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, intracocular 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 EGHB01 on retinal pathogenic neovascularization. To elucidate this, we examined the anti-angiogenic activity of EGHB01 in an oxygen-induced ischemic retinopathy (OIR) model. We also investigated whether EGHB01 inhibited the VEGF-mediated endothelial cell tube formation.
**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 (1×10⁶ 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 1×10⁶ 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. 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.

**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 isoelectin 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 isoelectin B4 (Vector Laboratories Ltd., Burlingame, CA, USA). The neovascular areas labeled with isoelectin 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 EGHB010 were 1.50 ± 0.13% and 0.51 ± 0.02%, respectively.

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**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 isoelectin 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 isoelectin 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).
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.

Table 1: Primer sequences for real-time PCR analysis

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<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>VEGF</td>
<td>Forward</td>
<td>5′-TCCTCCTATCTCACACCTATCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GACCCAGCGCCATATCC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5′-AACGACCTTCATTGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TCCACGACATCTCAGCAC-3′</td>
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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 (paenonilin and glycyrrhizin). Paenonilin 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, glycyr rhizin 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 glycyr rhizin.

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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REFERENCES


