCACCTATCC-3′
	Reverse	5′-GACCCAGCCAGCCATACCC-3′
GAPDH	Forward	5′-AACGACCCCTTCATTGAC-3′
	Reverse	5′-TCCACGACATACTCAGCAC-3′

DISCUSSION

Pathogenic angiogenesis is the primary cause of severe vision loss in several retinal degenerative diseases, including diabetic retinopathy and wet form AMD (Gehrs *et al.*, 2006). VEGF and its receptors serve an important role in the development of these retinal disorders (Aiello, 1997), and inhibiting angiogenesis by targeting VEGF has become a major focus in drug development (van Wijngaarden & Qureshi, 2008). In this study, we aimed to demonstrate the effect of EGHB010 on abnormal neovascularization in an OIR model. To the best of our knowledge, our work was the first to show that EGHB010 inhibits tube formation in HUVECs *in vitro* through a VEGF-mediated mechanism. In addition, EGHB010 significantly suppressed retinal neovascularization and VEGF mRNA expression in a mouse model of experimental OIR. Taken together, these results indicate that the inhibitory effect of EGHB010 on retinal neovascularization primarily stems from its potent anti-VEGF activity.

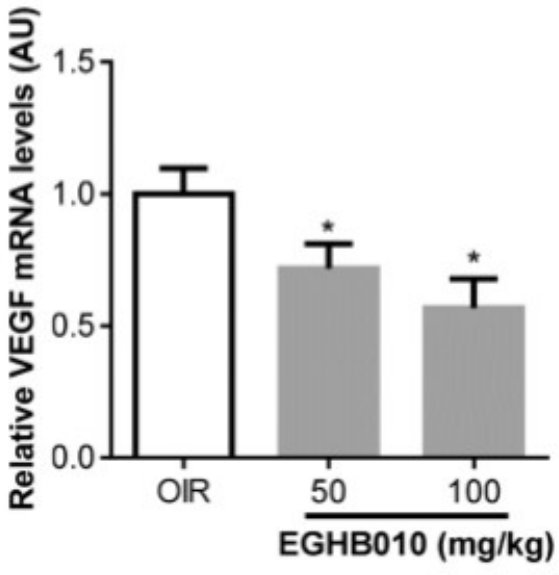

Fig. 5: Effects of EGHB010 on VEGF mRNA expression in OIR mice. Real-time PCR analysis of VEGF mRNA levels in OIR mice. VEGF mRNA expression was markedly reduced after EGHB010 treatment. The data are shown as the mean ± SEM, n = 7, *p<0.05 vs. OIR mice.

VEGF is a well-known vascular permeability and angiogenic factor that activates endothelial cell proliferation and angiogenesis (Shweiki *et al.*, 1992). In OIR mice, VEGF expression was suppressed during the hyperoxic phase (P7-P12) (Stone *et al.*, 1996). Once hyperoxia was terminated (P12-P17), hypoxia-driven up regulation of VEGF was observed even under normoxic conditions (Ashton, 1957; Ashton, 1966). Furthermore, in ischemic retinopathy, such as that present in diabetic retinopathy and neovascular AMD, the robust up regulation of proangiogenic VEGF expression leads to the activation of angiogenic signaling pathways and triggers neovascularization (Hoeben *et al.*, 2004). Numerous studies have suggested that VEGF has a key role in retinal vasculopathy, and its inhibition significantly blocks the pathogenic alterations in the retinal vasculature (Aiello *et al.*, 1994; Dorey *et al.*, 1996). Anti-VEGF agents were recently reported to exhibit beneficial effects in patients with proliferative diabetic retinopathy and neovascular AMD (Campochiaro, 2013; Dhoot & Avery, 2016).

In our study, there was no positive control (anti-VEGF drug) group. In several previous studies, anti-VEGF drugs, including ranibizumab and aflibercept, notably suppressed retinal neovascularization (Campa & Harding, 2011; Frampton, 2013; Garcia-Layana *et al.*, 2015). However, these anti-VEGF agents are humanized recombinant protein drugs designed for intraocular use. Thus, these agents are not the apt choice for a positive control drug.

EGHB010 is a standardized herbal extract. To the best of our knowledge, our study demonstrated the anti-angiogenic effects of EGHB010 *in vitro* and *in vivo* for the first time. Several studies have reported that certain crude herbal extracts and phytochemicals can inhibit pathogenic neovascularization in tumorigenesis (Ruma *et al.*, 2014; Yance & Sagar, 2006) and retinal neovascular diseases (Cao *et al.*, 2010; Hua *et al.*, 2011; Kumar Gupta *et al.*, 2013; Tanaka *et al.*, 2012). In traditional east Asian medicine, Paeoniae radix has been used to nourish blood, regulate menstruation, and alleviate pain. Glycyrrhizae radix has been used to suppress cough and detoxify several toxic substances. EGHB010 has been used to treat muscle contraction and cramps (Bensky *et al.*, 2004). It contains two major compounds (paeoniflorin and glycyrrhizin). Paeoniflorin prevented oxidative stress-induced apoptosis in human RPE cells (Wankun *et al.*, 2011) and reduced VEGF levels in the synovium of rats with arthritis (Zheng *et al.*, 2007). Glycyrrhizin inhibited neovascularization during tumor progression in mice (Kim *et al.*, 2013). It decreased VEGF generation in

retinal ganglion cells treated with advanced glycation end products (Lee *et al.*, 2012). In addition, glycyrrhizin has been known as a selective inhibitor of high-mobility group box-1, a potent proangiogenic molecule, and it attenuated ischemia-induced retinal neovascularization (Lee *et al.*, 2013). Although the detailed action mechanism of EGHB010 as a VEGF inhibitor is still not clear, it is suggested that the anti-angiogenic activity of EGHB010 may be due to the synergistic effects of paeoniflorin and glycyrrhizin.

CONCLUSION

This is the first study to provide evidence that EGHB010 inhibits experimental retinal neovascularization in ischemic retinopathy *in vivo*. In addition, *in vitro* studies showed that EGHB010 inhibits VEGF-induced tube formation in HUVECs. Further studies may be required to determine the feasibility of using EGHB010 for the treatment of patients with ischemic retinopathy.

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